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The Deleterious Effects of Glucocorticoids

From epidemiology to molecular perspective

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Abstract

Background: Cushing's syndrome (CS) is caused by prolonged and inappropriately excessive tissue exposure to glucocorticoids (GC) [1]. CS results in significant morbidity and excess mortality. Increased 11β-hydroxysteroid dehydrogenase type1 (11β-HSD1) activity at local tissue has been documented for adverse cortisol effects.

Aim: To explore the deleterious effects of systemic and local GC excess in man at molecular and epidemiological levels, which focuses on the outcomes that enable the quantification of disease burden and further avoidable premature death or morbidity.

Methods: The epidemiological studies focused on a meta-analysis of mortality and causes of death in endogenous and exogenous CS. Mortality is a crucial health problem, and meta-analyses systematically explore the issue. The molecular study investigates 11β-HSD1 expression in hypoxia in human dermal fibroblasts. This is the preliminary research of 11β-HSD1 role in ischaemic/diabetic wounds, the worldwide health burden. The understanding of 11β-HSD1 in hypoxic skin may yield a new treatment for diabetic/ischaemic wounds.

Results: The pooled proportion of death for endogenous CS was 5%, 4% in Cushing's disease (CD), 2% in adrenal adenoma, but 8% in bilateral adrenal hyperplasia. The standardised mortality ratio (SMR) was 3.0 for all CS. ACS was associated with a worse SMR than CD (p=0.003). Mortality was higher in publications published before 2000, active disease, and macroadenomas. Cumulative, average, and initial GC doses are associated with increased mortality in exogenous CS. Cardiovascular diseases, infection and malignancy, are the major contributors to deaths for all CS. Hypoxia increases 11β-HSD1 expression and activity in HDF, particularly in inflammatory conditions for the molecular study.

Conclusion: CS confirmed the association with an increase in mortality. The causes of death highlight the need for aggressive management. The 11β-HSD1 role in hypoxia requires further research in ischaemic or diabetic human skin with is the new hope for curing the wound.

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List of Abbreviation

£	pounds
11β-HSD	11β-hydroxysteroid dehydrogenase enzymes
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
17α-OHP	17α-hydroxyprogesterone
3β-HSD2	3β-hydroxysteroid dehydrogenase type 2
AA	adrenal adenoma
AC	adenylyl cyclase
ACE2	angiotensin-2
ACS	adrenal Cushing's syndrome
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
ADP	adenosine diphosphate
ADR/Adx	adrenodoxin/adrenodoxin reductase
AF-1	activation function 1
AF-2	activation function 2
ALDO	aldosterone
ANXA1	annexin A1
AP1	activator protein 1
AR	agonist receptor
ATIIR	angiotensin II type-2 receptor
ATP	adenosine triphosphate
AVP	arginine vasopressin
AZ	AZD4017, the 11β-HSD1 inhibitor
BAH	bilateral adrenal hyperplasia
C	Community
Ca2+	calcium
CBG	cortisol-binding globulin
CCAAT-EBP	enhancer-binding protein family
CCL	Chemokine (C-C motif) ligand
CD	Cushing's disease
CD163	Cluster of Differentiation 163
cDNA	complementary DNA
cGRE	composite glucocorticoid response element
CI	confidence interval
CO2	carbon dioxide

COX-2	cyclooxygenase-2
cPLA2	Cytosolic phospholipase A2
CREB	cAMP-response-element-binding protein
CRH	corticotrophin-releasing hormone
CRH-R1	CRH type 1 receptor
CS	Cushing's syndrome
CT	computerized tomography scan
Ct	cycle treshold
CVD	cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
CYP	cytochrome P450
DBD	DNA binding domain
df	degree of freedom
DFU	Diabetic foot ulcers
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DI	DerSimonian and Liard approach
DL	DerSimonian and Liard approach
DM	diabetes mellitus
DMEM	Dulbecco’s Modified Eagles Media
DNA	deoxyribonucleic acid
DOC	11-deoxycorticosterone
DUSP1	Dual-specificity phosphatase-1
E	expected deaths (chapter 3,4)
E	cortisone (chapter 5)
e.g.	exempli gratia
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
ES	effect size
FC	Free cholesterol
FCS	foetal calf serum
FEM	fixed effect models
FEM	fixed-effect model
FGF	fibroblast growth factor
FKBP51	FK506-binding protein 51

ftt	Freeman-Tukey Double arcsine transformation
GC	glucocortiocids
GCRG	glucocorticoid-responsive genes
GLMM	Generalised Linear Mixed Model
GLUT4	insulin-responsive glucose transporter 4
GM-CSF	Granulocyte/macrophage colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h, hr	hour
H6PD, H6PDH	hexose-6-phosphate dehydrogenase
HDAC6	histone deacetylase 6
HDF	human dermal fibroblasts
HDL	high-density lipoproteins
HPA	hypothalamic-pituitary-adrenal
HR	hazard ratio
HR	hinge region
HSD	hydroxysteroid dehydrogenase
HSD11B1	Hydroxysteroid 11-Beta Dehydrogenase-1
HSD11B2	Hydroxysteroid 11-Beta Dehydrogenase-2
HSL	hormone-sensitive lipase
hsp	heat-shock protein
HT	hypertension
I^2	Higgins's I^2 test statistic
IC50	half maximal inhibitory concentration
ICS	Inhaled corticosteroid
IFNϒ	interferonϒ
IGF-1	insulin-like growth factor 1
IkB-α	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha
IL	interleukin
IL-1	Interleukin-1
IL-1R2	interleukin-1 receptor 2
lncRNA	long non-coding RNA
iNOS	Inducible Nitric Oxide Synthase
IP3	inositol (1,4,5)-triphosphate
IP3R	inositol (1,4,5)-triphosphate receptor
IQR	interquartile range

IRF3/5	interferon regulatory factor 3/5
IRR	incident rate ratio
KO	Knock out
LBD	ligand-binding domain
LDL	low-density lipoproteins
LDLR	low-density lipoprotein receptor
LR	logistic regression
MACS	mild autonomous cortisol secretion
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
Med	medical record
mg/d	milligram/day
mGR	membrane glucocorticoid receptor
MIP	macrophage inflammatory protein
MKP1	mitogen activate protein (MAP) kinase phosphatase 1
MMP	matrix metalloproteinases
MOOSE	Meta-analysis Of Observational Studies in Epidemiology
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
n	no
NA	not applicable
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide-adenine-dinucleotide phosphate (oxidized)
NADPH	reduced nicotinamide-adenine-dinucleotide phosphate
NFAT	Nuclear Factor of Activated T Cell
NF-kB	Nuclear factor-kappa B
nGRE	negative glucocorticoid response element
NK	neurokinin
NLS	nuclear localisation signal
no, No	number
NR	not report
NTD	N-terminal transactivation domain
O	observed deaths

O2	oxygen
OR	odds ratio
P450c11AS	Aldosterone synthase
P450c17	17α-hydroxylase/17,20-lyase enzymes
PAPSS2	phosphoadenosine-5-phosphosulfate synthase type 2
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POMC	pro-opiomelanocortin
POR	P450 oxidoreductase
PPARγ2	peroxisome proliferator-activated receptor γ type 2
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
PRL	prednisolone
PRN	prednisone
PTGS-2	Prostaglandin-Endoperoxide Synthase 2
PVN	paraventricular nuclei
Q or Q-test	Cochrane's Chi-squared test
QUORUM	QUality Of Reporting Of Meta-analyses
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-B ligand
RANTES	regulated on activation, normal T cells expressed and secreted
real-time qPCR	Quantitative real-time polymerase chain reaction
REM	random effect model
REML	Restricted maximum-likelihood
Retro	Retrospective cohort
RNA	ribonucleic acid
Rnase	ribonuclease
S	secondary or tertiary or special care level
SCF	stem cell factor
SE or se	standard error
SERCA	sarco/endoplasmatic reticulum Ca 2+ -ATPase
SGN	spiral ganglion neuron
SLE	systemic lupus erythematosus

SLPI	secretory leucocyte peptidase inhibitor
SMR	standardised mortality ratio
SNP	single nucleotide polymorphism
SRBI	scavenger receptor B1
StAR	Steroidogenic-Acute-Regulatory-Protein
STAT5	signal transducer and activator of transcription 5
STROBE	Strengthening The Reporting of OBservational studies in Epidemiology
SULT2A1	sulfotransferase 2A1
T2DM	Diabetes mellitus type 2
T-bet	T cell-associated transcription factor
TGF-β	transforming growth factor β
tGRE	tethered glucocorticoid response element
THA	tetrahydro-11-dehydrocorticosterone tetrahydrodeoxycortisol
THB	THB, tetrahydrocorticosterone
THDOC	THDOC, tetrahydro-11-deoxycorticosterone
THE	tetrahydrocortisone
THF	tetrahydrocortisol
THS	tetrahydrodeoxycortisol
TLS	Tertiary Lymphoid Structures
TNF	tumor necrosis factor
TSC22D3	TSC22 Domain Family Member 3
TSS	transsphenoidal surgery
TTP	tristetraprolin
UK	United Kingdom
US	United States
v	random effects variance component (chapter 2)
V	Vehicle (chapter 5)
Var	variance
VCAM-1	vascular Cell Adhesion Molecule
VEGF	vascular endothelial growth factor
VEGFA	vascular endothelial growth factor A
VTE	venous thromboembolism
w	true weight
WH	wound healing
y	yes

ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis
μ	mean
T	Tau
T^2	Tau^2
$\chi 2$	Chi-squared test

Chapter 1

Introduction and background

1.1 Corticosteroids and physiology functions

1.1.1 Definition and classifications

Steroid hormones are classified by a chemical (Figure 1-1) and a biological basis and include corticosteroids (mineralocorticoids, glucocorticoids (GC)); sex hormones (dehydroepiandrosterone (DHEA), oestrogen, progesterone, androgens); and vitamin D[2]. Steroidogenesis occurs in classical steroidogenic glands (adrenal cortex and gonads)[3]; *de novo* steroidogenesis organs (placenta and brain)[4]; or non-steroidogenic or intracrine tissues (adipose tissue, thymus, skin and intestine)[5, 6]. The physiologic function of steroid hormones is mediated via receptors and their complexes for hormone signalling and genetic transcription responses[7]. Corticosteroids are synthesised from within the adrenal cortex; three classes of corticosteroids are differentiated by cytoarchitecture zone, specific zonal enzyme expression and physiological properties (Figure 1-2)[8]. Mineralocorticoids are synthesised from the outermost *zona glomerulosa (zG)* under the control of the renin-angiotensin-aldosterone system, potassium, or adrenocorticotropic hormone (ACTH). An active biological form in humans is aldosterone to regulate water and salt homeostasis[9, 10]. GCs are synthesised from the middle, and largest zone called *zona fasciculata (zF)*, under the regulation of ACTH with the active form in human being cortisol, to regulate carbohydrate metabolism stress responses, energy homeostasis, embryonic development, postnatal transitions, immunoregulation and inflammation[8]. Androgenic sex hormones are synthesised from the innermost layer, *zona reticularis* (*zR*), with the main products being DHEA or DHEA-sulfate (DHEA-S) and androstenedione[11, 12].

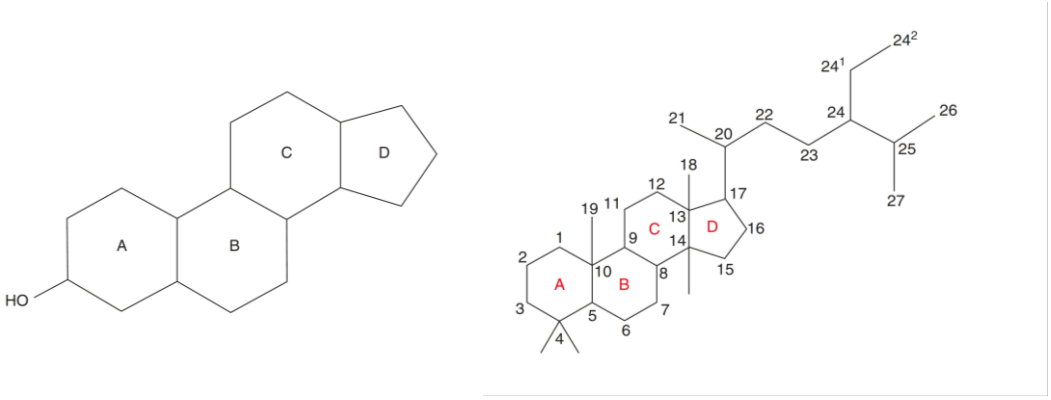


Figure 1-1. Steroid organic compound.

Structure demonstrated a four-membered hydrocarbon core with three cyclohexane rings (A-C) and one cyclopentane ring (D), perhydro-1,2-cyclopentenophenanthrene. A hydroxyl (OH) on the A ring is the basic structure of steroids. The first 17 carbons are the basic core structure in all steroids, with the additional carbon (18-27) as steroid side chains. The difference in steroid structures results from each adrenal cortical zone and their differential enzyme expressions. Mineralocorticoids and glucocorticoids consist of 21 carbon steroids with a hydroxyl on the 21st carbon, whereas androgens consist of 19 carbons[13].

1.1.2 Adrenal steroidogenesis

Adrenal steroidogenesis is the *de novo* hormone synthesis without pre-storage in the adrenocortical cells. It depends on ACTH binding to its specific cell surface G-protein-coupled receptor named melanocortin type-2 receptor[14] or angiotensin II binding to angiotensin II type I receptor or changing ion exchange (potassium) across cells mediated by transmembrane ion channels[15]. The timing of ACTH stimulation of adrenal steroidogenesis can be separated into three phases[14]. Firstly, ACTH stimulates adrenal gland hypertrophy and hyperplasia to prepare adrenal cells for steroidogenesis, taking several weeks to months. Secondary, ACTH stimulates genes transcription and increased steroidogenesis enzyme activity, which occurs over days. The third step takes 15-60 minutes following ACTH exposure enabling Steroidogenic-Acute-Regulatory-Protein (StAR) for cholesterol delivery to drive cortisol production[16]. Free cholesterol (FC) is the primary precursor derived from four sources[17].

(1**) Dietary low-density lipoproteins (LDL).** This is the primary source of FC that is transported to the adrenal cell surface via LDL receptor by endocytosis in the form of esterified cholesterol in lysosomes, followed by hydrolysis to produce FC[18].

(2) ***De novo* synthesis from acetate in the endoplasmic reticulum (ER) of adrenal cells.** This is an essential pathway to maintaining cholesterol balance under physiological and pathological conditions using acetyl coenzyme A[19].

(3) **Circulatory high-density lipoproteins (HDL).** The FC is up taken via scavenger receptor B1 and de-esterified by hormone-sensitive lipase[20]. This is a less important pathway in humans.

(4) **Hydrolysis** Cholesterol can be esterified into lipid droplets, and intracellular lipid droplets containing cholesterol esters can be re-esterified by hormone-sensitive lipase[20].

Firstly, steroidogenesis begins in the mitochondria, where FC is transported from the outer mitochondrial membrane into the inner mitochondrial membrane following interaction with StAR and cytochrome P450 (CYP) cholesterol side-chain cleavage enzyme or CYP11A1 to produce pregnenolone[21, 22]. This is the rate-limiting step of adrenal steroidogenesis when StAR (37 kDa) precursor protein is released from the ER immediately after ACTH stimulation or stress[23]. StAR requires a chaperone protein (glucose regulatory protein 78, which is located in the mitochondria-associated ER membrane, to fold and activate StAR (30 kDa)[22]. Following FC influx, two main groups of enzymes: CYP450 and hydroxysteroid dehydrogenases/ketosteroid reductase, together with their cofactors, are required for the intracellular biosynthesis of specific steroid hormones in different adrenal zones[21, 24, 25], as detailed below (Table 1-1)

Table 1-1. Cytochrome P450 enzymes and cofactors for adrenal steroidogenesis

Classification enzymes	Localisation	Enzyme	Gene	Electrons and molecular oxygen donors (cofactors)
CYP Type 1	Mitochondria	Cholesterol side-chain, P450scc	*CYP11A1*	flavoprotein (ferredoxin reductase) and an iron-sulfur protein (ferredoxin or adrenodoxin)
		11β-hydroxylase, P450c11	*CYP11β1*	
		Aldosterone synthase, P450c11AS	*CYP11β2*	
CYP Type 2	ER	17α-hydroxylase, P450c7	*CYP17A1*	flavoprotein (P450-oxidoreductase)
		21α-hydroxylase, P450c21	*CYP21A2*	
		P450 aromatase, P450aro	*CYP9A1*	

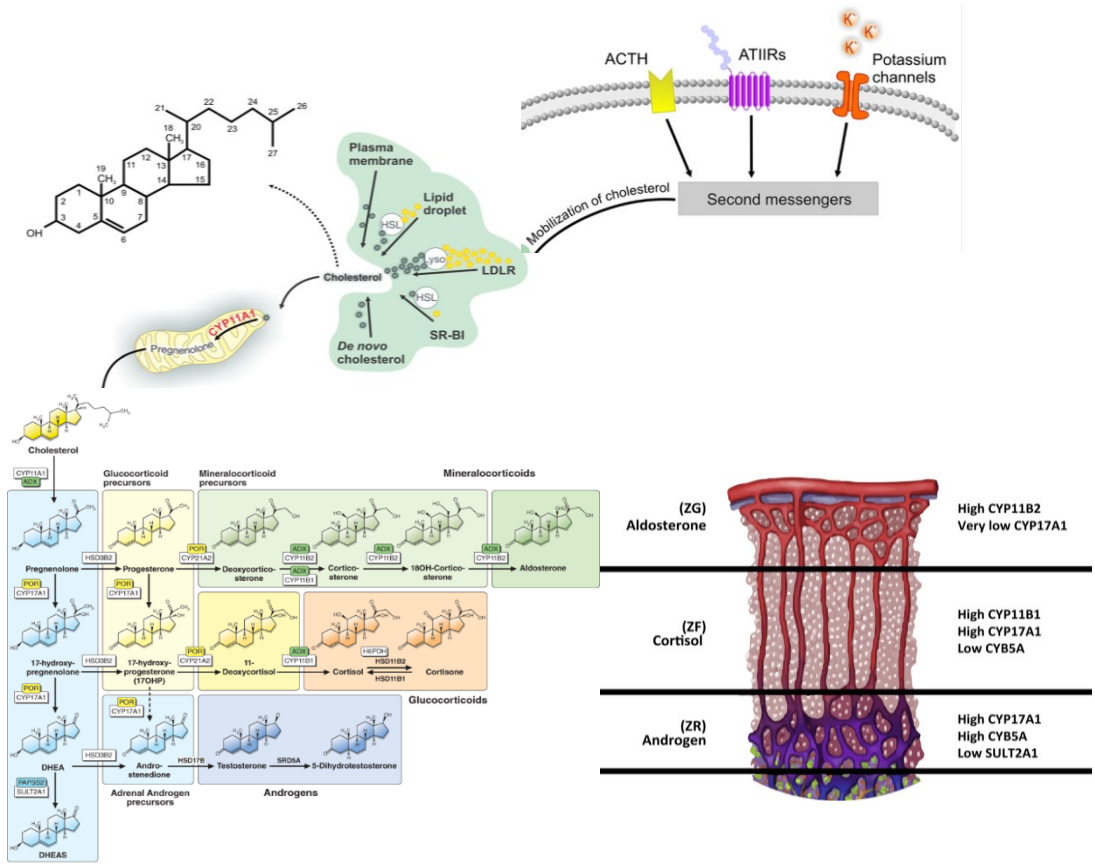


Figure 1-2. Pathways for adrenal steroidogenesis.

ACTH receptors expressed throughout the adrenal cortex play significant roles in regulating GC and androgen biosynthesis. In addition, angiotensin II type-2 receptor (ATIIR) and potassium channels, strongly expressed in zona glomerulosa (zG), regulate mineralocorticoid (aldosterone) production. When the receptors are activated, cholesterol from many sources: LDL via LDL-receptor (LDLR), HDL through scavenger receptor B1 (SRBI), de novo synthesis, lipid droplet or plasma membrane, is mobilised to mitochondria where side-chain cleavage enzyme (CYP11A1) cleaves the side chain of cholesterol. Then, the zonal-specific expression of enzymes ensures conversion of pregnenolone to aldosterone, cortisol and DHEA. ADX, adrenodoxin; CYP, cytochrome P family; CYP11B1, 11β-hydroxylase; CYP11B2, aldosterone synthase; CYP17A1, 17α-hydroxylase/17,20 lyase; CYP21A2, 21-hydroxylase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; H6PDH, hexose-6-phosphate dehydrogenase; DHT, dihydrotestosterone; HSD, hydroxysteroid dehydrogenase; HSD11B1, 11β-hydroxysteroid dehydrogenase type 1; HSD11B2, 11β-hydroxysteroid dehydrogenase type 2; HSD17B, 17β-hydroxysteroid dehydrogenase; HSD3B2, 3β-hydroxysteroid dehydrogenase type 2; HSL, hormone-sensitive lipase; PAPSS2, PAPS synthase type 2; POR, P450 oxidoreductase; SRD5A, 5α-reductase; SULT2A1, sulfotransferase 2A1; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis. Figure adapted with permission from Copyright Clearance Center's RightsLink®/ Elsevier, Adina F. Turcu & Richard J Auchus (2015) with license number 5225140316997[26] and Daniel B. Martinez-Arguelles & Vassilios Papadopoulos (2019) with license number 5225260614565[27], permission granted on January 9, 2022, by email.

1.1.2.1 Aldosterone biosynthesis

Angiotensin II via the renin-angiotensin-aldosterone system and hyperkalaemia are the main secretagogues for aldosterone secretion, acting via increased intracellular calcium[28]. Three essential enzymes are required to convert pregnenolone to aldosterone (Figure 1-3): (1) **3β-hydroxysteroid dehydrogenase type 2** (3β-HSD2, *HSD3B2*), which performs the irreversible conversion of pregnenolone to progesterone. (2) **21-hydroxylase** (P450c21, *CYP21A2*), which converts progesterone to 11-deoxycorticosterone (DOC). (3) **Aldosterone synthase** (P450c11AS, *CYP11B2*), present only in the *zG* and catalyses the final three steps of aldosterone synthesis: 11β-hydroxylation, 18-hydroxylation, and 18-methyl oxidation, converting corticosterone into aldosterone. Aldosterone, corticosterone, and DOC all have mineralocorticoid activity with aldosterone being the principal mineralocorticoid in man. *zG* expresses minimal 17α-hydroxylase/17,20-lyase enzymes (P450c17, *CYP17A1*), which convert their substrates to cortisol and androgens[3].

1.1.2.2 Cortisol biosynthesis

In the *zF* (Figure 1-3), pregnenolone in mitochondria is converted to 17α-hydroxyprogesterone (17α-OHP) via two pathways. In the main pathway, 3β-HSD2 converts pregnenolone to progesterone and 17α-hydroxylase enzyme hydroxylases progesterone into 17α-OHP. In the alternative pathway, 17α-hydroxylase enzyme converts pregnenolone into 17α-hydroxypregnenolone and then into 17-OHP by 3β-HSD2. Then 21-hydroxylase converts 17-OHP to 11-deoxycortisol, and 11β-hydroxylase converts 11-deoxycortisol to cortisol, the predominant GC[3].

1.1.2.3 Adrenal androgen biosynthesis

In the *zR* (Figure 1-3), 17-hydroxypregnenolone and 17-OHP can be converted into DHEA-S and androstenedione by 17,20 lyase (17α-hydroxylase). Although DHEA-S is predominantly made, some can be converted into androstenedione by 3β-HSD2 in *zR*. ACTH, not angiotensin II regulates adrenal androgen steroidogenesis and the development/ growth of the *zR*[3].

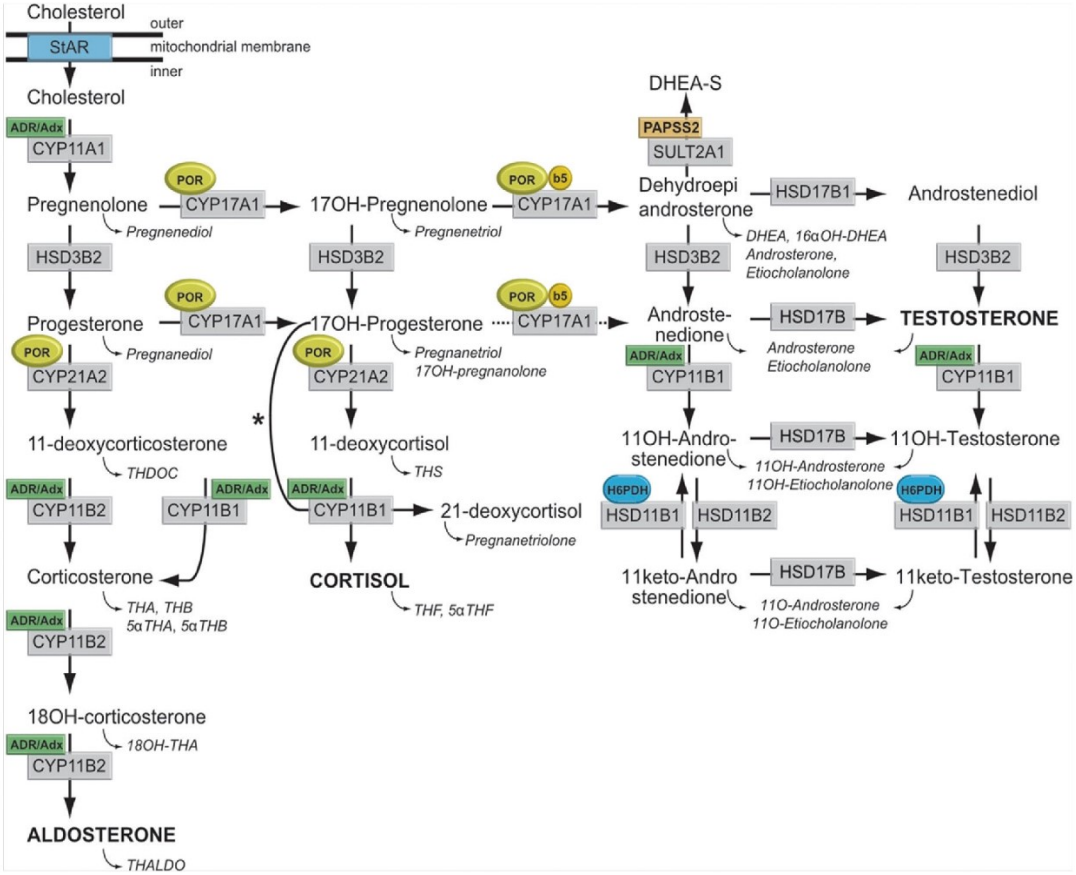


Figure 1-3. Steroidogenic pathways and enzymatic mechanisms convert pregnenolone to mineralocorticoids, glucocorticoids, and androgens in specific adrenal cortex zones.

The thick arrows indicate enzymatic processes involved by steroidogenic enzymes (grey boxes) and cofactors: green boxes represent adrenodoxin/adrenodoxin reductase (ADR/Adx); the orange boxes represent 3-phosphoadenosine-5-phosphosulfate synthase type 2 (PAPSS2); the yellow ovals represent P450 oxidoreductase (POR); the orange balls represent cytochrome b5, and the blue ovals represent coenzyme hexose-6-phosphate dehydrogenase (H6PD or H6PDH). The thin arrows denote the metabolites of steroid hormones. StAR, Steroidogenic-Acute-Regulatory-Protein; THA, tetrahydro-11-dehydrocorticosterone; THB, tetrahydrocorticosterone; THDOC, tetrahydro-11-deoxycorticosterone; THF, tetrahydrocortisol; THS, tetrahydrodeoxycortisol. Reproduced with permission from Copyright Clearance Center's RightsLink® BMJ Publishing Group Ltd, Bacila, I.A. et al.(2019) with license number 5225231209574[29], permission granted on January 2022, by email.

1.1.3 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis plays a master role in regulating cortisol synthesis, dynamic release and equilibration across a multisystem axis and negative-feedback loop between hypothalamus, pituitary, and adrenal glands to help maintain body homeostasis and the stress response[30] (Figure 1-4). Stress or external physiologic stimuli stimulate the HPA axis by neural and non-neural responses[31]. Cytokines including interleukin 1 (IL-1), tumour necrosis factor (TNF) and IL-6 are potent inducers of the HPA

axis[32]. Signals projected via the neural response located in the paraventricular nuclei (PVN) neurons and corticotroph cells in the hypothalamus release corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal vein. Furthermore, the suprachiasmatic nucleus or master of biological clock signals CRH neurons to stimulate CRH release with a circadian oscillator (pacemaker). CRH, augmented by AVP, activates CRH type 1 receptor (CRH-R1), vasopressin receptor 1B and adenylate cyclase in the anterior pituitary gland to synthesise pro-opiomelanocortin (POMC). POMC is cleaved into ACTH and other derivatives[33]. ACTH binds to the G protein-coupled melanocortin-2 receptors at the surface of *zF* or *zR* cells of the adrenal cortex to stimulate G protein activation, adenylyl cyclase activation, and finally cyclic adenosine monophosphate production to synthesis cortisol. POMC-ACTH is also regulated independently from CRH or AVP by inducing IL-1 or IL-2. Similarly, cortisol can be released from the adrenal cortex independently of ACTH by Toll-like receptor-2 and -4 in adrenocortical cells, especially in sepsis[34, 35].

GCs are released under physiological conditions with two main rhythms, ultradian and circadian[36, 37]. Ultradian rhythm refers to intra-pulse amplitude changed approximately every hour, and circadian rhythm is characterised by the variation of the amplitude of pulse or ultradian rhythm across the 24 hours with a peak in the morning and nadir or inactive phase in the late evening and night[37-39]. Adrenal GC and central circadian rhythms are powerful influencers of the molecular clock of peripheral tissues and resulting physiologic functions, as well as cognitive and stress responses[40]. The role of the ultradian rhythm is uncertain in man; in other mammalian species, it has been linked to sex differentiation, pregnancy, lactation, sleep, ageing and inflammation[41]. The dysregulation of circadian GC rhythm is demonstrated in many diseases, notably Cushing's syndrome (CS), adrenal insufficiency, cardiovascular diseases, psychiatric diseases, and metabolic syndrome[40]. Chronic disturbance of their action on biological clock genes is linked to increased susceptibility to cardiometabolic diseases, diabetes mellitus (DM), and malignancy[42]. However, the mechanism or causal relationships requires further investigation.

GC regulate many biological functions, including the stress response, and this is mainly mediated in target tissues by a low-affinity type II corticosteroid or glucocorticoid receptor (GR) and a high-affinity type I corticosteroid receptor or mineralocorticoid receptor (MR) (see section 1.1.4.1 and section 1.1.4.2)[43]. Finally, the HPA axis regulation of cortisol levels operates through a classical endocrine negative feedback control mechanism; the rapid elevation of cortisol inhibits CRH and ACTH release to re-set a normalised cortisol level[33]. Negative feedback mediated through GC binding to GR at PVN and anterior pituitary gland results in suppression of *CRH, CRH-R1*, and the *POMC* [39].

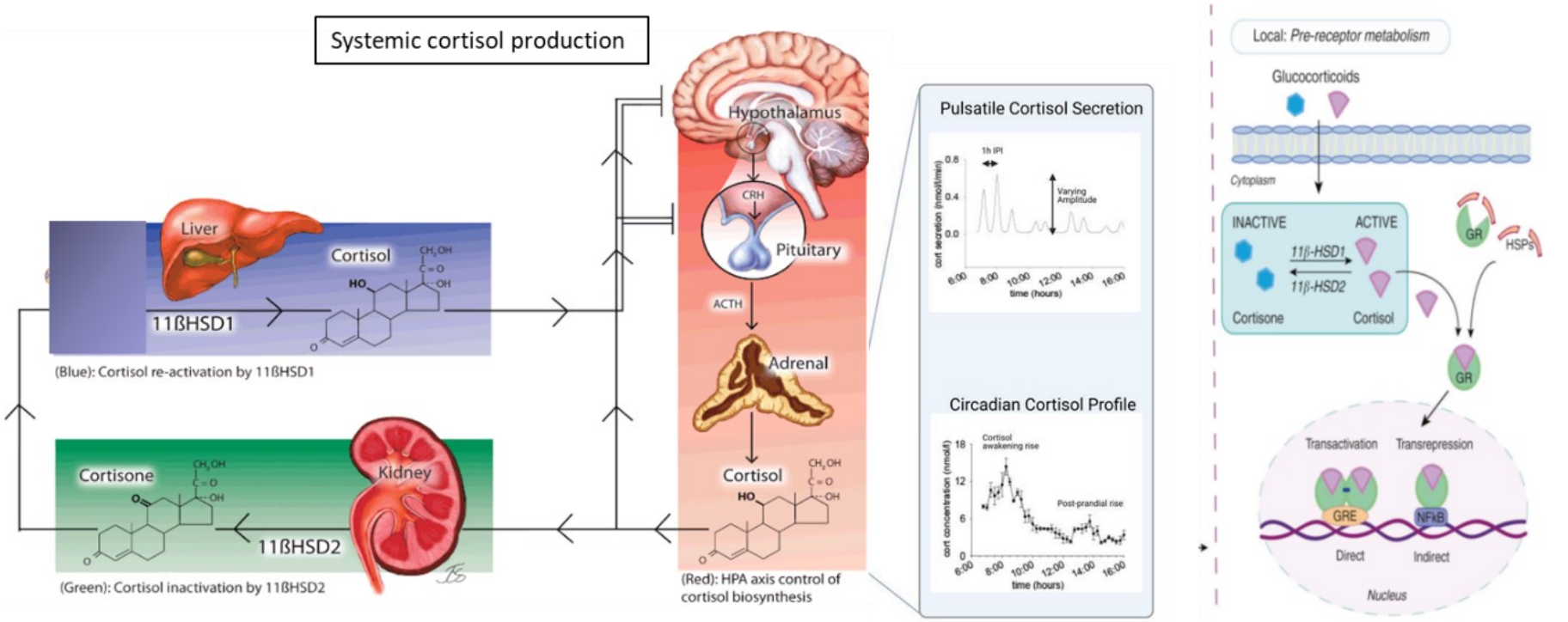


Figure 1-4. Systemic and local regulation of corticosteroids.

HPA axis regulates systematic cortisol production, whereas 11β-hydroxysteroid dehydrogenase enzymes (11β-HSDs) control local cortisol production and action together with glucocorticoid receptors (GRs). Under non-stress conditions, cortisol is released under ultradian and circadian rhythms. GRE, glucocorticoid response element; NF-kB, Nuclear factor-kappa B (photo modified from [44-46]). Reproduced with permission from Copyright Clearance Center's RightsLink® BMJ Publishing Group Ltd, Bacila, I.A. et al.(2019) with license number 5225231209574 permission granted on January 10, 2022, by email.

1.1.4 Corticosteroid receptors

Biological GCs mediate their functions through corticosteroid receptors, comprising GR and MR, which belong to the steroid-nuclear receptor family[47]. Both receptors share 94% homology in the deoxyribonucleic acid (DNA) binding domain (DBD), 50% for the ligand-binding domain (LBD), and two activation functions 1 and 2 (AF-1 and AF-2), which recognise the natural ligands (cortisol, corticosterone in rats, aldosterone and progesterone) but vary in binding affinities and biological activities of different ligands[48]. Without ligands, the receptors are located in the cytoplasm with multi-complex protein, chaperones, and immunophilins.

1.1.4.1 Glucocorticoid receptor

GRs are the ligand-inducible transcription factors encoded by *NR3C1* located on chromosome 5 (5q31); this gene consists of ten exons with three domains: the N-terminal transactivation domain (NTD), DBD and LBD (Figure 1-5)[49]. The hinge region connects DBD and LBD, allowing nuclear translocation[50]. The first exon is untranslated, while the second exon encodes the NTD. NTD is highly immunogenic and contains most phosphorylation sites and AF-1[51]. The AF-1 is the main transactivating domain interacting with co-regulatory proteins and can act spontaneously in the absence of LBD. The DBD encoded by exons 3 and 4 contains two zinc-finger motifs critical for GR dimerisation, nuclear translocation, and DNA binding selectivity. The first zinc finger recognises GC response elements (GREs) on target genes, whereas the second homodimerises the receptor. The LBD, which has the AF-2 domain active, is encoded by exons 5 to 9. The GR contains two nuclear localisation signals (NLSs): NLS1 (locate near the DBD-hinge) and NLS2[52], mediate nuclear import of GR at different velocities, rapid ($t_{1/2}$=4–6 min) and slow ($t_{1/2}$=45–60 min), respectively[53-55].

Although a single gene encodes the GR, alternative splicing in exon 1 results in GR variants, alternative translation initiation, and complex post-translational modification underpins the basis for multiple receptor isoforms[51]. Exon 9 encodes two further variants, resulting in GRα and GRβ, which share identical amino acids up to position 727[51]. The GRα and their isoforms contain 777 amino acids capable of binding to GCs and GREs with similar affinity. Additionally, they differ in cellular and tissue localisation[56]. GRβ is a 742 amino-acid protein that neither binds to nor targets the genes regulated by GCs but can dimerise with GRα, thereby acting as a negative regulator of GR action[53-55]. Moreover, GC resistance is associated with GRβ[57, 58] and other GR protein isoforms (GRY, GR-A, and GR-P)[50]. The variability of isoforms reveals unique tissue expression patterns and

gene regulatory profiles, plays a specific role in tissue-specific actions, and contributes to some diseases[59, 60]. For example, high levels of GRγ or other splice GR expression have been related to acute lymphoblastic leukaemia or a variety of malignancies [61].

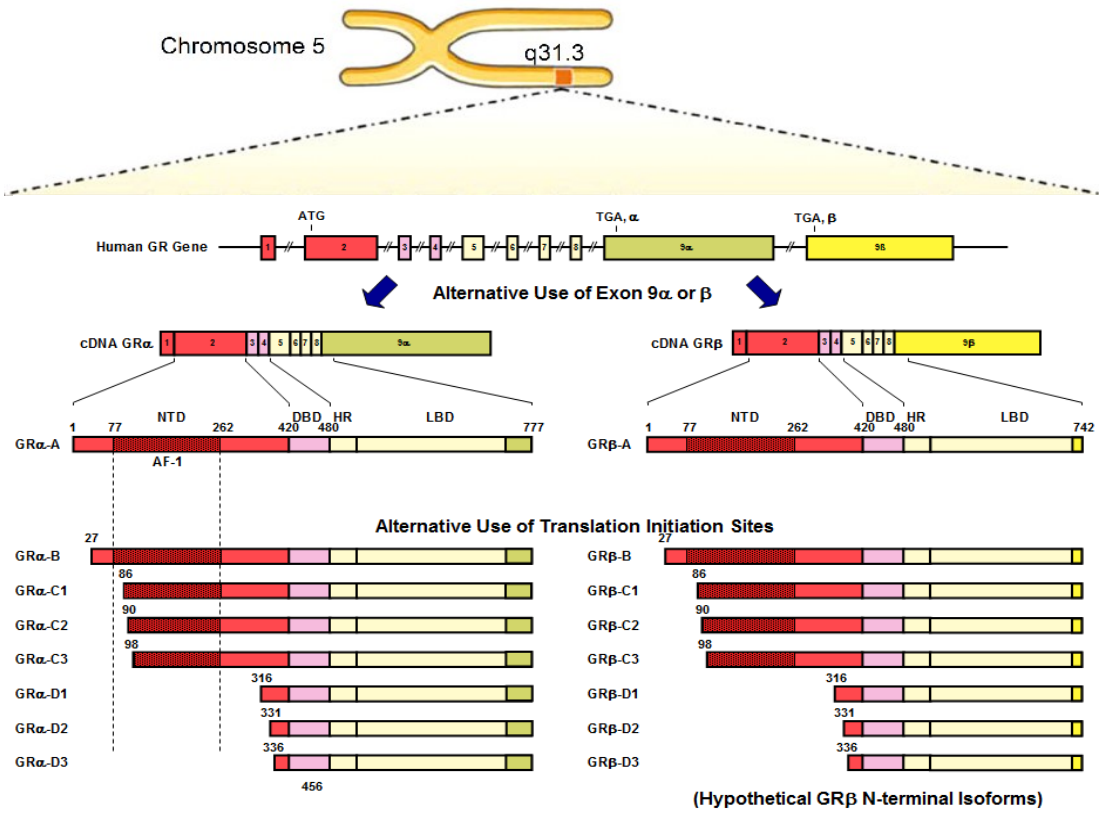
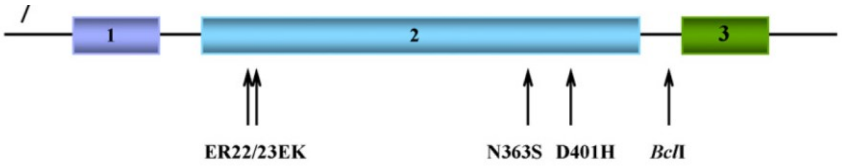


Figure 1-5. NR3C1 gene.

A nine-exon gene located on chromosome 5 is composed of four parts: N-terminal transactivation domain (NTD), DNA-binding domain (DBD), C-terminal ligand-binding domain (LBD) in orange, and a hinge region (HR). The terminal exons 9 (exon 9a and 9b) alternatively splice to produce the GRα and GRβ and their isoforms. GR, glucocorticoid receptor[27]. Reproduced with permission from Endotext by email. Nicolaides NC, Chrousos G, Kino T. Glucocorticoid Receptor. [Updated 2020 Nov 21]. In: Feingold KR, Anawalt B, Boyce A, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Figure 6. [GR isoforms produced through alternative...]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK279171/figure/glucocort-receptor.F6/.

Along with the splice variants, single nucleotide polymorphisms (SNPs) in the *NR3C1* may have functional effects on GC sensitivity[62, 63]**.** Clarifying the SNP responsible for GC activity advances GC therapy for various diseases. To date, there are over 2000 SNPs identified; a few of these SNPs are functionally relevant, for instance (Figure 1-6), 9β, *Bcl*I, ER22/23EK, and N363S[64]. *BCl*I (rs41423247) polymorphism is a C/G nucleotide substitution in intron 2, linked with a tissue hypersensitivity of GC action and associated with abdominal obesity [65, 66]. The *ER22/23EK* polymorphism with alteration at the DNA level of

GAG AGG to GAA AAG[1] is translated in glutamic acid-arginine (E-R) to glutamic acid-lysine (E-K) at two adjacent codons 22 and 23. This polymorphism is associated with relative GC resistance[67], more severity of autoimmune or inflammatory conditions, cognitive impairment, depression[68], and susceptibility to some infection. However, it increases favourable metabolic profiles[69, 70], protective cardiovascular complications, and increased longevity. The *TthII* I polymorphism, a C/T change 3807 bp upstream of the GR messenger ribonucleic acid (mRNA) start site, if combined with ER22/23EK polymorphism, increases GC resistance[71]. *N363S* polymorphism with alternate changing from asparagine (N) to serine (S) at codon 363 shows an increased trans-activating capacity *in vitro,* increased sensitivity to GCs *in vivo,* and significantly higher body mass index[62, 72].



Glucocorticoid Resistance

Decreased sensitivity to Glucocorticoids
Lower fasting insulin concentrations
Improved insulin sensitivity
Lower total and LDL cholesterol
Lower CRP concentrations
Lower risk of dementia
Fewer white matter lesions in brain
Longevity
Depression

Glucocorticoid Hypersensitivity

Increased sensitivity to Glucocorticoids
Increased insulin response to dexamethasone
Increased body fat, lower lean mass
Lower bone mineral density
Higher cholesterol and triglyceride concentrations
Higher incidence of coronary artery disease
Depression

Figure 1-6. *GR* polymorphisms with a review of their clinical correlations.

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1.1.4.2 Mineralocorticoid receptor

MR is a high-affinity nuclear receptor encoded by *NR3C2[73]*. LBD and DBD are structurally similar of 57% and 94% to GR (Figure 1-7)[74] such that cortisol, corticosterone, DOC and aldosterone are all agonist ligands for MR, whereas progesterone is a competitive MR antagonist[75] with similar affinities with Kd values between 0.5 and 3 nM. By contrast, GR showed Kd values of 20-70 nM, specifically cortisol and corticosterone[76]. MRs mediate the well-known classical effect of aldosterone, that is to stimulate transepithelial sodium transport and as such are expressed predominantly in the kidney, especially the distal renal tubules and cortical collecting ducts, salivary gland and distal colon, but also in the brain, heart tissues (cardiomyocytes, fibroblasts, and inflammatory cell), vascular endothelium, vascular smooth muscle cells and adipocytes[77]. *In vitro*, cortisol has a binding affinity to

[1] DNA has four bases: thymine (T), adenine (A), cytosine (C), and guanine (G)

MR similar to that of aldosterone[78]. In normal physiology, the plasma concentration of cortisol is 100-200 times over plasma aldosterone; however, in normal conditions, epithelial MR cannot be activated by cortisol due to 11β-hydroxysteroid dehydrogenase type 2 enzyme (11β-HSD2), which acts at a pre-receptor autocrine level to convert cortisol to inactive cortisone, thereby protecting the MR from illicit occupancy by cortisol[79, 80]. In tissues with low 11β-HSD2 activities, including the heart, hippocampus, and immune systems, cortisol is the preferred ligand occupying the MR[81]. The excessive amount of systemic cortisol (e.g., in CS) can swamp metabolism by 11β-HSD2, cause occupancy of the MRs and result in Na^+ retention, urinary K^+ and H^+ loss leading to mineralocorticoid hypertension (HT)[82].

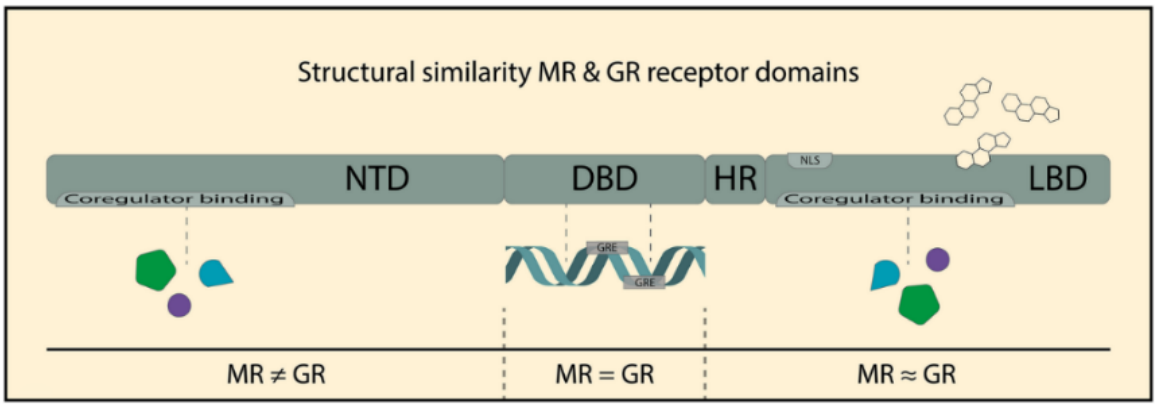

Figure 1-7. Comparing the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). *The highly conserved DNA binding domain (DBD) allows MR and GR to attach to a DNA glucocorticoid response element (GRE). The conserved ligand-binding domain (LBD) binds ligands that can activate both the MR and GR and translocate to the nucleus by nuclear localisation signal (NLS). Coregulator proteins may bind to both the LBD and the N-terminal transactivation domain (NTD). NTD is the most different domain between MR and GR, which modulate distinct transcription output [83]. Reproduced under the terms of the Creative Commons Attribution 4.0 International License, which permits unrestricted use from Figure 1 in Onno C. Meijer. et al. Cell Mol Neurobiol. 2019; 39(4): 539–549*

1.1.5 Glucocorticoid actions

GC production and response are affected by multiple factors, including the concentration of systemic ligand or free cortisol, which dynamically changes over a 24h period, local or extra-adrenal cortisol synthesis (which is mentioned in the following sections) or via 11β-HSD activity determining biologically active ligand and expression of the receptors, DNA proteins and cofactors[47, 84]. Under HPA axis control, the total amount of cortisol released by adrenal glands per day is approximately 5.7–11 mg/m^2/d or 9.5–15 mg/d (different measurements)[85-88] in a circadian and ultradian manner with a high intra- and

interpersonal variability[89]. The peak circulating cortisol is approximately 800 nM/L at 6:00 to 8:00 a.m., and the nadir concentration is as low as <50 nM/L in the late evening, nighttime[89, 90] (Table 1-2). During stress, cortisol secretion rates can rise to 150-200 mg[91]. For systemic cortisol, 80-90% of total cortisol is bound to cortisol-binding globulin (CBG) with high affinity (kDa 2.4×10^{-7} M; half-life of GC binding 5 days), 5-15% is bound loosely to albumin (kDa 5×10^{-5} M), and 4-5% is the free or biologically active form [70, 71]. The binding of cortisone to CBG is much less at approximately 50%, so free cortisol levels and cortisone are not too dissimilar. The liver synthesises CBG, which becomes saturated at a plasma cortisol level above 400-500 nM/L[92]. CBG serves as a primary gatekeeper, a reservoir with controlled release and delivery of GC to target tissues[93, 94]. Factors that affect CBG synthesis or GC-coupling, such as oestrogen, pregnancy, proinflammatory cytokines, cirrhosis or critical illness, lead to a discrepancy between biological free and total cortisol levels[95, 96]. In addition to the systemic cortisol level, 11β-HSDs can modulate intracellular cortisol levels as detailed in section 1.1.7[97, 98].

Lipophilic free cortisol rapidly diffuses through membranes and exerts its functions through binding to the LBD of GR in the cytosol, leading to a conformational change in the GR[47]. GC signalling pathways occur via classical genomic and alternative rapid non-genomic pathways[99, 100]. The criteria for distinguishing between genomic and non-genomic actions are based on studies that have evaluated GC effects with or without GR blocking agents or the immediate changes to basal Ca^{2+} levels[99]. As a result, achieving genomic effects by activating or repressing individual genes (e.g. anti-inflammatory) is a lengthy process (Table 1-3)[101]. On the other hand, non-genomic mechanisms produce effects with a quick onset; further details are mentioned insection 1.1.5.1 and 1.1.5.2[102].

Generally, the biological effects of GC mediated via GRs regulate energy homeostasis, stress responses, and inflammation[99], whereas via MRs result in fluid and electrolyte balance, haemodynamic homeostasis, and tissue repair[103]. Aldosterone, which circulates at a concentration three logs lower than GC (pmol/L), acts as a primary agonist for MR, whilst 11β-HSD2 protects against cortisol activity under normal conditions[104]. In states of cortisol excess or inadequate 11β-HSD2 activity, cortisol can occupy MR, with inappropriate MR activation in tissues causing excessive reactive oxygen species, inflammation, fibrosis, decreased insulin secretion and GLUT4[2] levels, increased adipogenesis and proinflammatory adipokines, decreased insulin metabolic signalling in liver, decreased lipolysis, decreased glycolysis, and decreased glucose uptake[105]. These

[2] insulin-responsive glucose transporter 4

may lead to cardiovascular damage, vasoconstriction, vascular remodelling, endothelial dysfunction, atherosclerotic diseases, HT, glomerulosclerosis, renal impairment, insulin resistance, hepatic steatosis, and type 2 DM[105, 106].

Table 1-2. **Circulating hormone levels and half-lives in normal humans**

Hormone	Trough	Peak	Acute stress	Plasma 1/2
ACTH				
pg/ml	5–15	10–50	40–80	19 min
pmol/L	1.1-3.3	2.2-11.0	8.8-17.6	
Cortisol				
µg/dL	4	16	20-35	60 min
nmol/L	110.4	441.4	551.8-965.7	

Table 1-3. **Glucocorticoid mediated gene expression[107]**[3]

Gene function	Gene
Decreased transcription	
Inflammatory cytokines	*IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, IL-15, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), SCF, TSL, CCL1*
Chemokines	*IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxin, CCL1, CCL5, CCL11, CXCL8*
Inflammatory enzymes	*Inducible Nitric Oxide Synthase (iNOS), cyclooxygenase-2 (COX-2), cPLA2*
Endothelin-1 receptors	*Neurokinin-, Endothelin-, and Bradykinin-receptors*
Adhesion molecules	*Intercellular adhesion molecule (ICAM-1), E-selectin, vascular cell adhesion molecule-1 (VCAM-1)*
11βHSD genes	*HSD11B2*
Increased transcription	
Inflammatory cytokines	*IL-1α, IL-1β, IL-6, IL-1 receptor antagonist, IL-1R2, Lipocortin-1 /annexin-1 (phospholipase A2 inhibitor), Clara cell protein (CC10, phospholipase A2 inhibitor), IkB-α (inhibitor of NF-kB), B_2-adrenoceptors, Secretory leukocyte inhibitory protein, MKP1, CD163*
Anti-inflammatory or inhibitory cytokines	*GC inducible leucine zipper*
11βHSD genes	*HSD11B1*
COVID-19-binding receptor	*ACE2*

[3]*ACE2, angiotensin-2; CCL, Chemokine (C-C motif) ligand; cPLA2, Cytosolic phospholipase A2; CD163, Cluster of Differentiation 163;; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; HSD11B1, Hydroxysteroid 11-Beta Dehydrogenase-1; HSD11B2, Hydroxysteroid 11-Beta Dehydrogenase-2; SCF, stem cell factor; IkB-α, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha; IL, interleukin; IL-1R2, interleukin-1 receptor 2; MCP, monocyte chemoattractant protein; MIP macrophage inflammatory protein; MKP1, mitogen activate protein kinase (MAPK) phosphatase 1; NF-kB nuclear factor-kappa B; NK, neurokinin; TLS, Tertiary Lymphoid Structures; RANTES, regulated on activation, normal T cells expressed and secreted.*

1.1.5.1 Genomic action of glucocorticoids

Unliganded GRα resides abundantly in the cytoplasm in a multiple-protein complex including heat-shock protein (hsp; e.g., hsp40, hsp70, hsp90), immunophilins (e.g., FKBP51), Hop and factors to prevent its degradation and assist in its maturation (Figure 1-8). ATP is required for the optimal function of ATPases hsp70 and hsp90, which cleaves ATP into ADP[99]. Despite the almost ubiquitous expression of GRs, different genetic and epigenetic mechanisms in distinct tissues result in specific effects that can be both beneficial and harmful (GC toxicity)[53, 108].

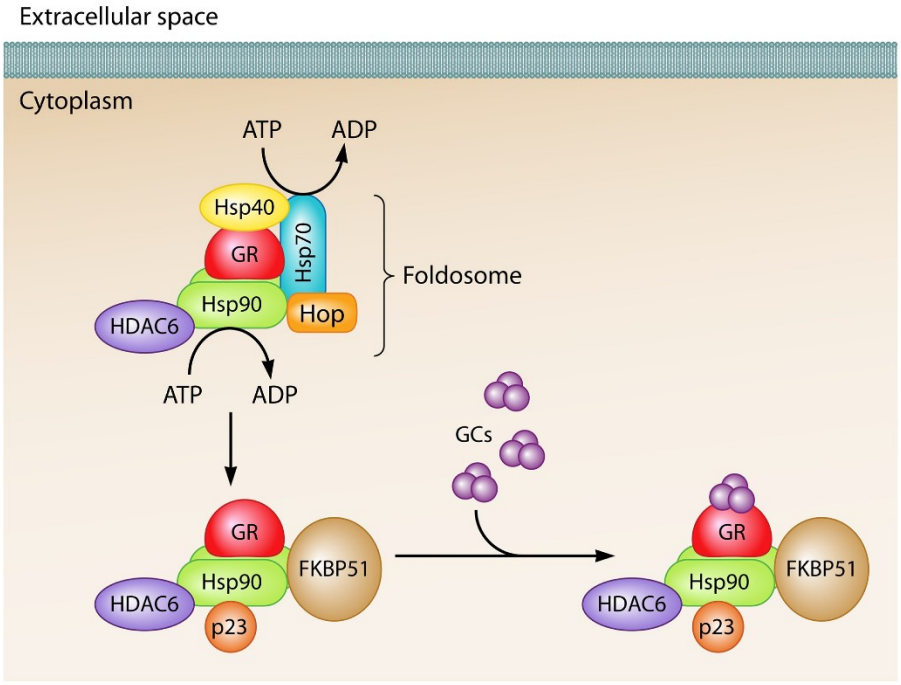

Figure 1-8. Glucocorticoid receptor complex.

ADP, adenosine diphosphate; ATP, adenosine triphosphate; GC, glucocorticoid; GR, glucocorticoid receptor; HDAC6, histone deacetylase 6; Hsp, heat shock protein; FKBP51, FK506-binding protein 51[4]. Reproduced with permission by ASM Journals and UK copyright law for noncommercial research from Figure 3 in Ioanna Petta. Microbiol Mol Biol Rev. 2016 Jun; 80(2): 495–522.

Following the binding of free cortisol (endogenous or synthetic GC) to cytosolic GRα, the activated GC-GRα complex becomes hyper-phosphorylated, dissociated from its protein complex, translocated to the nucleus and binding to sequences of DNA called the GC-response elements (GREs). GREs consist of a variant of the motif 5′-AGAACAnnnTGTTCT-3′, in which 'n' symbolises any nucleotide[99]. GREs are found in the promoter region of GC-

[4] FKBP51 is an intracellular protein or immunophilins that can act as cochaperone in Hsp90 machinery.

responsive genes and are classified as simple (+GRE), negative (nGRE), composite cGRE), ethered (tGRE) GREs (Figure 1-9) [48, 109]. Genomic effects manifest by enhancing or repressing target gene via three mechanisms: 1) GC-GR homodimers bind directly to +GREs or nGRE to activate or repress transcription, 2) interact with co-regulator proteins (which can function as coactivators, corepressors, anti-activators, and antirepressors) as a monomeric receptor that can bind to GREs or other regulatory transcription factors, in a process termed “tethering”, or 3) in a composite manner by direct GRE binding and interacting with transcription factors which bind to neighbour sites[110]. Anti-inflammation benefits from the transrepression of proinflammatory genes (Figure 1-10)[84]. GC suppresses inflammation by transactivating target genes encoding specific proteins[111]. Furthermore, TNFs produced during inflammation induce several proinflammatory cytokines, including IL-1, resulting in GC resistance[112]. The majority of GC adverse effects are mediated via transactivation[109].

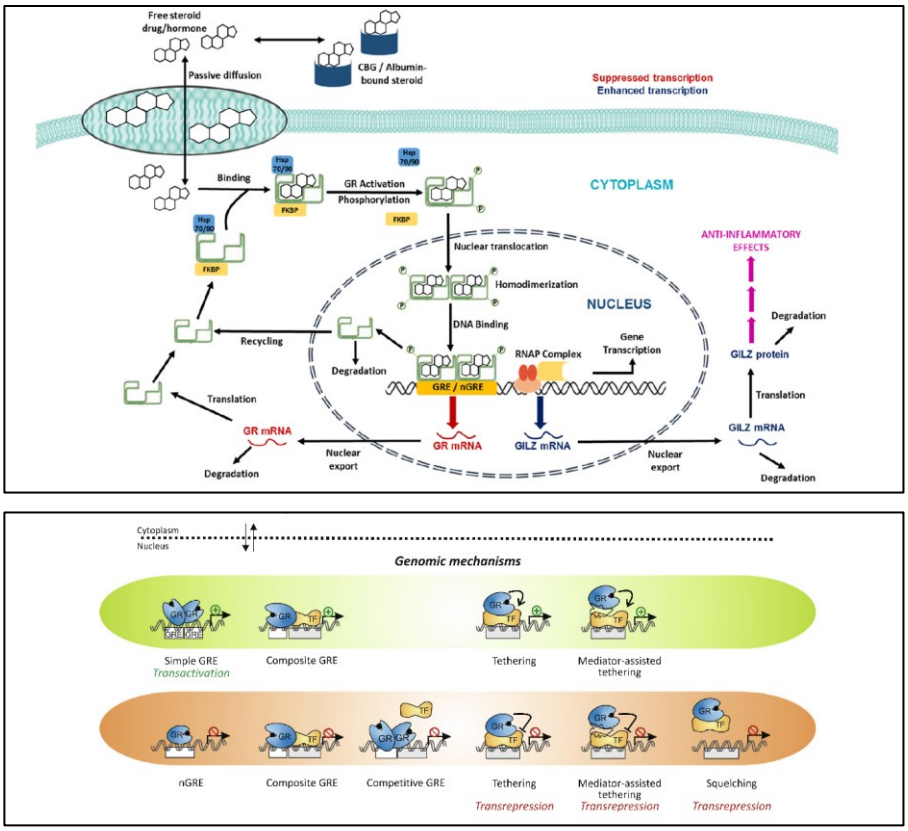

Figure 1-9. (Upper) Molecular and cellular mechanisms of corticosteroid action on regulating gene expression. (Lower) The genomic action of GCs regulates the expression of many genes by transactivation and transrepression[113].

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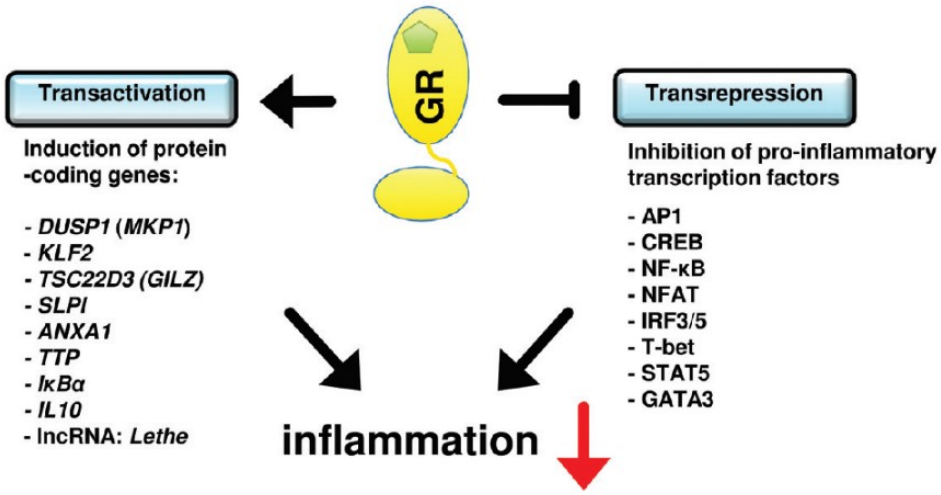

Figure 1-10. The dual molecular anti-inflammatory mechanisms of glucocorticoids: transactivation and transrepression[111].

ANXA1, annexin A1; AP-1, activator protein-1; CREB, cAMP-response-element-binding protein; DUSKP1, Dual-specificity phosphatase;IkB-α, NFAT, Nuclear Factor of Activated T Cell; NF-kB, nuclear factor-kappa B; lncRNA, long non-coding RNA; IL10, interleukin-10; IRF3/5, interferon regulatory factor 3/5; MKP1, mitogen-activated protein kinase phosphatase 1; SLPI, secretory leucocyte peptidase inhibitor; STAT5, signal transducer and activator of transcription 5 ;T-bet, T cell-associated transcription factor; TSC22D3, TSC22 Domain Family Member 3; TTP, Tristetraprolin. Note: Reproduced with permission from Copyright Clearance Center's RightsLink®Walter de Gruyter and Company, Sabine Hübner. et al.(2015) with permission granted on February 2022 by email.

1.1.5.2 Non-genomic action of glucocorticoids

In addition to genomic mechanisms, which usually occur in hours, GC can exert their effects more rapidly (within minutes) through non-genomic mechanisms[114]. The non-genomic actions are mediated by rapid changes to intracellular Ca2+ leading to inhibitory or potentiation of GC effects (Figure 1-11)[115]. These actions utilise the activity of multiple kinases, including phosphoinositide 3-kinase, serine/threonine-specific protein kinase, and mitogen-activated protein kinases (MAKPs)[100]. Non-genomic effects have also been shown to be mediated via membrane GR resulting in phospholipase A2 inhibition[100]. This GR independent effect is mediated through multiple signalling pathways in the cytoplasm and requires no protein synthesis[100].

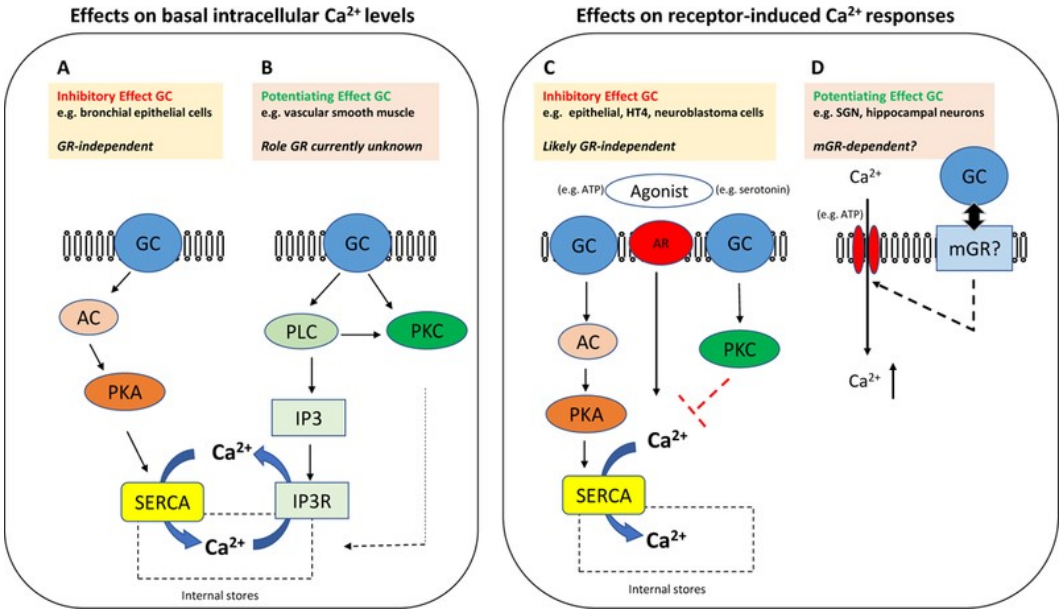

Figure 1-11. Non-genomic effects of GCs in different cell types[100].

AC, adenylyl cyclase; AR, agonist receptor; GC, glucocorticoid; GR, glucocorticoid receptor; IP3, inositol (1,4,5)-triphosphate; IP3R, inositol (1,4,5)-triphosphate receptor; mGR, membrane glucocorticoid receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; SERCA, sarco/endoplasmatic reticulum Ca $^{2+}$-ATPase; SGN, spiral ganglion neuron.Note: Reproduced with permission from Copyright Clearance Center's RightsLink® Elsevier, Reynold A. et al.(2019) with License number 5254270836070, permission granted on February 2022 by email.

1.1.6 Glucocorticoid metabolism

Cortisol has a half-life of 60 to 120 minutes in circulation and is a balance between cortisol production and metabolism[102]. A key enzyme here and a major focus of this thesis are the 11β-HSDs isozymes that mediate the interconversion of active cortisol to inactive cortisone[116]. As described in section 1.1.7, besides being a key step in cortisol clearance, this is also a key pre-receptor regulator of GC action depending upon the specific tissue expression and activity of 11β-HSDs[117]. The liver and kidney are the primary organs involved in GC metabolism and elimination from circulation[117]. Cortisol and cortisone are metabolised similarly in the liver by many enzymatic steps[117, 118]: 1) 5β-reductase enzyme to form 5β-dihydrocortisol and 5β-tetrahydrocortisol (THF) of tetrahydrocortisone (THE), 2) 5α-reductase enzyme to form 5α-dihydrocortisol and 5α-tetrahydrocortisol (allo-THF), 3) 6β-hydroxylase enzyme to form 6β-hydroxycortisol which appears predominantly during fetus and infant, and 4) 20β-oxoreductase enzyme convert cortisol to 20β-dihydrocortisol (Figure 1-12). The metabolites of cortisol and cortisone are excreted as sulfate or glucoronide conjugates via urine: 50% as THF, allo-THF, and THE; 25% as cortols/cortolones; 10% as C_{19} steroids; and 10% as cortolic/cortolonic acids and only 1% is

free form including cortisol, cortisone, 6β- and 20α/20β-metabolites of cortisol and cortisone[119, 120].

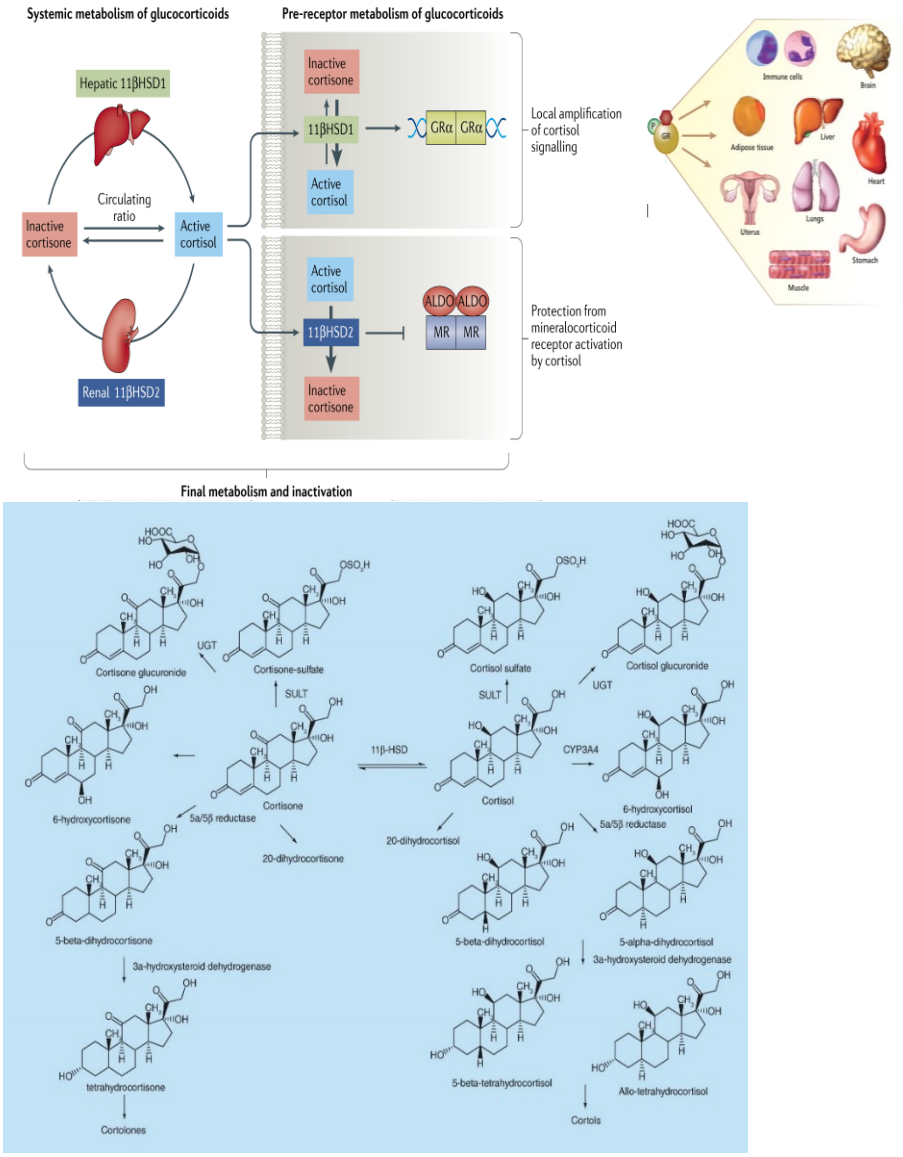

Figure 1-12. Corticosteroid metabolism in systemic circulation and pre-receptor regulation of action by 11β-HSDs.

Cortisol metabolites appear in the urine as 5α-tetrahydrocortisol, 5β-tetrahydrocortisol and tetrahydrocortisone via metabolism by 5α- or 5β-reductase and 3α-hydroxysteroid dehydrogenase. Cortisol can also be metabolised in the liver directly to 6β-hydroxycortisol and 20 β-dihydrocortisol. ALDO, aldosterone; MR, mineralocorticoid receptor; GRα, glucocorticoid receptor α. Reproduced with permission from Copyright Clearance Center's RightsLink®Springer Nature, Rowan S. Hardy et al (2020) with License number 5256390672665. Future Science Ltd., Fujioka, Naomi et al. (2018); permission granted on February 2022 by online and email.

1.1.7 11β-HSDs: tissue-specific modulation of glucocorticoid action

Along with cortisol synthesis by the HPA axis,11β-HSDs regulate the extra-adrenal cortisol production and regulation at the pre-receptor level[121]. They catalyze the conversion of hormonally active cortisol to inactive cortisone, often irrespective of cortisol levels in the blood[97, 116, 122]. In humans, 11β-HSD comprises two isoenzymes, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-HSD2, expressed in a tissue-specific manner and catalyze the interconversion of hormonally active cortisol and inactive cortisone (Figure 1-13, Table 1-4)[123]. The 11β-HSD expressions are strongly linked to the receptors[54]. 11β-HSD1 is abundant in tissue-rich GR, while 11β-HSD2 colocalizes with MR[116]. Though some instances do not exist, such as the placenta and fetal tissues show a high 11β-HSD2 expression in conjunction with GR[124], the hippocampus expresses both MR and GR[125] or the aortic smooth muscle cells express MR without 11β-HSD2[126, 127]. 11β-HSD1 has a low affinity for GC relative to 11β-HSD2. It catalyses both oxoreductase (conversion of cortisone to cortisol) and dehydrogenase activity (conversion of cortisol to cortisone) that depends on the availability of cofactor nicotinamide adenine dinucleotide (NAD) phosphate (NADP+) and reduced NADP (NADPH). In intact cells and tissue, oxoreductase activity predominates and depends on a high ratio of NADPH/NAPD+ concentrations generated by hexose-6-phosphate dehydrogenase (H6PDH or H6PD)[123, 128]. On the contrary,11β-HSD2 is a high-affinity NAD-dependent, largely unidirectional dehydrogenase that converts active cortisol to inactive cortisone and is expressed in mineralocorticoid responsive tissues, such as the distal nephron, colon and salivary glands to protect MR from cortisol action or excessive state or placenta which protect the foetus to exposure to maternal cortisol[116, 119]. 11β-HSD2 is also documented in some malignant tissues[129].

Cortisol, pro-inflammatory cytokines (e.g. IL-1β, IL-4, IL-5, IL-6, IFNY[5], TNFα)[130], and CCAAT/Enhancer binding protein[131] enhance 11β-HSD1 expression and activity[97]. On the other hand, oestradiol[132], growth hormone[133, 134], the liver X receptor agonists[135], and peroxisome proliferator-activated receptor (only in mice[136], not in human[137]) inhibit the expression[138].

11β-HSD1 activity has been linked to physiology and pathogenesis of deleterious effects of GC, e.g. DM[139], metabolic syndrome and central obesity[140-142], osteoarthritis[143], osteoporosis[144], ageing skin[145, 146] and cognitive decline[147, 148]

[5] Interferon ϒ

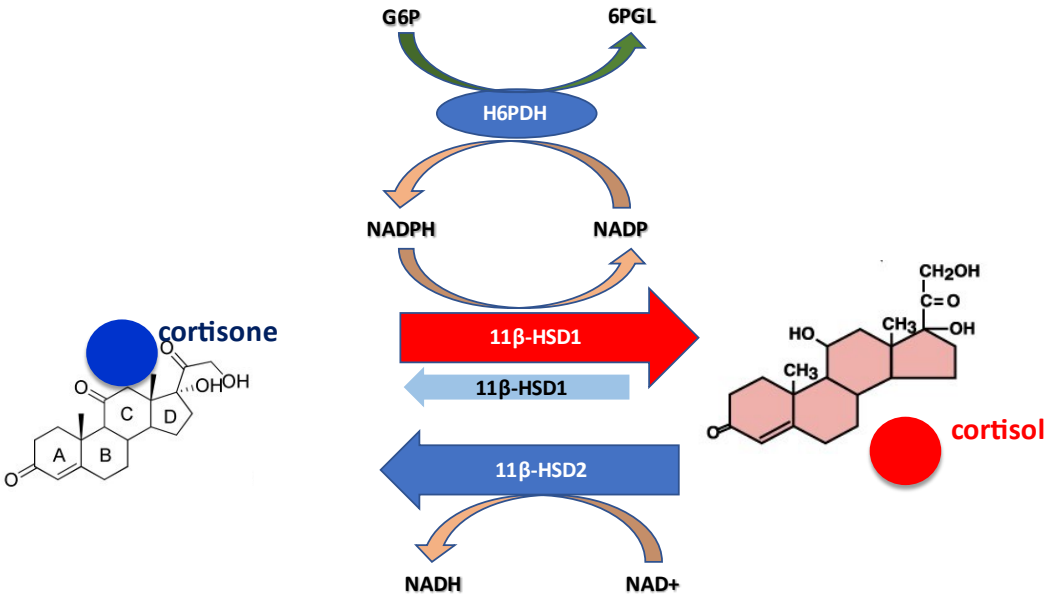

Figure 1-13. 11β-HSDs system.

11β-HSD1 exhibits both oxoreductase (red arrow; cortisone to cortisol) and dehydrogenase activities (blue arrow; cortisol to cortisone) in vitro, but in vivo, it mainly functions as an NADPH oxoreductase. It is co-expressed in the endoplasmic reticulum with hexose-6-phosphate dehydrogenase (H6PDH), which generates NADPH requisite for reductase activity. 11β-HSD2 exhibits mainly dehydrogenase activity (cortisol to cortisone)[149].

Table 1-4. Characteristics of 11β-hydroxysteroid dehydrogenase isozymes [104, 123, 150]

	11β-HSD1		11β-HSD2
Chromosome	1q32.2, 6 exons 30kb in length		16q22, 5 exons 6.2 kb in length
Gene	*HSD11B1*		*HSD11B2*
Protein	11β-HSD1		11β-HSD2
Molecular mass	34 kDa		44 kDa
Tissue expression	**Widespread** Liver Gonad (testis, oocyte, luteinised granulosa cells), Adipose tissue, proximal nephron, anterior pituitary, CNS- PVN, hippocampus, bone, adrenal cortex, GI tract (non-epithelial laminal propria), placenta (chorion, decidua, syncytiotrophoblast), eye (high trabecular meshwork, lens epithelium), vascular smooth muscle skin		**Discrete** Kidney (distal nephron, medulla, cortex), GI tract (parietal cells, sigmoid and rectal colon, surface mucosal epithelial cells), placenta (syncytiotrophoblast, extravillous cytotrophoblast), fetus (most of the tissues except testis), gonad (non-luteinised granulosa cells), lung (airway epithelium and adenocarcinoma), eye (non-pigmented ciliary epithelium), mammary gland, vascular smooth muscle, salivary glands and sweat glands skin
Location	ER, facing lumen		ER, facing cytoplasm
Binding affinity and activity ($K_{m,}$)	Low affinity Corticosterone: 1.83 ± 0.06 μM Cortisone 2-40 μM Cortisol 10-50 μM Prednisone 21 μM		High affinity Cortisol:50 nM (100 times that 11β-HSD1) Corticosterone: 5 nM Dexamethasone: 140 nM
Enzyme kinetics	In vitro bidirctional Invivo mainly reductase In tissue breakdown: dehydrogenase		Only dehydrogenase
	Reduction (predominant)	Oxidation	Oxidation
Substrates	Cortisone, Dehydrocorticosterone, Prednisone	Cortisol, Corticosterone, Prednisolone	Cortisol, Corticosterone, Prednisolone
Cofactor	NADPH	$NADP^+$	NAD^+
Coenzyme	H6PDH		-
Function	Generate active cortisol, facilitate GR response		Protect MR from cortisol

1.2 Therapeutic glucocorticoid use

Edward Kendall discovered compound E (17-Hydroxy-11-Dehydrocorticosterone), known today as cortisone, in the 1940s (Figure 1-14)[151, 152]. Subsequently, in an outstanding early paradigm for experimental-translational medicine, cortisone was used as a breakthrough treatment for patients with rheumatoid arthritis[153, 154]. Philip Hench, Edward Kendall and Tadeus Reichstein received the Nobel Prize for this remarkable advance in 1950[155]. In 1954, prednisone and prednisolone were developed as orally administered synthetic GCs. This was the birth of the use of steroids as anti-inflammatory agents, acting via the GR to repress the inflammatory and immune processes. Shortly afterwards, the adverse effects of cortisone and synthetic GC's were recognised[156]. However, seventy years later, synthetic GC with significant immunomodulatory and anti-inflammatory qualities are now employed to treat a variety of ailments, including inflammatory and immunologic disorders, allergies, cancer, transplantation, in-utero foetal lung maturity, COVID-19, replacement adrenal insufficiency and suppression for adrenal hyperplasia, seventy years later.[157]. Whilst highly effective, GCs also cause major morbidity and possibly increased mortality in their own right.

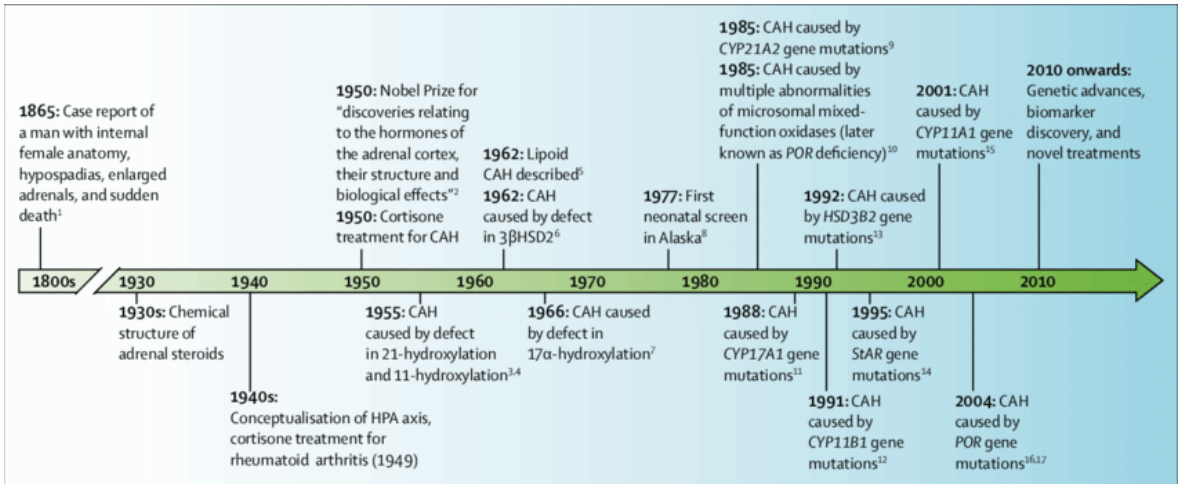

Figure 1-14. Timeline of GC discovery and development.

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1.2.1 Pharmacokinetics and pharmacodynamics of systemic glucocorticoids

Orally administered GCs are readily absorbed, with a bioavailability of 60-100% and a peak in serum within 2 hours. Inactive drugs (prednisone, cortisone) require 11-hydroxylation by 11β-HSD1 in the liver to convert to the active form (prednisolone, cortisol)[158]. GC action's duration depends on the biological half-life, which is classically assessed by ACTH suppression after administering a single dose of reference GC, not plasma half-life[47].

The duration of GC action has been categorised into short (8-12 hours), intermediate (24-36 hours) and long-acting (more than 36-48 hours)[159]. The actual duration of the effect or biological effect is longer than the duration of action because of the subsequent intracellular and nuclear actions[47]. Despite differences in names and chemical properties, synthetic GC's have similar biological effects, especially as they relate to anti-inflammatory and immune-modulatory effects (Figure 1-15, Table 1-5)[160, 161]. Combined genomic and non-genomic effects lead to different outcomes (Figure 1-16)[99]. The genomic effects exert their functions from a low dose (≤ 5 to 7.5 mg/d of prednisolone equivalent dose) where GR saturation is up to 50%; at a moderate dose (>7.5 to 30 mg/d), the GR becomes increasing saturated at 50 to 100% mediating transrepression and transactivation[162, 163]. Most of GR are fully occupied at approximately 30-40 mg of prednisolone dose and equivalent. When the dose reaches a higher level than the saturated dose (up to ≥ 100 mg/d or equivalent), anti-inflammatory effects are constant, but the transactivation actions increase susceptibility to adverse effects[162, 164, 165]. The non-genomic effects occur immediately at a high dose starting from 30 to 100 mg/d[162].

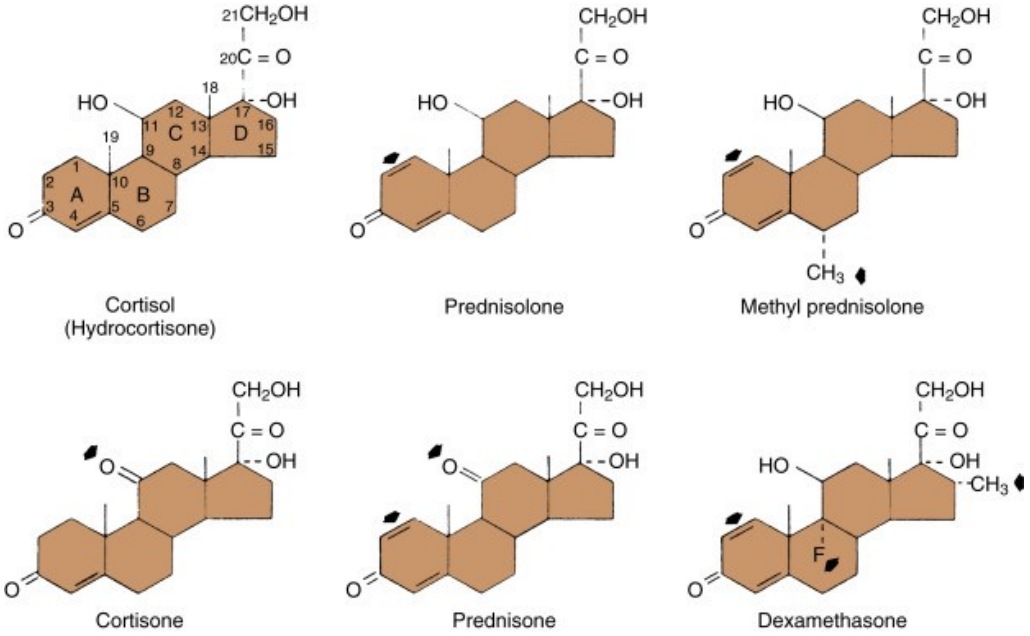

Figure 1-15. Structure of natural and synthetic glucocorticoids.

Table 1-5: Potency of glucocorticoid and mineralocorticoid activity for natural and synthetic corticosteroids[6] [160, 161, 166]

Oral	GC potency[7]	MC potency	Equivalent doses[8] (mg)	Plasma half-life (min)	Duration of action (h)
Short-acting					
Cortisone/ compound E	0.8	0.8	25	60	8–12
Cortisol/ compound F/ hydrocortisone	1.0	1	20	60	8–12
Intermediate-acting					
Prednisone	4	0.3	5	60	12-36
Prednisolone	4	0.3	5	200	12-36
Methylprednisolone [9]	5	0	4	180	12-36
Long-acting					
Triamcinolone	5	0.5	4	300	24-36
Dexamethasone	30	0	0.75	200	36–72
Betamethasone	30	0	0.6	200	36–72
Mineralocorticoids					
Fludrocortisone	10	250	0		24-36
Inhaled corticosteroid	**Receptor GR binding affinity relative to dexamethasone**	**Lung delivery (%)[10]**	**Oral Bioavailability (%)**	**Systemic Clearance (l/h)**	**Half-life (h)**
Beclomethasone (BDP/BMP) MDI	0.4/ 13.5	50-60	20/40	150/120	Unknown /2.7
Budesonide DPI	9.4	15-30	11	84	1.5–2.8
Ciclesonide MDI	0.12	50	<1	152	0.7–7
Flunisolide propionate DPI	1.8	68	20	58	1.6
Fluticasone MDI	18	20	≤ 1	66	3.1–14
Mometasone furoate DPI	23	11	<1	53	Unknown
Triamcinolone Acetonide MDI	3.6	22	23	45	3.6

[6] BDP, beclomethasone dipropionate; BMP, beclomethasone 17-monopropionate; DPI, dry-powder inhaler; h, hour; min, minute; GR, glucocorticoid receptor; GC, glucocorticoid; MC. Mineralocorticoid; MDI, metered-dose inhaler ; NA, not applicable

[7],[8]Equivalent to hydrocortisone

[9] intravenous form

[10] Therapeutic effects enhanced by decreased oral absorption, retention in the lung, and rapid systemic clearance

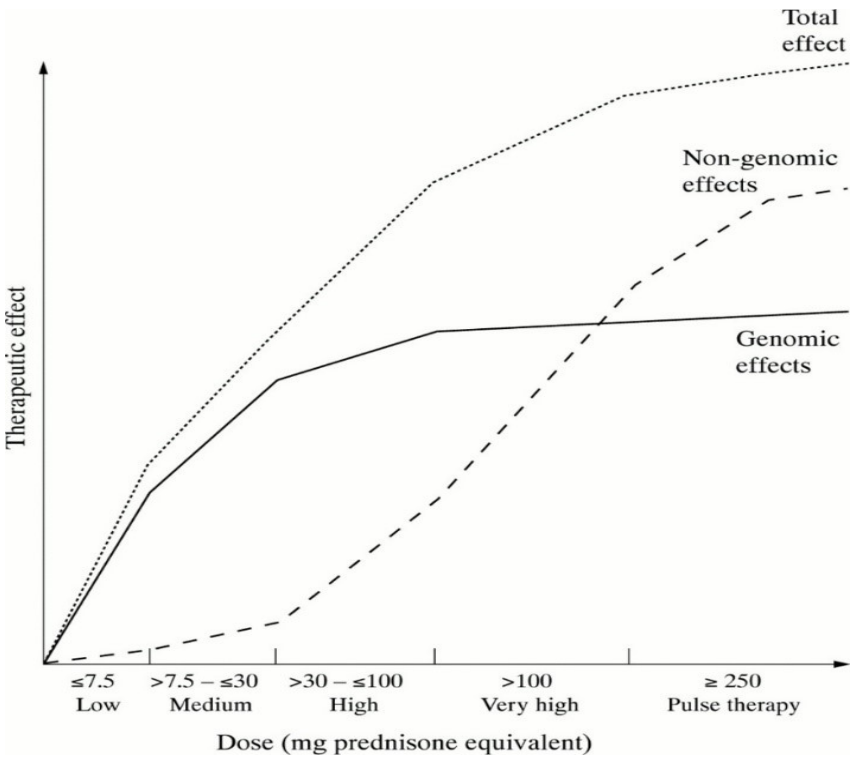

Figure 1-16. Standardised GC dosages, clinical applications, and the relationship with genomic and non-genomic actions [162, 163].

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1.2.2 Key physiology and pharmacology of glucocorticoid on inflammation, skin and metabolism

GCs maintain physiological homeostasis through a diverse array of actions maintaining metabolism, water and electrolyte balance, inflammatory and immune response, growth and development, visual system, cardiovascular function, mood and cognitive functions, reproduction and effects on the musculoskeletal systems[167]. All of the above are affected by every step mentioned above; GC synthesis that changes around a circadian and ultradian rhythm, 11β-HSD systems which control local cortisol and cortisone concentrations, the expression of GR and MR with their complexes to generate distinct transcriptional pathways and post-receptor translation processes to exert the various functions in tissues[59].

1.2.2.1 Cellular targets of glucocorticoid signalling

A genome-wide study for GC-responsive genes found expression distinctively in different cell types after endogenous or pharmacologic GC exposure (Figure 1-17). However, it shares similar protein kinase-driven signalling cascades and cytokine receptor signalling pathways[168], resulting in GC roles in health and diseases. The findings help explain mechanisms for GC action, complications and GC for personalised medicine[48].

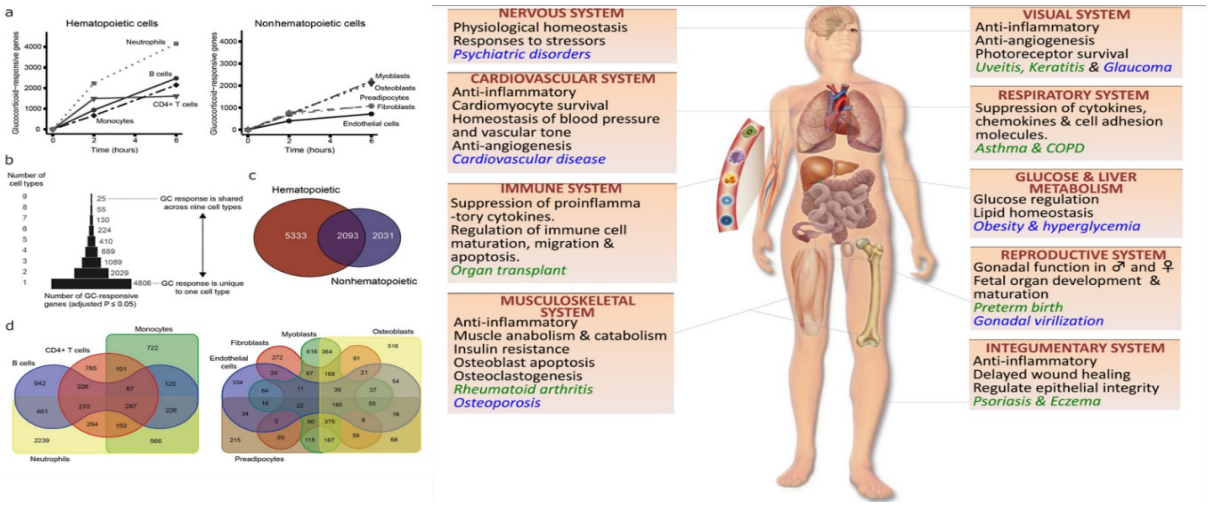

Figure 1-17. Left: The transcriptional response to glucocorticoid (GC) in different cell types

(a) Line plots of the number of glucocorticoid-responsive genes (GCRG) over time in each cell type. (b) Pyramid plots demonstrated GCRG at one or two time points (total 9,457 genes). The panel highlights just 25 GCRG shared across nine cell types. (c) Venn diagram demonstrating GCRG at one or two time points in haematologic and non-haematologic cells (total 9,457 genes) and d) in sub-type cells (d). Right: Roles of GC: physiological roles (black text), therapeutic roles (green text) and adverse outcomes of GC (blue text)[99, 168]. Reproduced with permission from 1. Copyright Clearance Center's RightsLink® Elsevier, Mahita Kadmiel et al.(2013) with License number 5256530533217, 2. Copyright Clearance Center's RightsLink® Rockefeller University Press, Franco, Luis M et al.(2019); permission granted on February 2022 by email.

1.2.2.2 Immunomodulatory and Inflammatory regulation

The inflammation progresses through multiple steps and increases inflammatory genes expression and proteins, mainly via nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)[169, 170]. Acute response to inflammation requires the recruitment of immune cells and vascular supplies[171]. Both natural and synthetic GC overcome inflammation through a complex interplay between GR-mediated transcriptional regulation and signal transduction within target tissues[167, 170, 172]. The evidence shows that GC act as both proinflammatory and anti-inflammatory effects; at a low dose of cortisol, the

immunostimulatory effects were observed, whilst anti-inflammatory effects occurred at the higher or therapeutic doses[173].

The physiological role of GC serves as proinflammatory agents to prepare the immune system for a quick response to stimuli or pathogens. GC mediates anti-inflammation during acute inflammation through GR, which physically interacts with NF-κB and AP-1 via transrepression mechanism (Figure 1-18)[174]. GC suppress COX-2, iNOS, and proinflammatory cytokines (IL-1β, IL-6, IL-12, IL-17, TNF, GM-CSF), NF-κB and intercellular adhesion molecule critical players in the inflammatory cascade[175]. It also inhibits pro-inflammatory macrophages, eosinophils, lymphocytes, mast cells, and dendritic cells, including phospholipase A2 (Figure 1-19)[47]. Moreover, GC inhibits the expression of adhesion molecules of endothelial cells, i.e. E-selectin, vascular endothelial growth factor (VEGF) and vascular cell adhesion molecule[167]. Furthermore, GC mediates the transactivation of anti-inflammatory genes and proteins, including IL-10 annexin-1 protein, which inhibits prostaglandin and leukotriene synthesis, lipocortin-1, glucocorticoid-induced leucine zipper; and reduces neutrophil migration to inflammatory sites[175]. Apart from the genomic mechanism, non-genomic effects explain some rapid actions post pharmacological treatment[176]. GC stimulates monocytes and macrophages at the final inflammatory state to resolve inflammation, cellular clearance and restore homeostasis[177].

The body responds to inflammation, not only by adrenal steroidogenesis, which drives systemic GC action, but local endogenous also regulates inflammatory signals, of which TNF and IL-1β induce the 11β-HSD1 expression[178]. The endogenous GC and synthetic GC exert the same effects on immune and inflammation. However, fewer potent mineralocorticoid effects, higher potency of anti-inflammation than endogenous cortisol due to longer half-life, improve absorption with the parenteral route, and reduced binding to CBG, thereby diffusing more rapidly cells[47, 179-181]. The anti-inflammation also depends on the cell type, disease, dose and timing of application[182].

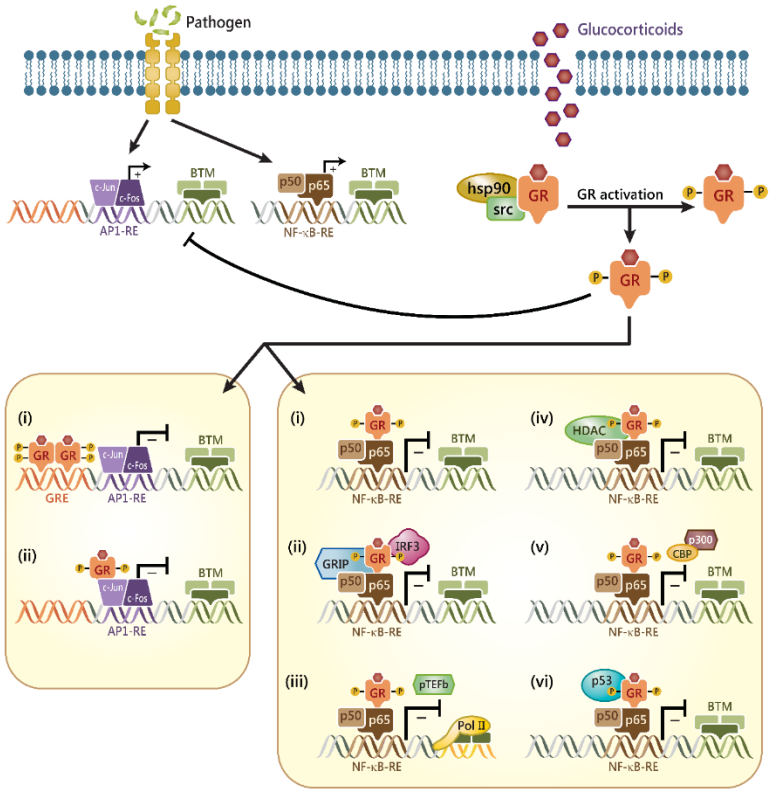

Figure 1-18. Transrepression mechanism of glucocorticoid exerts anti-inflammation by suppressing the AP-1 and NF-κB expression[173].

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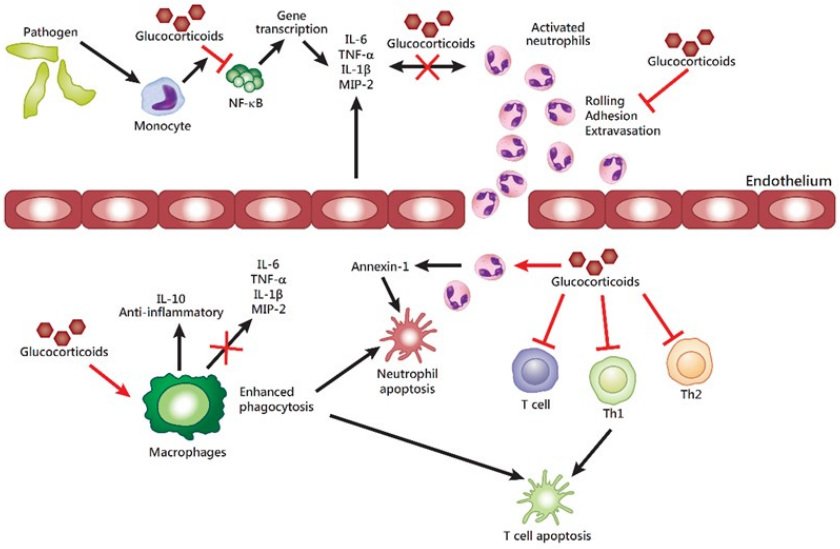

Figure 1-19: Anti-inflammatory effects of GC[173]

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1.2.2.3 Metabolic effects

GC is the essential catabolic hormone in supplying adequate energy to critical organs during stress. GC regulate glucose homeostasis by increasing hepatic gluconeogenesis and glycogenolysis; enhancing the effect of glucagon and epinephrine; inhibiting peripheral glucose utilisation; decreasing uptake of carbohydrates and glycogenesis; and acting as counterregulatory to insulin[183]. B-cells are inhibited and α-cells are increased by cortisol for insulin and glucagon release, respectively[183]. Furthermore, GC increases protein breakdown from muscle enhance lipolysis and appetite to mobilise peripheral substrates for gluconeogenesis[184].

1.2.2.4 Skin homeostasis

GC regulates skin homeostasis notably through inflammatory and metabolic processes, proliferation and differentiation[185]. Local receptor expression together with 11βHSDs help maintain the equilibrium of active GC[186]. Lipogenesis, cellular adhesion, apoptosis, formation of stratum corneum, differentiation of keratinocytes ensuring an intact skin barrier function are all critical functions of GCs[31]. The effects of GC on the normal wound healing (WH) process, together with 11β-HSD1 expression and activity, are discussed in greater detail in Chapter 5.

1.2.3 Therapeutic use of glucocorticoid

Data of nationwide long-term GC use has been reported from some countries, including the United Kingdom (UK), Denmark, France, the United States (US) and Sweden, and ranges from 0.9-3.0% of the population[187-190]. Therapeutic use of GCs can be categorised as "physiological" replacement therapy or suppressive "supra-physiological" therapy for immunomodulatory and anti-inflammatory purposes across both acute and chronic conditions and used to promote fetal lung maturation. The factors that affect GC efficacy and toxicity include[118]; (1) pharmacokinetics and pharmacodynamics of GC[191], (2) GC absorption: drugs containing aluminium or magnesium decrease GC absorption by 40%, (3) GC clearance with, for example, 33% increased clearance in children aged < 12-year-old[192], (4) Hepatic metabolism including CYP system, mainly the CYP3A4 subfamily, (5) Tissue-specific regulation of GC via 11β-HSD1 with increased expression increasing local cortisol levels, (6) 5α/5β-reductases, enzymes for cortisol clearance modulate differences in cortisol availability in different hepatic diseases[193], e.g. non-alcoholic fatty liver disease[193], (7) CBG which is altered by oestrogen and in thyroid disease, nephrotic syndrome or haemodialysis patients[194], (8) GR activation[195], and (9) Timing and pattern of GC delivery and prescription as shown in Table 1-5. Replacement therapy is used to treat

patients with adrenal insufficiency and aims to mimic endogenous GC release in response to normal or stress conditions[196]. The therapeutic goals of GC are primarily to decrease inflammatory and immunoregulatory pathways in order to control a variety of diseases on an acute or long-term basis. The ideal synthetic GC would have the highest efficacy, the lowest mineralocorticoid activity, and the fewest side effects[196]. To minimize adverse effects, novel GC agents, selective GR agonists, and modulators have been created[195, 197]. The limitation of therapeutic GC use is GC resistance[198] and adverse effects[199]. GC resistance is the inadequate disease response, usually due to acquired causes, and in the minority due to mutation of the GR-encoding genes[71]. Adverse effects are discussed in sections 1.3 and 1.4. Human and in vitro studies have addressed these questions with genomics, transcriptomics, and other omics approaches in recent years[200].

1.3 Dysregulation of GC

This thesis will be to focus on the harmful complications of GC excess states. Prolonged and inappropriate GC action from either an endogenous or exogenous cause can compromise target organ responses and result in CS[201]. GC resistance arises concurrently with GC adverse effects, gives critical information about the molecular processes behind these medicines[202] (Table 1-6). In the short term, GC can induce hyperglycaemia, salt and water retention with HT and oedema, gastrointestinal bleeding, acute psychosis, susceptibility to infection, hypokalaemia, venous thromboembolism (VTE) and HPA axis disturbance[191]. Long-term GC use presents various symptoms and signs and systemic complications termed CS, described further in section 1.4.

Table 1-6. Clinical manifestation of glucocorticoid hypersensitivity/ excess or resistance/deficiency

Organ	GC excess or hypersensitivity	GC deficiency or resistance
Central nervous system	Insomnia, anxiety, depression, defective cognition	Fatigue, somnolence, malaise, defective cognition
Liver	Gluconeogenesis, lipogenesis	Hypoglycemia
Fat	Accumulation of visceral fat (metabolic syndrome)	Loss of weight
Cardiovascular	Hypertension	Hypotension
Bone	Stunted growth, osteoporosis	Steroid withdrawal arthropathy
Inflammatory and immunity	Immune suppression, anti-inflammation, vulnerability to certain infections and tumours	Inflammation, autoimmunity

1.4 Cushing's syndrome

1.4.1 Definition, classification, and epidemiology

CS is a disease caused by prolonged and excessive exposure of tissues to GCs[1]. The aetiologies are due either to endogenous (where cortisol is the "offending" GC) or exogenous sources[203-206]. Exogenous CS is the most common cause of CS, with an estimated 1-3% prevalence[187-190] from anti-inflammatory or immunosuppressive use of GC in chronic conditions such as asthma, autoimmune, inflammatory, and neoplastic diseases. Prolonged exposure via any route such as oral, injection, inhalation, or topical application with supraphysiologic doses can cause CS. Oral therapy is the most common route for exogenous CS. The incidence of endogenous CS is much lower, estimated to be 0.7-4.5 cases per million per year in different populations[187-190], with a prevalence of 40-80 cases per million population [207-209]. Affected patients can be of any age, but the usual diagnosis is between 20 and 50 years[210]. Female sex is predominant compared to men with an F: M ratio of 3-15:1[211]. Despite the rare incidence of endogenous CS, timely diagnosis and management difficulties represent a significant challenge for the practising clinician/ endocrinologist, particularly in reversing long-term morbidities and excess mortality [212].

The pathogenesis of endogenous CS is classified as either ACTH-dependent or ACTH-independent. 70-80% of CS are ACTH-dependent; of these, 75-80% originated from pituitary adenomas or so-called "Cushing's disease" (CD), 10-15% are caused by ectopic ACTH production from any neuroendocrine tumours, and less than 1% caused by CRH-producing adenomas[211]. Conversely, ACTH-independent CS or adrenal CS (ACS) are caused by unilateral adrenal lesions in 90% of cases and bilateral adrenal tumours in the remainder of cases[213]. Benign adenomas account for 80% of unilateral cases, with 20% of patients being adrenocortical carcinomas[214].

1.4.2 Clinical features

CS is characterised by a variety of signs and symptoms based on the duration of exposure, severity / "dose" of exposure, and tissue susceptibility to GC. Features include a rounded and plethoric face, rapid weight gain, truncal obesity, thin skin, purplish striae, easy bruising, delayed WH, fatigue, muscle weakness, secondary amenorrhea, hirsutism, and HT [205]. Truncal obesity is the most common presenting feature of endogenous CS[211]. All of these manifestations are often full-borne forms, whereas clinical presentation of the mild form of CS is broad, and diagnosis is challenging, especially in individuals with underlying obesity, diabetes, depression, secondary osteoporosis and HT[210]. Delay in diagnosis may

lead to multisystem involvement and long-term complications. High morbidity and mortality from chronic GC exposure are therefore not uncommon even in people with mild CS. The clinical features of CS are shown in Table 1-7.

Table 1-7. Clinical characteristics of Cushing's syndrome [1, 204, 215]

Clinical features	Frequency (%)
Dermatologic changes	
Skin changes (round face, facial plethora, and skin atrophy)	80–90
Hirsutism	70–75
Violaceous striae	55–65
Easy bruising	45–65
Gonadal dysfunction	
Decreased libido	25–90
Menstrual irregularity	75–80
Bone and musculoskeletal	
Muscle proximal weakness	60–80
Osteoporosis	40–75
Avascular necrosis in femoral head	5–10
Metabolic changes	
Increased weight (centripetal obesity, supraclavicular region, and upper back)	95-100%
Hypertension	70-85
Obesity	40–95
Dyslipidemia	40–70
Glucose intolerance or DM	50–80
Neuropsychological disorders	
Psychiatric symptoms	50–70
Immunocompromised host	
Increased infections and decreased wound healing	15–30
Kidney	
Renal calculi	15–20
Cardiovascular diseases	
Venous thromboembolism	10–20
Specific for Cushing's disease	
Headaches	0–37
Visual problems (bitemporal hemianopsia)	0–33
Other anterior pituitary hormone deficiencies	0–25
Alterations with severe hypercortisolism	
Weight reduction (with ectopic ACTH secretion by malignancy)	10–50
Hypoalbuminemia	15–35
Skin hyperpigmentation	10–15
Hypokalemia and metabolic alkalosis	4–10

1.4.3 Morbidity and mortality in Cushing's syndrome

Prolonged exposure to hypercortisolism results in a broad range of deleterious effects [47]. The long-term complications associated with CS include cerebrovascular and cardiovascular diseases[216, 217], uncontrolled DM, osteoporosis, psychiatric complications, hypercoagulable states[218] and infections. These conditions may persist for several months and years despite remission of endogenous CS. Overall, these morbidities are manifested as increased mortality reported for CS. The plethora of CS-induced morbidities is demonstrated below (Figure 1-20).

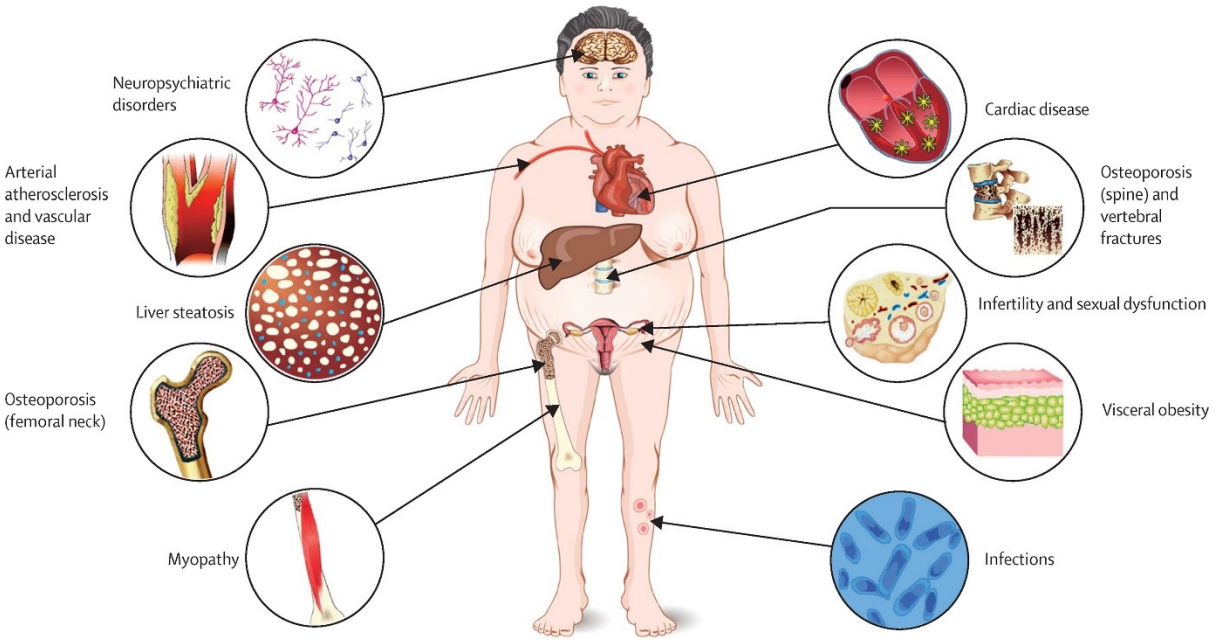

Figure 1-20. Overview of glucocorticoid-associated side effects.

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1.4.3.1 Mortality associated with CS

Most studies of mortality associated with endogenous and exogenous CS have reported increased estimates[219-227]. The standardised mortality ratio (SMR) for endogenous CS ranged between 2.2 to 4.8[219, 220, 225, 226, 228]. Despite remission, the overall SMR for all-cause mortality was 1.61 (95%CI 1.23-2.12), with long-lasting metabolic and vascular pathology being the main contributing factors leading to CV events and mortality[229]. However, there are no published systematic reviews that assess overall mortality and specific causes of death across all types of CS. Addressing this gap in knowledge base was a major aim of this thesis.

1.4.3.2 Morbidity and adverse effects

1.4.3.2.1 *Metabolic disorders*

Metabolic manifestations of CS include increased total adipose fat and visceral fat, decreased total subcutaneous fat, changes in adipokine secretory pattern (elevated leptin, resistin, TNF-α and IL-6, reduced adiponectin), insulin resistance spectrum from pre-diabetes to overt DM, sleep apnea syndrome, dyslipidaemia, and hepatic steatosis (Figure 1-21) [230]. The meta-analysis reported GC-induced hyperglycaemia or new-onset DM in 32.3% and 18.6% cases, respectively [231]. The incidence of GC induced DM in users of GC's in the UK was 12.2 per 1000 person-years (95% CI 11.9,12.4) and was strongly correlated to increased dose [232]. The independent risks also related to continuous exposure (odds ratio (OR) 2.0, 95%CI 1.29, 3.1), older age (OR 1.40, 95%CI 1.06, 1.84) and body mass index (OR 1.87, 95% CI 1.03, 3.38 [233]). The specific high-risk groups included those with a history of gestational DM, a family history of DM, concomitant treatment with mycophenolate mofetil and abnormal fasting glucose or glucose intolerance [234].

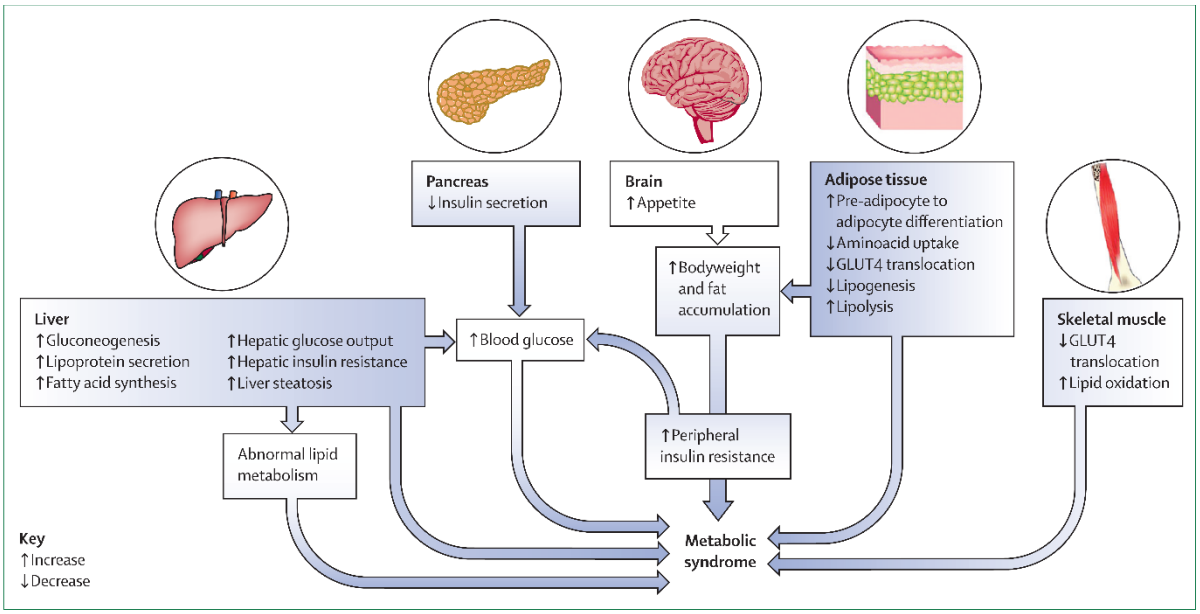

Figure 1-21. The pathogenesis of GC related metabolic side effects and clinical consequences.

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1.4.3.2.2 Cardiovascular diseases

Cardiovascular disease is the commonest cause of reported death in CS[226]. The pathogenesis involves hypercoagulable states[235], insulin resistance[236, 237], dyslipidaemia[238], HT[239, 240], sympathovagal imbalances, arterial stiffness[241], endothelial dysfunction with an increase of endothelin[242, 243], homocysteine, vascular endothelial growth factor, cell adhesion molecules, IL-8 and osteoprotegerin, hypokalaemia and hypomagnesemia[244, 245]. Those led to complications; systemic arterial HT, atherosclerotic vascular diseases (hazard ratio (HR) 2.1, 95% CI 0.5, 8.6)[227], stroke (HR 4.5, 95% CI 1.8, 11.1), arrhythmia [246] and cardiac dysfunction (HR 6.0, 95% CI 2.1, 17.1) (Figure 1-22)[227, 244]. Danish population studies demonstrated HR in endogenous CS of 3.7 (95% CI 2.4, 5.5) for myocardial infarction and 2.0 (95% CI 1.3, 3.2) for stroke [227]. ACS had higher HR compared to normal age- and sex-matched controls with ratios for coronary artery disease and stroke, of 17.5 (95%CI 11.8, 26.0) and 14.4 (95% CI 8.9, 23.1), respectively[247]. A 2- to 4-fold increased risk of cardiovascular disease in patients using 7.5 mg or more of prednisolone[248-251]. Recent Clinical Practice Research Datalink (CPRD) UK reported the incidence of all-cause cardiovascular disease (CVD) to be 24.8 per 1,000 person-years (95% CI 24.4, 25.2) with correlation to higher dose of GC[252]. In subjects using <5.0-mg daily dose, HRs for type-specific CVDs were 1.69 (95% CI 1.54, 1.85) for atrial fibrillation, 1.75 (95% CI 1.56, 1.97) for heart failure, 1.76 (95% CI 1.51, 2.05) for acute myocardial infarction, 1.78 (95% CI 1.53, 2.07) for peripheral arterial disease, 1.32 (95% CI 1.15, 1.50) for cerebrovascular disease, and 1.93 (95% CI 1.47, 2.53) for abdominal aortic aneurysm[252].

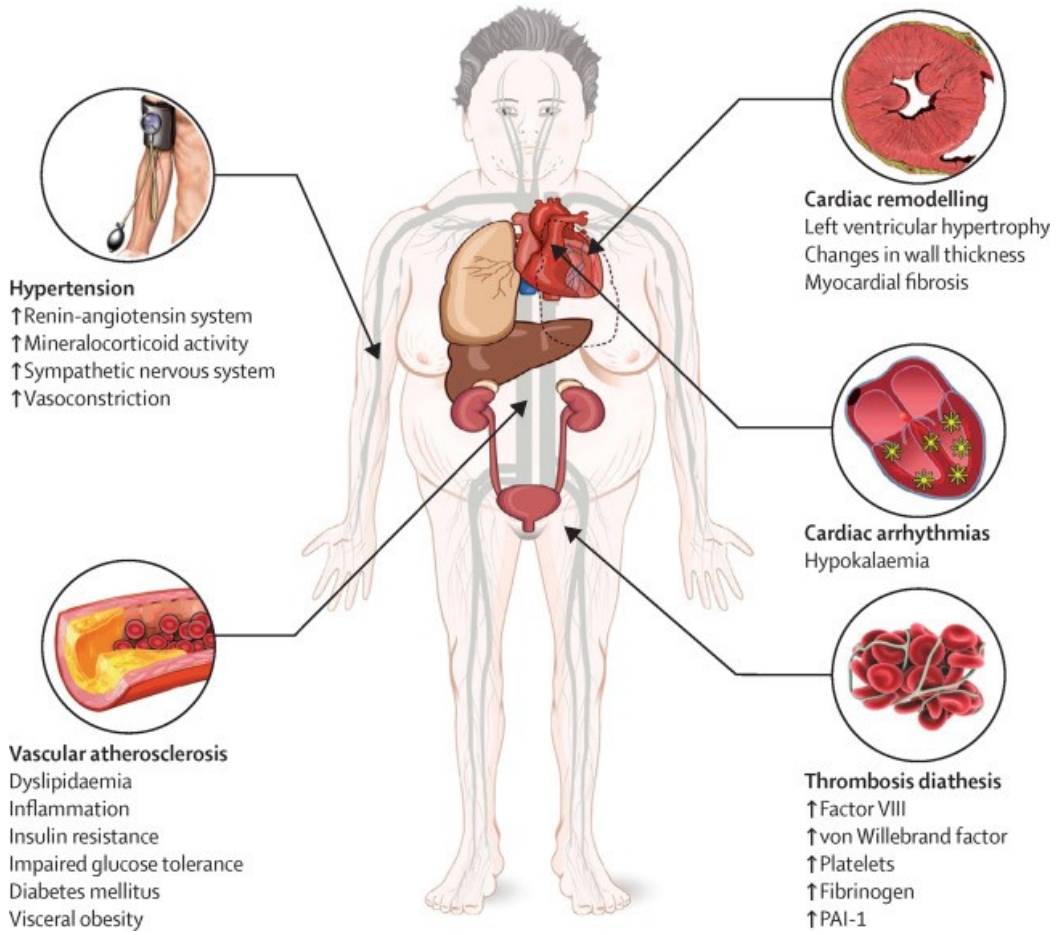

Figure 1-22. Mechanism of increased cardiovascular risk mediated by hypercortisolism[244].

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1.4.3.2.3 Hypertension

The pathogenesis of arterial HT is multi-factorial and is mediated by cortisol inappropriately activating MR following saturation of 11β-HSD2 (endogenous only) or occupancy of GR[253]. Enhanced vascular tone through direct actions on nitric oxide, catecholamines, atrial natriuretic peptide, increased oxidative stress, activation renin-angiotensin-aldosterone system, increased plasma volume and cardiac output, and cardiac hypercontractility are key[245]. Here there are subtle differences between endogenous CS (prevalence of HT 30-82%)[222, 225, 227, 254], and chronic exogenous GC use (prevalence rates <20%)[255]. The incidence of HT in chronic GC use from the CPRD during 1992-2019 was 87.6 per 1000 person-year (95%CI 83.0, 92.4) in subjects taking GC dose ≥ 7.5 mg/d[256].

1.4.3.2.4 Venous thromboembolism

Hypercortisolism increases VTE incidence, pulmonary embolism, and cerebral venous sinus thrombosis[257] in both endogenous and exogenous CS[258, 259]. The activation of coagulation factors, including factors VIII, IX, XI, and von Willebrand factor, increased fast-activating plasminogen activator inhibitor 1, impairing the fibrinolytic system, enhancing oxidative stress with platelet activation underpinned the mechanism of VTE[260-268]. For endogenous CS, the OR of spontaneous VTE in CS compared to the normal population was 7.82 (95%CI 15.24-20.85)[266]. Exogenous GC use increased the risk of VTE 3-fold[269] (adjusted incident rate ratio (IRR) 2.31; 95%CI 2.18-2.45) [218] in current users of oral GC compared with nonusers. The risk associated with new users, especially over the first three months (adjusted IRR 3.06; 95%CI 2.77-3.38), was higher than for continuing use (adjusted IRR 2.02; 95%CI 1.88-2.17) compared to former use patients (adjusted IRR 0.94; 95%CI 0.90-0.99). The risk was also higher for cumulative GC dose over 1g (adjusted IRR ranged 1.6 to 1.98) compared to cumulative dose less than 1g (adjusted IRR 1.00; 95%CI 0.93-1.07)[218].

1.4.3.2.5 Immunoregulatory defects and infection

GC are highly effective in suppressing inflammation, but in the longer term this too can have deleterious effects[173]. One mechanism might increase GR-β expression in inflammatory cells following long-term GC use, leading to ineffective GC treatment and aggravating toxicity[270]. GC excess directly impacts the innate and adaptive immune response and indirectly through vascular damage and hyperglycemia, leading to increased susceptibility to infection (Figure 1-23)[212]. Recent CPRD data reported that infection occurred in 55.7% of patients taking GC for a median of 4.8 years, of which 26.7% required hospitalisation and 7.3% died within 7 days, and 8.7% died within 30 days. The most common sites of infection were lower respiratory tract infections (27.3%), conjunctivitis (18.6%) and herpes zoster (7.4%). Causes of infection related to mortality were pneumonia (52.6%), urinary tract infection (3.0%) and peritonitis (2.2%)[271].

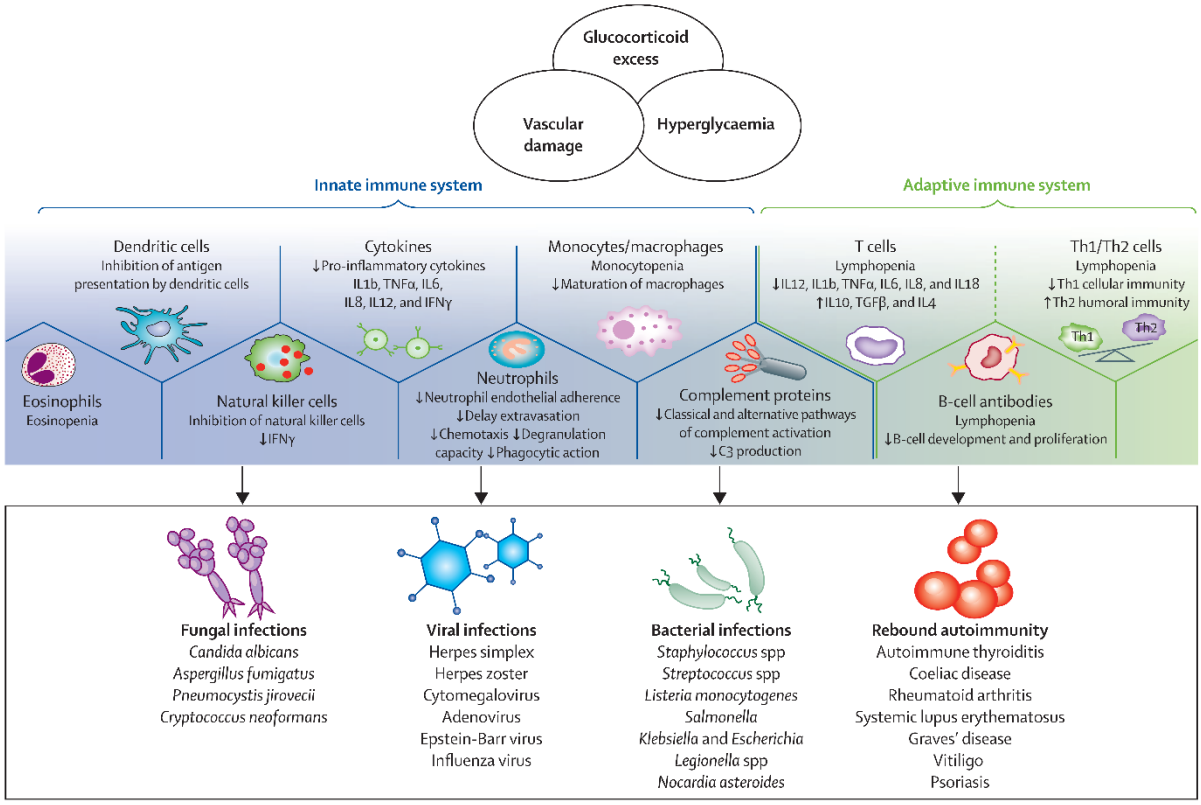

Figure 1-23. Immune dysregulation and infectious susceptibility due to Cushing's syndrome[212].

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1.4.3.2.6 Musculoskeletal system

Hypercortisolism inhibits osteoblast maturation by blocking Wnt/β-catenin, related nuclear factors, type 1 collagen synthesis, increasing apoptosis of osteoblasts and osteocytes and increased osteoclast activities. Suppression of bone formation is a rapid effect mediated by suppression of osteocalcin secretion[272]. GC also indirectly affects bone quality by increasing urinary calcium excretion and GC-induced secondary hypogonadism[273]. GC also induce myopathy and can cause avascular necrosis (Figure 1-24)[212]. Endogenous CS and bone disorder had been reported for osteopenia for 40-78%, osteoporosis for 22-57% and fractures for 11-76%. For exogenous GC, so-called GC-induced osteoporosis is the most common cause of secondary osteoporosis, with a prevalence of 0.5 to 1.0%. It results in susceptibility to fracture with a risk ratio at any site of approximately 1.6-1.75, 3-fold higher for vertebral fracture than hip fracture[274-277]. The fracture risk is strongly related to cumulative GC dose is greater than 1 gram or if the daily dose is higher than 15 mg[278].

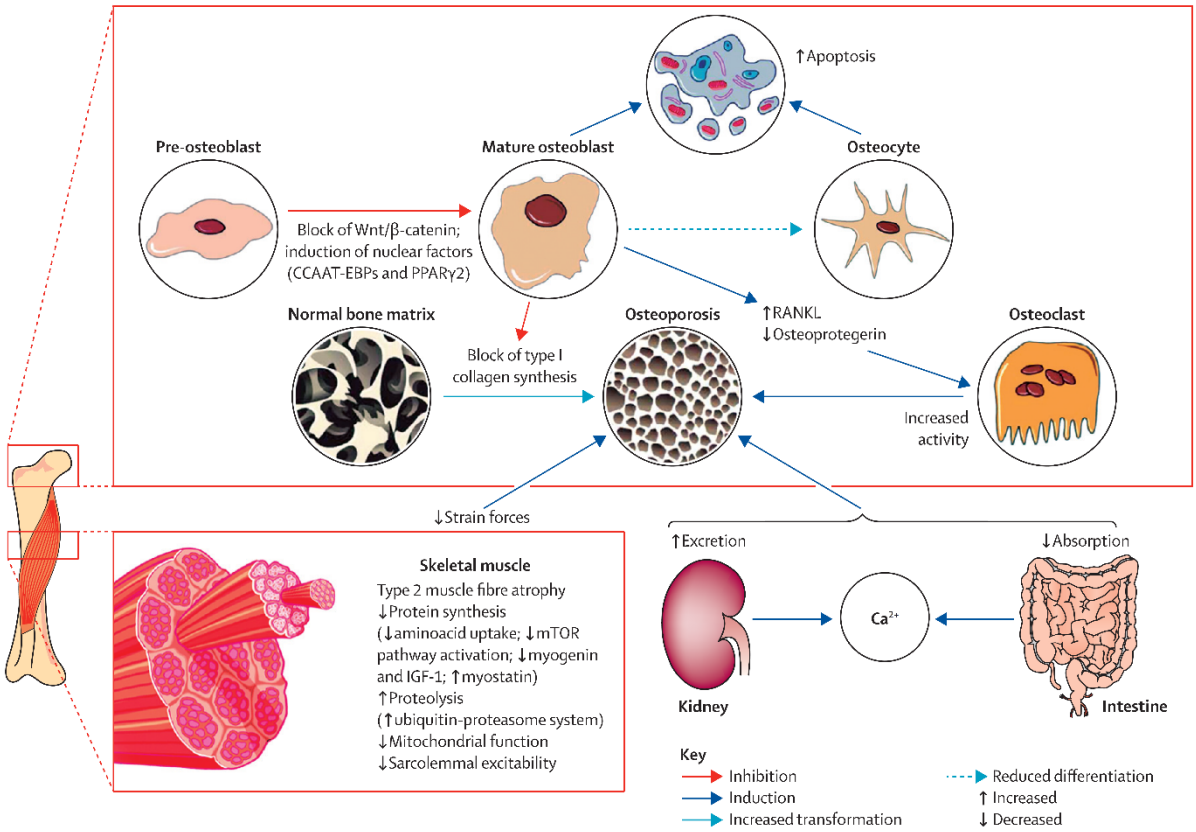


Figure 1-24. Pathogenesis of GC induced musculoskeletal dysfunctions.

Ca2+,calcium; CCAAT-EBP, enhancer-binding protein family; IGF-1, insulin-like growth factor 1; mTOR, mechanistic target of rapamycin; PPARγ2, peroxisome proliferator-activated receptor γ type 2; RANKL, receptor activator of nuclear factor kappa-B ligand [212]. Reproduced with permission from Copyright Clearance Center's RightsLink® The Lancet Diabetes & Endocrinology, Rosario Pivonello et al.(2016) with License number 5261470962822, permission granted on March, 2022 by email.

1.4.3.2.7 Neuropsychiatric side effects

Chronic GC exposure leads to structural and functional brain disorders and impairs quality of life [279-281]. The structural changes were reported as 90% cerebral and 74% cerebellar atrophy[282], decreased hippocampus volume, white matter abnormalities, and neurochemical alteration[283, 284]. Patients experience mood changes, irritability, depressive disorders, cognitive decline, memory loss, psychosis, dementia, and delirium [285, 286]. For endogenous CS, the prevalence of major depression was 50-80%[287, 288], anxiety was 66%, and bipolar disorder 30%[289]. Exogenous GC users report a wider range of incidence from 2% to 60%, depending on underlying disease diagnosis and duration/dose of GC[290].

1.4.3.2.8 Dermatologic effects

The skin is the body's largest organ. Thus, abnormalities in the integument system are the most prevalent for CS (~60-90%)[254]. The catabolic nature of GC promotes protein breakdown, enhances lipolysis, attenuates apoptosis, and inhibits inflammatory, immunologic and healing processes[230, 291]. So the dermatologic manifestations in CS or chronic topical GC use are the best visible demonstration of the catabolic effects of GC and offer discrimination in diagnosing CS from, say, simple obesity[292].

Skin manifestations include atrophy with all skin's compartment hypoplasia, loss of subcutaneous connective tissue and elasticity, cutaneous transparency, purple striae >1-2cm in diameter, telangiectasia, bruising, dry, dysfunctional skin barrier and delayed WH[292, 293]. Skin also manifests as a consequence of metabolic disorders, including facial acne, hirsutism, acanthosis nigricans, or susceptibility to a fungal skin infection [292]. These effects are driven by deregulation of multiple skin cell functions, including inhibition of epidermal cell division, flattening dermo-epidermal junctions and loss of keratinocytes[294], keratinocyte growth factor inhibition[295, 296], suppression of fibroblast proliferation[297], inhibition of type-I and-III collagen gene expression[298], and increased collagen degradation by matrix metalloproteinases (MMP)[242]. There is a loss of lipid barrier function caused by increased transepidermal water loss[299]. All of the manifestations can be found in topical GC or CS.

The deleterious effects of topical GC depend on the GC type, potency, and vehicle; the application techniques, including frequency, duration, and occlusion; the underlying skin disease and distributions; and patient's characteristics[300]. Skin atrophy is one hallmark of topical GC use applied in the skin, and there is sufficient histological and molecular evidence indicating GC adverse effects[301]. The epidermis changes include: thinning of epidermis and fattening of the dermo-epidermal junction from decreased keratinocyte differentiation, proliferation, migration and re-epithelization; and enhanced keratinocyte maturation, as early as three to fourteen days after GC treatment[302]. Additionally, the epidermis lost its skin barrier function due to increased transepidermal water and electrolyte loss, stratum corneum shrinkage[303], lipid depletion, and a reduction in the number of lamellar bodies intercellular lamellae[304]. Dermis found the reduced synthesis and induced degradation of the extracellular matrix (ECM), MMP-1, MMP-2 Type 1 and type 3 collagen synthesis, and hyaluronic contents)[303, 305]. In vivo, GC applied topically can activates GR and translocates into the nucleus within 6 hours and remained inside the nuclei for 24 hours. GC suppresses keratin genes with decreased mRNA level during 12-h treatment and further decreases during 24- and 48-h mediate via transactivation of GR and MR[306].

For another route of GC use, such as ICS use in asthma, low to moderate doses demonstrate decreased skin collagen synthesis as early as six to twelve weeks of treatment[307, 308]. Visible skin atrophy or bruising due to ICS is related to high dose, ageing, longer duration than 1-2 years, concomitant oral GC use, and ultra violet exposure[309-311].

Endogenous hypercortisolism induced by physiological stress can compromise the stratum corneum, impair skin permeability, or alter skin morphology of the epidermis, hair follicle, sweat sebaceous glands of human skin[312]. The visible skin manifestations of endogenous CS are promising. However, to our knowledge, there are a few investigations in skin alterations caused by endogenous CS, particularly molecular studies[313, 314]. Endogenous CS had been shown to reduce hyaluronic acid synthetase (HAS)-1, 2 and 3 in a non-reversible manner during eucortisolism [315]. The type-1, -2 or -3 collagen, and elastin mRNAs expressions, compared to topical GC use [247], increased and correlated with GH levels, which is possible from the adaptive response for restoring atrophic skin [315]. However, growth hormone receptors and IGF-1 R mRNA are decreased. The discovery may provide light on the pathogenesis of CS skin atrophy associated with hyaluronic acids rather than COL genes. Locally produced proinflammatory cytokine mRNAs (IL1β and TNFα) are increased. The skin mRNA expressions of *HSD11B1* and *HSD11B2* in endogenous CS were higher than the normal population. Despite being in remission, CS treatment did not reverse skin alterations over the research period. These findings indicate that endogenous cortisol significantly impairs skin function for a long time after cortisol normalization. For exogenous CS, type-1 and -3 collagen were suppressed[316]. GCs contribute to cutaneous skin malignancy also inconsistent: three observed positives in lymphoma patients[317, 318] and others [319]; and others had negative association[320-322]

Any differences between endogenous and exogenous CS in skin manifestation are largely unknown. In a small study comparing skin findings between endogenous (n=19) CS, exogenous CS (n=16) and normal population (n=15), stria, acne, hypertrichosis, alopecia, and fungal infection were more prevalent in exogenous CS. However, hirsutism was the highest finding in endogenous CS, probably related to concomitant increases in adrenal androgens. The prevalence of skin manifestation in subtypes of endogenous CS was unaltered[254].

1.4.3.2.9 Ophthalmic side effects

The most common ophthalmologic diseases associated with CS are cataracts and glaucoma. Other findings were mydriasis, ptosis, central serous chorioretinopathy, herpetic keratitis and cytomegalovirus retinitis[323]. The prevalence of cataracts found in chronic GC

users was 11%-15%, with many types including posterior subcapsular and cortical cataracts[324]. Intraocular pressure increase associated with GC use occurs in 18%-36% of patients and is usually reversible after discontinuing GC treatment for 2-4 weeks[325].

1.5 Dysregulation of 11β-HSD1 and local cortisol excess

11β-HSD1 expression in key metabolic tissues has been linked to central obesity, Type 2 DM (T2DM), HT, and hepatic steatosis, so-called "metabolic syndrome". Visceral adiposity is one of the key factors linking the insulin resistance found in diabetes, obesity and CS spectrum. Studies have demonstrated an increase in 11β-HSD1 expression and activity in omental adipose tissue[326] further after GC exposure. Here insulin and local cortisol production synergised to increase the differentiation of preadipocytes to adipocytes[140, 327]. However, whilst adipose 11β-HSD1 was increased in obese subjects, hepatic 11β-HSD1 reductase activity was decreased in parallel with increasing body mass index. These findings supported the relationship between excessive tissue generation of cortisol by 11β-HSD1 and insulin resistance conditions. Phase II clinical trials using selective 11β-HSD1 inhibitors in patients with DM [328-330] and hepatic steatosis[331] have shown beneficial results, but not to the magnitude to support phase III studies. At the skin level, 11β-HSD1 was shown to play a key role in WH, with 11b-HSD1 knock out mice having accelerated WH and the above selective inhibitors also improving WH in man. Age and DM were linked, associated with enhanced 11β-HSD1 expression in the skin[145]. A key pathogenetic mechanism in skin ulceration is hypoxia. Focusing on the effects of GC on the skin, chapter 5 in this thesis details the effect of hypoxia on 11β-HSD1 expression and activity in human skin cells, which may benefit in the management of diabetes-induced skin ulceration.

1.6 11β-HSD1 inhibitors

Increased 11β-HSD1 enzyme locally may contribute to elevated intracellular cortisol levels and local toxicity. Inhibiting the 11β-HSD1 enzyme has emerged as a novel therapeutic target in many diseases. There are both natural and synthetic 11β-HSD1 inhibitors. The natural form including carbenoxolone[332-334], liquorice[335], curcumin[336], green tea extracted epigallocatechin-3-gallate[337], resveratrol[338], citrinal B[339] and tanshinone 2a[340]. All are poor bioavailability, rapid metabolism and less specific to 11β-HSD1 oxoreductase activity. Thus, 11β-HSD1 inhibitors ideally selectively lower tissue cortisol while not affecting normal plasma cortisol[341]. The promising studies were developed in pre-clinical stages in the settings of DM[342-349], metabolic syndrome[350-355], obesity[356-359], Alzheimer’s Disease[148, 360-362], and post-traumatic stress disorder[363] before being translated into clinical trials.

In human studies, many 11β-HSD1 inhibitors (AZD4017, BI135585[364], MK0736, MK0916, UE2343, S-707106[365], SPI-62[366], RO5093151, RO5027383, INCB13739, ABT384, ASP3662, UI-1499) are under investigation in patients with hypertension, T2DM, metabolic syndrome, CS [365], and Alzheimer's disease. So far, only phase II human studies of 11β-HSD1 inhibitors have been reported. Clinical trials have either been ineffective for the test condition or of limited effectiveness, suggesting difficulties translating basic research into the clinical environment.

Tissue cortisol levels are regulated not only by 11β-HSD1, but also by HPA-axis. Prolonged inhibition of 11β-HSD1 is considered to mediate HPA axis activation. One potential side-effect of the compensatory increase in ACTH brought about by 11β-HSD1 inhibition is the possibility of ACTH-mediated androgen, DHEA, DHEAS, and androstenedione excess. The following sections go over the 11β-HSD1 inhibitors in clinical trials focused on clinical potential of 11β-HSD1 inhibitors.

1.6.1 Diabetes, obesity and metabolic syndrome

The majority of clinical trials concluded that there was no statistically significant difference in main outcome between 11β-HSD1 and placebo. INCB13739, a T2DM therapy, significantly decreased HbA1c levels across time and dosage. All three parameters (fasting plasma glucose, HOMA-IR, and body weight) reduced. ACTH and DHEAS levels were dramatically raised in INCB13739, although no symptoms were seen[62]. MK-0916-treated T2DM patients with obesity had no significant difference in fasting plasma glucose or two-hour postprandial glucose levels compared to placebo. HbA1c, body weight, systolic and diastolic blood pressure, and systolic and diastolic blood pressure were all lowered with the highest MK-0916 dose, although LDL-C rose[285]. MK-0736 treatment resulted in a substantial reduction in diastolic blood pressure in hypertensive individuals. Following therapy with the maximum dosage of MK-0736, body weight, LDL, and HDL cholesterol levels decreased. However, MK-0736 considerably raised DHEA, DHEAS, and androstenedione[305]. After 14 days of therapy with T2DM (N = 72), BI135585 suppressed hepatic 11HSD1[302]. Other medications, such as RO-151 or RO-838 in combination with metformin, were tested in T2DM. No impact was seen on mean daily or fasting plasma glucose levels. Although it had a weight-reducing effect at the maximal dosage [63]. AZD4017 showed no significant effect on the key objectives of liver fibrosis, weight, liver enzymes or lipids, or insulin sensitivity in a 12-week randomized, double-blind, placebo-controlled trial of fatty liver patients. The mean liver fat percentage, on the other hand, was considerably improved in T2DM patients with fatty liver[306]. In T2DM patients treated with RO5093151, the mean NFALD, total body fat, and visceral fat reduced considerably during a

12-week period, however the unfavorable effects were greater in the RO5093151 group[268].

1.6.2 Wound healing

In a double-blind, placebo-controlled phase 2 study, oral AZD4017 was found to reduce wound size and improve skin integrity in iatrogenic wounds of patients with T2DM[272].

1.6.3 Idiopathic Intracranial Hypertension

AZD4017 compared to placebo over 12 weeks reduced the lumbar puncture pressure significantly compared to placebo with phase II trial[367].

1.6.4 Alzheimer's Disease (AD)

ABT-384 was used for mild AD with primary endpoint was the change of Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog). The result showed no differences seen between ABT-384 and placebo [67]. Xanamem™ trial for mild-to-moderate AD had shown no statistical difference on either the primary outcomes (ADAS-Cog and the Alzheimer's Disease composite cscore (ADCOMS))[68].

1.6.5 Cushing's syndrome

S-707106 administered for 24 weeks in CS and autonomous cortisol secretion resulted in effective insulin sensitizer, antisarcopenic and antiobesity[365].

1.7 Thesis aims

This thesis aims to explore the deleterious effects of GC in two parts:

Part 1: A Systematic review and meta-analysis of mortality in CS

Chapter 2 describes the intensive systematic review methodology, including tools and software selection for every step, bias assessment, and tools modification. In this part, the literature on mortality in patients with endogenous CS and exogenous GC use were systematically reviewed together with meta-analysis and meta-regression analysis as a statistical tool. The meta-analysis and meta-regression analysis methodology specified to single proportion data, including fitted models, were performed and described. In doing so, new statistical advances are reported. The results of systematic reviews and meta-analyses are presented in Chapter 3 for endogenous CS and Chapter 4 for exogenous CS.

Part 2: 11β-HSD1 as a mediator of GC toxicity in dermal fibroblasts

In this part, I explored the regulation and functional consequences of 11β-HSD1 activity in primary human dermal fibroblasts (HDF) as a model of cellular GC toxicity. Skin cells, including HDF, are highly sensitive to GC excess, with skin thinning and striae being rate-limiting discriminatory features of CS. My findings reveal evidence to support the development of 11β-HSD1 inhibitors as new therapeutics in patients with skin ulceration associated with vascular insufficiency (e.g. DM, atherosclerosis and ischaemic wounds). These findings are presented in Chapter 5.

The overall conclusions from the findings presented in this thesis are discussed in Chapter 6

Chapter 2

Methodology Systematic Review, Meta-analysis and meta-regression of single proportions

2.1 Background

A systematic review aims to be transparent, reproducible and updatable, and address well-defined questions[368, 369]. The process includes (1) methodology for defining the research questions with a **PICO** or **PECO** format (**P**atient/**P**roblem/**P**opulation; **I**ntervention/**E**xposure; **C**omparison and **O**utcome), (2) determining the types of studies to answer research questions, (3) comprehensive search of the literature, (4) study screening, (5) critical appraisal of the studies to be included in the systematic review; (6) synthesise the studies and assess for homogeneity; and (7) disseminate the outcome of the review along with detailed, transparent documentation of each step[370, 371].

In the past, traditional review methods focused only on the p-value to explore the studies' statistical significance[372]. The p-value depends on the sample size without taking the weight of the studies, the magnitudes of the outcomes, reproducibility or replicability, and may lead to misuse of statistical inference[373, 374]. Methods for meta-analysis allow demonstration of the direction and magnitude of the effects across the studies regarding the estimated "effect size" (ES)[375]. A meta-analysis technique was used by Blaise Pascal around the 17th century, followed by the astronomers and mathematicians such as Gauss and Laplace during the 18th and 19th century, which published in George Biddell Airy's textbook[376, 377]. Since then, Airy's method was applied and used by the British statistician Karl Pearson for inoculation against typhoid fever, and the same for Ronald Fisher and Cochrane [377]. The terminology "meta-analysis" was introduced to statistical theory by Glass[378]. Nowadays, meta-analysis is applied and used strictly to synthesise a systematic review to ensure reproducibility and reduced bias.

Meta-analysis methodology conceptually refers to a statistical synthesis of primary studies by systematic selection, integration and pooling the results of two or more scientific studies to derive conclusions on a particular research question[379]. Furthermore, meta-analysis evaluates the robustness of the effects across all studies, assesses and improves the precision of the evidence, evaluates the ascertainment of publication bias, answers the inconsistent question, settles the controversies or generates new hypotheses and conclusions[380]. The process includes two stages: 1) a summary statistic for describing the study effect of the individual study and describing the ES in the same way across the studies

and 2) a combination of the estimated ES of individual study by using weighted average under the assumption of the variability of treatment effects (fixed or random effect models: FEM or REM, respectively). The confidence interval (CI) can demonstrate the precision of the study, which is influenced by the sample size and standard error (SE)[381]. Furthermore, the research's heterogeneity and consistency may be evaluated to corroborate the therapy effects. If there is the heterogeneity, statistical approaches and procedures are used to determine the cause of the heterogeneity.

There are recommendations and guidelines to conduct the meta-analysis properly. These include Cochrane review [370], Meta-analysis Of Observational Studies in Epidemiology (MOOSE) [382], Strengthening The Reporting of OBservational studies in Epidemiology (STROBE) [383], QUality Of Reporting Of Meta-analyses (QUORUM) [384] or Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) [385, 386], http://www.equator-network.org/ and http://www.nlm.nih.gov/services/research_report_guide.html. The most common included studies for meta-analysis are randomised controlled trials studies which included comparison arms. On the other hand, single-armed studies in a meta-analysis are usually observational retrospective or prospective studies that aim to demonstrate the nature of disease or harm of treatment. Thus, a meta-analysis of proportion without comparison usually applies for meta-analysis of incidence or prevalence. The clinical importance of these systematic review and meta-analysis types is to allow clinicians, social carers, or policymakers to understand the disease's burden and develop strategies for a broad range of research, health care policies, and clinical practice guidelines [387]. The quantity of systematic literature reviews on prevalence has risen over the decades, but analysing these single-arm proportions presents several difficulties [388]. Furthermore, the meta-analysis of data presented as a proportion is challenging, including the methodology utilised, the limits of software or programs for single-arm proportion meta-analysis and the exclusion of publications with a proportion of 0 or 1. Borges et al. reviewed the prevalence methods used in 152 studies and demonstrated that despite growth in the number of publications, the limitations and variability of the methodology used have persisted (Table 2-1) [389].

Table 2-1. Methods used for meta-analysis from Borges et al.[389]

Characteristic	Description
Methods approach	**Classic: 151 (99.3%)**
	Bayesian: 1 (0.7%)
Model	**Random-effects: 141 (93.4%)**
	Fixed-effects: 7 (4.6%)
	Other: 2 (1.3%)
	Not reported: 7 (4.6%)
Variance estimator (REM meta-analysis, n=141)	DerSimonian and Laird: 30 (21.3%)
	Hartung-Knapp-Sidik-Jonkman: 4 (2.8%)
	Restricted maximum-likelihood: 1 (0.7%)
	Not reported: 106 (75.2%)
Transformation	**Freeman-Tukey double arcsine: 32 (21.1%)**
	Logit: 5 (3.3%)
	Log: 4 (2.6%)
	Raw: 2 (1.3%)
	Arcsine: 1 (0.7%)
	Arcsine square roots: 1 (0.7%)
	Not reported: 107 (70.4%)
Heterogeneity assessment	**Subgroup analysis: 89 (58.6%)**
	Meta-regression: 57 (37.5%)
	I^2 : 144 (94.7%)
	Galbraith plot: 4 (2.6%)
	Other (e.g. influence analysis, outliers): 54 (35.5%)
Publication bias	Begg's test: 26 (17.1%)
	Egger test: 54 (35.5%)
	Funnel plot: 56 (36.8%)
	Doi plot: 4 (2.6%)
	Trim and fill: 7 (4.6%)
	LFK index: 4 (2.6%)
	Not reported: 79 (52.0%)
Prediction interval	**Yes: 3 (2.0%)**
	Not reported: 149 (98.0%)
Software	**STATA: 83 (54.6%)**
	R: 29 (19.1%)
	Comprehensive Meta-analysis: 14 (9.2%)
	MetaXL: 11 (7.2%)
	MedCalc: 5 (3.3%)
	Review Manager: 3 (2.0%)
	Open Metanalyst: 3 (2.0%)
	StatsDirect: 3 (2.0%)
	MedScale: 1 (0.7%)
	Not reported: 5 (3.3%)

In this chapter, the single-arm proportion meta-analysis methodology with binary events was evaluated and subsequently applied in this thesis. There are different statistical methods for pooling results, and there is no single perfect or best method. This chapter will discuss the importance of different methods, assumptions and software for the single-arm proportion (binary data). Several methods aimed at expanding the initial meta-analysis in Chapters 3 and 4 were examined. Finally, the approaches and the outcomes of various assumptions were compared.

2.2 Aims for systematic reviews and meta-analysis

We aimed for a systematic review and meta-analysis of all-cause and specific causes of mortality amongst patients diagnosed with benign endogenous or exogenous CS and examined the factors associated with mortality.

2.3 Protocol, registration, and guidance

The protocol, data collection, and report were prospectively designed according to the principles of the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement (www.prisma-statement.org) [390, 391] and PRISMA harms checklist [392], and detailed the specific objectives, criteria for eligible articles, the bias and quality assessment criteria, as well as the outcomes of interest. The protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO), approval reference CRD42017067530 (Appendix 2.1).

2.4 Eligibility criteria

Criteria for study inclusion and exclusion were designed for chapter 3 (endogenous CS) and chapter 4 (exogenous CS).

2.4.1 Endogenous CS

2.4.1.1 Criteria for study inclusion

Original studies reporting the numbers of death or SMR in adults with endogenous CS were eligible for inclusion. Other inclusion criteria were:

Study publications: reports written in English and published in scientific journals between 1945 and March 2019. The secondary search in Pubmed was performed in the last week of January 2021 for updating the eligible publications between April 2019 and January 2021.

Study design: cohort studies of patients with endogenous CS studies

Study population: Adult patients (≥18 years of age) diagnosed with endogenous CS.

Study sample size: The minimum sample size was 10 participants in order to minimise the risk of selection bias from small sample sizes articles[393][11].

2.4.1.2 Criteria for study exclusion

Articles were excluded if they were conducted amongst a non-adult population, or non-human studies; they were case reports, case series, conference abstracts without an accompanying full-text article, book chapters, systematic reviews or clinical guidelines; exogenous CS; they were conducted amongst patients with high mortality conditions, including adrenal cell carcinoma, malignancy pituitary tumours or ectopic CS; and articles solely reporting the long-term follow up of patients with CS in remission.

2.4.2 Exogenous CS

2.4.2.1 Criteria for study inclusion

Study publications: reports written in English and published in scientific journals between 1945 and March 2019 and full-text available were eligible

Study design: Retrospective and observational studies (ie, cohort, case-control) or prospective trials of only oral GC used arm were selected when they reported the association on GCs used were enrolled.

Study population: Adult patients (≥18 years of age) with exogenous CS. Exogenous CS was defined amongst patients treated with oral GCs at a dose equivalent to ≥ 5 mg of prednisolone for at least three weeks, and at least 90% of the overall study population was reported to have taken GCs. If less than 90% of total population in the article had taken GCs, the study was enrolled if the mortality in the subgroup of exclusive GCs users was known.

Study sample size*:* The minimum sample size was 50 participants in order to minimise the risk of selection bias from small sample sizes articles[393][11].

2.4.2.2 Criteria for study exclusion

Publication outputs were excluded if they were from a non-adult population or non-human studies; if they were case reports, case series, conference abstracts, , abstracts published without an accompanying full-text article, book chapters, systematic reviews or guidelines; if they had specific pathologies and diseases or high mortality conditions such as

[11] This was done to counteract the effect of "small-study effects," which can bias the results. Smaller studies typically report more positive intervention effects than larger studies, indicating a greater degree of heterogeneity between studies. The cut-off level was agreed upon by the meta-analysis team, and sample sizes of 10 are typically used to exclude participants from small studies in rare disease.

malignancy; organ, stem cell or bone marrow transplantations, infections treated with GCs (e.g. human immunodeficiency virus, malaria, sepsis, tuberculosis or viral hepatitis) or intensive care patients; if they had alcohol or liver-related diseases, such as cirrhosis, alcoholic hepatitis or autoimmune hepatitis and if they were treated with non-oral GCs or using GCs for supplement or replacement therapy, such as adrenal insufficiency, critical illness-related corticosteroid insufficiency (CIRCI) or traumatic brain injury.

2.5 Outcome measures

2.5.1 Primary outcome

Primary outcomes were the SMR or the proportion of deaths from any cause (all-cause mortality) reported at the maximum duration of follow-up.

2.5.2 Secondary outcome

Secondary outcomes were the SMR or number of deaths by specific cause of death.

2.6 Search strategy

2.6.1 Identifying studies-information sources

2.6.1.1 Endogenous CS

The electronic literature search was performed using a combination of well-defined terms for CS (Cushing*, Adrenal tumo*, Adrenal adenoma*, Adrenocortical adenoma*, glucocorticoid producing adenoma*, glucocorticoid producing tumo*, Cushing's disease, ACTH producing tumo*, ACTH-secreting tumo*, ACTH-producing adenoma*, pituitary tumo*) and terms for the study outcome (death and mortality) with restriction to human articles and publications in English, but not the year of publication. The full structured search strategy using medical subject headings and keyword terms is presented in **Error! Reference source not found.** With the support of a specialised librarian, I conducted the search through several databases from inception to 31 March 2019: PubMed/MEDLINE (1966 to 31 March 2019), Cochrane Database of Systematic Reviews, EMBASE (1974 to 31 March 2019), the web of science (1900 to 31 March 2019) and CINAHL (1981 to 31 March 2019). An initial search was performed in July 2017, and it was last updated in April 2019 and January 2021.

2.6.1.2 Exogenous CS

The electronic literature search was performed using a combination of well-defined terms for CS, including any Cushing* or types of oral GCs and the study outcome (death and mortality) with restriction to human articles and publications in English, but not a year of publication. The complete structured search strategy using medical subject headings and keyword terms are described in Appendix 2-2. One of the authors (PL) conducted the search through several databases from inception to 31 March 2019: PubMed/MEDLINE (1966 to 31 March 2019), Cochrane Database of Systematic' Reviews, EMBASE (1974 to 31 March 2019), the web of science (1900 to 31 March 2019) and CINAHL (1981 to 31 March 2019). An initial search was performed in July 2017, and it was last updated in April 2019.

2.6.2 Complementary search methods

The reference lists of eligible articles or relevant systematic reviews of diseases treated with long-term GCs were also screened to identify other potentially eligible studies. In the case of missing relevant data, the authors of relevant publications were contacted by e-mail to obtain the information. The results of the search were de-duplicated using Endnote version X9 (Thomson Reuters, Philadelphia, PA, USA), Rayyan—a web and mobile app for systematic reviews (https://rayyan.qcri.org/)[393] and Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia (available at www.covidence.org). All steps details were recorded, including the following:

- Databases searched plus the specific years or other limitations specified
- Subject headings and keywords used for each database
- Total number of articles displayed for each search strategy
- Number of articles that met inclusion criteria that were finally selected

2.7 Review procedures

The articles identified from every search engines were uploaded into EndNote and checked for article duplications. After the removal of article duplications, the articles were uploaded into a web-based data screening tool named Rayyan (http://rayyan.qcri.org)[393] for secondary de-duplication as well as title and abstracts screening. All titles and abstracts were screened at the first stage by one reviewer, Padiporn Limumpornpetch (PL), and verified by a second reviewer Mar Pujades Rodriguez (MPR). The potentially relevant articles were uploaded to Covidence (https://www.covidence.org: the electronic systematic reviewer tool developed by Cochrane) for full-text screening. Firstly, PL assessed eligibility through full-text screening by applying the inclusion and exclusion criteria of the review. The

reasons for exclusions were recorded. Then either two reviewers, MPR or Paul Stewart (PS), independently reassessed the eligibility through full-text screening again. Disagreements were resolved by consultation with MPR, PS and Ann Morgan (AM); adjudicated by PS.

The potential duplication of reports was further explored by simultaneously assessing and comparing articles reporting on the same diseases by the same authors or different authors in the same institutes/ hospitals and the overlap in years of follow-up covered by the studies. This included the assessment of duplication by overlapping reports from single, national and international multisite studies. For multiple studies reporting the same cohort, the following criteria for final study inclusion were: 1) the longest follow-up of outcomes that met the inclusion criteria; 2) the most recent publication and the largest population; 3) the most generalizability of findings; 4) the transparency in reporting, and 5) the lowest bias in mortality ascertainment.

For endogenous CS, using the above criteria, some articles that reported the SMR had been excluded if there were the articles fitted to the above criteria (longest followed up and largest population). The article which reported the SMR was also included for only the SMR meta-analysis group, not for proportion of deaths.

2.8 Data extraction and management

Standardised data extraction forms were initially designed in paper and then implemented, tested and revised using Microsoft® ACCESS version Office® 365. The following data were obtained by one reviewer (PL). We contacted the authors by e-mail to obtain the original, non-report information and relevant missing data. Extracted data were reviewed and cross-checked against the electronic records by both reviewers (PL and MPR). Disagreements between 2 reviewers were resolved by discussion and consensus and consultation with two other investigators (PM and AM) when additional clinical expertise was required.

2.8.1 Endogenous CS

Pubmed identification (PMID), first author, country, hospital and publication year, study design, sample size, age, sex, type of CS and specific information for subtypes of CS, level of care, data source and period of recruitment or observation; the treatment; time-related to mortality report (e.g. perioperative period or long-term follow up); the follow-up period; and mortality data, including specific causes of death. If the article included multiple groups of patients with the separated mortality outcome (e.g. ACS and pituitary CS or UK patients and Greece patients, which reported mortality separately), data were extracted for

each group separately if the numbers of each group were at least 10, instead of pooling the data. The multicentre or multi-nation, or nationwide publications were re-checked for the possibility of duplication with the cohorts from individual countries or institutes. The European cohorts, including European Cushing's Disease Survey Group (data were collected between 1975-1990 from 25 institutions throughout Europe) [394] and ERCUSYN (data were collected between 2000-2017 from 25 institutions throughout Europe)[395] were also checked for patient duplication in the same process**.** Because the information from the European Cushing's Disease Survey Group was published several decades before this study and restricted to perioperative mortality, and there were new publications with longer outcomes from individual institutes, then the publications during this period were chosen under the institutes or nation reports. All the European cohorts from the individual institutes or countries were re-checked and updated again after the publication by ERCUSYN[395]. The publications from European countries, including the UK, were excluded if the patients were potentially reported from the ERCUSYN centres during 2000-2017 (https://www.ercusyn.eu/centers-ercusyn-europe/). Furthermore, we asked the authors for the duplications, including the coordinators and the authors who conducted the local studies in each institute or published the articles (Prof. Susan M Webb, Alicia Santos, Prof.John AH

Wass, Christian J Strasburger, John Newell-Price, Antoine Tabarin). Eleven articles (1022 CD, 280 ACS, and 70 combined CS patients) were excluded after ERCUSYN publication (Figure 2-1, Table 2-2). For outcome information, SMR, numbers of death and the causes of death were extracted.

Figure 2-1. ERCUSYN study

Table 2-2. Excluded articles as they were duplicated population with ERCUSYN study (2000-2017)

1st author (year)	Country	No. patients	Observation period	Mean age at diagnosis [Median]	No. of women (%)	Etiology									Mean or [Median] follow-up in years	No. of deaths (%)
						Cushing's disease				ACS				Unknown		
						Total	microadenoma	macroadenoma	Unknown / mixed	Total	Adenoma	Bilateral lesion	Other ACS			
Arnardottir, 2011 [396]	Iceland	19	1955-2009	44	14 (73.7)	19	0	0	19	0	0	0	0	0	NR	5 (26.3)
Yaneva, 2013[220]	Bulgaria	240	1965-2010	36	82 (34.2)	240	0	0	240	0	0	0	0	0	7.1 [7.1]	66 (27.5)
Yaneva, 2013[220]	Bulgaria	84	1965-2010	38	76 (90.5)	0	0	0	0	84	84	0	0	0	[4.17]	12 (14.3)
Yaneva, 2013[220]	Bulgaria	11	1965-2010	43	8 (72.7)	0	0	0	0	11	0	11	0	0	[5.5]	2 (18.2)
Ntali, 2013[219]	Greece	58	1962-2009	[46]	52 (89.7)	0	0	0	0	58	0	0	58	0	[2]	1 (1.7)
Ntali, 2013[397]	Greece	129	1962-2009	[40.5]	104 (80.6)	129	113	16	0	0	0	0	0	0	[5.5]	13 (10.1)
Terzolo, 2014[398]	Italy	70	1991-2011	NR	NR	51	0	0	51	19	11	8	0	0	NR	10 (14.3)
Dimopoulou, 2014[399]	Germany	120	1992-2012	50	96 (80.0)	120	88	32	0	0	0	0	0	0	6.3	2 (1.7)
Torales, 2014[400]	Spain	19	2005-2012	55.7	NR	19	0	0	19	0	0	0	0	0	4.58	0 (0.0)
Reincke, 2015[401]	Germany	59	1990-2014	NR	NR	0	0	0	0	59	46	13	0	0	NR	1 (1.7)
Aranda, 2015[402]	Spain	41	1974-2011	34	35 (85.4)	41	29	6	6	0	0	0	0	0	14 [6.68]	3 (7.3)
Villeon, 2016 [403]	France	68	1994-2011	[38]	65 (95.6)	0	0	0	0	68	0	0	68	0	[3.5]	0 (0.0)
Solak, 2016[404]	Croatia	33	2007-2014	[38]	27 (81.8)	33	23	10	0	0	0	0	0	0	[2.33]	1 (3.0)
Brichard, 2018[405]	Belgium	71	1996-2017	43	57 (80.3)	71	58	13	0	0	0	0	0	0	6.87	0 (0.0)

2.8.2 Exogenous CS

Data were extracted for Pubmed identification (PMID), first author, country, hospital and publication year, study design, sample size, age, sex, level of care, data source and period of recruitment or observation; the follow-up period and the mortality data included specific causes of death. Exogenous GC information included underlying disease treated by GC, GC dose, and pattern: cumulative doses, average mean doses, maintenance doses, starting doses, the last follow-up GC dose were extracted. The duration of GC use and duration of follow-up were extracted separately to maximise the completeness of GCs exposure information. If the article included multiple groups of patients (e.g. SLE in the UK and SLE in Germany), instead of pooling, the data were extracted separately (if the numbers in each group had at least 50 in exogenous CS). For outcome information, all SMR, numbers and percentage of death and the causes of death were extracted. We contacted the authors for obtaining the relevant missing data

2.9 Assessment of risk of bias in the included study

Existing tools for bias assessment[406] were reviewed, and the Risk Of Bias In Non-randomized Studies - of Interventions (ROBIN-I) tool[407] was chosen and modified for evaluation of the risk of bias in this systematic review separately between endogenous CS and exogenous CS. This tool assesses seven domains related to confounding, selection of participants into the study, classification of interventions, deviations from intended interventions, missing data, measurement of outcomes and reporting. The risk of bias judgement in each and overall domain options was: 1. Low risk of bias (the study is comparable to a well-performed randomised trial with regard to this domain); 2. Moderate risk of bias (the study is sound for a non-randomised study with regard to this domain but cannot be considered comparable to a well-performed randomised trial); 3.Serious risk of bias (the study has some important problems in this domain); 4. Critical risk of bias (the study is too problematic in this domain to provide any useful evidence on the effects of intervention); and 5. No information on which to base a judgement about the risk of bias for this domain. The highest risk of bias for any criteria was used to reflect the overall risk of bias for the study. Then the pilot articles for endogenous CS were drawn on the preliminary bias assessment with the objective of considering how the articles might be assessed the bias risk. The last step was re-testing and amending the tool. The modified ROBIN-I is shown in Appendix 2-3. The risk of bias assessments was presented as a summary plot and traffic light plot using the robvis tool (https://mcguinlu.shinyapps.io/robvis/) to generate the info-graphic [408].

2.10 Data synthesis

2.10.1 Simple statistical methods use for meta-analysis

Numerous meta-analyses of proportions are conducted using the traditional two-step procedure. Firstly, each study's proportion estimate is often transformed to improve its approach to the normal distribution, as needed by the assumptions of traditional meta-analysis models. Second, a meta-analysis is conducted using the transformed scale, and the resulting result is then back-transformed to the original proportion scale, which spans from 0% to 100% (or proportion of 0 to 1). Meta-analyses are usually performed using straightforward statistical techniques, with pooled estimates derived as weighted averages. The ease with which these techniques are calculated obscures the distributional assumptions that underlie them.

2.10.2 Defining the type of data or interesting outcome and effect size

In this study, the outcome of interest was the SMR and the proportion of deaths from the single group (CS patients). The SMR was calculated as a weighted average of the mortality rate or SMR±95%CI in the individual studies. If an SMR with 95% CI was not provided, then it was calculated from the reported observed (O) and expected (E) deaths, as SMR = OE and its 95%CI = SMR±1.96(O/E)[409].

The proportion of deaths represented the number of deaths in CS patients, either exogenous or endogenous CS, calculated by the number of deaths divided by total sample size. The data in this meta-analysis is the single-armed meta-analysis without comparison groups. The probability of death is the binary outcome in which each unit of the patient has only two possible chances "survive" or "death". The proportion of deaths in each study is always between 0 and 1; including the summation over categories always equals 1. So, the natural distribution for modelling of this data is the binomial distribution[410] (numbers of success in a sample) which is given by:

$$\int(y;p) = \binom{n}{y} p^y (1-p)^{n-y}$$

for y = 0,1,…,n, p ∈ (0,1).

The proportion (p) of deaths in each study is $p = \frac{r}{n}$

where r is the number of deaths and n is the total number of CS patients.

The variance of a binomial random variable or single-proportion or prevalence is

$$Var(p) = \frac{p(1-p)}{N}$$

Where p is the proportion or prevalence of deaths, and N is the sample size.

2.10.2.1 The effect size framework and model for binomial data

In a meta-analysis, the ES is the result of interest that has been created as the standard scale for all studies in order for them to be comparable, standardised, pooled across studies, and tested for outcome heterogeneity[375]. Different models may lead to different estimated ES and standard errors (SE)[411, 412]. There are three frameworks of binomial data modelling as described below (Table 2-3

).

2.10.2.1.1 *Untransformed proportion*

This approach is not appropriate to use because the proportion estimates are not distributed normally. Problems frequently occur for rare events or small samples sizes. The Wald-type confidence intervals (CIs) of untransformed proportions may be found outside the range of 0 to 1[412, 413].

2.10.2.1.2 *Approximate likelihood approach or transformations*

Transformation of statistical variables stabilises variance to facilitate the computation of tail sums of the distribution with the aid of the normal probability integral[414]. This framework approximates the within-study variability with a normal distribution. There were two transformation techniques used: Canonical transformations for proportions (logit transformations) and variance stabilising transformations for proportions (arcsine transformations)[415]. The approximate method relies on the approximation of normal distribution instead of the true nature of binomial or Poisson distributions[414]. There are some bias and poor statistical properties if the proportion is close to zero or one, or where there are small sample sizes or rare events[416]. By using Canonical transformation, the logit transformation of proportion expands value close to zero or one[417]. The variance stabilising transformation for binomial data is the Freeman-Tukey Double arcsine transformation[418]. Meta-analysis can then be done in several different ways to produce the pooled estimates e.g. the inverse variance method of transformed proportion as study weight. For presentation in the original probability scales, the pooled transformed ES and CI were back transformed to a proportion for easy interpretation.

The transformation method is the most popular selection of meta-analysis framework because of easy accessibility to computation with supporting software and no need for statistical expertise[389]. The default of some programmes in the statistical analysis tools provides a user-friendly approach, especially for applying Freeman-Tukey Double arcsine transformation as the tools for meta-analysis of proportion which approach to 0 and 1[419]. However, the Freeman-Tukey Double arcsine transformation use may lead to the indirect

conclusion if the data is not the real normal distribution and the true nature of this data is beta-binomial or a binomial distribution[420, 421]. The limitation of the inverse arcsine transformation is the erratic values for domain close to 0 or 1[422]. The transformation methods demonstrated in Table 2-3

The logit transformation equation solved the ES outside 0 to 1, but it could not stabilise the variance[423]. Freeman-Tukey double arcsine transformation can solve the problems of CI and stabilisation of variance. For this reason, the double arcsine transformation is the preferable method for transformation of the proportion data[415, 423], although recent publications have raised some controversial issues[421]. The controversy when the proportions equal to 0 (an impossible event) or 1 (a certain event) lead to the sampling variance equal to 0. Cochrane recommended solving and managing these data by adding a constant number (e.g. 0.5%)[423, 424]. This Cochrane recommendation works where a small number of studies have 0 or 1 proportions. If there are many articles where proportions equal to 0 or 1, or the sample size in each article is small, the reliability of the pooled ES will be distorted and overestimated. For example, consider a report in which 50 or 100 patients died at a rate of 0%; the proportions altered when constant numbers were added from 0% to 1% or 0.50%, respectively. The better solution is to transform data by the Freeman-Tukey transformation recommended by Barendregt et al[423]. Schwarzer et al. published on Freeman-Tukey double arcsine transformation problems lack weighting of the sample sizes [421]. The controversy of choosing the transformation arcsine-based transformation methods for proportions was also published [389, 420]. Generalised Linear Mixed Models (GLMM) with logit transformation was recommended because they fully accounted for within-study uncertainties, critical for small sample sizes and rare events [425].

Table 2-3. Definition and properties of prevalence transformations[12]

Transformation	estimate	Approximate variance	Comments
Canonical transformations			
log	$\log(a/n)$	$\frac{1}{a}-\frac{1}{n}$	Infinite estimate and variance for zero events
logit	$\log\left(\frac{a/n}{1-a/n}\right)$	$\frac{1}{a}+\frac{1}{n-a}$	The ES outside 0 to 1 problem is solved, but it could not stabilise the variance.
variance stabilising transformations			
arcsine	$arcsin\sqrt{\frac{a}{n}}$	$\frac{1}{4n}$	Variance stabilising; defined for zero events
Double arcsine	$arcsin\sqrt{\frac{a}{n+1}}+arcsin\sqrt{\frac{a+1}{n+1}}$	$\frac{1}{4n+2}$	Outperforms arcsine for small prevalences; Sample size needed in back-transformation

[12] a, number of events; and n, total sample size.

2.10.2.1.3 True nature framework (exact likelihood approach)

The beta-binomial[426] is the ideal to allow for uncertainty in proportions, but the process for doing this and the software were limited at the time I undertook this study.

2.10.2.1.4 Two stages approach by fusing the approximate and exactly likelihood approaches

The first step is to model the data using the binomial distribution, and the logit transformation is used to model the heterogeneity. The approach was chosen for our meta-analysis by *metapreg* program, which was the most fitted assumption for this research outcome. ***Metapreg,*** https://ideas.repec.org/c/boc/bocode/s458693.html***,*** is Stata module to compute fixed- or random-effects meta-analysis and meta-regression of proportions developed by Victoria Nyawira Nyaga[427].

2.10.2.2 Mortality estimation and pooling of effect sizes

In summary for this thesis: in each study, the primary outcome was reported as the proportion of death calculated by the number of death divided by the sample size of GC use. This proportion was obtained as the observed ES. The goal of reporting meta-analysis is to summarise the magnitude and direction of intervention effects (GC) on mortality in the population across the studies. True ES or estimated population ES from the individual article was computed under the *metapreg* program. For the subgroup of GC used in exogenous CS or subgroup of endogenous CS: patients which including age, percentage of women, the proportion of death, duration of follow-up, and duration of GC use were reported in the study characteristics as the weighted mean of each group. The weight for each group calculated by the proportion of patient in the study divided by total patients in each group.

2.10.3 Statistical models for aggregate data

This is the process that should be considered before combining the overall effects. The model of choice for the meta-analyses should be decided by prior assumptions of heterogeneity or tests of homogeneity. There are at least three variable sources of heterogeneity to be considered: sampling error, study-level (within-study) characteristics, and between-study variation. There are three models of choice: the FEM, REM and mixed effect, used in meta-analyses which depends on the assumptions of the nature of the studies. The assumption leads to the different mathematic model to combine the results and interpretation. There are two aetiologies of ES variability of the primary studies: (1) within-study variance (σ_i^2) caused by the sampling error in the selection and (2) between-studies variance (τ^2) caused by sampling error in the selection of the studies. If all of the included

studies share the same true ES and all the variability between ES is only from sampling error ($\tau^2 = 0$), the FEM is appropriately applied for this assumption. In other aspects, if the variability between ES is beyond sampling error with including research methodology or population effects, which lead to differences in the true ES across the study, the REM is the most appropriate assumption for analysis. In REM, there are two levels of error from the estimate of the true ES (1) the individual study in a specific population and (2) from the estimate of the overall mean of true effect by combining the true ES across the studies.

To test the study's homogeneity, the FEM assumes that all k studies share a common mean θ. A statistical test for the homogeneity of study means is equivalent to test the hypothesis as the following: H_0: $\theta = \theta_1 = \theta_{2=} =\ldots= \theta_k$ against H_A: At least one θ_l different. If H_0 is not rejected, the common mean across k studies are the same, or the between-study variation is small. If the H_0 is rejected, the random-effects model should be applied and exploring the subgroups, covariates or causes of heterogeneity.

There are several mathematical estimators or methods to estimate between-study variance or τ^2 [428]e.g. DerSimonian and Liard approach (DL)[429, 430], Hartung and Knapp method[431-433], method of moment estimators[434], maximum likelihood estimators[435], or restricted maximum likelihood[436].

For the REM, the common method for combining the estimated ES is the DL; this is implemented as the default method in many software[437]. However, using this method for proportion or binomial data caused bias, underestimating the true between-study variances, especially when the between-study variance is large[438]. The selection of the appropriate summary statistic methods is a subject of debate due to conflicts in the relative importance of mathematical properties and the ability to interpret results intuitively. The selection of methods for meta-analyses of binary outcomes is considered from the consistency of effect, easy interpretation and mathematical properties[439]. Higgins and team compared nine variance estimators for REM and recommended restricted maximum likelihood to estimate the heterogeneity variance over other methods[440].

The true effect of mortality from CS varied across studies in the meta-analysis of due to differences in underlying disease, age, female prevalence, co-morbidities, co-interventions, the severity and chronicity of the diseases, the duration of GC use or CS disease activity, the study duration, and the year of management. All of the aforementioned explanations were fitted to the mixed effect with binary outcomes, and the DL model estimation was used in these meta-analyses.

2.10.4 Methods of combining the effect sizes

Several methods combine the ES, e.g. inverse-variance weighting, Mantel-Haenszel, Peto, or DL method[441]. For a REM, to make the comparable ES, all the outcomes from multiple independent studies must be weighted by generating the SE[441]. The SE is the direct index of ES precision which is influenced by the sample size and is calculated CI. Basically, the optimal weighted ES for single proportions is calculated generally by using the inverse variance weighted method model or GLMM[442].

2.10.5 The inverse variance weighted method model

This is the most popular and common method for general meta-analysis. Under the assumptions of the inverse-variance weighted method obtained the unbiased and minimum variance estimator, so-called the uniformly minimum variance unbiased estimator[443]. This method generates the weight to each study by inversing the variance of the estimated ES as the following equation:

$$w_i = \frac{1}{se_i^2 + v_\theta}$$

Where w is the true weight; se is the standard error; v is the random effects variance component.

For the FEM meta-analysis, the weighted average formula is:

$$Generic\ inverse - varince\ weighted\ avaerage = \frac{\sum y_i\left(1/SE_i^2\right)}{\sum\left(1/SE_i^2\right)}$$

where y_i is the estimated ES in the i[th] study, $SE_i^2$is the se of that i[th] estimated ES.

The variance of the proportion of deaths from the individual study can be calculated by:

$$Var(p) = \frac{p(1-p)}{N}$$

Where p is the proportion of deaths, and N is the sample size of CS.

Under inverse variance weighted method model, the pooled estimated ES (P) of single-arm proportion is equal to:

$$P = \frac{\sum_i \frac{p_i}{Var(p_i)}}{\sum_i \frac{1}{Var(p_i)}}$$

Where p_i is the proportion of deaths at i[th] study.

With SE is:

$$SE(P) = \sqrt{\sum_i \frac{1}{Var(p_i)}}$$

And CI of the pooled ES is:

$$CI_{\gamma}(P) = P \pm Z\propto_{/2} SE(P)$$

Where $Z\propto_{/2}$ denotes the appropriate factor from the standard normal distribution for the desired confidence percentage ($Z_{0.025}$=1.96).

The assumption of the value was the normal distribution which α and $Z_{0.025}$ equalled 0.05 and 1.96, consequently. The equation works very well if p is around 0.5. There were some problems when the equations were applied for proportions that closed to 0 or 1, which resulted in a variance of nearly 0[444]. When calculating the ES by using the inverted variance method model, the ES or weighted ES of those studies will be large. The CI from the extreme proportion (nearly 0 or 1) will exceed one or minus value. A proportion of 0 or 1 simplifies to a zero variance leads to an infinite weighting. The studies whose proportion were 0 or 1 resulted in the inadmission of the studies[444]. So the appropriate selection of analytic framework and model is essential for the meta-analysis of binomial data.

This analysis applies *metapreg* program in STATA, which highlights the integration of regression analysis by using mixed-effects logistic meta-regression with the binomial assumption data[427]. As the variance of a binomial variable is p(1-p)/N[13], so the both p and n play a role in the telling the precision. The more the precise a study is, the more weight it has. The distribution of a binomial distribution is $[(N!)/(N-n)!n!](p^n)(1-p)^{(N-n)}$ To estimate p, we maximize the log likelihood of $[(N!)/(N-n)!n!](p^n)(1-p)^{(N-n)}$. The maximization is done iteratively. The maximized equation each time is calculated in a way to see the “weights” used in each iteration. The weighting is not explicit in the forest plot because parameter estimation is an iterative procedure. Therefore, even though the forest plot displays equal weights for the individual studies, weighting is indeed done.

2.10.6 Heterogeneity assessment and interpretation

The heterogeneity of the component studies was identified for three aspects, included clinical, methodological and statistical heterogeneity[379]. Clinical heterogeneity resulted from the population, intervention, outcome definitions, duration of therapy, follow-up, study methodology, and publication bias[445]. The descriptive information in each article can be used to detect clinical heterogeneity. If clinical heterogeneity is present, the overall statistical heterogeneity may or may not exist. Methodological and statistical heterogeneity was analysed according to the study design.

The clinical heterogeneity was stratified by disease subgroup (ACS and CD), disease activity (active vs remission; the criteria were extracted from the reported articles and

[13] N=Total number of CS, n=Total number of deaths, 1-p = Probability of alive, p= probability of death on a single study.

summarised in), pituitary size in CD (macroadenoma vs microadenoma), and gender. Surgical techniques and perioperative care have improved significantly with time. Consequently, mortality related to surgery (perioperative) was considered a contributing factor to the overall mortality and taken into account in the analyses. Perioperative mortality was defined as deaths that occurred within 30 days of a surgical procedure. The publication period was also evaluated in a subgroup analysis further to assess advances in diagnosis, treatment and care.

Measuring statistical heterogeneity is an important initial step in the meta-analysis and can be displayed as Cochran's Q, τ^2, $I^2$and predictive intervals[446]. The test of heterogeneity also influences the model selection for combining the results[447]. REM is the best fit based on presenting of heterogeneity between studies[447]. And if homogeneity is present across all studies, then FEM was the most suitable model[447]. The heterogeneity is not ignored; rather, it is documented, the underlying causes are investigated, and suitable statistical techniques have been used[448]. The benefits and disadvantages of the various heterogeneity tests are addressed in Table 2-4.

2.10.7 Cochrane's Chi-squared test (Cohran's Q statistic)

Cochran's Q is the statistical heterogeneity test based on a null and an alternative hypothesis[449]. The null hypothesis states the populations are homogeneous, and the variation arises from sampling error. The statistic test performed is the chi-squared (χ2) test statistic (χ2 distribution), *k*-1 (k is the number of primary studies), degrees of freedom, and p-value from *k* studies. Q is the weighted sum of squares on a standardised scale, reported together with the p-value[450]. The strength of the Q-test is dependent on the number of included articles, and a small number of studies has low power to reject the null hypothesis[450]. This method has the lowest power for detecting heterogeneity, and so the threshold value for statistical significance was 0.1. Using this cut off to reject the null hypothesis may increase the type I error or false-positive conclusion[451]. Q-test will not detail the magnitude nor causes of the heterogeneity[452]. Because of the inaccuracy and low statistical power, the I² test was used to demonstrate the magnitude of heterogeneity.

2.10.7.1 Higgins's I² test statistic

The I² *was* developed by Higgins[452] and represent the percentage of variation across the studies due to real heterogeneity, rather than occurring by chance, which ranges between 0 to 100% (from no to maximum heterogeneity)[453].

The equation used to calculate I² is $\mathrm{I}^2 = 100\%\, X\, \frac{(Q-df)}{Q}$

Where Q is Cochran's heterogeneity statistic, df is the degrees of freedom (number of studies minus 1).

The approach to interpretation I^2, as proposed by Higgins and Thompson, refers to 0% meaning no heterogeneity, whereas an I^2 of around 25%, 50% and 75% indicates low, medium, and high degrees of inconsistency or heterogeneity, respectively[452, 453]. Cochrane also published an alternative heterogeneity stratification by I^2 interpretation guide: minimal or might not be important for I^2 0% to 40%, moderate heterogeneity 30% to 60%, substantial heterogeneity for I^2 50% to 90% and considerable heterogeneity for I^2 90% to 100%[379]. Statistically, significant heterogeneity usually equates with I^2>50%. Despite being the most popular tool, I^2 cannot provide complete information about the heterogeneity, such as variation in ES[454]. One limitation of using only I^2 to quantify the heterogeneity is that it may be misleading for observational studies[455, 456]. Consequently, the exploration of heterogeneity in a meta-analysis often uses more than one method and investigates their sources by subgroup or meta-regression analyses[457]. So in the presence of statistical heterogeneity, the next step is to explore the causes of heterogeneity by re-checking the correctness of data extraction, subgroup and meta-regression analysis.

2.10.7.2 Tau^2 (τ^2)

$\boldsymbol{\tau^2}$ represents the between-study variance, and Tau ($\boldsymbol{\tau}$) is the estimated standard deviation of underlying true effects across studies[428]. $\boldsymbol{\tau^2}$ is not used itself as a measure of heterogeneity but is used in two other ways: (1) to compute $\boldsymbol{\tau}$; and (2) to assign weights to the studies in the meta-analysis under the REM[453]. $\boldsymbol{\tau}$ is is used for computing the prediction interval[458].

Table 2-4. The heterogeneity test

Measure	Advantage	Disadvantage
$\boldsymbol{\tau^2}$	τ^2 : SD of the between study variation (on the scale of the original outcome)	Difficult to interpret for clinical applications, especially when τ^2 belongs to outcomes that were transformed and analysed on another scale eg. log scale
	τ^2 is used to calculate the prediction interval	Imprecise for a small number of studies
$\boldsymbol{I^2}$	I^2 presents the inconsistency between the study results and quantifies the proportion of observed dispersion that is real, that is, due to between-study differences and not due to random errors	Difficult to interpret for clinical context or clinical application
	I^2 reflects the extent of overlap of the CIs of the study effects	Ambiguous values as its size depends on sample size - With very large studies, even tiny between-study differences in ES may result in a high I^2 ; - With small (imprecise) studies, very different treatment effects can yield an I^2 of 0.
	I^2 represents the inconsistency on a standardised scale between 0 and 100, therefore it can be compared with recommended thresholds for low or high inconsistency	
CI	in a REM contains highly probable values for the summary (mean) treatment effect.	CI gives no information on the range of true treatment effects.
Prediction intervals	A REM provides highly probable values for the true treatment effects in future settings, if those settings are similar to the conditions explored in the meta-analysis.	Conclusions drawn from the prediction interval are based on the assumption that τ2 and the study effects are normally distributed
	Comparable with clinically relevant thresholds to see whether they correspond to benefit, null effects or harm	The estimate of the prediction interval will be imprecise if the estimates of the summary effect and the τ2 are imprecise, for example, if they are based on only a small number of studies and if the sample sizes are small.
	It can be used to estimate the probability that the treatment in a future setting will have a true-positive or true-negative effect and to perform better power calculations	

2.10.8 Graphic presentation

2.10.8.1 Forest plot

Meta-analysis results are visualised as a forest plot, which was developed in the 1980s by Richard Peto's team[459, 460]. The forest plot is the tool to visualise the estimated effect and CI for the individual and combined studies of meta-analysis[452, 461]. Generally, the squares of different sizes in forest plot display the positions of the point estimates with the weighted proportion of individual study. The CI displayed as the horizontal line run symmetrically through the squares represents the study's precision. The main vertical line across zero means no effect or 0% mortality for the single-arm meta-analysis of proportion. The overall estimated ES and CI are displayed as the diamond shape at the bottom of the forest plot[462].

2.10.8.2 Prediction intervals

Prediction intervals are the range of true ES calculated by using τ and mean (μ), which are used to predict the expected 95% of the true effects in future studies to lie within μ±1.96 τ[448]. Thus, the equation of predicted interval was $\mu \pm t\sqrt{\tau^2 + \mathrm{SE}(\mu)^2}$ (as t is 97.5 percentile of a t-distribution, df was the degrees of freedom which was equal to numbers of studies– 2, and therefore at least 3 studies are required to calculate this statistic[448].

2.10.9 Subgroup analyses

The subgroup analysis aims to investigate the causes of heterogeneity by considering the heterogeneity factors and group them[457]. If differences in the subgroups' outcomes were observed, the interpretation and application of overall results should be undertaken with caution. Furthermore, a statistical test was conducted to examine whether the ES of the subgroups differed significantly from each other. Usually, these subgroup analyses were performed using a mixed-effects model whereby the ES within the subgroups were pooled with a REM and the test to determine whether the ES between the subgroups differed significantly from each other was performed using a FEM[463].

According to the differences in study-level variables and reports, subgroup analyses for endogenus and exogenous CS were planned to explore possible reasons for heterogeneity.

2.10.9.1 Endogenous CS

Subgroup analyses were planned according to the following variables: (1) subtypes of CS patients: CD, ACS (adrenal adenoma (AA), bilateral adrenal hyperplasia (BAH) and

mixed types of ACS) and combined types of CS; (2) disease activity of CD: active and remission; (3) pituitary size: microadenoma vs macroadenoma; (4) perioperative mortality versus longer-term mortality; (5) follow-up duration; (6) study period (or published time) which represented different management protocols for CS patients; and (7) operative procedures

2.10.9.2 Exogenous CS

Subgroup analyses were planned according to the following variables: (1) dose of GCs reported as cumulative dose (g/d), average whole-time follow-up dose (mg/d), maintenance dose (mg/d), initial treatment dose (mg/d) and last follow-up dose (mg/d); (2) treatment duration; (3) underlying disease indication for GC treatment; (4) duration of follow-up.

2.10.10 Sensitivity analyses

Sensitivity analysis is the method to prove that the synthesis data of systematic review are robust and not depend on arbitrary reports or unclear reasons or decisions or studies at high risk of bias[464]. The sensitivity analyses apply as the leave-one-out method in the subsets of *N* studies[465]. The technique removes one study out of N studies per times and runs the meta-analysis on the remaining N-1 studies. The process will repeat for *N* times which equal to the total number of studies. Under the assumption that is removing one result, is not affected the overall results. *N* meta-analyses results will be explored for the likelihood of consistency and homogeneity. In our study, sensitivity analyses were conducted by removing the high risk of bias or poor study quality.

2.10.11 Meta-regression analyses

Meta-regression analysis is a more advanced meta-analysis technique whereby linear regression is performed to investigate statistical heterogeneity between estimated effects of various studies in conjunction with covariates[466]. For the single-arm meta-analysis of proportion, meta-regression was used to explore whether a linear association existed between the variables and the estimated ES and the direction of association[467]. The advantage of meta-regression over performing a subgroup analysis was that one or more covariates could be applied[468]. The associations found in the meta-regression can be used to generate hypothesis and not in themselves proof of causality. Meta-regression was used to explore potential sources of heterogeneity when the I^2 was higher than 25%[466]. In general meta-regression analyses should only be conducted when the number of included studies is at least 10 and the number of covariates that was chosen was based on rules of 10 articles per 1 covariate[469]. This heterogeneity can be attributed to

systematic differences in methodology, studied population, and/or the length of follow-up or study.

Meta-regression analyses performed in this thesis used the mixed-effects logistic regression model[470] fitted with the covariates. The heterogeneity between groups was tested formally by running the model with and without the covariate of interest and then performing a likelihood ratio test[471]. The p-value of ≤ 0.05 meant that the random component was likely to have influenced the model and should not be ignored. The programmes was developed by Victoria and used for analysis under the mixed-effects logistic regression model fitted with the covariates (*metapreg*)[427]. Whereas the SMR is the relative risk data, then *metan*[472] for continuous ES (SMR) with 95%CI and was used for proportion data.

2.10.11.1 Selection of covariates for meta-regression analysis

The selection of appropriate covariates and models for meta-regression was based on the aims of each research question 1) explanatory, 2) exploratory, or 3) prediction. The initial aims when exploring mortality in CS were to explain the estimated effect of CS and proportion of deaths or SMR in endogenous and exogenous CS. Using meta-regression, significance was determined first by univariable analysis (P⩽0·05 was considered significant).

2.10.11.1.1 *Endogenous CS*

Clinical subtype of CS; publication period; perioperative period; duration of follow-up and study duration were considered as the covariates for meta-regression analysis.

2.10.11.1.2 *Exogenous CS*

Underlying disease treated by GC, GC dose, duration of treatment and duration of the study was considered as the covariates on estimates in the meta-regression analysis. GC doses in exogenous CS were extracted from the studies, which were grouped into cumulative dose, average mean dose, maintenance dose, initial dose, and last follow-up dose. The analysis was done for all types of GC prescription.

2.10.12 Publication bias

Publication bias aimed to consider the probability that a positive result influenced publication of the study[473]. Publication bias is a very serious issue for the validity and generalisability of the conclusions made. This bias occurs when small studies are statistically non-significant, are not submitted for publication by the authors. Publication bias can lead to

over-or underestimated pooled ES. One study demonstrated a 15% increased in ES compared to the inclusion, and exclusion of unpublished studies[474].

The publication bias can be analysed using a visualisation technique where funnel plots are created and tested using Egger's test[475]. This is a qualitative assessment with reviewer-dependent interpretation. The x-axis displays the point estimate ES from individual studies, and the y-axis displays the selected precision measurement, such as SE,variance or sample size. Precision increases in relation to the sample size and vice versa. Using these assumptions, small studies are displayed scattered at the periphery of the plot, which causes over-or under-estimated ES. The symmetrical and inverted funnel plots are the ideal visualised graph for unbiased studies. Asymmetrical or skewed funnel plots demonstrated the potential for bias which requireed further exploration and explanation; publication bias is only one of many sources of bias[476]. Other potential sources of asymmetrical funnel plots [477] include: 1) selection biases: publication bias and other reporting biases or biased inclusion criteria; 2) true heterogeneity: the size of the effect differs according to study size or intensity of intervention or differences in underlying risk; 3) data irregularities: poor methodological design of small studies, inadequate analysis or fraud; 4) artefact: heterogeneity due to poor choice of outcome measure; or 5) occurring by chance. It is important to take asymmetrical funnel plot into account for interpretation of pooled estimated ES. At the present time, there is lack of guidance regarding the use, and interpretation of,funnel plots for proportional data.

2.11 Summary of advantages and disadvantages of meta-analysis software that were used for single proportion analyses

Meta-analysis software for data synthesis included RevMan, STATA, R, Meta-Analyst, Comprehensive Meta-Analysis (CMA). The software was used to analyse the proportion of CS and found both advantage and limitation (Table 2-5. Table 2-6, Figure 2-2).

Table 2-5. Comparison of available software tests for single proportion data

Software	STATA16 (*Metaprop*)	STATA16 (*Metapreg*)	STATA16 Built-in program (*Meta*)	Comprehensive Meta-Analysis
Cost	Commercial, paid	Commercial, paid	Commercial, paid	Commercial, paid
User-friendly	moderate	moderate	moderate	Yes
Assumption of data distribution	Normal	**Binomial**	Normal	Normal
Data transformation methods	the double-arcsine transformation and logit	Untransformed, logit and the double-arcsine transformation	logit	logit
Specific for proportion single arm	Yes	Yes	There are non-admission articles if the outcome is zero	Yes
Pooled ES methods (package or macro)	Yes	Yes	Yes	Yes
Bulit-in meta-regression	No	Yes (random-effects logistic regression)	Yes	Yes

Table 2-6. Examples of results (percentage of deaths due to exogenous Cushing's syndrome) analysed using various software programmes.

Software	Metapreg without covariate	Metapreg with covariate use	Metaprop	Meta (DL)	Meta (REML)
Framework	beta-binomial	beta-binomial	ftt	ftt by metaprop	ftt by metaprop
Methods assumption	Binomial-normal distribution	Binomial-normal distribution	Normal-normal distribution	Normal-normal distribution	Normal-normal distribution
Method for transformation	No	No	Yes	Yes	Yes
Method of pooling the ES	logistic regression	logistic regression	DerSimonian-Laird(DL)	DerSimonian-Laird(DL)	Restricted maximum-likelihood (REML)
Co-variate	No	Disease group	NA	NA	NA
Software	Metapreg	Metapreg	Metaprop	Meta	Meta
Overall	0.12	0.12	0.14	0.17	0.17
Vasculitis	0.12	0.18	0.2	0.22	0.23
Connective tissue diseases	0.12	0.1	0.12	0.14	0.14
Inflammatory disease	0.12	0.07	0.09	0.13	0.13
Haematologic diseases	0.12	0.28	0.28	0.28	0.28
Respiratory diseases	0.12	0.06	0.06	0.07	0.07
I^2 (overall)	89.11	NA	98.26	88.51	84.89
Tau^2(overall)	1.41	1.23	NR	0.16	0.16
Prediction interval	[0.01, 0.68]	[0.01, 0.60]	NA	[-0.125, 0.456]*	[-0.082, 0.414]*

Note * the prediction interval for the proportion data should be in the range of 0 to 1. ftt, Freeman-Tukey Double arcsine transformation; REML, Restricted maximum-likelihood; DL, DerSimonian-Laird

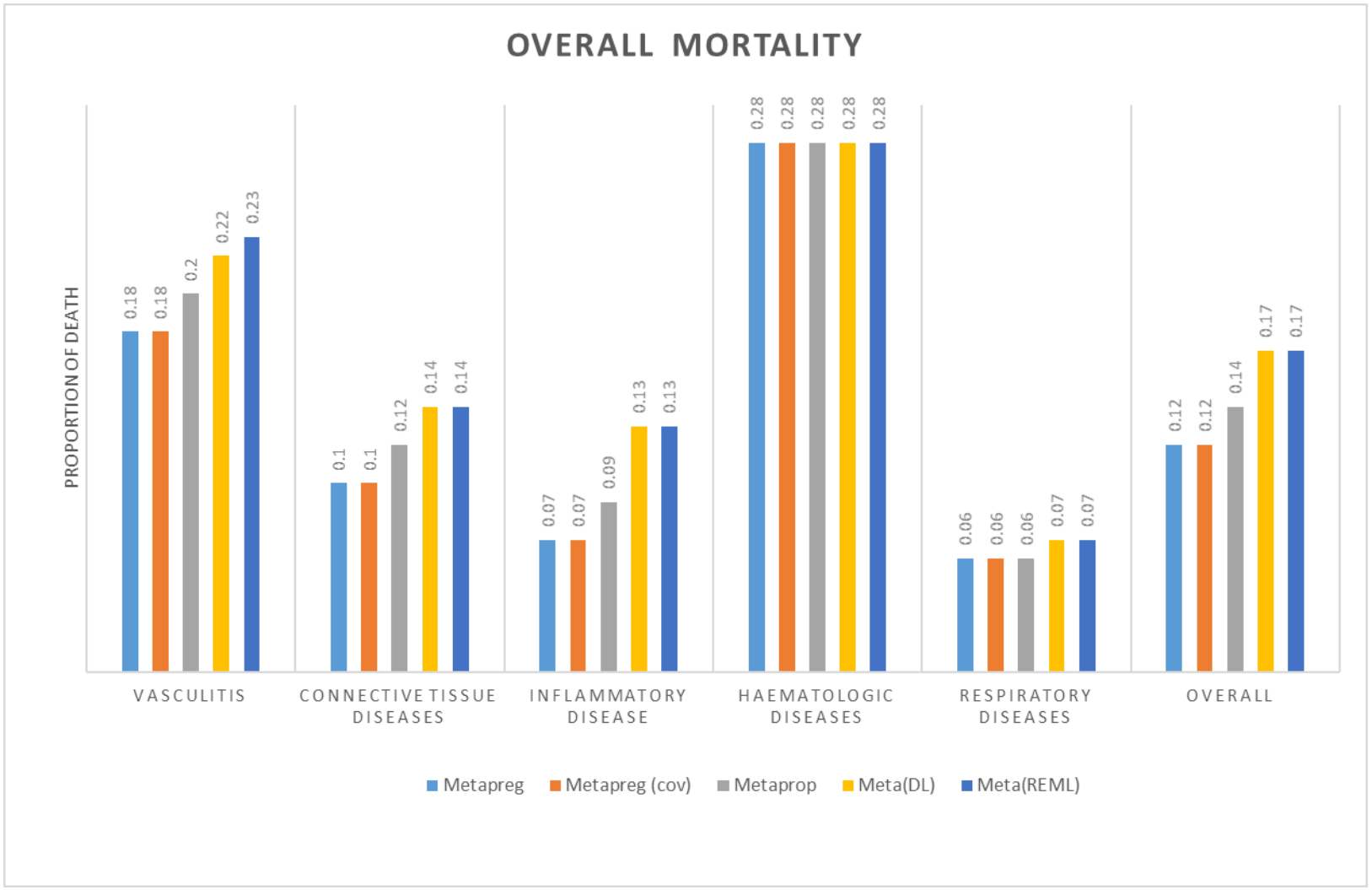


Figure 2-2. Bar graph demonstrated the results by using different program

2.12 Software selection

Statistical analyses were performed using STATA version 16.1 (Stata Corp., College Station, TX, USA). For the descriptive data, the continuous variables and proportion of deaths were presented as a weighted mean across studies. The SMR was calculated as a weighted average of the mortality rate or SMR ± 95%CI for the individual studies. If an SMR with 95%CI was not provided, then it was calculated from the reported observed (O) and expected (E) deaths, as SMR = OE and its 95%CI = SMR±0.96(O/E). The meta-analysis of SMRs was analysed using the ***metan*** command[478] as the best-fitting program for calculating SMR±95%CI. The pooled SMR used the inverse-variance weighting of log-SMR from each study to calculate random-effects summary estimates and forest plots were produced using the exponential the log-SMR for each study. The proportion of deaths was calculated from the number of deaths divided by the total CS patients reported in the article. A pooled ES and meta-analysis of the proportion of deaths were analysed under the assumption of binomial distribution with a REM by using ***metapreg*** command[479].The strength of the metapreg programme was that the model could be fitted to our data which was assumed to be from a binomial distribution. It also allowed covariates (e.g. underlying disease treated by GC or subgroups of CS) that may impact the results to be included in the model and produced outcomes that theoretically were closed to natural or true data.

2.13 Strength and limitation of the meta-analysis

The strengths of undertaking a meta-analysis were 1) to impose a discipline on the process of gathering the data across the selected studies; 2) to improve precision and the ability to answer research questions not posed by the individual studies[480]; 3) to generate the strong evidence by combining the outcomes of interest; 4) capability for exploring the relationships across studies, which are obscured by the individual study or other synthesis methods; 5) prevent the under-or over-interpretation of the different outcomes across the studies; 6) suitable for handling large numbers of published studies and the heterogeneity of study outcomes, which is very difficult in traditional reviews.

The weaknesses are 1) methods not widely accessible to non-specialists; 2) the data is extracted at study and not individual patient level; 3) the clinical and statistical heterogeneity of the included studies; 4) selection bias; 5) validity of included studies; and 6) publication bias.

Chapter 3
The effect of endogenous Cushing's syndrome on all-cause and cause-specific mortality: A systematic review and meta-analysis

3.1 Introduction

Endogenous CS refers to inappropriate hypercortisolism caused by either ACTH hypersecretion or autonomous adrenal cortisol hypersecretion[1, 481]. These are rare diseases with limited epidemiological data, and the publications are mostly restricted to CD[221, 482]. Studies from across the world show an overall incidence of all cause CS between 1.8 and 3.2 cases per million people per year [208, 483], with a prevalence of 57-79 cases per million person-years[208, 224, 483, 484]. CD is the most common subtype accounting for 70% of endogenous CS, followed by ACS 20-25% and ectopic CS 5-10% [485]. The incidence of subtypes of CS is 0.6-2.6 per million per year for CD[208, 221, 482, 483, 486], 1.27 per million per year for all ACS[487], 0.3-0.7 per million per year for benign ACS[208, 224, 483, 487], 0.2 for adrenocortical carcinoma and 0.8 for ectopic CS[208, 221, 396]. The prevalence was 39.1 per million population for CD[221] and 23.4 per million for ACS[487]. Despite the rare incidence of endogenous CS, the difficulties in diagnosing and managing it represent a significant challenge in terms of long-term morbidities and mortality [212]. The long-term complications include cerebrovascular and cardiovascular diseases[216, 217], uncontrolled DM, osteoporosis, psychiatric complications, hypercoagulable states[218] and infections that translate to increased mortality.

The average survival from the onset to death in the historical case series reported by Harvey Cushing was five years [488], with earlier studies of CS reporting 5-year mortality of 50% [489]. The SMR in non-malignant endogenous CS was approximately 1.7-4.8 times higher than the general population[225] and was considerably greater in patients with persistent diseases[225]. Regardless of remission, morality remained higher than the general population[229]. The predictors or risk factors for mortality were cardio- and cerebrovascular disease, (which included ischaemic heart disease, atrial fibrillation, heart failure and peripheral vascular disease), longer-duration of hypercortisolism exposure, delay in diagnosis, persistent disease, DM[229], HT, male, advanced age at diagnosis, a high preoperative plasma ACTH level[225, 490] and multiple treatments[229]. SMR was 5-13.8 for active disease reducing to 2.72 (95%CI 1.88-3.95, $p<0.001$) 10 years after remission[18]. For the known causes of death in CS patients, the most common causes were cardiovascular complications, infection and metabolic complication such as DM.

Over the past 90 years, Harvey Williams Cushing incredibly solved the puzzle of the patient symptoms that arose from pituitary basophil adenoma and secondary adrenal hyperplasia, named later as "*Cushing's syndrome*". Numerous advances in the field include a greater understanding of and ability to diagnose different subtypes of CS, early detection and confirmation of disease, genetic pathogenesis, multi-modality treatment, including surgery and radiation therapy. Together with new medical therapies, we have improved patient outcomes and can now even achieve a "cure". Most studies of all-cause and specific mortality associated with endogenous CS have reported increased estimates[219-227]. However, the pooled SMR of CS remains unclear[226]. Due to the rare incidence of disease with small numbers of patients, single CS cohort studies have insufficient power to analyse mortality data. A previous systematic review and meta-analysis of CS reported in 2012 identified seven publications with a total CS of 797 patients. The analysis was limited to a majority of CD (688 patients) with an SMR of 1.23 (95% CI 1.9, 4.8; p=0.06), and a small proportion of AA (109 patients) with SMR of 1.9 (95% CI 0.93, 3.91; p=0.38). This meta-analysis could not demonstrate a statistically significant difference in CD or AA mortality compared to the general population [226]. To date, no published systematic reviews and meta-analysis have assessed the overall mortality and specific causes of death across all the different subtypes of CS.

3.2 Results

3.2.1 Study selection

A total of 11,527 articles were retrieved in the database search, including 4,637 through PubMed/Medline, 2,548 through Web of Science, 3,586 through EMBASE, 378 through EBSCO (CINAHL) and through references of included articles. 5,492 duplicated articles were excluded. 6,035 articles were included for titles and abstract screening. After screening the articles by title and abstract, 4,720 articles were excluded, leaving 1,315 articles for full-text detailed review. Reasons for exclusion were summarised in Figure 3-1. 61 articles were excluded due to duplication in reporting the same or an overlapping population.

In total, 92 articles were retained. The SMR analyses included 14 articles reporting 20 patient cohorts containing 3,691 patients. Eighty-two articles describing 92 patient cohorts containing 19,181 CS reported the number (or proportion) of deaths. These articles were included in this systematic review and meta-analysis.

For articles that reported the number of deaths, forty-nine study cohorts reported CD (14,971 patients), 24 study cohorts reported ACS (2,304 patients), and 19 study cohorts reported on combined types of CS (1,906 patients). The SMR analyses were performed separately from the proportion of deaths and involved 13 CD cohorts (2,160 patients) and 7 ACS cohorts (1,531 patients). Five articles[208, 220, 222, 491, 492] were included only for SMR analyses because they included duplicated patients in articles that reported the proportion of deaths with extended periods. Under these circumstances, the basic characteristics of articles that reported the proportion of deaths and articles that reported SMR were presented separately.

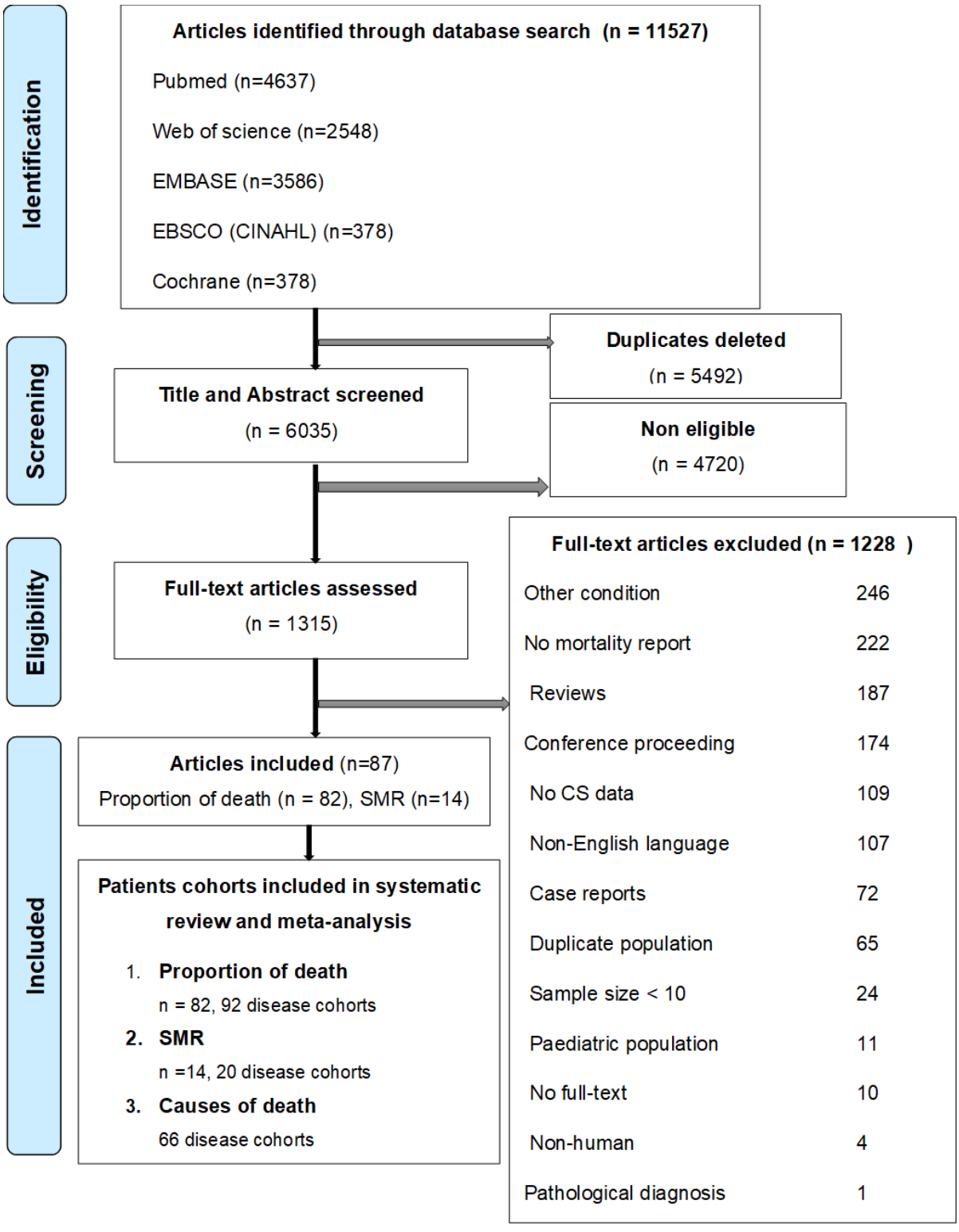


Figure 3- 1. PRISMA flow diagram[493]

3.2.2 Study characteristics

The main characteristics of included studies are shown in Table 3-1. The studies were all cohort studies and published from 1952 to 2021. The sample size ranged from 13 to 5527 patients. Of the total 19181 patients, 84.7% (16250 patients) had CD, 15.2% (2,912 patients) had ACS, and 0.1% (19 patients) were unknown (combined adrenal or pituitary sources).

Table 3- 1. Characteristics of the studies reporting proportion of death included in the systematic review of endogenous CS[14]

1st author (year)	Country	No patients	Observation period	Age at diagnosis mean or [Median] years	No women (%)	Resource [Level of care]	Aetiology									Follow-up mean or [Median] in years	No. of deaths (%)	Analysis		
							CD				ACS				Unknown			SMR	Proportion of death	Causes of death
							Total	microadenoma	macroadenoma	Unknown/mixed size	Total	Adenoma	Bilateral lesion	Other ACS						
Plotz, 1952[489]	US	32	1932-1951	31.0	26 (81.3)	Med [S]	32	0	0	32	0	0	0	0	0	3.5	17 (53.1)	n	y	y
Poutasse, 1953[494]	US	24	1933-1952	NR	NR	Med [S]	0	0	0	0	24	2	22	0	0	NR	6 (25.0)	n	y	y
Sprague, 1953[495]	US	45	1945-1952	35.0	37 (82.2)	Med [S]	0	0	0	0	45	0	45	0	0	NR	7 (15.5)*	n	y	y
Roberts, 1961[496]	US	44	1939-1960	32.0	38 (86.4)	Med [S]	0	0	0	0	44	8	36	0	0	NR	3 (6.8)*	n	y	y
Taft, 1970[497]	Australia	33	1956-1969	40.0	NR	Med [S]	0	0	0	0	33	4	29	0	0	NR	7 (21.2)	n	y	y
Orth, 1971[498]	US	17	1952-1969	37.0	13 (76.5)	Med [S]	0	0	0	0	17	17	0	0	0	NR	3 (17.6)	n	y	y
Orth, 1971[498]	US	64	1952-1969	35.0	36 (56.3)	Med [S]	64	0	0	64	0	0	0	0	0	NR	5 (7.8)	n	y	y
Welbourn, 1971[499]	UK	35	1953-1968	37.0	25 (71.5)	Med [S]	0	0	0	0	35	6	29	0	0	NR	13 (37.1)	n	y	y
Mjolnerod, 1974[500]	Norway	67	1955-1971	38.5	56 (80.0)	Med [S]	3	0	0	3	67	9	58	0	0	6.5	10 (14.3)	n	y	y

[14] Abbreviation: NR refers to no report; No. refers to numbers; C refers to community care; CD refers to Cushing's disease; ACS refers to adrenal CS; S refers to secondary or tertiary or special care level; * refers to perioperative-death (death within 30 days of post-operation); y refers to causes of death were reported; Med refers to medical records; n refers to no cause of death reported

Lawrence, 1976[501]	US Russia	41	1958-1972	NR	NR	Med [S]	41	0	0	41	0	0	0	0	0	NR	2 (4.9)	n	y	y
Prinz, 1979[502]	US	18	1968-NR	27.0	16 (88.9)	Med [S]	18	0	0	18	0	0	0	0	0	NR	0. (0.0)*	n	y	n
Hamberger, 1982[503]	US	74	1970-1979	[50]	NR	Med [S]	0	0	0	0	74	24	50	0	0	NR	4 (5.4)*	n	y	y
Ross, 1985[504]	UK	57	1966-1985	40.6	49 (86.0)	Med [S]	0	0	0	0	57	8	49	0	0	20	21 (36.8)	n	y	y
Welbourn, 1985[505]	UK	79	1953-1980	37.2	53 (67.1)	Med [S]	79	0	0	79	0	0	0	0	0	10	23 (29.1)	n	y	y
Watson, 1986[506]	US	40	1970-1979	[42]	29 (72.5)	Med [S]	40	0	0	40	0	0	0	0	0	0.08	2 (5.0)*	n	y	y
Nakane, 1987[507]	Japan	100	1977-1984	34.0	70 (70.0)	Med [S]	100	76	17	7	0	0	0	0	0	3.2	3 (3.0)	n	y	y
Sarkar, 1990[508]	US	38	1975-1989	NR	30 (79.0)	Med [S]	12	0	0	12	26	20	6	0	0	NR	1 (2.6)	n	y	n
Grabner, 1991[397]	Norway	109	1950-1987	36.0	NR	Med [S]	109	0	0	109	0	0	0	0	0	[12.5]	29 (26.6)	n	y	y
McCance, 1993[24]	Ireland	24	1972-1991	46.0	20 (76.9)	Med [S]	26	0	2	24	0	0	0	0	0	[5.25]	1 (4.2)	n	y	y
Etxabe, 1994[221]	Spain	49	1975-1992	39.6	46 (93.9)	Med [S]	49	0	0	49	0	0	0	0	0	6.59 [4.67]	5 (10.2)	y	y	y
Favia, 1994[509]	Italy	43	1975-1991	47.5	34 (79.1)	Med [S]	43	0	0	43	0	0	0	0	0	NR	4 (9.3)	n	y	y
Zeiger, 1994[510]	US	19	1983-1993	35.6	16 (84.2)	Med [S]	10	0	0	10	9	0	9	0	0	4.58	1 (5.3)	n	y	y
Heerden, 1995[511]	US	66	1981-1991	44.0	NR	Med [S]	24	0	0	24	42	33	9	0	0	NR	3 (4.5)*	n	y	n
Chapuis, 1996[512]	France	78	1980-1995	41.0	58 (74.4)	Med [S]	78	0	0	78	0	0	0	0	0	NR	2 (2.6)*	n	y	y

Imai, 1996[513]	Japan	30	1957-1994	25.7	21 (70)	Med [S]	30	0	0	30	0	0	0	0	0	16.8	5 (7.1)	n	y	y
Imai, 1996[513]	Japan	70	1957-1994	35.5	64 (91.4)	Med [S]	0	0	0	0	70	70	0	0	0	16.8	5(7.1)	n	y	y
Imai, 1996[513]	Japan	13	1957-1994	30.9	10 (76.9)	Med [S]	0	0	0	0	13	0	13	0	0	16.8	4 (30.7)	n	y	y
Feleke, 1998[514]	Ethiopia	16	1985-1995	[24.0]	14 (87.5)	Med [S]	9	0	0	9	7	0	0	7	0	NR	5 (31.3)	n	y	y
Lo, 1999[515]	China	14	1981-1996	45.0	NR	Med [S]	0	0	0	0	14	13	1	0	0	NR	0 (0.0)*	n	y	n
Pikkarainen, 1999[516]	Finland	22	1981-1996	NR	19 (86.4)	Med [S]	0	0	0	0	22	20	2	0	0	NR	2 (9.1)	y	y	y
Pikkarainen, 1999[516]	Finland	44	1981-1996	NR	39 (88.6)	Med [S]	44	38	5	1	0	0	0	0	0	NR	8 (18.1)	y	y	y
Swearingen, 1999[491]	US	161	1978-1996	38.0 [38.0]	129 (70.1)	Med [S]	161	161	0	0	0	0	0	0	0	8.7 [8.0]	6 (3.7)	y	y	n
Chee, 2001[517]	UK	61	1980-1997	37.3	45 (73.8)	Med [S]	61	0	0	61	0	0	0	0	0	[6.9]	3 (4.91)	n	y	y
Lindholm, 2001[208]	Denmark	73	1985-1995	[41.1]	50 (68.5)	Med [S]	73	0	0	73	0	0	0	0	0	[8.1]	7 (9.6)	y	n	n
Lindholm, 2001[208]	Denmark	37	1985-1995	[38.3]	33 (89.2)	Med [S]	0	0	0	0	37	37	0	0	0	[7.1]	4 (10.8)	y	n	n
Salomon, 2001[518]	France	37	1995-2000	49.3	NR	Med [S]	16	0	0	16	21	21	0	0	0	2	0 (0.0)	n	y	n
Rees, 2002[519]	UK	54	1980-2000	41.3	42 (77.8)	Med [S]	54	0	0	54	0	0	0	0	0	[6]	4 (7.40)	n	y	y
Valeri, 2002[520]	Italy	18	1995-2001	52.4	NR	Med [S]	0	0	0	0	18	18	0	0	0	0.01	1 (5.6)*	n	y	y
Yap, 2002[521]	UK	97	1969-1998	39.1	78 (80.4)	Med [S]	97	76	10	11	0	0	0	0	0	7.7	1 (1.0)	n	y	y
Chen, 2003[522]	US	162	1973-1993	NR	NR	Med [S]	162	133	29	0	0	0	0	0	0	5	4 (2.5)	n	y	y

Hammer, 2004[492]	US	289	1975-1998	[37.0]	239 (82.6)	Med [S]	289	140	60	89	0	0	0	0	0	[11.1]	25 (8.7)	y	n	n
Hoybye, 2004[523]	Sweden	35	1990-1999	40.0	26 (74.3)	Med [S]	35	32	2	1	0	0	0	0	0	6	2 (5.7)	n	y	y
Meyer, 2004[524]	Germany	41	1987-2001	47.3	36 (87.8)	Med [S]	4	0	0	4	37	0	0	37	0	4.6	4 (9.8)	n	y	y
Porpiglia, 2004[525]	Italy	21	1993-2002	45.7	NR	Med [S]	16	0	0	16	5	0	5	0	0	0.02	0 (0.0)*	n	y	n
Atkinson, 2005[526]	UK	63	1979-2000	40.3	36 (57.1)	Med [S]	63	0	0	63	0	0	0	0	0	9.6	4 (6.3)	n	y	y
Hara, 2005[527]	Japan	14	1999-2003	49.0	NR	Med [S]	0	0	0	0	14	12	2	0	0	4	2 (14.3)	n	y	n
Iacobone, 2005[528]	Italy	50	1980-2000	43.9	46 (92.0)	Med [S]	0	0	0	0	50	0	0	50	0	11.2	3 (6.0)	n	y	y
Shah, 2006[529]	India	69	NR	NR	NR	Med [S]	69	53	14	2	0	0	0	0	0	NR	5 (7.2)*	n	y	n
Dehdashti, 2007[530]	Canada	25	2004-2007	42.0	19 (76.0)	Med [S]	25	18	7	0	0	0	0	0	0	[1.4]	0 (0.0)*	n	y	n
Dekkers,2007[222]	Netherlands	74	1977-2005	39.1	56 (75.7)	Med [S]	74	63	11	0	0	0	0	0	0	[12.8]	12 (16.2)	y	n	n
Mishra, 2007[531]	India	11	1990-2005	28.0	9 (81.8)	Med [S]	4	0	0	4	12	11	1	0	0	[4.0]	0 (0.0)	n	y	y
Patil, 2007[532]	US (nation)	3525	1993-2002	NR	NR	Med [S]	3525	0	0	3525	0	0	0	0	0	NR	25 (0.7)*	n	y	n
Rollin, 2007[533]	Brazil	108	1989- NR	34.0	83 (76.9)	Med [S]	108	71	17	20	0	0	0	0	0	6	1 (0.9)*	n	y	n
Gil-Ca´rdenas, 2008[534]	Mexico	51	1995-2005	37.0	NR	Med [S]	33	0	0	33	18	0	0	18	0	NR	2 (3.9)	n	y	y
Hofmann, 2008[535]	Germany	426	1971-2004	39.4	325 (76.3)	Med [S]	426	387	39	0	0	0	0	0	0	6.0 [5.6]	3 (0.7)	n	y	y
Lezoche, 2008[536]	Italy	59	1994-2005	52.8	NR	Med [S]	0	0	0	0	59	59	0	0	0	0.01	1 (1.7)*	n	y	y

Liao, 2008[537]	Taiwan	23	2000-2005	35.2	22 (95.7)	Med [S]	0	0	0	0	23	0	0	23	0	NR	0 (0.0)*	n	y	n
Porterfield, 2008[538]	US	253	1995-2005	NR	NR	Med [S]	196	0	0	196	57	54	3	0	0	0.1	0 (0.0)*	n	y	n
Wang, 2009[539]	Taiwan	18	1997-2008	45.7	NR	Med [S]	0	0	0	0	18	18	0	0	0	NR	0 (0.0)*	n	y	n
Bolland, 2011[224]	New Zealand (nation)	37	1960-2005	41.0	33 (89.2)	Survey [S]	0	0	0	0	37	37	0	0	0	[3.1]	3 (8.1)	y	y	n
Bolland, 2011[224]	New Zealand (nation)	30	1960-2005	45.0	22 (73.3)	Survey [S]	30	0	30	0	0	0	0	0	0	[6.9]	5 (16.7)	y	y	n
Bolland, 2011[224]	New Zealand (nation)	158	1960-2005	36.0	122 (77.2)	Survey [S]	158	158	0	0	0	0	0	0	0	[7.5]	19 (12.0)	y	y	y
Clayton, 2011[225]	UK	60	1958-2010	38.2	51 (85.0)	Med [S]	60	0	0	60	0	0	0	0	0	[15.0]	13 (21.7)	y	y	y
Stuijver, 2011[258]	Netherlands	473	1990-2010	42.3	363 (76.7)	Med [S]	360	0	0	360	113	95	18	0	0	[6]	7 (1.5)	n	y	n
Ali, 2012[540]	UK	19	2000-2010	50.6	NR	Med [S]	0	0	0	0	0	0	0	0	19	NR	0 (0.0)*	n	y	n
Hassan-Smith,2012[541]	UK	60	1988-2009	[40]	57 (71.3)	Med [S]	80	0	0	80	0	0	0	0	0	[10.9]	13	n	y	y
He, 2012[542]	China	93	2003-2010	38.0	85 (91.4)	Med [S]	0	0	0	0	93	93	0	0	0	NR	1 (1.1)	n	y	y
Honegger,2012	Germany	83	1998-2011	46.0	NR	Med [S]	83	72	11	0	0	0	0	0	0	3.2	0 (0.0)	n	y	n
Alexandraki, 2013[543]	UK	135	1961-2001	39.2	102 (75.5)	Med [S]	135	103	28	4	0	0	0	0	0	15.9	4 (3.0)	n	y	y
Dekkers, 2013[227]	Denmark (nation)	343	1980-2010	[43.8]	257 (74.9)	Med [S]	211	0	0	211	132	0	0	132	0	12.1	74 (21.6)	n	y	n

Loyo-Varela, 2013[544]	Mexico	62	1973-2011	NR	52 (83.9)	Med [S]	62	60	2	0	0	0	0	0	0	NR	1 (1.6)	n	y	y
Ntali, 2013[219]	UK	16	1962-2009	[45.5]	NR	Med [S]	0	0	0	0	16	0	0	16	0	[5.0]	1 (6.3)	y	y	y
Ntali, 2013[219]	UK	182	1962-2009	[39.5]	137 (75.3)	Med [S]	182	159	23	0	0	0	0	0	0	[12.0]	26 (14.3)	y	y	y
Yaneva,2013[220]	Bulgaria	240	1965-2010	36.0	197(82.1)	Med [S]	240	0	0	240	0	0	0	0	0	[8.8]	66 (27.5)	y	N	N
Yaneva,2013[220]	Bulgaria	84	1965-2010	38.0	76 (90.5)	Med [S]	0	0	0	0	84	84	0	0	0	[4.2]	16 (19.0)	y	n	N
Yaneva,2013[220]	Bulgaria	11	1965-2010	43.0	8 (72.7)	Med [S]	0	0	0	0	11	0	11	0	0	[5.5]	2(18.0)	y	n	N
Ammini, 2014[545]	India	250	1985-2012	28.0	NR	Med [S]	215	185	30	0	35	30	5	0	0	NR	4 (1.6)	n	y	y
Conzo, 2014[546]	Italy	16	2003-2013	43.6	12 (75.0)	Med [S]	0	0	0	0	16	15	1	0	0	4.7	0 (0.0)	n	y	n
Lo, 2014[547]	Phillipines	15	2005-2011	[26]	14 (93.3)	Med [S]	8	0	0	8	7	0	0	7	0	NR	1 (6.7)	n	y	y
Wilson, 2014[548]	Australia	50	1971-2007	[41]	38 (76.0)	Med [S]	50	0	16	34	0	0	0	0	0	5.5	2 (4.0)	n	y	n
Zeiger, 2014 [549]	Spain	26	1982-2009	37.1	21 (80.8)	Med [S]	26	22	4	0	0	0	0	0	0	12	0 (0.0)	n	y	n
Prajapati, 2015[550]	India	17	1991-2013	28.8	NR	Med [S]	13	0	0	13	4	0	4	0	0	[6.7]	3 (17.6)	n	y	y
Shirvani, 2015[551]	Iran	96	1997-2012	31.4	73 (76.0)	Med [S]	96	78	18	0	0	0	0	0	0	3.67	0 (0.0)	n	y	n
Wilson, 2015[552]	US (nation)	5527	2002-2010	NR	NR	Survey [S]	5527	0	0	5527	0	0	0	0	0	NR	25 (0.5)*	n	y	n
Sarkar, 2016[553]	India	64	2009-2014	31.9	51 (79.7)	Med [S]	64	53	11	0	0	0	0	0	0	1.7	2 (3.1)	n	y	y

Cebula, 2017[554]	France	230	2008-2013	42.0	188 (81.7)	Med [S]	230	176	54	0	0	0	0	0	0	1.8	0 (0.0)*	n	y	n
Espinosa-de-los-Monteros, 2017[555]	Mexico	89	1991-2014	[34]	77 (86.5)	Med [S]	89	76	8	5	0	0	0	0	0	[6.3]	5 (5.6)	n	y	y
Johnston, 2017[556]	US	101	2005-2014	[47]	73 (72.3)	Med [S]	101	74	27	0	0	0	0	0	0	[4.3]	6 (5.9)	n	y	y
Losa, 2017[557]	Italy	75	1994-2015	41.4	NR	Med [S]	75	0	0	75	0	0	0	0	0	[6.5]	3 (4.0)	n	y	y
Powell, 2017[558]	Uzbekistan	150	2000-2013	NR	82 (54.7)	Med [S]	131	0	0	131	9	0	0	9	0	NR	10 (7.1)	n	y	y
Mortini, 2018[559]	Italy	496	1990-2016	40.1	390 (78.6)	Med [S]	496	390	106	0	0	0	0	0	0	4.2	1 (0.2)	n	y	y
Martínez, 2019[560]	Spain	119	1980-2016	[38.0]	100 (84.0)	Med [S]	119	62	10	47	0	0	0	0	0	[7.3]	11 (9.2)	n	y	y
Nagendra, 2019[561]	India	21	2005-2018	39.3	NR	Med [S]	14	0	0	14	7	0	7	0	0	[6.1]	7 (33.3)	n	y	y
Ragnarsson, 2019[486]	Sweden (nation)	502	1987-2014	43.0	387 (77.1)	Registry [C]	502	0	0	502	0	0	0	0	0	[13.0]	133 (26.5)	y	y	y
Saini, 2019[562]	India	60	2000-2015	[24.5]	45(75.0)	Med [S]	60	34	18	8	0	0	0	0	0	[3.3]	5 (8.3)	n	y	y
Vala, 2019 (ACS)[395]	Europe (57 centres)	385	2000-2017	44.4	NR	Med [S]	0	0	0	0	385	0	0	385	0	NR	6 (1.6)	n	y	y
Vala, 2019 (CD)[395]	Europe (57 centres	1045	2000-2017	44.4	NR	Med [S]	1045	0	0	1045	0	0	0	0	0	NR	23(2.2)	n	y	y
Ahn, 2020[487]	Korea (nation)	1127	2002-2017	44.8	886 (78.6)	Med [S]	0	0	0	0	1127	0	0	1127	0	[9.7]	74 (6.6)	y	y	n
Guarald, 2020[563]	Italy	151	1998-2017	41.0	107 (70.9)	Med [S]	151	80	35	36	0	0	0	0	0	7.7[7.4]	1 (0.7)	n	y	y
Roldán-Sarmiento, 2021[564]	Mexico (nation)	172	1979-2018	33.0	154 (89.5)	Med [S]	172	136	32	4	0	0	0	0	0	[7.5]	18 (10.5)	y	y	y

3.2.2.1 SMR

In total, 14 publications were included, representing 20 disease cohorts. Five articles were included for SMR analyses alone and were omitted from the proportion of deaths analysis due to duplicated populations in the proportion of death articles[208, 220, 222, 491, 492], as described in Table 3-2. Of these 20 cohorts comprising 3,691 patients, 13 were CD cohorts with 2,160 patients and 7 ACS cohorts with 1,531 patients. There were more subcategories of SMR reported in some enrolled articles, which included SMR of active CD (n=262)[208, 219, 222, 224, 225, 486], SMR of remission CD (n=1234)[208, 219, 222, 224, 225, 486, 541], SMR of microadenoma (n=332; active vs remission) [219, 491], SMR of macroadenoma (n=60; active vs remission)[219, 224], SMR of AA (n=158)[208, 220, 224] and SMR of BAH (n=20)[220, 224]. For causes of death, four cohorts reported SMR for ischaemic cardiovascular diseases, and two cohorts reported SMR for infection. The references for expected numbers of deaths are shown in the footnote of Table 3- 2.

Table 3- 2. Baseline characteristics of articles reporting standardised mortality ratio[15] in CD

Study	Country	Obs period	Age[16]	No. CS	No. death	Follow-up[17]	CS subtypes	SMR (95% CI)
CD								
Etxabe,1994[221]	Spain (multi-centre)	1975-1992	39.6	49	5	6.6(4.7)	unknown 100%	3.8 (2.5-17.9)[18]
Pikkarainen,1999[516]	Finland (single centre)	1981-1996	44.6	44	8	NR	micro 86.4%, macro 11.4%, unknown 2.3%	2.7 (0.9-5.3)[19]
Swearingen, 1999[491]	US (single centre)	1978-1996	38 (38)	161	6	8.7(8.0)	micro 100%	1.0 (0.4-2.2)[20]
Lindholm, 2001[208]	Denmark (nationwide)	1985-1995	(41.1)	73	7	(8.1)	unknown 100%	1.7 (0.7-3.5), proven[21]
			(51.1)	26	11	(8.1)		unproven: 11.5 (5.7, 20.5)
			(38.5)	45	1	(9.1)		remission: 0.3 (0.01-1.7)
			(46.4)	20	6	(10.0)		active : 5.1 (1.9-11.0)

[15] AA, adrenal adenoma; ACS, adrenal CS; BAH, bilateral adrenal hyperplasia; CD, Cushing's disease; CI, confident interval; CS refers to Cushing's syndrome; micro refers to pituitary microadenoma; macro refers to macroadenoma; No. refers to number of; Obs refer to observation; Ref refers to reference; SMR refers to standardised mortality ratio; yr refers to years; US refers to United States; UK refers to United Kingdom

[16] Mean or (median) in years

[17] Mean or (median) in years

[18] Ref for expected no of death: age and sex group structures (Direccion de Informacion Sanitaria y Evaluacion (1989) La mortalidad en la Comunidad Autonoma del Pais Vasco, 1987. Sistema Vasco de Informacion Sanitaria (SISVA), 6.)

[19] Ref for expected no. of death: life tables for the expected mortality of the whole population for 1986-90 obtained from Statistics Finland.

[20] Ref for expected no of death: age- and sex adjusted sample of the U.S. population

[21] Ref for expected no of death: age- and sex specific mortality rates for Denmark 1991–1995

Study	Country	Obs period	Age[22]	No. CS	No. death	Follow-up	CS subtypes[23]	SMR (95% CI)
CD								
Hammer, 2004[492]	US (single centre)	1975-1998	(37.0)	289	25	(11.1)	micro 48.4%, macro 20.8 %, unknown 30.8%	1.4 (1.0-2.1)[24]
			(37.0)	236	17	(11.1)		remission: 1.2 (0.7-3.4)
			(37.0)	53	7	(11.1)		active : 2.8 (1.4-11.0)
Dekkers,2007[222]	Netherlands (single centre)	1977-2005	39.1	74	12	12.8	micro 85.1%, macro 14.9%	2.4 (1.2-3.9)
			39.1	59	7	12.8		remission: 1.8 (0.7-3.8)
			39.1	15	5	12.8		active: 4.4 (1.4-9.1)
Bolland,2011[224]	New Zealand (nationwide)	1960-2005	39.0	188	24	NR	micro 84.0%, macro16.0%	3.2 (2.6-3.8)[25]
			36.0	158	19	(7.5)		micro: 3.2 (2.0, 4.8))
			45.0	30	5	(6.9)		macro: 3.5 (1.3, 7.8)
			36.0	117	NR	(7.5)		micro (remission): 3.1 (1.8, 4.9)
			36.0	37	NR	(7)		micro (active): 2.4 (0.4, 7.8)
			45.0	14	NR	(7.5)		macro (remission): 2.5 (0.4, 8.3)
			45.0	19	NR	(6.9)		macro (active): 5.7 (1.4, 8.3)
			36.0	158	19	(7.5)		micro: 3.2 (2.0, 4.8))
Clayton,2011[225]	UK (single centre)	1958-2010	(38.2)	60	13	(15.0)	unknown 100%	4.8 (2.8-8.3)[26]
			(38.5)	54	8	(17.5)		remission: 3.3 (1.7, 6.7)
			(46.0)	6	5	(15.0)		active: 16.0 (6.7, 38.4)

[22] Mean or (median) in years

[23] Abbreviation: micro refers to croadenoma; macro refers to macro adenma

[24] Ref for expected no of death: age and sex, divided into 5-yr age groups, were obtained from the U.S. Bureau of Census 1995, Monthly Vital Statistics Report 43

[25] Ref for expected no of death: probability of each individual dying during follow-up using data from the Statistics New Zealand: New Zealand life tables (2000-2002) (http://www.stats.govt.nz)

[26] Ref for expected no of death: age, sex, and calendar year-specific mortality rates in the general population of England and Wales

Study	Country	Obs period	Age	No. CS	No. death	Follow-up	CS subtypes	SMR (95% CI)
CD								
Hassan-Smith,2012[541]	UK (single centre)	1988-2009	(40.0)	80	13	(10.9)	unknown 100%	3.2 (1.7-5.4)[27]
			(40.0)	52	5	(10.9)		remission: 2.5 (0.8, 5.8)
			(40.0)	20	4	(10.9)		active: 16.0 (6.7, 38.4)
Yaneva,2013[220]	Bulgaria (single centre)	1965-2010	36.0	240	66	(8.8)	unknown 100%	1.9 (0.7-4.1)[28]
Ntali,2013[219]	UK (single centre)	1962-2009	(39.5)	182	26	(12.0)	micro 87.4%, macro 12.6%	9.3 (6.2-13.4)[29]
			(39.5)	155	13	(12)		remission: 10.8 (6.0, 18.0)
			(39.5)	23	5	(12.0)		active: 9.9 (3.6, 21.9)
			(39.5)	155	19	(12.0)		micro (remission): 7.6 (4.7, 11.7)
			(39.5)	19	3	(12)		micro (active): 6.5 (1.7, 17.8)
			(39.5)	23	5	(12)		macro: 15.6 (5.7, 34.6)
			(39.5)	7	2	(5.0)		macro (active): 45.5 (7.6, 150.2)
Ragnarsson,2019[486]	Sweden (nationwide)	1987-2014	43.0	502	133	(13.0)	unknown 100%	2.5 (2.1-2.9)[30]
			41.0	419	89	(15)		remission: 1.9 (1.5, 2.3)
			56.0	40	22	(4)		active: 6.9 (4.3, 10.0)
Roldán-Sarmiento,2021[564]	Mexico (single centre)	1979-2018	33.0	172	18	(7.5)	micro79.1%, macro 21.9%	3.1 (1.9-4.8)[31]
			33.0	83	8	(7.5)		remission: 1.4 (0.6, 2.6)
			33.0	29	8	(7.5)		active: 1.4 (0.6, 32.6)

[27] Ref for expected no of death: age, sex, and calendar year-specific mortality rates in the general population of England and Wales

[28] Ref for expected no of death220. Yaneva, M., K. Kalinov, and S. Zacharieva, *Mortality in Cushing's syndrome: data from 386 patients from a single tertiary referral center.* Eur J Endocrinol, 2013. **169**(5): p. 621-7.: age and sex mortality rates in the Bulgarian general population (official data may be found at http://www.nsi.bg/otrasal.php?otrZ19)

[29] Ref for expected no of death: age, sex, and calendar year-specific mortality rates in the general population of England and Wales

[30] Ref for expected no of death: general Swedish population for every calendar year and 5-year age group

[31] Ref for expected no of death: age, sex, calendar year-specific mortality rates for the general population of England and Wales

Study	Country	Obs period	Age	No. CS	No. death	Follow-up	CS subtypes	SMR (95% CI)
ACS								
Pikkarainen,1999[516] (combined)	Finland (single centre)	1981-1997	NR	22	2	NR	AA 90.9%, BAH 9.1%	1.4 (0.2-4.9)
Lindholm, 2001[208] (adenoma)	Denmark (nationwide)	1985-1995	(38.3)	37	4	(7.1)	AA 100%	3.5 (1.0-8.9)
Bolland,2011[224] (combined)	New Zealand (nationwide)	1960-2005	39.0	46	6	NR	AA 80.4%, BAH 19.6%	**10.0 (5.8-14.1)**
		1960-2005	41.0	37	3†	(3.1)		AA 7.5 (1.9, 20.0)
		1960-2005	41.0	9	3	(5.7)		BAH 14.0 (3.7, 40.0)
Yaneva,2013[220] (adenoma)	Bulgaria (single centre)	1965-2010	38.0	84	16	(4.2)	AA 100%	1.7 (0.2-6.0)
Yaneva,2013[220] (BAH)	Bulgaria (single centre)	1965-2010	43.0	11	2	(5.5)	BAH 100%	1.1 (0.2-6.3)
Ntali,2013[219] (combined)	UK (single centre)	1962-2009	(45.5)	16	1	(12.0)	unknown 100%	5.3 (0.3-26.0)
Ahn, 2020[487] (combined)	Korea (nationwide)	2002-2017	44.8	1127	74	(9.3)	AA 96.9%, BAH 3.1%	3.0 (2.4-3.7)[32]
CS (Combined AD and ACS) NB: Duplicated patients from the above data								
Pikkarainen,1999[516]	Finland (single centre)	1981-1996	44.6	76	10	NR	Combined	2.0 (0.9-5.3)
Lindholm, 2001[208]	Denmark (nationwide)	1985-1995	(41.4)	139	23	(8.1)	CD (proven) 52.5%, CD (unproven) 18.7% , ACS(AA) 28.8%	3.68 (2.3-5.3)
Bolland,2011[224]	New Zealand (nationwide)	1960-2005	39.0	234	36	(6.4)	CD 80.3%, AA 15.8%, BAH 3.9%	4.1 (2.9-5.6)
Yaneva,2013[220]	Bulgaria (single centre)	1965-2010	38.0	335	84	(7.1)	CD 71.6%, AA 25.1%, BAH 3.3%	2.2 (1.1-4.1)

[32] Ref for expected no of death: age- and sex-matched 2015 Korean National Health and Nutrition Examination Survey (KNHANES)

3.2.2.2 Proportion of death

The CS subtypes was shown in Table 3-3. 97% of patients were recruited from secondary or tertiary hospitals, and only 3% of patients were from community studies. Overall, 92.3% of the studies were retrospective cohorts, 4.3% were prospective cohorts, and 3.4% were combined retrospective and prospective cohorts. In addition, 93.5% of studies were based on analysis of medical records, 5.4% on survey data and 1.2 % on medical registries.

For pituitary tumour status, subtypes of CD were established as microadenoma in 2802 cases (17.3%), macroadenoma in 605 cases (3.7%), and unknown adenoma size for 12,843 patients (79.0%). For ACS, 23.9% (697 patients) had AA, 13.9% (404 patients) had BAH, and in 62.2% (1811 patients) no subtype could be identified (Figure 3- 2).

For articles reporting subtypes of CS with a number of deaths, 93 cohorts were classified into five disease cohorts: CD cohort (n=49), AA cohort (n=7), BAH cohort (n=2), combined ACS cohort (n=15), and a combined analysis for all types of CS (n=20). One cohort was restricted solely to the number of deaths for CD microadenoma patients [491]. Twenty-one cohorts, comprising 10,274 patients, reported the number of deaths during the peri-operative period (less than 30 days post-operative period), whereas 71 disease cohorts, including 8,907 patients, reported long-term mortality. Sixty-one cohorts, including 7,148 patients, reported on the causes of death.

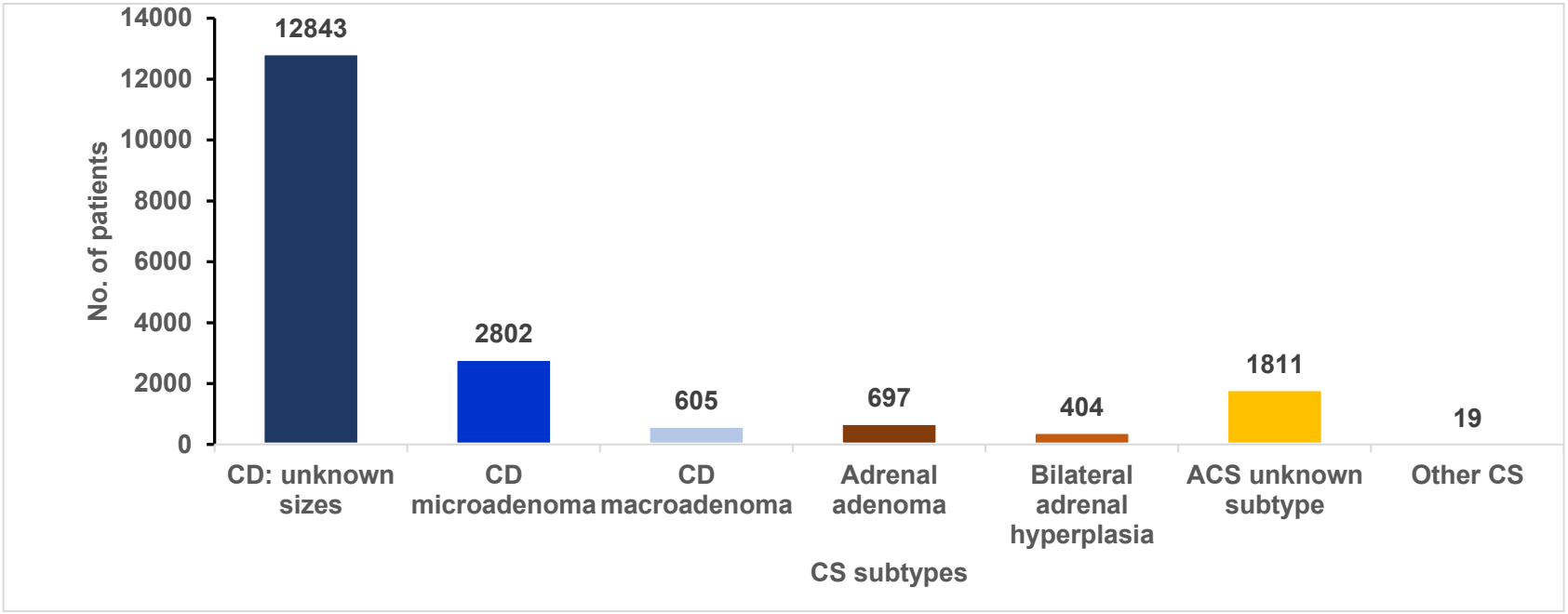


Figure 3- 2. Aetiology of Cushing's syndrome.

Abbreviation: ACS, adrenal Cushing's syndrome; CD, Cushing's disease; CS, Cushing's syndrome; NA, not applicable; No, number.

Table 3- 3: Characteristics of the study cohort broken down as reported subtype of Cushing's Syndrome

Type	No. Study	No. patients (range)	Mean age at diagnosis (range)	No. women (%)* (range)	Mean or (median) follow-up in years (range)	No. deaths (%)* (range)
All disease cohorts	92	19181 (13-5527)	40.9 (27.5-52.8), N=68	7317 (60.5) ({16-390), N= 65	6.4 (0.01-20), N=36 (8.4) (0.01-15), N=28	775 (4.0) (0-133)
CD cohorts	49	14971 (18-5527)	40.4 (25.7-47.5) N=34	3453 (59.4) (16-390), N=41	5.8 (0.1-16.8), N=21 (8.4) (1.4-15), N=20	477 (3.2) (0-133)
Pituitary microadenoma	1	158	36	122	(7.5)	19
Pituitary macroadenoma	1	30	45	22	(6.9)	5
Combined CD	47	14783 (18-5527)	40.5 (25.7-47.5), N=32	3309 (59.4) (16-390), N=39	5.8 (0.1-16.8), N=21 (8.4) (1.4-15.0), N=18	341 (3.1) (0-133)
ACS cohorts	24	2304 (13-1127)	43.2 (30.9-52.8) N=21	1339 (66.2) (10-886), N=14	13.5 (0.01-20), N=7 (8.4) (0.01-9.7), N=4	167 (7.2) (0-74)
AA	7	312 (17-93)	41.8 (35.5-52.8) N=7	195 (80.9) (13-85), N=4	13.4 ({0.01-16.8), N=2 (8.4) (0.01-3.1), N=2	14 (4.5) (0-5)
BAH	2	58 (13-45)	34.1 (30.9-35) N=2	47 (65.7) (10-37), N=5	16.8, N=1	11 (19.0) (4-7)
Combined ACS	15	1934 (14-1127)	43.8 (31.0-49.0) N=12	1097 (63.9) (12-886), N=8	13.4 (4.0-20.0), N=4 (8.4) (5.0-9.7), N=2	142 (6.2) (0-74)
Combined types of CS cohorts	19	1906 (15-473)	38.7 (28.0-50.6) N=13	877 (56.6) (9-363), N=10	6.2 (0.02-12.1), N=8 (8.4) (4.0-6.7), N=4	131(6.9) (0-74)

Abbreviation: N or No refers to the number of study cohorts; range refers to the number of patients across studies; * refered to weighted mean; Abbreviation: AA refers to adrenal adenoma; ACS refers to adrenal Cushing's syndrome; BAH refers to bilateral adrenal hyperplasia; CD refres cushing's disease;

3.2.3 Demographic characteristics

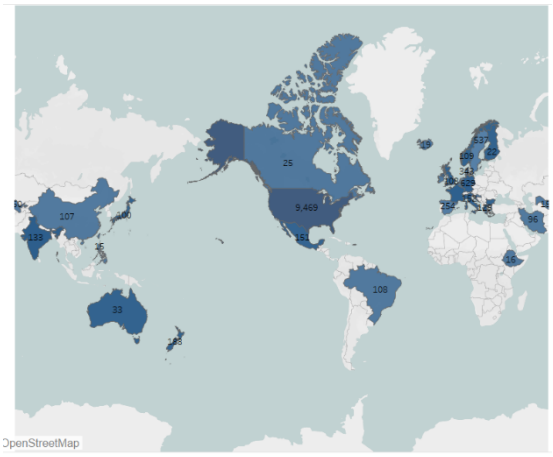
3.2.3.1 Standardised mortality ratio

In a total of 14 articles, four articles used the observation of death from nationwide CS patients including Denmark (73 CD, 37 AA patients)[208], New Zealand (188 CD, 46 ACS patients)[224], Sweden (502 CD patients)[219], and Korea (1227 ACS patients)[487]. Other publications compridsed patients from Spain (multi-centre, 49 CD patients)[221], Finland (44 CD, 22 ACS patients)[516], US (450 CD patients)[491, 492], Netherlands (74 CD patients)[222], UK (322 CD, 16 ACS patients)[219, 225, 541], Bulgaria (240 CD, 96 ACS patients)[220], and Mexico (172 CD patients)[564]

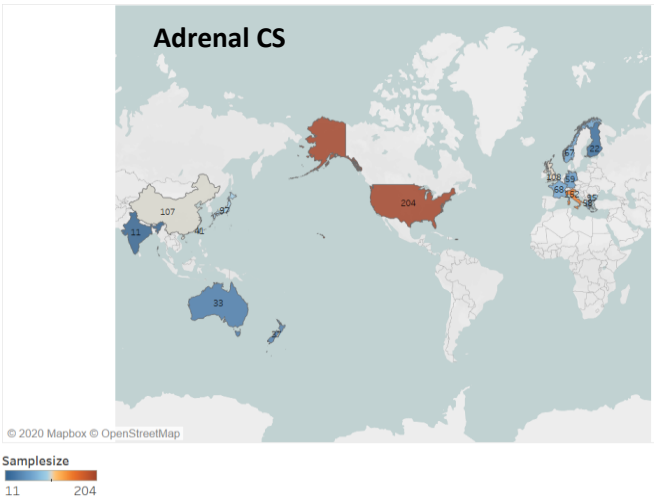
3.2.3.2 Proportion of deaths

The studies were conducted worldwide (Figure 3- 3 A-D). Seven articles including ten study cohorts, reported nationwide data from Sweden[486], Mexico[564], US[532, 552], New Zealand[224], Korea[487] and Denmark[227]. Multiple institutes in European countries were also included in the ERCUSYN Consortia[395]. Regarding the geographical distribution of the studied patients; ***North America (18 disease cohorts, 10074 patients)*** including the US (17 cohorts, 10049 patients) and Canada (1 cohorts, 25 patients); followed by ***Europe (39 disease cohorts, 5665 patients)*** including the UK (13 cohorts, 938 patients), Italy (9 cohorts, 929 patients), Germany (3 cohorts, 550 patients), Sweden (2 cohorts, 537 patients), Netherlands (1 cohort studies,473 patients), France (4 cohorts, 345 patients), Denmark (1 cohort, 343 patients), Spain (3 cohorts, 194 patients), Norway (2 cohorts, 179 patients), Belgium (1 cohort, 71 patients), Finland (2 cohorts, 66 patients), Ireland (1 cohort, 24 patients), and multiple institutes in Europe by Ercusyn (2 cohorts, 1430 patients); ***Asia (20 studies, 2250 patients)*** including India (7 cohort studies,497 patients), Japan (5 cohort studies, 227 patients), Uzbekistan (1 cohort study, 140 patients), China (2 cohort studies, 107 patients), Iran (1 cohort study, 96 patients), Taiwan (2 cohort studies, 41 patients), Phillipines (1 cohort study, 15 patients), and Republic of Korea (1 cohort, 1127 patients); ***Australia (6 studies, 561 patients)*** including New Zealand (4 cohort studies, 478 patients), Australia (2 cohort studies, 83 patients); ***South America (5 cohort studies, 482 patients)*** including Brazil (1 cohort studies, 108 patients), Mexico (4 cohort studies, 374 patients); ***Africa*** only Ethiopia (1 study, 16 patients); and ***mixed*** US-Russia for 1 cohort study (41 patients), respectively.

A. Overall distribution of Cushing's syndrome



B. Adrenal Cushing's syndrome



C. Cushing's disease

D. Mixed types of Cushing's syndrome

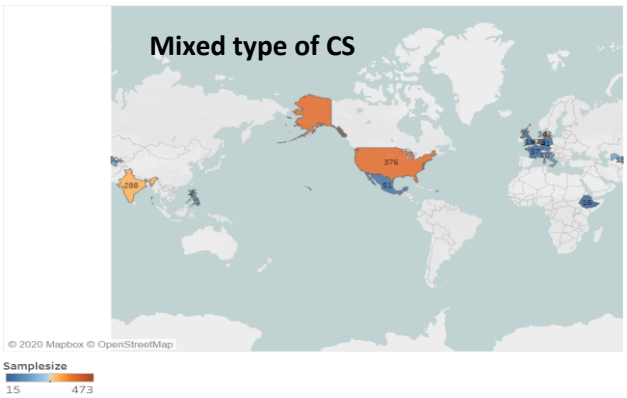


Figure 3- 3: Geographic distribution of Cushing's syndrome (CS) and subtype of CS reported in A) Total patients B) Adrenal CS C) Cushing's disease and D) Mixed types of CS.

3.2.4 Patient characteristics

3.2.4.1 **Standardised mortality ratio**

The reported mean age (described in 11/20 studies) varied between 33 and 44.8 years, with a weighted mean of 41.3 years. The percentage of women (20/20 studies) ranged from 68.5 to 93.9%, with the weighted percentage of women being 63.8%. Median follow-up was reported in 15/20 studies and ranged between 4.2 to 15 years. For SMR, 13 CD cohorts included 1,953 patients and 368 deaths with a weighted mean age of 39.1 years (7/13 studies range 33-43-years), and weighted percentage of women of 64.7% (20/20 studies), varying between 68.5 to 93.8%. 19 of 20 studies enrolled patients before the year 2000.

3.2.4.2 Proportion of deaths

The mean age of patients included in the eligible studies ranged from 27.5 to 52.8 years. The overall weighted mean age at diagnosis was 40.9-year-old (68/92 studies). The weighted mean age in studies reporting CD was 40.4 years (34/49 studies), ACS was 43.2 years (21/24 studies), and combined types of CS was 38.7 years (13/19 studies). The majority of patients were women (60.5% of all cohorts (65/92 studies)), 59.4% for CD cohorts (41/49 studies), 66.2% for ACS cohorts (14/24 studies) and 63.9% for combined CS cohorts (8/15 studies). The average duration of follow up across all studies ranged between 30 days (peri-operative outcomes) and 20 years, with a weighted mean follow-up of 6.4 years.

3.2.5 Risk of bias and quality of evidence

Results of the bias assessment (ROBIN-1) for each of the seven components examined are presented in Figure 3-4, Appendix 3-1. Overall, 43% of studies had a low risk of bias, 45% moderate risk, 2% serious risk, and in 10% the bias assessment was inconclusive. A serious risk of bias was defined by a high risk of confounding selection bias and bias associated with intervention classification. Concerning the different components of bias assessed, 73% of articles had a low risk of confounding or competing for bias; 80% were low in selection bias, 18% had a moderate risk of selection bias and 1% had a serious risk of bias. 81% of articles clearly defined CS diagnosis, whereas 19% of articles did not clearly report the diagnosis. Concerning intervention deviation (e.g. patients receiving different management along the period of studies), 86% of articles had low risk, 13% had a moderate risk of bias, and for 1%, there was no information. 91% of articles had low risk of missing data in completing patient follow-up, and 9% of articles had moderate, which referred to other missing data or patient follow-up. All articles had a low risk of bias related to outcome measurement and reporting.

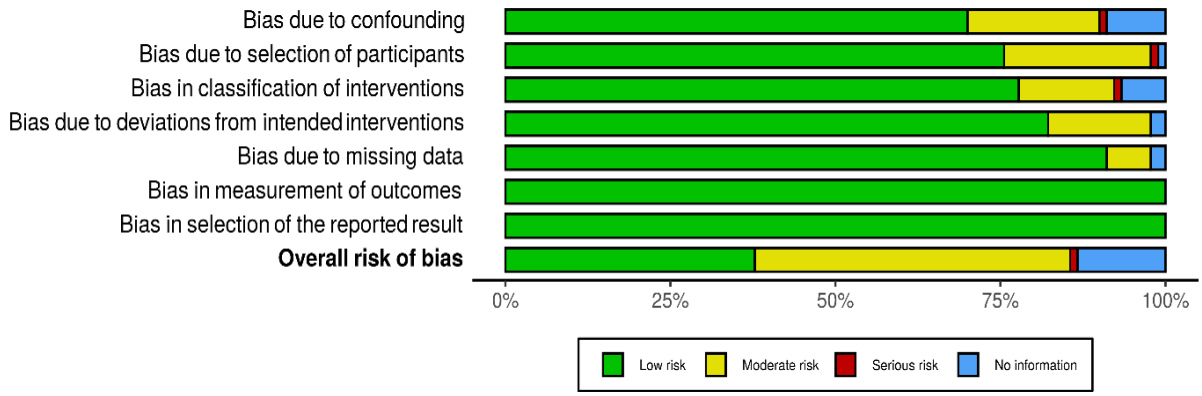


Figure 3- 4. Summary of results of the bias assessment for each study included in the systematic review.

3.2.6 Outcomes: standardised mortality ratio

3.2.6.1 Pooled analysis: standardised mortality ratio of all-cause mortality

SMR was the first outcome of interest, representing the numbers of CS death in the study compared to the expected number of deaths in an age- and sex-matched normal population. A value greater than one means that CS patients are more likely to die, and a value less than one means that they are less likely to die. Fourteen articles (20 patient cohorts) reported SMR in different patient cohorts, which included CD cohorts (n=13; 2,160 patients), and ACS cohorts (n=7; 1,531 patients). The pooled SMR of all CD and ACS irrespective of disease activity in a REM was 3.00 (95%CI 2.3-3.9; I^2=80.5% with the estimated predictive interval of 1.2-7.8; Figure 3-5).

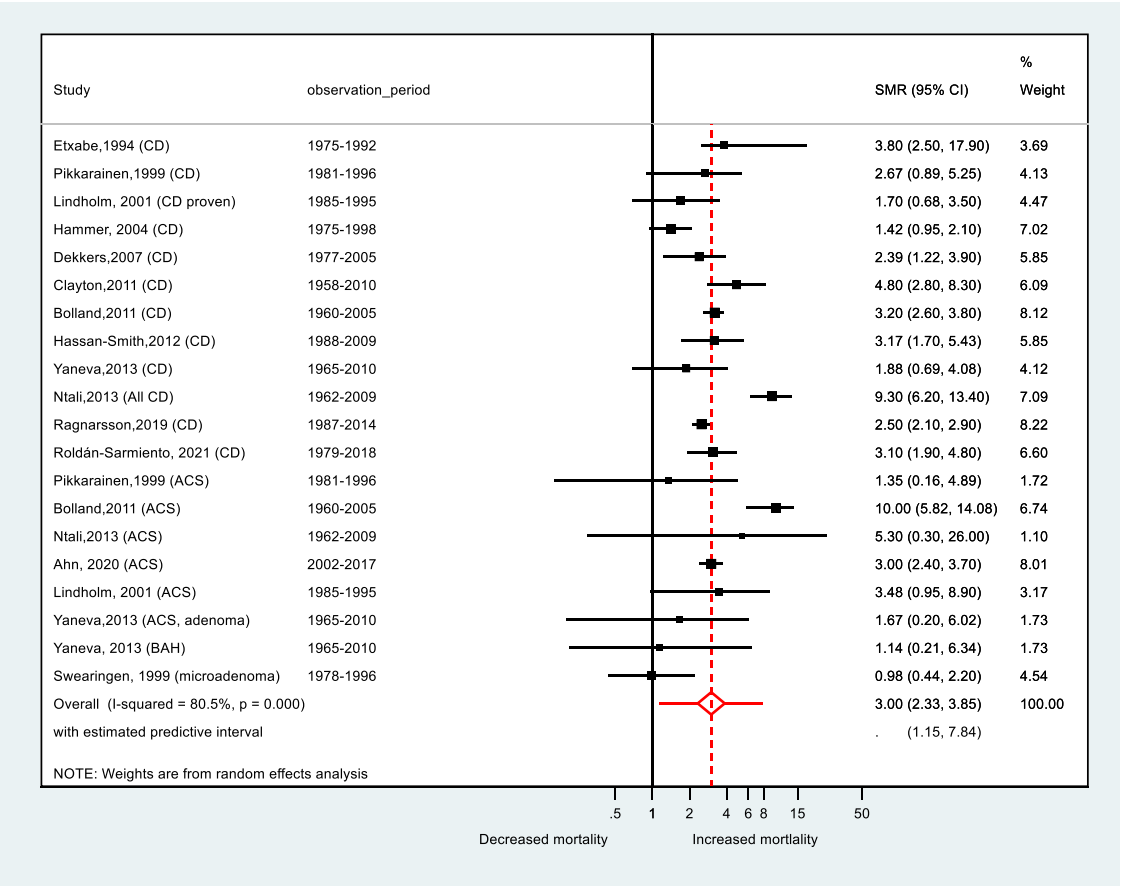


Figure 3- 5. Forest plot presenting standardised mortality ratio (SMR) all-cause mortality of all types of Cushing's syndrome.

ACS, adrenal Cushing's syndrome; BAH, bilateral adrenal hyperplasia; CD, Cushing's disease; CI, confident interval.

3.2.6.2 Pooled analysis: standardised mortality ratio of Cushing's syndrome's subtypes

SMR was analysed for the subtypes of CD and ACS (Figure 3-6). The pooled SMR for CD was 2.8 (95%CI 2.1-3.7 I^2=81.7% with an estimated predictive interval of 1.03-7.58), significantly lower than that of ACS (SMR: 3.34; 95% CI 1.68, 6.63; I^2=77.9% with an estimated predictive interval of 0.46-6.63) (p=0.003). Not surprisingly, the analysis showed high heterogeneity of both CD and ACS, further analysis for the subgroup of CD including activity or size (in the following section 3.2.6.3), the subgroup of ACS (AA and BAH) and year of study or CS management were also explored. When ACS was dissected further, the pooled SMR for AA was 4.06 (95%CI 1.89-8.73; I^2=7.6%) and 4.32 (95%CI 0.4-50.2; I^2=82.1%) for BAH, a difference that was not statistically significant (p=0.51).

3.2.6.3 Factors associated with SMR of Cushing disease (disease activities, pituitary size and gender)

When assessing CD mortality by disease activity (), the pooled SMR of active disease was 5.7 (95%CI 3.7-8.7; I^2=49.0% with an estimated predictive interval of SMR of 1.8-17.8), compared to an SMR in remission of 2.3 (95%CI 1.3-4.0; I^2=83.4% with an estimated predictive interval of 0.36-14.38) (p<0.001). The heterogeneity (I^2) was higher in the remission group, and the predictive interval of mortality for both groups shows wide ranges. One explanation, as illustrated in might be the different definition of CD activity (clinical features and/or laboratory data) and period of evaluation (Table 3-4). Additionally, once disease activity is established, active CS becomes more visible and constant without additional treatment. In comparison, the remission state may change over time in terms of disease activity. This is something to bear in mind in reporting CD-related activity analysis. Three articles reported the SMR for different disease activities for pituitary microadenoma and macroadenoma [219, 224, 491]. The SMR for pituitary macroadenoma was 7.4 (95%CI 1.7-31.9 I^2=81.2%), compared to an SMR for microadenoma of 1.9 (95%CI 0.6-5.9; $I^{2=}$84.4%) (p=0.004). For patients with microadenoma and active disease, SMR was 4.4 (95%CI 1.7-11.4; I^2=6.0%) and 4.9 (95%CI 2.0-11.8; I^2=85.2%) for those in remission. The SMR of active macroadenoma was 15.3 (95%CI 2.01-117.1; I^2=77.9%). There were no SMR data for macroadenoma subjects in clinical remission. The impact of gender on CD mortality showed no statistical difference (p=0.64) between male (SMR 2.20; 95%CI 1.41-3.43; I^2=54.6) vs female (SMR 2.16; 95%CI 0.96-4.84; I^2=86.3%).

Table 3- 4. Definition of remission in included SMR studies

Study	Criteria for cure / active (upper/ lower)
Lindholm, 2001[208], Denmark (nationwide)	**Remission**: At 12–180 days after the operation: plasma cortisol at 30 min after IV 250 mg ACTH < 18 mg/dL (500 nmol/L) and/or UFC <18 mg/24 h (50 nmol); or patient became panhypopituitary or at 0.5 yr after the first operation: UFC values < 90 mg/24 h
	Recurrence: UFC ≥ 90µg/24 h at any time during follow-up
Hammer, 2004[492], US (single centre)	**Initial postoperative remission:** basal or dexamethasone-suppressed plasma cortisol level ≤ 5 µg/dl (≤140 nmol/liter) determined within the first week after surgery; **Within 6 months after surgery:** low or normal plasma or urinary cortisol, resolution of clinical features, and no additional therapy **Long-term**: plasma cortisol after a 1-mg dexamethasone test of ≤ 5 µg/dl (140 nmol/liter) or a normal 24-h UFC at last follow-up, and no undergone additional therapy
	Recurrence: elevated postoperative cortisol levels or underwent additional therapy of CD within six months or required bilateral adrenalectomy after surgery
Dekkers,2007[222], Netherlands (single centre)	**Remission:** Normal suppression of serum cortisol levels to 1 mg oral dexamethasone (cortisol < 100 nmol/l the following morning) and normal 24 h UFC in two consecutive samples
	Persistent or relapse: failure to fulfil biochemical criteria for remission 3–6 months after the first operation
Clayton,2011[225], UK (single centre)	**Remission:** Clinical resolution of symptoms and clinical signs and biochemical normalization of urinary steroid excretion, restoration of plasma cortisol suppression by low-dose dexamethasone, and mean normal plasma cortisol day curve for subjects on metyrapone, within 3y after treatment *or* bilateral adrenalectomy with subsequent GC replacement
	Active disease: failure to achieve these targets within 3 y
Hassan-Smith,2012[541], UK (single centre)	**Remission:** **Initial outcome**: remission was defined by a morning postoperative cortisol level ≤1.8 µg/dl (50 nmol/liter) (measured between d 4th and wk 6th). **Long-term outcome**: cure defined those patients with ongoing absence of hypercortisolism at last follow-up.
	Recurrent disease: defined on biochemical grounds (raised UFC or failure of cortisol suppression on dexamethasone suppression test) and on clinical grounds after initial remission **Persistent disease** was defined biochemically by a postoperative cortisol ≥ 1.8 µg/dl (50 nmol/liter)
Ntali,2013[219] UK (single centre)	**Remission:** 'undetectable' 0900h serum cortisol (according to local assays) after pituitary surgery, adrenalectomy or removal of an ectopic ACTH-producing tumour, or patient was on a GC replacement

	Remission following pituitary radiotherapy: normal 24 h urine cortisol levels or a mean serum cortisol in the range 150–300 nmol/l on a 5-point cortisol day curve or develop ACTH deficiency
	Active disease: Clinical and biochemical assessment: elevated 24-h urine cortisol and lack of suppression of serum cortisol on overnight or low dose dexamethasone suppression tests
Ragnarsson,2019[486] Sweden (nationwide)	**Remission:** based on a review of medical records from clinical visits and included the resolution of clinical features of CS, UFC, salivary or serum cortisol at midnight, cortisol suppression after dexamethasone suppression test, adrenal insufficiency, and/or bilateral adrenalectomy
	Not mentioned
Roldán-Sarmiento, 2021[564] Mexico (single centre)	**Remission**: postoperative serum cortisol <2 µg/dL and requiring GC replacement therapy with subsequent normal 24-hour UFC after GC withdrawal **Disease control** in patients with a persistent or recurrent disease on medical therapy was defined as normal 24-hour UFC (<140 µg/d)
	Uncontrolled CD: high serum or 24-hour UFC in at least 2 consecutive measurements, the inability to suppress serum cortisol <1.8 µg/dL after overnight dexamethasone suppression test

Subgroup	no. of study	no. of patients	no. of death	SMR	95%CI	Prediction interval	Q	I^2	Tau^2	P value*
Article report mortality										NA
CD or ACS (overall)	20	3691	503	3.00	2.33, 3.85	1.15, 7.84	97.6	80.5	0.19	
Combined CS	4	782	153	3.26	2.39, 4.45	1.13, 9.44	4.71	36.3	0.04	
Types of CS (No report activity of diseases)										0.003
CD	13	2160	368	2.80	2.11, 3.72	1.03, 7.58	65.6	81.7	0.18	
ACS	7	1531	135	3.34	1.68, 6.63	0.46, 6.63	27.2	77.9	0.48	
Activity of CD (after treatment or last follow-up)										<0.001
Active	8	206	62	5.70	3.73, 8.73	1.83, 17.8	13.7	49	0.17	
Remission	8	1103	148	2.28	1.29, 4.01	0.36, 14.38	42.1	83.4	0.48	
Size of Pituitary adenoma (CD)										0.004
Microadenoma	2	319	25	1.86	0.59, 5.91	NA	6.41	84.4	0.59	
Macroadenoma	2	53	10	7.38	1.71, 31.93	NA	5.31	81.2	0.91	
Microadenoma activity (CD)										0.790
Active (microadenoma)	2	56	3+NR	4.40	1.70, 11.41	NA	1.06	6	0.03	
Remission (microadenoma)	2	272	19+NR	4.88	2.03, 11.76	NA	6.73	85.2	0.34	
Active disease with size of CD										0.110
Active microadenoma	2	56	3 +NR	4.40	1.70, 11.41	NA	1.06	6	0.03	
Active macroadenoma	2	26	2+NR	15.33	2.01, 117.1	NA	4.52	77.9	1.68	
Subtypes of ACS										0.512
Adrenal adenoma	3	158	23	4.06	1.89, 8.73	NA	2.16	7.6	0.04	
Bilateral adrenal hyperplasia	2	20	5	4.32	0.37, 50.17	NA	5.59	82.1	2.58	
Gender (all CD)										0.640
Female	3	NR	NR	2.20	1.41, 3.43	NA	4.41	54.6	0.08	
Male	2	NR	NR	2.16	0.96, 4.84	NA	7.29	86.3	0.29	
Study source										
CD										0.199
Nationwide	3	809	176	2.71	2.14, 3.44	0.23, 32.43	5.11	60.9	0.02	
Institute	9	1302	187	2.77	1.67, 4.59	0.47, 16.27	58.5	86.3	0.49	
ACS										0.091
Nationwide	3	1398	114	4.82	1.9, 12.22	NA	23	91.3	0.57	
Institute	4	133	21	1.71	0.69, 4.2	0.24, 12.34	1.28	0	0	

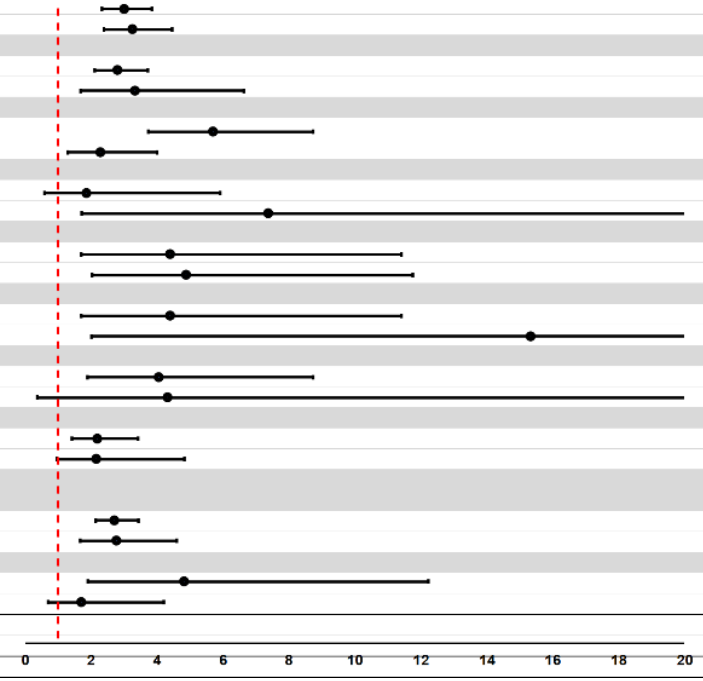


Figure 3- 6. Forest plot of SMR sorted by subgroups of CS and factors contributing to mortality.

*Note: *The heterogeneity between sub-groups analysed by metan is taken only from the inverse-variance fixed-effect model. Abbreviation: AA refers to adrenal adenoma; ACS refers to adrenal Cushing's syndrome; BAH refers to bilateral adrenal hyperplasia; CD refers to Cushing's disease; CI refers to confident interval; CS refers to Cushing's syndrome; NA refers to not applicable; No. refers to number*

3.2.7 Outcomes: proportion of deaths

3.2.7.1 Meta-regression analysis: proportion of death

The overall proportion of death in CS was analysed, taking account of the clinical heterogeneity of CS subtypes. Three meta-analyses fitted with a generalized linear model for the binomial family with a logit link were examined; model 1) meta-analysis in which cooperating CS subtypes were used as the covariate: this is referred to meta-regression analysis; model 2) basic meta-analysis in which particular subtype articles (eg, CD, AA, BAH) were included without considering the covariates. The results were comparable to traditional meta-analysis with subgroup analysis. However, this is more precise since the analysis based on binomial data (model 3 meta-analysis) ensured that all articles were considered in the analysis. The results of this analysis under *metapreg* program were different from model 2 since they revealed the same proportion of death values across the subtypes of CS. This can be explained by the fact that the analysis performed using linear meta-regression included the covariate rejecting null-hypothesis testing (so no effect of subtypes on the results). All articles were then pooled, and the analysis was calculated while ignoring the significance of subtypes. In summary, model 3 is equivalent to a simple meta-analysis without subgroup influence, with the outcome displaying a single value throughout the analysis.

The overall proportion of death results showed a value of 0.05 (without covariate model or simple meta-analysis: 95%CI 0.03-0.06; I^2 58.3%), and 0.05 (with covariate model: 95%CI 0.03-0.07 with an estimated predictive interval of 0.0-0.64) (Figure 3- 7). Even though a heterogeneity value I^2 of 58.3% is acceptable, the predictive interval for the proportion of death is large. Subtypes of CS (CD, ACS or combined CS) were considered a factor contributing to the heterogeneity observed between studies. The LR test for heterogeneity between subtypes was borderline significant (p= 0.05 with Tau^2 1.81). Considering the clinical heterogeneity of the CS subtype, the meta-regression with the CS subtype model was fitted (full forest plot detailed in Appendix 3- 2). The proportion of deaths adjusted by CS subtype was 0.04 (95%CI 0.03-0.06) for CD, 0.02 (95%CI 0.01-0.05) for AA, 0.09 (95%CI 0.03-0.26) for BAH, 0.08 (95%CI 0.04-0.15) for combined ACS, and 0.04 (95%CI 0.02-0.08) for combined all types of CS. The analysis also demonstrated the OR of deaths compared to baseline CD cohorts.

Subtypes	Model	Covarites use (publication enrolled)	No. Studies	No. patients	No. Deaths	Proportion of death	95%CI	Predictive interval	I² (%)	Tau²	Odds Ratio	P value of odds ratio
CD			**49**	**14971**	**477**							
	1	Yes†				**0.04**	**0.03, 0.07**	**0.00, 0.63**	NR		1.00	1.00
	2	No* (only CD cohorts)				0.04	0.03, 0.06	0.00, 0.66	66.11	2.03		
	3	No*(analyse all studies with subgroup analysis)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		
AA			7	312	14							
	1	Yes†				**0.02**	**0.01, 0.05**	**0.00, 0.44**	NR		0.42	0.06
	2	No* (only AA cohorts)				0.04	0.02, 0.09	0.00, 0.66	29.83	0.43		
	3	No* (analyse all studies with subgroup analysis)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		
BAH			2	58	11							
	1	Yes†				**0.09**	**0.03, 0.26**	**0.00, 0.82**	NR		2.01	0.23
	2	No* (only BAH cohorts)				0.19	0.11, 0.31	0.00, 0.66	0.00	0.00		
	3	No* (analyse all studies with subgroup analysis)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		
Combined ACS			15	1934	142							
	1	Yes†				**0.08**	**0.04, 0.15**	**0.00, 0.76**	NR		1.69	0.21
	2	No* (only combined ACS cohorts)				0.08	0.04, 0.15	0.00, 0.66	59.82	1.28		
	3	No* (analyse all studies with subgroup analysis)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		
CS			19	1906	131							
	1	Yes†				**0.04**	**0.02, 0.08**	**0.00, 0.64**	NR		0.98	0.95
	2	No* (only comined all CS cohorts)				0.04	0.02, 0.09	0.00, 0.66	50.09	2.01		
	3	No* (analyse all studies with subgroup analysis)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		
All type of CS			92	19181	775							
	1	Yes†				**0.05**	**0.04, 0.06**	**0.00, 0.64**	NR	1.81	NA	NA
	2	No* (All CS cohorts)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		
	3	No* (analyse all studies with subgroup analysis)				0.05	0.03, 0.06	0.00, 0.66	58.29	1.86		

Figure 3- 7. The proportion of deaths and 95% CI in subtypes of CS.

†refers to the analysis under the equation of †logit(p) = mu + ß_1*subtype of CS (with covariate); *refers to the analysis under the equation of logit(p) = mu + study (No covariate). I^2 for BAH is unreliable because only two articles were enrolled. Model 1 is meta-regression analysis with covariate; model 2 is meta-analysis of CS subtypes; model 3 is the results of meta-regression without covariate. AA, adrenal adenoma; BAH, bilateral adrenal hyperplasia; CD, Cushing's disease; CI, confident interval; CS, Cushing's syndrome; No, number.

3.2.7.2 Mortality trends over the period of study

The analysed articles changed with time, moving from a predominance of adrenal based CS to CD and subtypes of ACS in latter years, especially post-2000 (Figure 3- 8). Transsphenoidal surgery and magnetic resonance imaging were introduced in 1978 and 1980 respectively. These methods have improved diagnosis and treatment, particularly in patients with CD. The discoveries of modern diagnostic testing provide an early and accurate diagnosis; surgical methods and medical treatment also advance, all of these supported by the volume and information of publications, particularly after 2000 as shown in Figure 3-8. For the reasons stated previously and due to the availability of data, the year 2000 was chosen to investigate the difference in mortality in endogenous CS. Because of this, analyses focussed on studies reporting before and after 2000. However, the analysis of SMR articles found that publication before and after 2000 did not represent the SMR between two periods because of the overlap of patients; SMR publications at post-2000 involved patients with the disease before 2000 (Figure 3-9). However, the evidence of changes in mortality is more evident for the proportion of death data (Figure 3- 10). The proportion of deaths was 10% for studies published pre-2000 compared to 3% post-2000 with a decrease of 71% in the proportion of deaths. Moreover, these findings were consistent (approximately 70% reduction) for all subtypes of CS ($p<0.05$) except BAH, for which there were insufficient publications after 2000. The proportion of deaths decreased significantly in CD (OR 0.31, $p<0.001$), AA (OR 0.29, $p =0.02$) and combined ACS (OR 0.24, $p=0.01$).

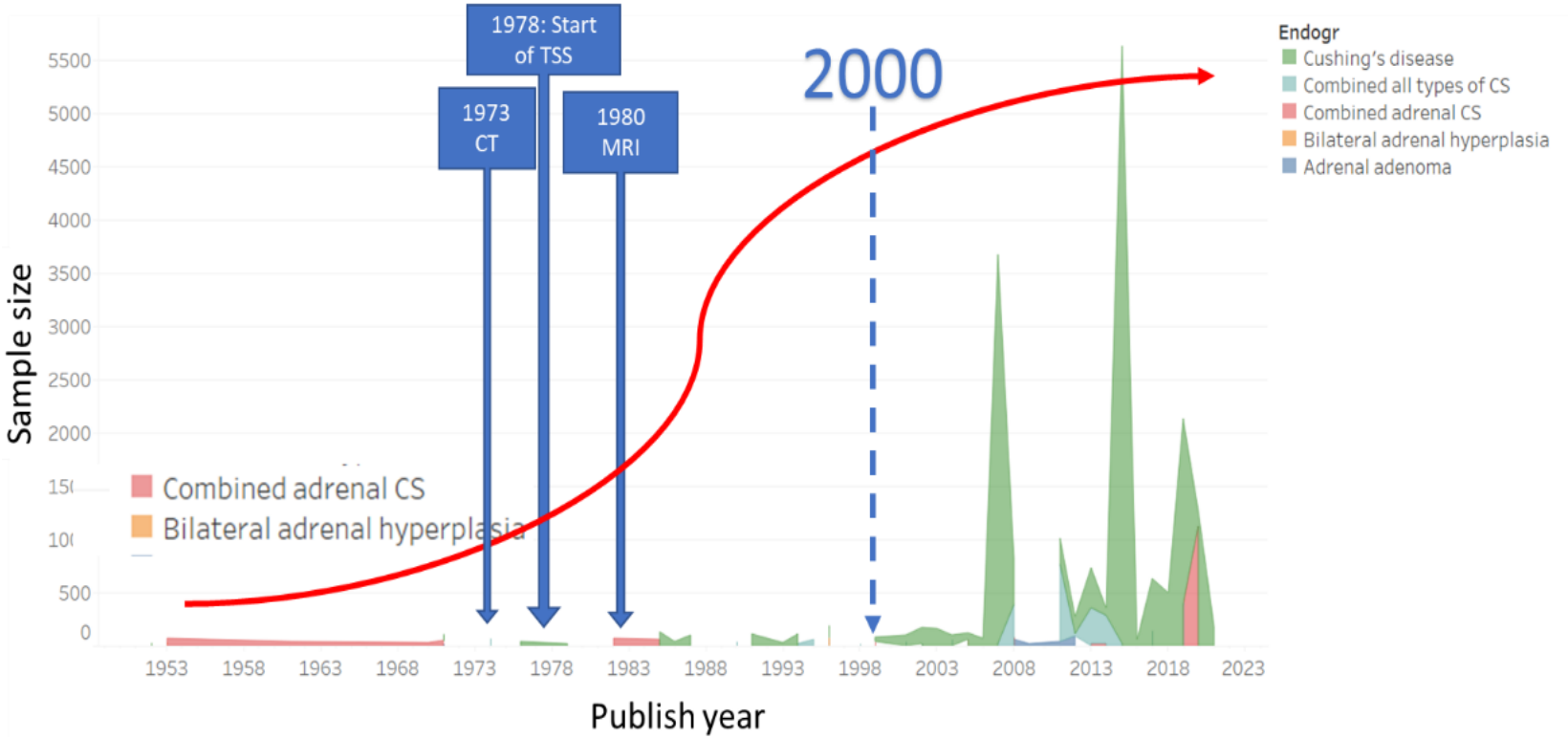


Figure 3- 8. Publications of subtypes of Cushing's syndrome over time; year of publication.

CT, computerized tomography scan; CS, Cushing's syndrome; MRI, magnetic resonance imaging; TSS, transsphenoidal surgery

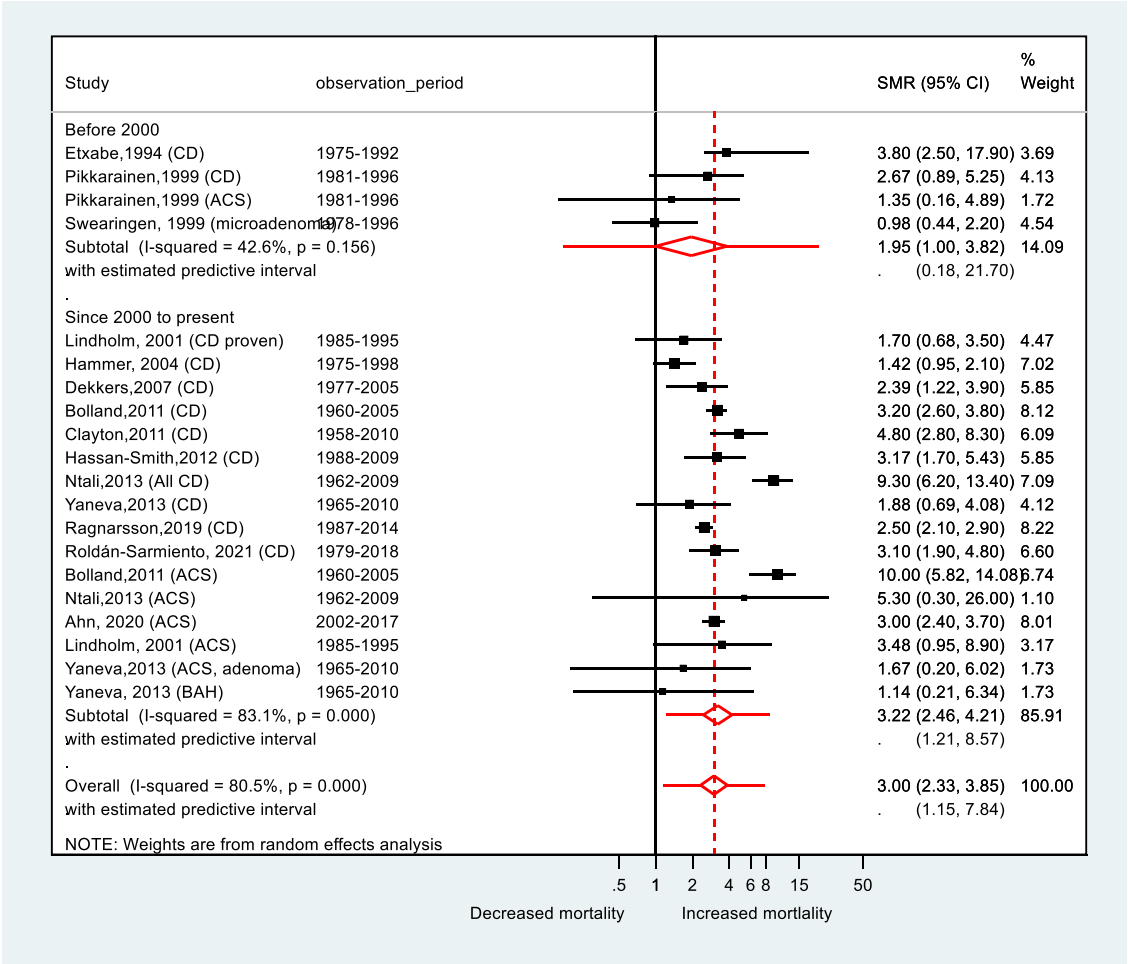


Figure 3- 9. SMR of CS stratified by published year.

ACS, adrenal Cushing's syndrome; AA, adrenal adenoma; BAH. Bilateral adrenal hyperplasia; CD, Cushing's disease; CI, confident interval; CS, Cushing's syndrome; No., number; VS, versus.

Group*	No. of study	No. of patients	No. of death	Proportion of death (95%CI)	Predictive interval	odds ratio (post vs pre 2000)	P value
All CS cohorts						0.29	**<0.001**
Before 2000	31	1410	202	0.1 (0.07, 0.16)	0.01, 0.69		
Since 2000 -2021	61	17771	573	0.03 (0.02, 0.05)	0.00, 0.39		
Cushing's disease cohorts						0.31	**<0.001**
Before 2000	14	753	109	0.1 (0.05, 0.19)	0.0, 0.77		
Since 2000 -2021	35	14218	368	0.03 (0.02, 0.05)	0.0, 0.57		
Adrenal adenoma cohorts						0.29	**0.02**
Before 2000	2	87	8	0.09 (0.05, 0.17)	0.03, 0.22		
Since 2000 -2021	5	225	6	0.03 (0.01, 0.16)	0.01, 0.06		
Bilateral adrenal hyperplasia cohort						NA	NA
Before 2000	2	58	11	0.19 (0.11, 0.31)	NA		
Since 2000 -2021	0	0	0				
Combinded adrenal CS report cohort						0.24	**0.01**
Before 2000	8	303	56	0.15 (0.08, 0.26))	0.03, 0.47		
Since 2000 -2021	7	1631	86	0.04 (0.02, 0.08)	0.01, 0.17		
Combined reports of all types of CS cohort						0.59	0.48
Before 2000	5	209	18	0.06 (0.02, 0.22)	0.0, 0.85		
Since 2000 -2021	14	1697	113	0.04 (0.02, 0.09)	0.0, 0.73		

Figure 3- 10. Proportion of death stratified by published year (before 2000 vs after 2000)

3.2.7.3 Peri-operative mortality vs long-term mortality

The growth in knowledge in CS management over the study window encompassing this meta-analysis can be expected to translate to improvement in patient outcomes and cure rates. Perhaps surprisingly, no articles reported SMR during the peri-operative period. Our study had the power to evaluate this. Thus, the proportion of deaths was taken for the evaluation of peri-operative deaths. To start, a hypothesis test was performed to determine whether there was no difference in the meta-analysis model when the covariate (peri-operative vs long-term follow-up) was included (equals meta-regression analysis) or omitted (equals basic subgroup analysis in meta-analysis). The model comparisons indicated that there was a significant difference between models with and without the covariate ($p<0.05$), indicating that appropriate analysis required incorporating the covariate in the model. The proportion of death adjusted by covariate model was chosen after the heterogeneity testing for covariate effect to the mortality.

The proportion of deaths in the peri-operative period (less than 30 days post-surgery) in any CS cohorts was 0.01 (95%CI 0.01-0.03), compared to long-term mortality of 0.06 (95%CI 0.00-0.05; tau^2 1.52, $p<0.01$) (Figure 3-11 and Appendix 3-3). The publications before 2000 demonstrated significantly higher mortality compared to post-2000 for both peri-operative vs long-term periods in All CS, CD, combined ACS and combined reported of all types of CS. Furthermore, there was no further increase in long-term deaths compared to peri-operative death post-2000.

Group*	No. of study	No. of CS	No. of death	Proportion of death	95% CI	τ^2	P value
All CS cohort				0.04	0.03, 0.06	1.52	<0.01
Peri-operative death	21	10274	77	0.01	0.01, 0.03		
Long-term	71	8907	698	0.06	0.05, 0.09		
All CS cohort (Before 2000)						0.69	<0.001
Peri-operative death	8	379	19	0.04	0.02, 0.08		
Long-term death	23	1031	183	0.15	0.11, 0.21		
All CS cohort (since 2000-2021)						1.28	<0.001
Peri-operative death	13	9895	58	0.01	0.00, 0.02		
Long-term death	48	7876	515	0.04	0.03, 0.06		
CD cohorts (Before 2000)						0.84	<0.001
Peri-operative death	3	136	4	0.02	0.01, 0.10		
Long-term death	11	617	105	0.14	, 0.23		
CD cohorts (2000-2021)						1.43	<0.001
Peri-operative death	6	9484	56	0.01	0.00, 0.02		
Long-term death	29	4734	312	0.04	0.02, 0.06		
AA cohort (Before 2000)						NA	NA
Peri-operative death	0	0	0		NA		
Long-term death	2	87	8	0.09	0.05, 0.17		
AA cohorts (2000-2021)						0.19	0.62
Peri-operative death	3	95	2	0.02	0.00, 0.09		
Long-term death	2	130	4	0.03	0.01, 0.10		
BAH cohort (Before 2000)						NA	NA
Peri-operative death	1	45	7	0.19	0.11, 0.31		
Long-term death	1	13	4	0.09	0.03, 0.26		
Combinded adrenal CS report cohort (Before 2000)						0.06	<0.001
Peri-operative death	3	132	7	0.05	0.02, 0.11		
Long-term	5	171	49	0.28	0.20, 0.37		
Combinded adrenal CS report cohort (2000-2021)						NA	NA
Peri-operative death	1	23	0		NA	NA	NA
Long-term death	6	1608	86	0.04	0.02, 0.08	NA	NA
Combined reports of all types of CS cohort (Before2000)						NA	NA
Peri-operative death	1	66	1	0.02	0.00, 0.08		
Long-term death	4	143	17	0.11	0.05, 0.23		
Combined reports of all types of CS cohort (2000 -2021)						<0.001	1.3
Peri-operative death	3	293	0	0.00	0.00, 1.00		
Long-term death	11	1404	113	0.06	0.03, 0.12		

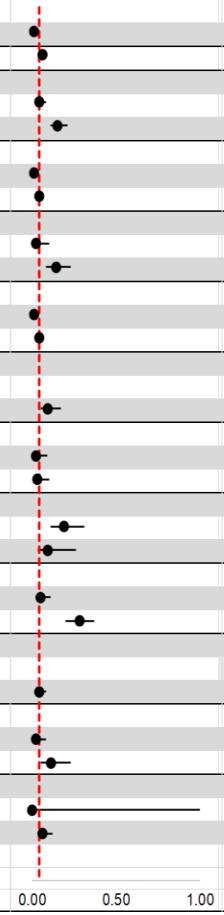


Figure 3- 11. Proportion of deaths in the peri-operative period vs long-term mortality in all subtypes of CS

Abbreviations: AA refers to adrenal adenoma; BAH refers to bilateral adrenal hyperplasia; CD refres Cushing's disease; CI refers to confident interval; CS refers to Cushing's syndrome; No. refers to number; NA refers to not applicable.

3.2.7.4 Peri-operative deaths

Peri-operative deaths for CS subtypes were explored (Figure 3-12). The overall proportion of deaths within the first 30 days of surgery in articles reported before 2000 was 0.04 (95%CI 0.02-0.09) compared to 0.01 (95%CI 0.00-0.01) post-2000 – an 83% decrease in the proportion of deaths after 2000 (p<0.01). Additionally, perioperative mortality fell dramatically by 89% in combined ACS after 2000 (p=0.03). However, among patients with CD, who constituted the majority of the patients in our analysis, peri-operative mortality was not substantially different before and after 2000 (0.02; 95%CI 0.01-0.1 vs 0.01; 95%CI 0.01-1.00) (p=0.21). In summary, both the ACS and total CS groups demonstrated a considerable reduction in perioperative mortality, but not CD specifically. The proportion of death occurring before and after 2000 has not approached zero, especially in CD. Despite improving all aspects of CD care, this remains a key component of the overall mortality, particularly in patients with CD.

Peri-operative death (within 30 days)	No. of study	No. of CS	No. of death	Proportion of death(95% CI)	Predictive interval	Odds compared to before 2000 (percentage of decrease from 2000)	P value
All CS cohort	**21**	**10274**	**77**	0.01 (0.01, 0.02)	0.0, 0.07		<0.01
Before 2000	8	379	19	0.04 (0.02, 0.09)	0.01, 0.19	1.00	
Since 2000-2021	13	9895	58	0.01 (0.00, 0.01)	0.01, 0.04	0.17 (83%)	<0.01
Cushing's disease cohorts	**9**	**9620**	**60**	0.01 (0.01, 0.02)	0.00, 0.09		0.21
Before 2000	3	136	4	0.02 (0.01, 0.10)	0.00, 0.26	1.00	
Since 2000-2021	6	9484	56	0.01 (0.00, 0.02)	0.00, 0.07	0.33 (67%)	0.21
Combined any adrenal CS cohort (ACS, BAH, Combined)	**8**	**295**	**16**	0.04 (0.02, 0.08)	0.01, 0.10		0.03
Before 2000[1]	4	177	14	0.08 (0.04, 0.14)	0.04, 0.16	1.00	
Since 2000-2021[2]	4	118	2	0.02 (0.00, 0.07)	0.00, 0.10	0.21 (89%)	0.03

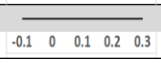


Figure 3- 12. Peri-operative mortality in subtypes of CS[33]

[33] Abbreviations: ACS refers to adrenal Cushing's syndrome; BAH refers to bilateral adrenal hyperplasia; CI refers confident interval; CS refers to Cushing's syndrome; No. refers to number; NA refers to not applicable.

3.2.7.5 Peri-operative mortality and operative procedure

The type of operative procedure is likely to be one factor impacting peri-operative mortality. Twenty-one cohorts that reported peri-operative deaths and operative procedures were analysed to explore this in more detail. I encountered a number of problems with the metapreg program that could not operate complicated variable models, and so the metaprop with Freeman-Tukey Double arcsine transformation was used as an alternative approach. Transsphenoidal surgery (6 CD cohorts) was shown to have the lowest proportion of deaths 0.01 (95%CI 0.00-0.01, I^2=70.7%), whereas adrenalectomy for any type of CS was 0.03 (95%CI 0.00-0.05, I^2=22.0%, n=13). Interestingly this was similar whether the CS aetiology was CD (95%CI 0.00-0.08, n=2) or ACS (0.04 95%CI 0.01-0.08, I^2=38.0%, n=8) (Table 3-5).

Table 3- 5. Operative procedure and peri-operative mortality[34]

Peri-operative death and procedures*	No. of study	No. of CS	No. of death	Proportion of deaths (95% CI)	Predictive interval	I^2	P value
All CS cohort	**21**	**10274**	**77**	0.03 (0.02, 0.06)	0.0, 0.61	71.83	<0.01
Transsphenoidal surgery	6	9484	56	0.01 (0.0, 0.01)	0.0, 0.02	70.7	<0.01
Adrenalectomy	13	519	21	0.03 (0.01, 0.05)	0.0, 0.08	22.0	0.22
Combined treatment or unspecified	2	271	0	0.00 (0.0, 0.0)	NA	NA	NA
CD cohorts	**8**	**9602**	**60**	0.00 (0.00, 0.01)	0.0, 0.52	72.0	0.01
Transsphenoidal surgery	6	9484	56	0.00 (0.00, 0.01)	0.0, 0.02	72.0	<0.01
Adrenalectomy	2	118	4	0.03 (0.0, 0.08)	NA	NA	0.03
ACS cohorts							
Adrenalectomy	8	295	16	0.04 (0.01, 0.08)	0.0, 0.15	38.0	0.13
Combined CS cohorts							
Adrenalectomy	3	106	1	0.00 (0.0, 0.04)	NA	NA	NA

[34] Abbreviations: ACS refers to adrenal Cushing's syndrome; BAH refers to bilateral adrenal hyperplasia; CI refers confident interval; CS refers to Cushing's syndrome; No. refers to number; NA refers to not applicable. The analysis performed with metan program.

3.2.7.6 Trends towards changes in CS deaths versus duration of follow-up

Long-term mortality was evaluated against the duration of follow-up, excluding the peri-operative death window of 30 days. The duration of the follow-up was reported as either mean or median data, but as only three papers described, a mean follow-up period analysis was undertaken for median follow-up. In the case of SMR, fifteen articles disclosed the median follow-up period, with the majority (13/15) following up patients for more than five years (ranging between 3.1 to 17.5 years). The overall SMR of CS patients followed-up for more than five years was 2.3 (95%CI 1.7, 3.2; I^2=42.2%; predictive interval 1.03-5.2), and more than ten years was 3.5 (95%CI 2.0, 6.3; I^2=90.8%, predictive interval 0.5-25.4) (p=0.57) (Figure 3-13).

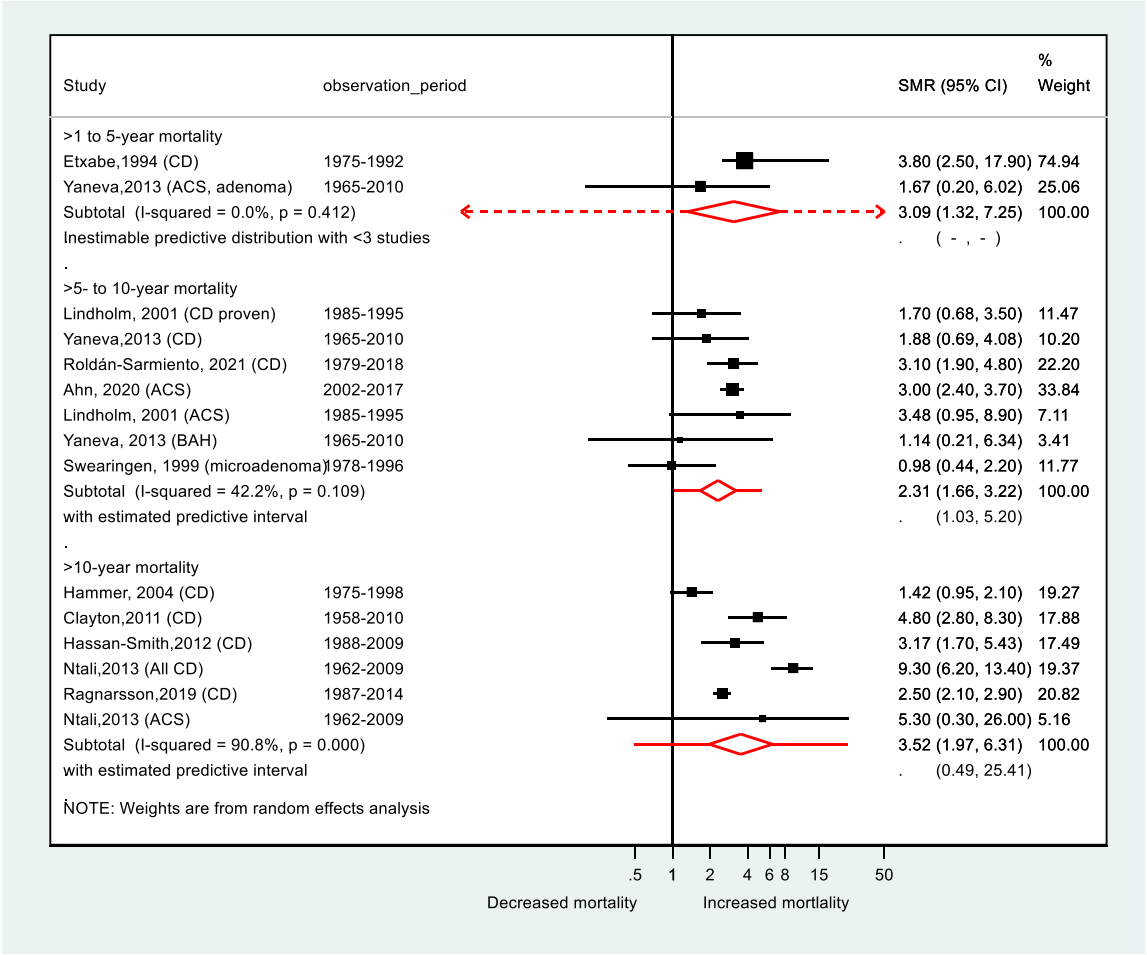


Figure 3- 13. SMR of CS stratified by median duration of follow-up.

SMR of CD for a follow-up duration window of between 5-10 years and more than 10 years was 1.9 (95%CI 1.1-3.2; I^2=53.6%, predictive interval 0.3-14.7) and 3.4 (95%CI 1.9-6.3; I^2=92.6%, predictive interval 0.3-34.5) respectively, but this was not statistically significant (p=0.11) (Figure 3-14). We were unable to stratify the SMR of ACS because of the limited numbers of studies (n=1 for < 5 years and > 10 years and n=3 for 5-10 years).

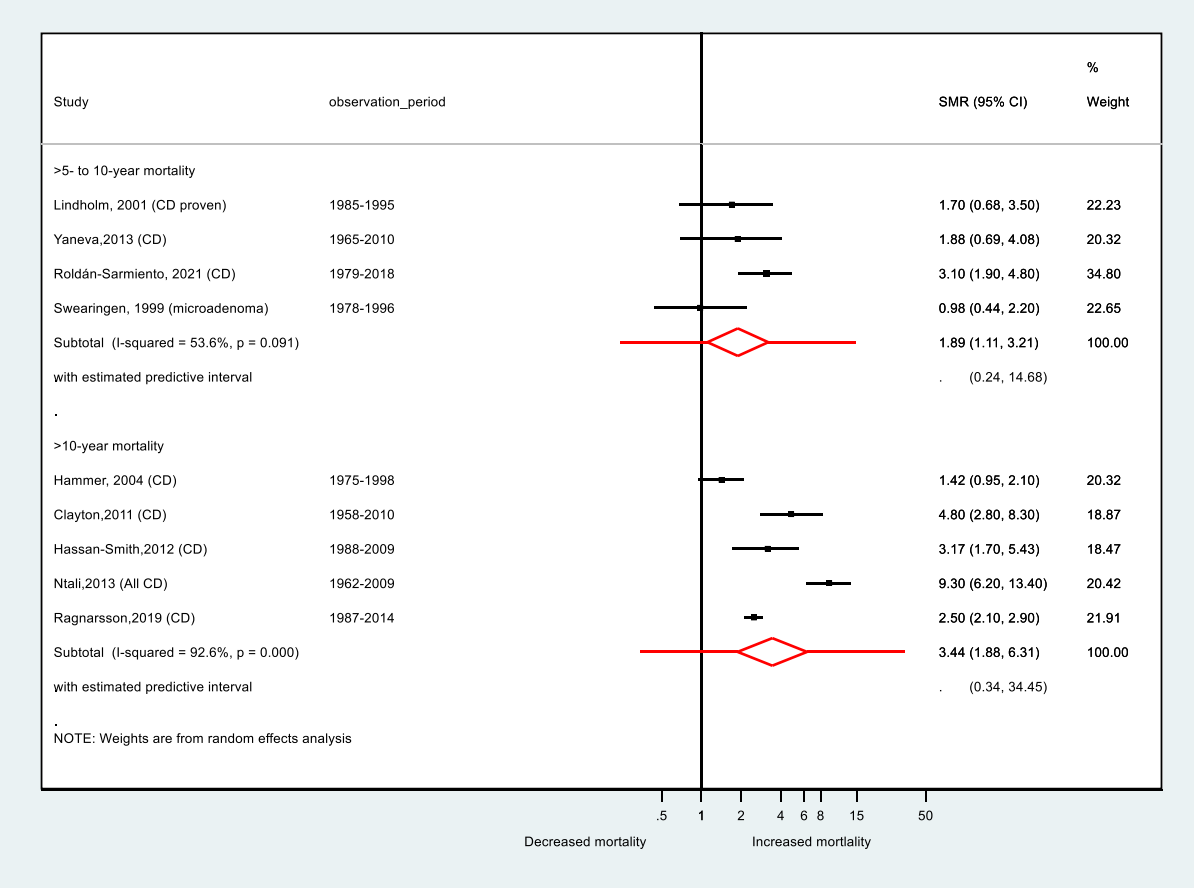


Figure 3- 14. SMR of CD stratified by a median duration of follow-up.

For the proportion of deaths analyses, all studies reported follow-up with a mean or median duration of ≥ 1 year. A separate analysis of duration in mean and median was conducted. Model comparison with or without mean follow-up in years (continuous data) by leave-one-out LR test demonstrated p=0.01. This disproved the null hypothesis that mean follow-up had no impact on deaths. Meta-regression analysis with incorporate mean followed up was considered. Then 5-year stratification duration was taken for meta-regression analysis for all CS and subtypes of CS cohorts. After adjusting the follow-up period (in mean), there was only a trend towards increased deaths after ten years for all CS cohort analyses (p=0.05). Otherwise, there were no statistically significant changes in CS mortality with different mean duration follow-up periods (Figure 3-15).

Long-term death* (mean duration ≥ 1 year)**	No. of study	No. of CS	No. of death		Proportion of death(95% CI)	Predictive interval	Odds compared to 1-5 years	P value
All CS cohort					**0.03 (0.02, 0.06)**	**0.0, 0.87**		**0.17**
> 1 to 5 year	14	1441	36		0.02 (0.01, 0.05)	0.0, 0.79	1.00	
> 5 to 10 year	10	1128	52		0.03 (0.01, 0.10)	0.0, 0.88	1.94	0.37†
> 10 year	8	724	119		0.09 (0.02, 0.27)	0.0, 0.95	4.88	0.05†
CD					**0.02 (0.01, 0.05)**	**0.0, 0.96**		**0.515**
> 1 to 5 year	8	1263	27		0.01 (0.00, 0.05)	0.0, 0.94	1.00	
> 5 to 10 year	9	1058	42		0.03 (0.01, 0.09)	0.0, 0.97	2.53	0.34†
> 10 year	3	191	12		0.04 (0.00, 0.31)	0.0, 0.99	3.78	0.32†
Combined any adrenal CS cohort (ACS, BAH, Combined)					**0.11 (0.04, 0.24)**	**0.01, 0.66**		**0.31**
> 1 to 5 year[1]	2	30	2		0.05 (0.01, 0.29)	0.0, 0.74	1.00	
> 10 year[2]	4	190	33		0.15 (0.09, 0.18)	0.0, 0.76	2.78	0.33†
				0 0.2 0.4				

Figure 3- 15. Proportion of deaths in CS stratified by mean duration of follow-up.

** random-effects logistic regression fitted with the duration of follow-up in mean, ** refers to no cohort that reported death during 1 month to 1 year,[1] refers to no AA and BAH cohorts; [2] refers to it included combined ACS (30 patients, two deaths), and †compared to baseline mean duration >1 to 5 year.*

However, the proportion of deaths in all CS or CD cohorts was significantly increased for a median follow-up duration beyond ten years compared to less than five years (p=0.01) (Figure 3-16). The meta-analysis of stratification of median duration of follow-up could not be performed for ACS and mixed types of CS cohorts because of insufficient study numbers.

Long-term death* (median duration ≥ 1 year)**	No. of study	No. of CS	No. of death	Proportion of death(95% CI)	Predictive interval	Odds compared to 1-5 years	P value
All CS cohort	**27**	**3336**	**399**	**0.07 (0.05, 0.10)**	**0.02, 0.21**		**0.01**
> 1 to 5 year	7	304	21	0.06 (0.03, 0.12)	0.02, 0.20	1.00	
> 5 to 10 year	15	2099	164	0.06 (0.04, 0.09)	0.02, 0.817	0.96†	0.93
> 10 year	5	933	214	0.20 (0.12, 0.34)	0.06, 0.49	3.43†	0.01
CD	**20**	**2529**	**303**	**0.08 (0.06, 0.11)**	**0.03, 0.17**		**<0.001**
> 1 to 5 year	4	235	16	0.06 (0.03, 0.12)	0.02, 0.18	1.00	
> 5 to 10 year	11	1361	73	0.05 (0.03, 0.08)	0.02, 0.13	0.87†	0.76
> 10 year	5	933	214	0.21 (0.13, 0.32)	0.08, 0.42	3.38†	0.01
Combined any adrenal CS cohort (ACS, BAH, Combined)	**3**	**1180**	**78**	**0.07(0.04, 0.13)**	**NA**		**0.78**
> 1 to 5 year[1]	2	53	4	0.08 (0.03, 0.18)	**NA**	1.00	
> 5 to 10 year[2]	1	1127	74	0.07 (0.05, 0.08)	**NA**	0.87†	0.78
Combined any CS cohort	**4**	**527**	**18**	**0.08(0.02, 0.27)**	**NA**		**0.7**
> 1 to 5 year	1	16	1	0.06 (0.00, 0.30)	NA	1.00	
> 5 to 10 year	3	511	17	0.09 (0.02, 0.34)	NA	1.97†	0.71

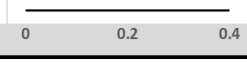


Figure 3- 16. Proportion of deaths in CS stratified by median duration of follow-up.

** random-effects logistic regression fitted with the duration of follow-up in mean, ** refers to no cohort that reported death during 1 month to 1 year,[1] refers to no AA and BAH cohorts; [2] refers to it included combined ACS (30 patients, two deaths), and †compared to baseline median duration >1 to 5 year.*

3.2.8 Outcome: causes of death

The causes of death in CS were reported in 68 study cohorts and included 592 deaths from 7,255 patients (Table 3-6, Figure 3-17). Twenty-five study cohorts did not report the causes of death, 14 studies had no deaths (879 CS patients), and 11 studies had 219 deaths from 12,179 patients, but no details on the cause of death.

The most common cause of death was vascular disease (that included coronary atherosclerotic heart disease, cerebrovascular disease, and VTE) which resulted in 43.4%, 43.7%, 38.6% and 47.1% of total deaths for all CS, CD, ACS, and combined CS type patients, respectively. Cardiovascular disease was the major contributor to death in 27.4%, 29.4%, 21.6%, and 23.5% of all patients, CD, ASC, and combined cohorts. The second commonest cause of death was an infection in 12.7%, 11.5%, 15.9%, and 15.3% of all CS, CD, ASC, and combined cohorts. Thirdly, malignancy was the cause for 10.6%, 11.5%, 4.2% and 12.9% in all CS, CD, ACS, and combined CS type patients, respectively. VTE was reported for all CS of 4.4, which was comparable to CD (3.4%) and ACS (3.4%). However, the combined subtypes of CS reported in the papers showed the greatest fatality rate due to VTE (10.6%).

It was notable that there were also reported deaths from gastrointestinal diseases (3.2%) – be it pancreatitis, gastrointestinal bleeding or unspecified gastrointestinal disorders; active CS (3.5%), adrenal insufficiency (3.0%) and suicide (2.2%). Adrenal insufficiency and suicide as a cause of death was higher in ACS patients compared to all CS cohorts, CD and combined subtype reports. An unknown cause of death was found in 15.5% of the articles.

Table 3- 6. Reported cause of death in CS studies and breakdown by subtype

Causes of death	Total (%)	CD (%)	ACS (%)	Mixed (%)
	n=68	n= 38	n=17	n=13
No. patients	**7255**	**5253**	**1055**	**947**
Cardiovascular disease	257 (43.4)	183 (43.7)	34 (38.6)	40 (47.0)
Atherosclerotic cardiovascular disease	162 (27.4)	123 (29.4)	19 (21.6)	20 (23.5)
Cerebrovascular diseases	69 (11.7)	46 (11.0)	12 (13.6)	11 (12.9)
Venous thromboembilsm	26 (4.4)	14 (3.3)	3 (3.4)	9 (10.6)
Infection	75 (12.7)	48 (11.5)	14 (15.9)	13 (15.3)
Malignancy	63 (10.6)	48 (11.5)	4 (4.5)	11 (12.9)
Active diseases	21 (3.5)	17 (4.1)	2 (2.3)	2 (2.4)
Gastrointestinal disorder	19 (3.2)	14 (3.3)	4 (4.5)	1 (1.2)
Pancreatitis	5 (0.8)	3 (0.7)	2 (2.3)	0 (0.0)
Gastrointestinal bleeding	5 (0.8)	3 (0.7)	1 (1.1)	1 (1.2)
Unspecified gastrointestinal causes	9 (1.5)	8 (1.9)	1 (1.1)	0 (0.0)
Adrenal insufficiency	18(3.0)	8 (1.9)	7 (8.0)	3 (3.5)
Suicide and psychosis	13 (2.2)	8 (1.9)	5 (5.7)	0 (0.0)
Surgery	9 (1.5)	7 (1.7)	2 (2.3)	0 (0.0)
Respiratory failure	8 (1.4)	5 (1.2)	3 (3.4)	0 (0.0)
Renal failure	5 (0.8)	5 (1.2)	0 (0.0)	0 (0.0)
Other hemorrhage	3 (0.5)	1 (0.2)	1 (1.1)	1(1.2)
Multi-organ failure	2 (0.3)	1 (0.2)	1 (1.1)	0 (0.0)
DM	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)
Trauma	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)
Musculoskeletal	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)
Amyloidosis	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)
Hemolysis	1 (0.2)	0 (0.0)	1 (1.1)	0 (0.0)
Sudden death	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)
Nelson's syndrome	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)
Undetermined	92 (15.5)	68 (16.2)	10 (11.4)	14 (16.5)
No. total death	**592 (8.2)**	**419 (8.0)**	**88 (8.3)**	**85 (9.0)**

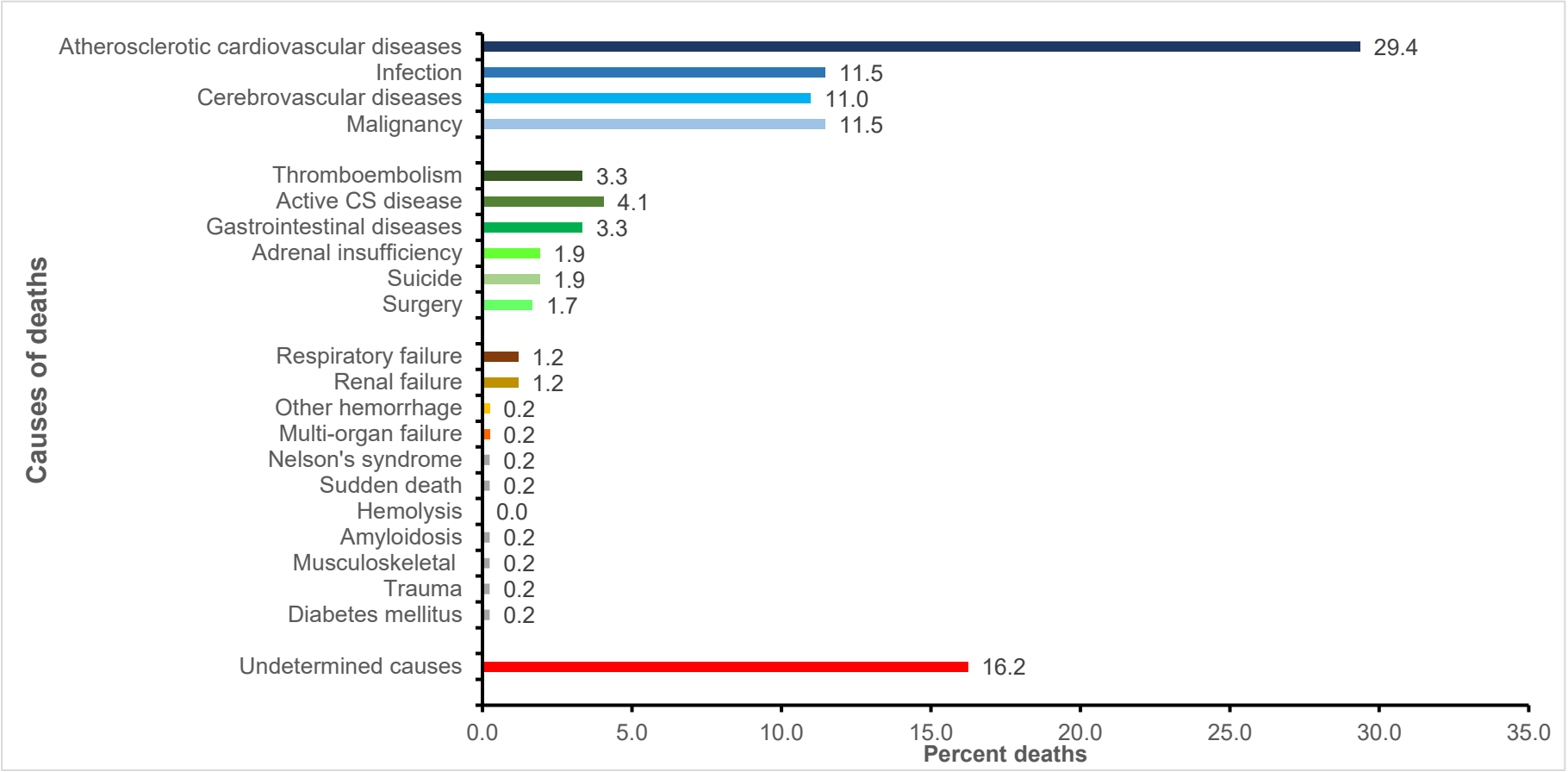


Figure 3- 17. Causes of death in Cushing's syndrome.

Only cardiovascular disease and infection provided the necessary numbers to conduct a meta-analysis to determine SMR of specific causes of death. All of the underpinning data were from CD cohorts. The pooled SMR values for cardiovascular disease and infection were 5.53 (95%CI 2.51-12.21; I^2=81.5%;Figure 3-18) and 8.5 (95%CI 1.65-43.42; $I^{2=}$36.1; Figure 3-19), respectively.

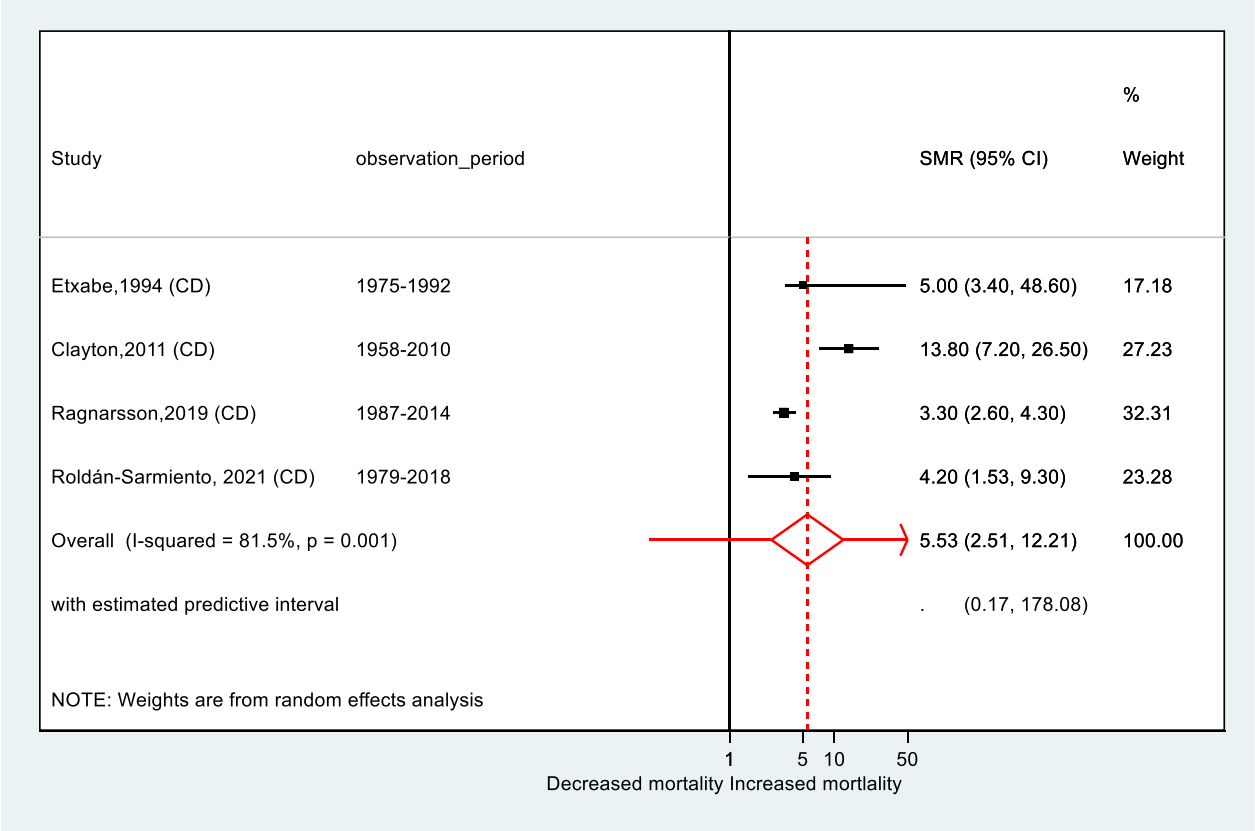


Figure 3- 18. Forest plot demonstrated SMR of cardiovascular disease

Abbreviations: CI refers confident interval; CD refers to Cushing's disease; ES refers to effect size. The analysis performed with metan program.

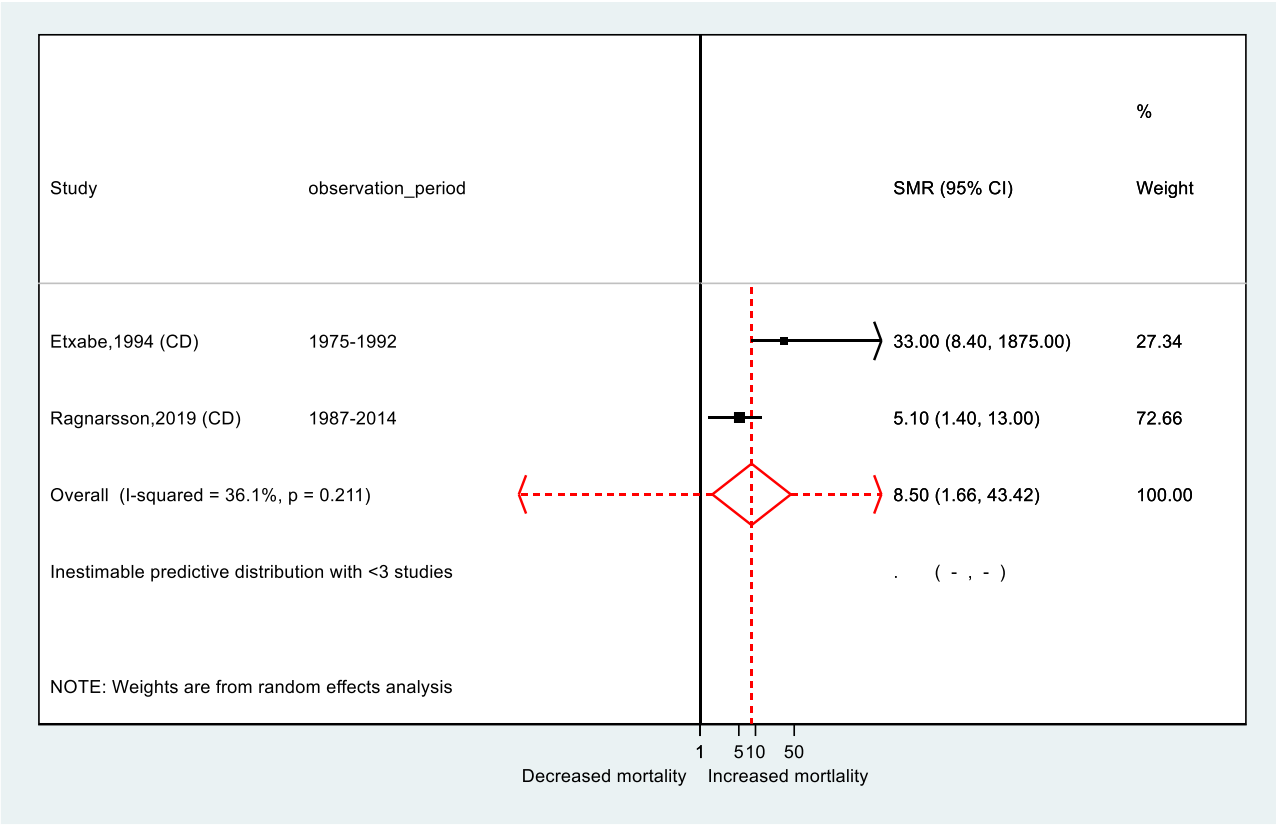


Figure 3- 19. Forest plot demonstrated SMR of infection

Abbreviations: CI refers confident interval; CD refers to Cushing's disease; ES refers to effect size. The analysis performed with metan program.

More detail on the cause of peri-operative causes of death were explored. Twenty-one study cohorts reported peri-operative death, including 10,274 patients with 77 deaths. In addition to the articles reporting only peri-operative deaths, we looked intensively for the timing of deaths in the long-term follow-up articles. There were an additional 30 deaths that occurred within 30 days of the first treatment, nine deaths within 60 days of the first treatment and 26 deaths within 90 days of treatment. None of these patients was duplicated across the studies. The most common reported causes of death during the 30 day peri-operative period were infection 11.3%, VTE 7.0%, CVD 5.6%, bleeding 2.8%, pancreatitis 2.1%, adrenal insufficiency 2.1%, respiratory failure 2.1%, intracerebral haemorrhage 0.7%, malignancy 0.7%, Nelson's syndrome 1%, however, the cause was unknown for 62.7%. Regarding infection, pneumonia was reported in 39.4%, meningitis in 14.1%, urinary tract infection in 2.8%, peritonitis in 2.8%, wound infection and candidiasis 1.4% each, and unspecified sources in 25.8%. Although malignancy was referred to as the third common cause of death, the aetiology of malignancy was known for only 18 of 55 cases: 6 of these were pituitary carcinomas, five pulmonary malignancies, three colon or rectal cancers, and one each for prostate, uterus, pancreas and medullary thyroid cancer.

3.2.8.1 Meta-regression analysis for prediction of overall death

To explore the prediction of death, the univariate analysis demonstrated that duration of follow-up, cardiovascular diseases, cerebrovascular diseases, VTE, malignancy, infection, active diseases, gastrointestinal diseases, adrenal insufficiency, surgery and suicide had a statistically significant effect on the proportion of deaths (Table 3-7). Additionally, the meta-analysis of SMR of CD in women (2.20; 95%CI 1.41-3.43; I^2=54.6%) vs men (2.16; 95%CI 0.96-4.84; I^2=86.3%) was statistically different (P=0.02) (Figure 3-20).

Table 3- 7. Cause of death and results of univariate regression

Covariate	No. study	Coefficient (95% CI)	P value
Mean age at diagnosis	68	-0.003 (-0.006, 0.0002)	0.07
Women	65	-0.00003 (-.0002, .0001)	0.73
Duration of follow-up in mean (year)	28	0.02 (0.01, 0.02)	<0.001
Duration of follow-up in median (year)	36	0.01 (0.004, 0.01)	0.001
Causes of death			
Atherosclerotic cardiovascular diseases	68	0.01 (0.004, 0.01)	<0.001
Cerebrovascular diseases	68	0.02 (0.01, 0.02)	<0.001
Infection	68	0.01 (.01, .02)	0.001
Venous thromboembolism	68	0.02 (0.001, 0.04)	0.036
Malignancy	68	0.01 (0.003, 0.01)	0.002
Active diseases	68	0.03 (0.01, 0.05)	0.001
Gastrointestinal disorder	68	0.03 (0.01, 0.04)	0.002
Adrenal insufficiency	68	0.08 (0.05, 0.11)	<0.001
Surgery	68	0.06 (0.01, 0.11)	0.011
Suicide and psychiatric diseases	68	0.04 (0.02, 0.06)	<0.001

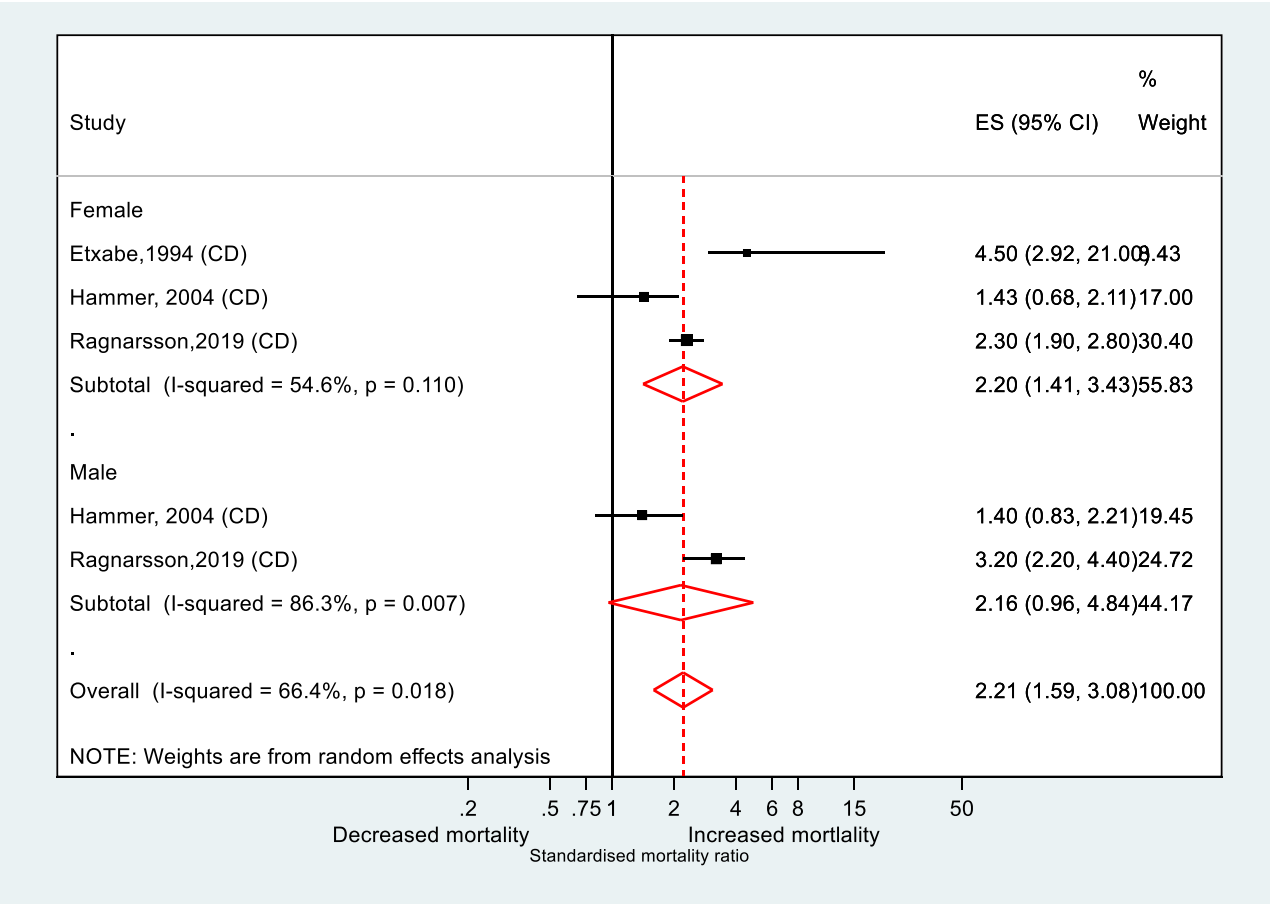


Figure 3- 20. Impact of gender upon SMR.

3.2.8.2 Sensitivity Analyses

Sensitivity analyses were conducted for the primary outcome by excluding studies with one serious bias, 11 inconclusive bias and 43 moderate biased articles. In total, 37 articles with a low bias were analysed. The findings were similar to those found in all of the included papers. Additionally, we conducted further analyses by eliminating severe and inconclusive bias publications, and the findings remained unchanged (Table 3-8). In summary, the quality of the research did not influence the results.

Table 3- 8. The sensitivity analysis for the quality of the studies

Sensitivity analysis		No. of study	no. of patients	no. of death	Proportion of death	95%CI	P value*
All studies (no exclusion)	ACS	24	2304	167	0.05	0.01, 0.08	
	CD	49	14971	477	0.05	0.02, 0.09	
	Mixed all types of CS	19	1906	131	0.04	0.00, 0.22	
	Overall	**92**	**19181**	**775**	**0.04**	**0.02, 0.07**	**0.77**
Quality of study: only low bias articles	ACS	12	1870	123	0.03	0.01, 0.08	
	CD	22	12278	325	0.05	0.02, 0.09	
	Mixed all types of CS	3	285	6	0.03	0.00, 0.22	
	Overall	**37**	**14433**	**454**	**0.04**	**0.02, 0.07**	**0.98**
Quality of study: low and moderate bias articles	ACS	19	2111	155	0.05	0.02, 0.09	
	CD	44	14385	469	0.05	0.03, 0.08	
	Mixed all types of CS	17	1836	129	0.05	0.02, 0.1	
	Overall	**80**	**18332**	**753**	**0.05**	**0.04, 0.07**	**0.94**

3.2.8.3 Publication bias

The funnel plots of SMR are illustrated by the log of SMR on the x-axis against SE on the y-axis. The visual graph demonstrated the symmetrical distribution of articles reported below and above the mean ES. Conversely, asymmetrical funnel plots were shown in articles reporting a number of deaths for all study cohorts and subtype of CS articles (Figure 3-20, Figure 3=21, Figure 3-22). The use of publication bias in meta-analyses of single-armed proportions may not be suitable. Because the data are non-comparative; the results (incidence or prevalence) are used as parameters rather than comparison studies (conditions, treatment, or methods), which are inherently inconsistent; there are no "negative" or "undesirable" results or study characteristics such as significant levels that could result in publication bias[565]. For these reasons, these conventional analytical approaches may not correctly account for the asymmetric distribution of ES shown on funnel plots. For asymmetrical plots, publication bias was merely one of many possible explanations (111). Additionally, funnel plots to identify publication bias are predicated on the premise that larger studies are less susceptible to publication bias than smaller research. Factors other than publication bias in this study may produce a skewed funnel plot, such as clinically significant heterogeneity.

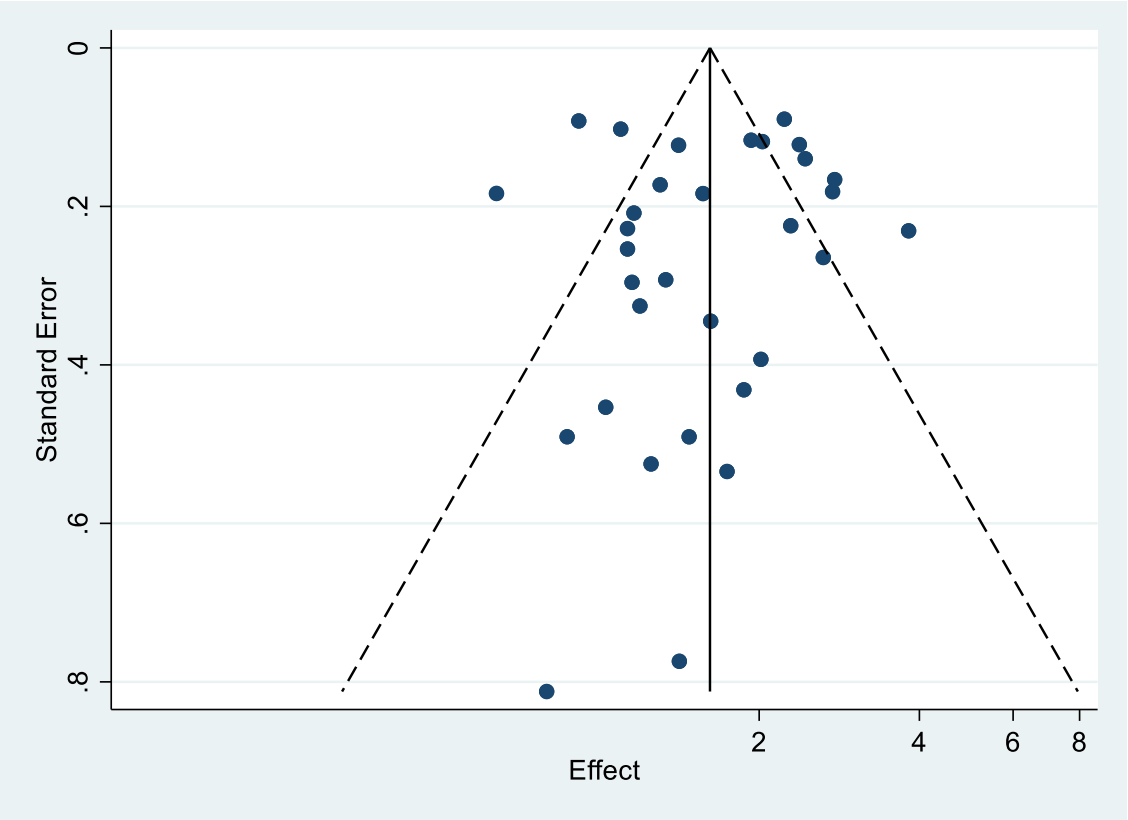


Figure 3- 21. Funnel plot of studies reporting SMR in Cushing's syndrome cohorts

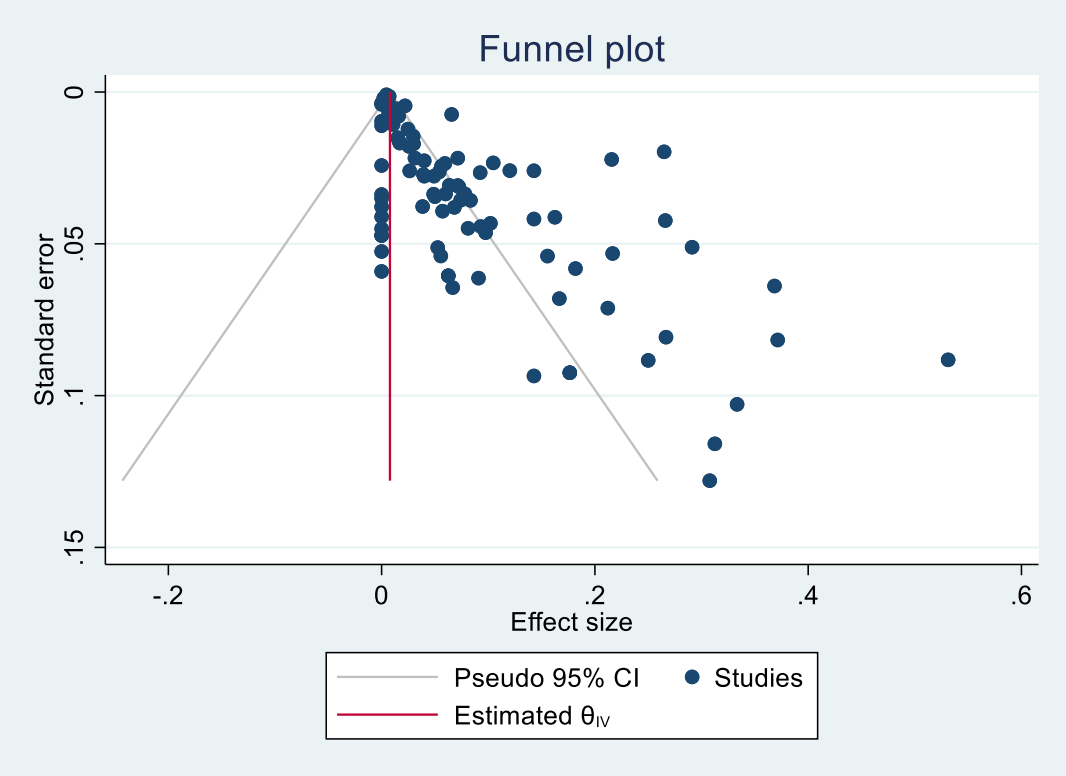


Figure 3- 22. Funnel plot of all studies reporting the proportion of death in Cushing's syndrome cohorts

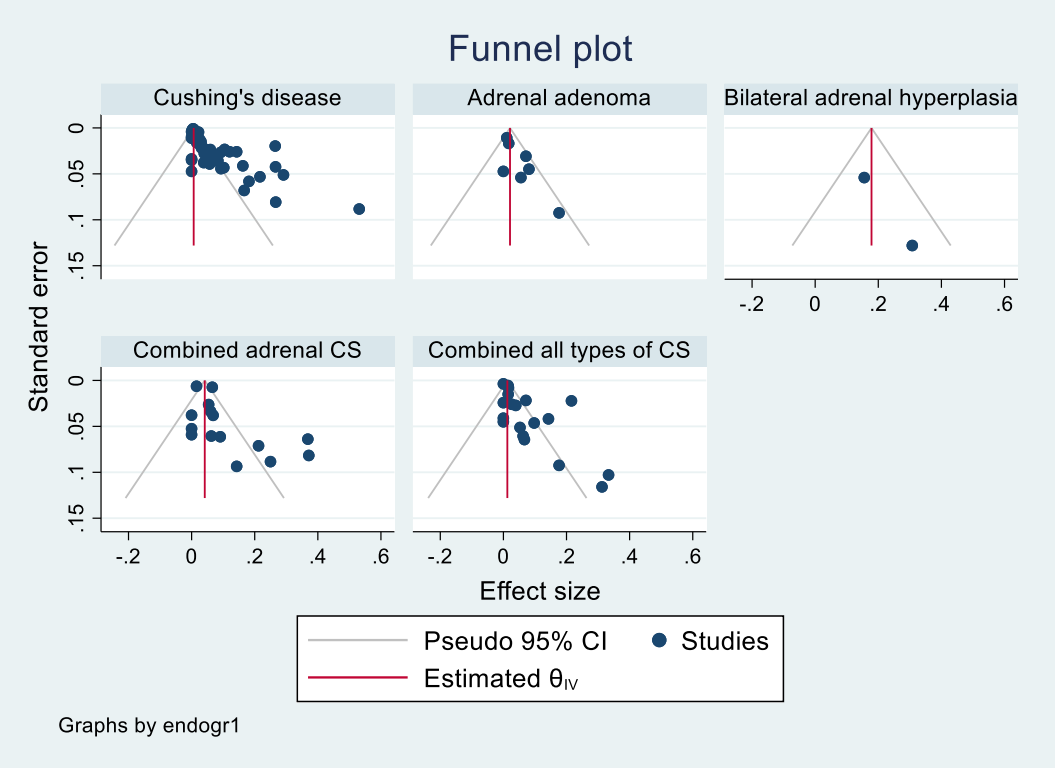


Figure 3- 23. Funnel plot of studies reporting the proportion of death stratified by subtypes.

3.3 Discussion

This study delivers the largest and the most inclusive systematic review, meta-analysis, and meta-regression of mortality in CS ever undertaken and enables us to understand the real-world mortality in this disease. This analysis extends the findings of a previous report, which included seven research studies comprising 779 patients with CS from 1994 to 2007[226]. By comparison, the current study spanned from 1952 to 2021 and included 19,181 CS patients. Additionally, we defined mortality from all benign CS subtypes and examined both SMR and the proportion of deaths. Pooled SMR for all subtypes of CS is 3.0 and is significantly worse in ACS compared with CD patients (3.3 vs 2.8; p=0.003). The evidence was more substantial than the previous meta-analysis [226], in which the mortality revealed an increase in overall CD (SMR: 1.8; 95% CI 1.3-2.7) and a higher value with persistent diseases (SMR: 3.73; 95%CI 2.31, 6.01). However, the previous meta-analysis showed no evidence to suggest elevated mortality associated with CD in early remission (SMR: 1.23; 95%CI 0.51-2.97) or AA (SMR: 1.9; 95%CI 0.9-3.9)[226].

This meta-analysis, conducted almost ten years later, demonstrated higher SMR across subtypes of CS. Our finding considered the possibility of incompletely reversible effects of CS in remission stages, resulting in increased mortality. The first explanation is the timely diagnosis of CS. Detection of CS on clinical grounds alone may be too late for reversible molecular or vascular damage after treatment. This is supported by a 6.5-year follow-up of CS in New Zealand; despite 80-90% of patients undergoing remission, mental illness, osteoporosis, dyslipidemia, and DM were not reversible after treatment. Furthermore, ischaemic heart disease, cerebrovascular disease, and hypopituitarism all deteriorated throughout the follow-up period[224]. The importance of early detection can be explained by the finding of mild autonomous cortisol secretion (MACS or formerly referred to as "subclinical CS") with subclinical manifestations. The data provides evidence for the detrimental effects of GCs prior to the appearance of clinical features. MACS has been shown to be associated with an increased risk of cardiovascular disease[566-573] and mortality[567, 574, 575], atrial fibrillation[570], DM[568, 571, 572, 576], visceral obesity[577], HT[568, 570-573], dyslipidaemia[568], frailty[578], low muscle mass[577] and reduced bone turnover[579] and osteoporosis[580]. This highlight the importance of subtle CS that can exacerbate cardiometabolic conditions. Ideally, CS should be identified early and treated appropriately to normalise cortisol.

To analyse the impact of disease activity on mortality, this research found that patients with active disease had a significantly higher SMR than those in remission (5.7 vs 2.3; p=0.001) with a median follow-up ranging from 7.5 to 15 years[208, 222, 225, 486, 492, 541, 564]. Despite being in remission, mortality was 2.3 times higher than age and the gender-matched general population. The results corroborated a previous meta-analysis of mortality in CD patients, which included eight cohort studies with a total of 766 patients and revealed a pooled SMR of 2.5 (95% CI 1.4-4.2; P

0.001) in treated CD patients, compared to an SMR of 4.6 (95% CI 2.9-7.3) for patients who remained uncured following transsphenoidal surgery[228]. An additional multi-centred study indicated increased mortality in CD with an overall SMR of 1.6 after more than ten years of remission and risk association with DM and cardiovascular complications [229]. Our data did not allow us to examine disease duration before diagnosis, disease severity, co-morbidities, or the duration of hypercortisolemia, which is critical for considering and minimizing poor long-term outcomes. However, this most recent data still highlights the issue; the active or persistent disease had a larger SMR than those in remission, ranging from 2.2 to 5.5 vs 1.2, respectively.[225, 226, 228]. Moreover, our meta-analysis also demonstrated the burden of SMR in macroadenoma vs microadenoma (7.4 vs 1.9, p=0.004) patients. These data are also supported by the meta-analysis of outcomes of pituitary surgery for CD, where microadenomas are more likely to enter remission vs macroadenomas (83% vs 68%, $p < 0.01$)[581].

It was not possible to undertake a proportion of death analysis with different disease activity in CS due to the substantial heterogeneity in published reports, which included dynamic changes in disease activity with multimodality approaches over study periods, incomplete patient information for further investigation, the management or disease conditions, variable time points stated for active or remission disease, post-operative vs follow-up vs censor period and unknown death. Here the primary challenge in terms of CD remission was the wide variety of criteria utilized across studies, time periods (1962–2018), regional guideline implementation, laboratory tests, and normal reference ranges (Table 3-4).

For ACS subtypes, few researchers had addressed the SMR of ACS compared to other subtypes of CD. A previous meta-analysis reported SMR for adrenal adenoma of 1.90 (95%CI 0.93-3.91; 72 patients). Our study reported SMR of 3.34 (95%CI 1.68-6.63; 1531 patients) for combined ACS, 4.06 (95%CI 1.89-8.73; 158 patients) for adrenal adenoma and 4.32 (95%CI 0.37, 50-17; 20 patients) for BAH. In the meta-analysis, the 95 % CI expresses the uncertainties associated with the SMR and indicates a range of values within which we can have confidence in any real impact. In this meta-analysis, the 95% CI in all subgroups of adrenal oriented CS (combined ACS:1.68-6.63 with n=1,531; AA: 1.89-8.73 with n=158 and; BAH: 0.37-5.73) was wider than CD. These might be explained by 1) samples zies or size of the studies, 2) the risks of events and time to event outcomes; 3) the period of patient enrolment, mostly before 1990 for ACS and involved pre-CT, pre-MRI, and pre-transsphenoidal surgery[208, 219, 220, 224, 516] which may contribute to higher mortality.

Our research provides a chance to examine the dynamic changes in CS mortality over recent decades. We accumulated a significant dataset on the proportion of deaths that comprised more articles (82 articles, 92 study cohorts and 19,181 patients) than SMR. These articles included

both historical and modern era articles encompassing a range of diagnostic and management approaches in CS. The overall proportion of deaths was significantly decreased post-2000 (3% vs 10% pre-2000, p <0.001) and were consistent for CD, AA, and combined ACS. Surgery is the mainstay of the initial treatment of CS. Our findings would seem to show that the proportion of deaths was higher following adrenalectomy for CD or ACS (3% or 4%) compared to transsphenoidal surgery for CD (1%). These findings should be taken cautiously since the introduction of transsphenoidal surgery for pituitary adenoma began in the mid-1970s to the late 1990s. Historically, CS was diagnosed clinically and treated with bilateral adrenalectomy. Secondly, adrenalectomy in CD can be a late treatment option for severe or life-threatening CS. A decrease in peri-operative deaths was also demonstrated after 2000 for all CS and combined ACS cohorts. Thus, our meta-analysis identified peri-operative death (fatalities within 30 days after treatment) as a risk factor for deaths occurring before and after 2000. We tried to explore the peri-operative complications, factors or events that might predict peri-operative mortality, but the limitation of information prevented this.

Focusing on peri-operative mortality, our study provides considerable insight into the causes of death, highlighting infection, VTE, and cardiovascular disease as the main causes of death during the peri-operative period. Despite the “unknown” causes of death during the peri-operative period, the additional known causes of death identified in this study allow for the potential to prevent future peri-operative deaths in an era of precision medicine and skilled surgical- multidisciplinary management. The peri-operative death analyses compare favourably with a study reporting acute life-threatening complications in CS occurring within the first year of operation [582]. Here, 23% of patients were hospitalized for acute complications before CS was suspected, and half of the complications occurred after the first surgery. Complications comparable to our findings included infection (CD 27.7% vs. ACS 17.2%), VTE (CD 14.8% vs. ACS 15.2%), ischaemic cerebro- and cardiovascular events (CD 11.9% vs. ACS 5%), cardiac arrhythmias (CD 6% vs ACS 3%) and adrenal crisis (CD 5% vs. ACS 6.1%). Furthermore, the additional peri-operative deaths in our study, including active CS, pancreatitis, adrenal insufficiency, bleeding tendency, respiratory failure, suicide and surgery, provide significant information about the possible association between an elevated or deficient cortisol level. Additionally, it is noteworthy that many of these causes should be preventable.

Notable surgical-related complications were tissue fragility and bleeding tendency, which were ascribed to hypercortisolism. The role of medical treatment to normalise cortisol levels before surgery has been proposed and has been found to be of benefit in ectopic CS but of limited value in terms of morbidities and remission outcomes for CD[583].

In our study, the VTE death rate for overall CS was 4.4% and comparable between CD (3.4%) and ACS (3.4%); however, the combined CS cohort was elevated to 10.6%. Recent meta-analysis described OR of VTE in CS compared to general population at 17.8 (95%CI 15.2-20.8; $p < 0.00001$); and CS undergoing surgery without vs with anticoagulant prophylaxis to be 0.34 (0.19-0.36; $p < 0.001$) vs 0.26 (95%CI 0.07-0.11, $p < 0.001$), respectively[266]. Prescribing anticoagulants for patients with CS is likely to be beneficial.

Suicide is one of the leading causes of death in CS. This study raises concerns about neuropsychiatric problems in CS, which are common manifestations and can be debilitating [286]. The neuropsychiatric manifestations in CS are depression (55–81%)[288, 289, 584], panic attacks (53%)[584], anxiety (12-79%)[584], sleep disorders, psychosis (8%) and cognitive impairment[584, 585]. Depression is one of the leading causes of suicide in the general population and the most common psychiatric problem in CS, as mentioned above. 17% of CS patients had suicidal ideas, and suicide is a common cause of death at the early follow-up. Until now, the extent to which remission of CS may fully cure neuropsychiatric problems is debatable[279]. Some publications report on the improvement of mental disorders after cortisol normalization. Regrettably, several papers, including nationwide cohorts, showed that mental problems remained an issue following remission [224, 288, 586-588]. This might be explained by irreversible effects of prior excessive exposure to GC, including decreased hippocampal volumes, enlarged ventricles, and cerebral atrophy[282, 589-592], leading to an aged brain[593]. The grey- and white matter changes are not fully reversible and are associated with psychiatric symptoms and cognitive deficits[283]. Furthermore, psychiatric manifestations are reported as the first common disorders in CS [584]. Thus, early CS diagnosis in psychiatric illness is preferable, despite the known, challenging biochemical problems in this context.

Adrenal insufficiency is a preventable cause of death if CS patients are treated with adequate GC replacement during the postoperative period in the suspected remission phase[594]. We could not identify the period of events or subtypes of CS or surgery due to a lack of individual patient data.

Follow-up cohorts between 1 to 10 years did not demonstrate any influence of follow up on death. However, when the median duration of follow-up was more than ten years, there was a significantly increased mortality compared to less than five years follow-up for all CS and CD cohorts. Clearly, two periods of mortality impacted the frequency of fatalities within 90 days after treatment and CS with a follow-up of more than ten years. For early deaths, ERCUSYN showed that 49% of CD or ACS mortality occurred within 90 days after first treatment[395]. Interestingly, death may be attributed to acute complications, which rose dramatically during the postoperative period, accounting for 60% of total deaths[582]. Additionally, during the first 90 days of therapy, dynamic

changes in cortisol levels from hypercortisolism to adrenal insufficiency (due to HPA axis suppression or hypopituitarism) were offered as supportive evidence for early mortality. Furthermore, death after ten years might be explained by the recurrence of CS or morbidities associated with CS through long-term cortisol exposure. The recurrence rate was supported by one-third of CD encountering relapse rate during a 10-year period after surgery, 18% (95%CI 14–22; p<0.01) of CD had recurrence at 50.3±24.0 months after surgery (range, 3.0–205.0 months), and 28% (95%CI 16–42; p<0.01), at 17.8±15.0 months after surgery (range, 2.0–76.0 months) [581].

In our study, atherosclerotic cardio- and cerebrovascular diseases, infection and malignancy were the leading causes of death across long-term follow-up. The causes of death can be compared to the report of CS morbidities in the Swedish National Patient Register with high standardized incidence ratios (SIRs) of VTE of 4.9 (95%CI 2.6-8.4), stroke of 3.1 (95%CI 1.8-4.9), and sepsis of 6.0 (95%CI 3.1-10.6)[595]. In comparison, the SIR for myocardial infarction was 3.6-4.4 (95%CI 1.2-11.4) and was significantly higher in the first three years before diagnosis. There is supporting evidence that excess cortisol states increase atherosclerotic risks and pathogenesis of CVD events[596]. These include DM and insulin resistance [581], HT[240], dyslipidaemia[238, 597], reduced coronary flow [598], hypercoagulable states, and arthrofibrosis [599]. The increased incidence of infections may be explained by the immunosuppressive effects of hypercortisolism, DM or hyperglycaemia together with vascular insufficiency from CS. The limited amount of detail as it relates to the type of infection/ organism or opportunistic infection in our study will hopefully trigger further studies; in the interim empirical treatment, especially in life-threatening situations, should be given to prevent/ treat underlying infection.

3.3.1 Strength

The strength of our study is the reach of what is a worldwide CS database, including national publications from USA, Europe, Denmark, Sweden, Korea, New Zealand, and Mexico. The research is the largest systematic review and meta-analysis of studies of endogenous CS reported to date. The novelty of this work is the development of a methodological pipeline for the meta-analysis of single-arm proportion data by using the *metapreg* program, and this is the first utilisation of this comprehensive tool. The assumption from the program was binomial distribution fitted with a generalized linear mixed model[425] with a logit link as recommended by Schwarzer et al.[421] and Stijnen *et al*. [600]. I believe this analysis is superior to the classic meta-analysis of proportion data that used the inverse variance method or Freeman-Tukey double arcsine transformation[420, 423], which assumed data as a normal distribution. The Freeman-Tukey double arcsine method was suggested to be the cause of the seriously misleading results in meta-analysis with different sample sizes that underpinned our meta-analysis[421]. We also presented the prediction interval together

with the ES and 95%CI. 95%CI as the precision index for estimated mean, which was the property of the sample, not population. Thus prediction intervals help us to anticipate future outcomes.

For the duplicated subjects in different patient cohorts, we put a great deal of effort into excluding duplicate patient cohorts if the same authors, hospitals, cities, or countries presented across the whole article. Individual e-mails were sent to the corresponding- or co-authors to confirm the possibility of duplication. The studies reported from multi-centre studies were also checked for overlapping subjects reported from their individual centres. The centre that reported multiple publications was checked for an overlapping period to reassure that the patients' mortality was not counted more than once. We enrolled all articles over a long period of follow-up that enabled us to appreciate CS management's timeline further.

3.3.2 Limitation

The meta-analysis was performed at the study and not the individual patient level. This limited the possibility to explore the true differences in subtypes of CS characteristics, confounding factors, especially comorbidity and co-intervention, the disease activity, recurrence of diseases, treatment, and mortality. The selected articles may encounter selection bias because the review was restricted to articles published in English language and peer-reviewed journals. The mortality outcomes may be underestimated if patients die before diagnosis and treatment. The information during the peri-operative period was scanty for exploring the prediction of deaths. The causes of death should be interpreted with caution because 16% of CS in the articles reported “unknown” as the cause of death, and 219 deaths out of 11,300 CS patients from 25 articles failed to report causes of death. Despite known causes, the real aetiology could not be confirmed: for example, organ failure or the consequence of active CS. Sources of death certificates or information were also included in several methods. Our data could not explore the duration of disease before diagnosis, the severity of the diseases, including co-morbidities, or the duration of hypercortisolemia, which are all crucial factors for reducing adverse long-term outcomes.

3.4 Implementations of finding and future research

CS treatment recommendations are clear: normalization of hypercortisolism, prevention or control of the comorbidities, long-term disease monitoring and control without recurrence, and elimination of pituitary compression on the adjacent structures while saving normal pituitary function for CD. Based on the work presented in this chapter, we suggest adding the additional recommendation of reversal of the unacceptable increase in mortality and specific intervention to reduce peri-operative mortality. This will raise awareness, for example, of prophylactic treatment for infection and thromboembolic disease prevention. Here screening for

cardiovascular/cerebrovascular disease, risk stratification, considering medical therapy as the bridging therapy for hypercortisolism before surgery, appropriate cortisol replacement therapy for adrenal insufficiency state should also be considered.

Aggressive cardiometabolic management may benefit at least 50% of CS patients for long-term mortality. The early detection of recurrence of CS needs greater effort, including long-lasting follow-up for CS. A concerning issue is the risk of suicide, and psychological evaluation may improve the management of all CS patients.

The next step for my research will be to explore the long-term GC effects on different tissue responses and to translate these into better patient investigation and care. More research will define predictive factors of mortality during peri- and within-90 days that may improve outcomes. I aim to study potential biomarkers to identify early CS to minimise long-term exposure to excessive cortisol.

An interesting network meta-analysis can be performed to examine multiple treatments for CS. The individual patient meta-analysis may help to understand the causes of deaths better. Such CS networks need to be established across nations in a similar manner to the pan-European database ERCUSYN to explore the differences in patient characteristics and outcomes. Greater concern for infection will give us more direction for therapeutic intervention and control of CS. For malignancy, the third most common cause of death, we lacked the details of cancer aetiology and SMR. However, the extracted data demonstrated the unpleasant diagnosis of pituitary carcinoma. For other malignancies, further studies are required to ascertain whether cancers are truly more prevalent in CS due to CS management or immune dysfunction. Data will be forthcoming on the impact of COVID-19 on mortality in CS, with the likelihood that CS patients are more susceptible to infection. This highlights the need to continue to evaluate the natural history or causes of death in CS across Covid infection and the immune response after covid infection or covid vaccination in patients with CS.

Chapter 4
The effect of exogenous Cushing's syndrome on all-cause and cause-specific mortality in real-world settings: A systematic review and meta-analysis

4.1 Introduction and research concept

GCs are widely used to treat autoimmune, inflammatory and haematological diseases. The prevalence of oral GC usage amongst the adult population worldwide ranges between 0.7 to 17%, depending upon indication, dose, duration and age group[187, 190, 601-603]. While the advantages of GC usage are obvious as it relates to suppression of the underlying inflammation, with GC prescription rates growing across several decades, the negative effects of GC are a serious concern, particularly for long-term use, as outlined in Chapter 1. This type of CS, referred to as iatrogenic or exogenous CS, is the most prevalent form.

In general, individuals who take GC for an extended period of time develop CS, which is determined by a combination of GC-specific characteristics, such as dose, duration of exposure, route of administration, and preparation, and patient-specific characteristics, such as protective effects and risk factors[71, 604, 605]. The definition of long-term or chronic GC use in the literature is variable, largely dependent upon the GC exposure contributing to adverse effects (e.g. HPA axis suppression or clinical manifestation). A hallmark of exogenous CS is suppression of the HPA axis, defined by dose (prednisolone equivalent) and duration of GC used. Broadly speaking, adrenal suppression is rare following exposure to any dose for less than seven days[606] but has been reported with doses as low as 5-7.5 mg/d given for over three weeks[607], or 40 mg/d for a week. A higher dose may certainly cause HPA axis suppression[608-610]. However, long-term use of oral GC as low as 2.5-5.6 mg/d can suppress the HPA axis[610-612]. The HR for adrenal dysfunction and mortality increased by 1.07 (95%CI 1.04-1.09) for every 5 mg increase per day and by 2.25 (95%CI 2.15-2.35) for every 1000 mg cumulative prednisolone-equivalent dosage over the preceding year[613]. Different GC doses drive different genomic and non-genomic pathways (detailed in section 1.1.5.1 and 1.1.5.2) that play critical roles in determining GC's therapeutic versus toxicity effects (detailed in section 1.2.1)[614].

GC-dose has been demonstrated to contribute to both morbidity and mortality[615, 616]. Generally, the higher the dose and the more prolonged the usage of GCs results in more side effects[617]. However, variable dose-response relationships have been reported, including cumulative dose, average daily dose, maintenance dose, or starting dose protocol, limiting the patterns contributing to deaths[618].

The clinical features of exogenous CS are similar (but not identical) to those of the classically described endogenous CS [605, 619] and lead to multiple co-morbidities [613] that have been discussed in Chapter 1. Only a limited number of studies have explored mortality from chronic GC use[613]. Many publications have described the relationship between underlying disease treated by GC and mortality rather than specific effects of the GC per se. The top three causes of death reported in patients who were prescribed oral GCs over 5.5 years were cardiovascular disease (32.4%), malignancy (21.0%) and infection (13.3%)[613]. GC dosage associated with all causes and cardiovascular mortality was reported in rheumatoid arthritis (RA) patients: HR 1.07 per mg of prednisone per day (95% CI 1.05-1.08) or HR 1.74 (95%CI 1.25-2.44) for minimum cumulative GC dose at 40 mg[620]. More prolonged exposure to GC (> ten years) despite a low dose also increased mortality[621].

As recognised by the multidisciplinary European League Against Rheumatism (EULAR) task force and US equivalents, there is limited knowledge on which to assign an evidence-based approach for long-term GC and morbidity or mortality [604]. Consequently, most international guidelines for GCs and disease treatment are led by expert recommendations rather than robust evidence-based information. Invariably data on the GC pattern of dose prescription or dose-response threshold on mortality and causes of death are lacking. The evidence base for exogenous CS-related morbidity and mortality were often of low quality because of the study design (mostly observational studies), high risk of confounding factors (underlying diseases, co-intervention, co-morbidities, limitation of therapeutic effects from GC resistance), inadequate information of GC exposures especially the dynamic changes of prescription and different models of risk attribution [604]. There are also limitations in the numbers of clinical trial studies for GC-only use patients compared to GC-naïve patients in the current studies reported for patients with autoimmune or inflammatory disorders.

This study is the first comprehensive systematic review and meta-analysis of chronic oral GC related to mortality from exogenous CS

4.2 Results

4.2.1 Study selection

The search strategy initially yielded 109,511 potential studies, including 31,696 through PubMed/Medline, 57,002 through EMBASE, 12,197 through the web of science, 5,150 through Cochrane and 3,466 through EBSCO (CINAHL), of which 84,715 duplicate studies were excluded using an EndNote and Rayyan web-based tool. After removing duplicates, 24,796 studies were screened for title and abstract using Rayyan web-based tool, which excluded 22,222 articles. A total of 2,574 full-text articles were assessed for eligibility, of which 2,451 articles were excluded. From 123 included articles, seven articles were deleted owing to their critical quality[622-628]. Consequently, the meta-analysis included 116 articles with 128 study cohorts and 51380 patients who satisfied the criteria for inclusion (Appendix 4-1). Figure 4- 1 illustrates the search method and selection procedure.

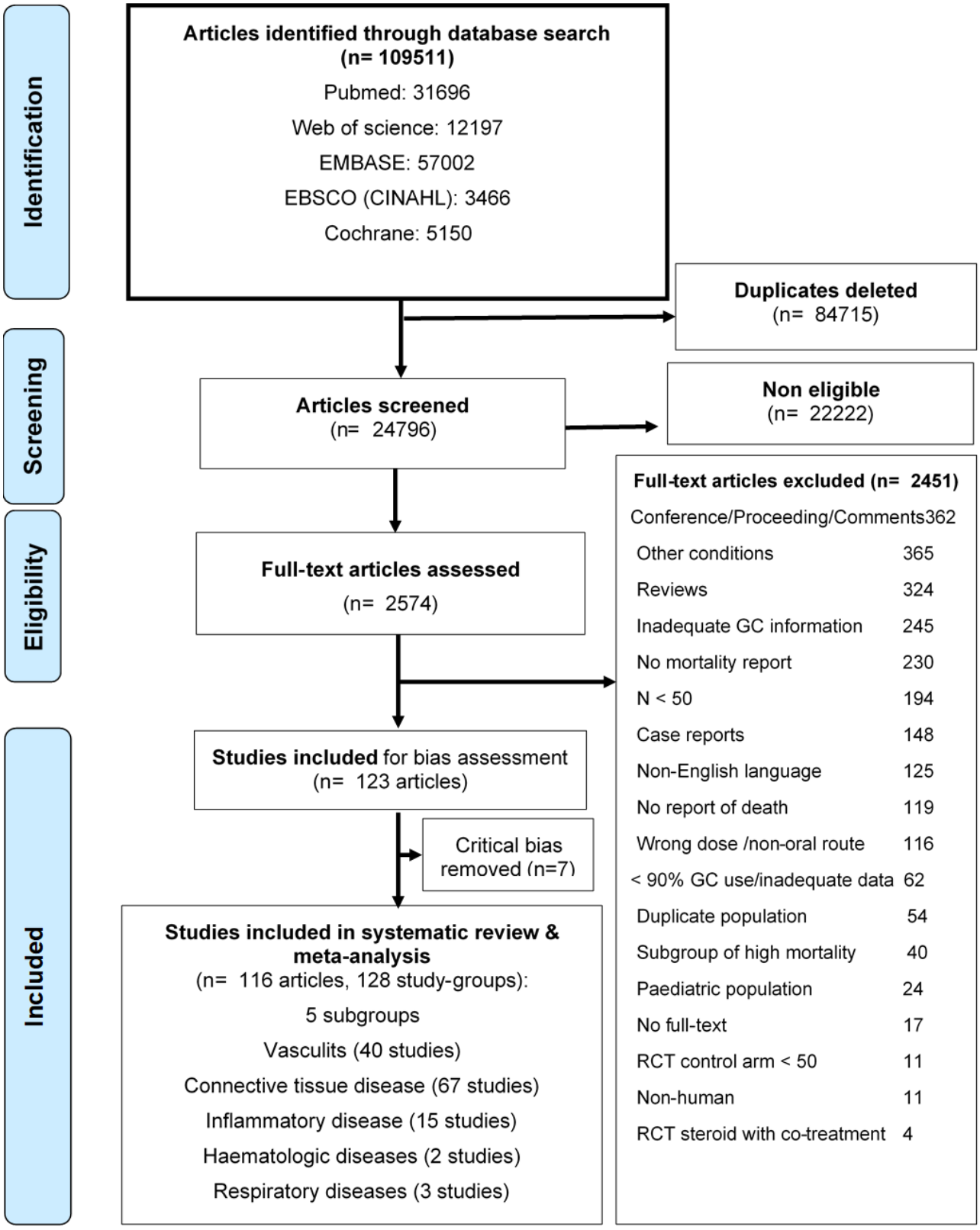


Figure 4- 1. Prisma flow diagram for searching for exogenous CS and mortality [493]

4.2.2 Study characteristics

The main characteristics of the selected studies are summarised in Table 4-1and Appendix 4-1). The studies were published from 1966 to March 2019 (Figure 4-2). Population size substantially varied, ranging from 50 to 13770 patients with a median sample size of 107 patients. The number of chronic GC-use patients was reported the most in studies from the UK and Europe, followed by the US, Korea and China (Figure 4- 3 and Appendix 4-2). The data sources were from medical records (95.3%) or medical registries (4.7%). The level of care was mostly from secondary or tertiary centres (94.5%), less so the community (5.5%). The study designs included 105 retrospective cohort studies, nine prospective cohort studies, one case-control study, one cross-sectional study, one other cohort study and 11 clinical trial studies.

The mean age of the population was reported in 77.3% of outputs and ranged from 22.1 to 81.0 years with a median age of 49.1 years. Women represented a proportion of the population ranging from 1.3 to 100%, with median percentages of women being 64.0%. The underlying disorders treated with chronic GC were classified into five groups according to their pathogenesis:

1) ***vasculitis group*** consisted of giant cell arteritis (10 studies), Takayasu's arteritis (1 study), ANCA-associated vasculitis (24 studies), anti-glomerular basement membrane disease (1 study), central nervous system vasculitis (1 study), and medium and small-vessel vasculitis (4 studies);

2) ***connective tissue disease group*** consisted of systemic lupus erythematosus (SLE) (30 studies), bullous diseases (13 studies), dermatomyositis and polymyositis (10 studies), glomerulonephritis (1 study), IgA nephropathy (1 study), IgM nephropathy (1 study), nephrotic syndrome (3 studies), idiopathic pulmonary fibrosis (2 studies), myasthenia gravis (1 study), sarcoidosis (2 studies), and autoimmune thrombocytopenia (3 studies);

3) ***inflammatory disease group*** consisted of inflammatory bowel disease (3 studies), polymyalgia rheumatica (2 studies), RA (8 studies), and adult-onset still's disease (2 studies);

4) ***haematologic disease group*** consisted of aplastic anaemia (1 study) and Evan's syndrome (1 study);

5) **respiratory disease group** consisted of asthma (2 studies) and chronic obstructive pulmonary disease (1 study).

From chronologically published articles (1966-2019) and from a sample size perspective this increased during the last five years for inflammatory diseases, vasculitis and connective tissue diseases (Figure 4-2).

Table 4- 1. Study characteristics[35]

Diseases	No. of studies	No. of patients (range)	Mean age at diagnosis*	No. of women (%)*	Mean follow-up in years*	Mean duration GC use in months*	Total deaths (%)*
Vasculitis							
Large vessel vasculitides[36]	11	6509 (50-5011)	72.4 n=10	4595 (73.2) n=9	5.2 n=5	32.6 n=3	925 (14.2)
Medium to small vessel vasculitides	29	4349 (50-595)	55.2 n=18	2037(48.0) n=28	4.3 n=15	30.9 n=9	1035 (23.8)
Total	40	10858 (50-5011)	67.5 n=28	6632 (63.0) n= 37	4.6 n=20	31.4 n=12	1960 (18.1)
Connective tissue diseases							
All SLE	30	7636 (50-1918)	30.5 n=24	4828 (90.2) n=25	6.5 n=21	32.9 n=7	707 (9.3)
Bullous diseases	13	1650 (51-316)	64.2 n=11	571 (53.6) n=10	9.3 n=10	24.1 n=8	380 (25.1)
Dermatomyositis/ polymyositis	10	1576 (53-467)	44.9 n=8	1054 (72.1) n=10	7.6 n=4	59.3 n=4	276 18.9)
Glomerular diseases[37]	7	525 (56-122)	41.4 n=6	154 (38.1) n=5	5.3 n=6	15.7 n=2	38 (7.2)
Sarcoidosis	2	289 (62-152)	39.5 n=2	160 (74.8) n=2	4.0 n=1	33.0 n=2	22 (10.3)
Idiopathic pulmonary fibrosis	2	183 (83-100)	53.6 n=2	107 (58.5) n=2	6.8 n=1	26.9 n=2	68 (37.2)
Myasthenia gravis	1	113	68.5 n=1	NR	NR	54.6 n= 1	9 (8.0)
Total	67	11648 (50-1918)	37.8 n=54	67990 (78.8) n=53	5.7 n=42	36.8 n=26	1500 (12.9)
Inflammatory disease							
RA	8	26970 (112-13770)	55.5 n=8	10537 (79.8) n=7	6.9 n=5	43.1 n=6	4393 (16.3)
Inflammatory bowel disease	3	377 (73-158)	59 n=1	110 (29.2) n=3	0.1 n=1	78 n=1	9 (2.4)

[35] * refers to the calculation weighted percentage; no. or n refers to numbers of; NR refers to not report; GC refers to glucocorticoids; SLE refers lupus erythematosus

[36] GCA and Takayasu

[37] Nephrotic syndrome, IgA nephropathy, IgM nephropathy, Glomerulonephritis

Polymyalgia rheumatica	2	408 (134-274)	70.5 n=1	266 (65.2) n=2	5.3 n=1	20.2 n=1	68 (16.7)
Still's disease	2	154 (54-100)	42.5 n=2	105 (68.2) n=2	3.1 n=2	NR	15 (9.7)
Total	15	27909 (54-13770)	55.5 n=12	11018 (77.9) n=14	43.3 n=8	6.8 n=9	4485 (16.1)
Haematologic disease							
Aplastic anaemia	1	56	NR	NR	NR	2.0 n=1	19 (33.9)
Evan's syndrome	1	68	56.4 n=1	41 (60.3) n=1	NR	4.8 n=1	16 (23.5)
Total	2	124 (56-68)	56.4 n=1	41 (60.3) n=1	NR	3.5 N=2	35 (28.2)
Respiratory diseases							
Asthma	2	415 (170-245)	48.0 n=1	253 (61.2) n=2	NR	26.5 N=2	26 (6.3)
COPD	1	80	68.1 n=1	3 (3.8) n=1	0.25 n=1	2.0 N=1	2 (2.5)
Total	3	495 (80-245)	53.0 n=2	256 (51.7) n=3	0.25 n=1	27.0 n=3	28 (5.7)
Overall	**128**	**51374 (50-13770)**	**49.2 (22.1-81.0)**	**226 (64.0) N=110**	**5.0 N=77**	**29.4 N=51**	**8037 (15.6)**

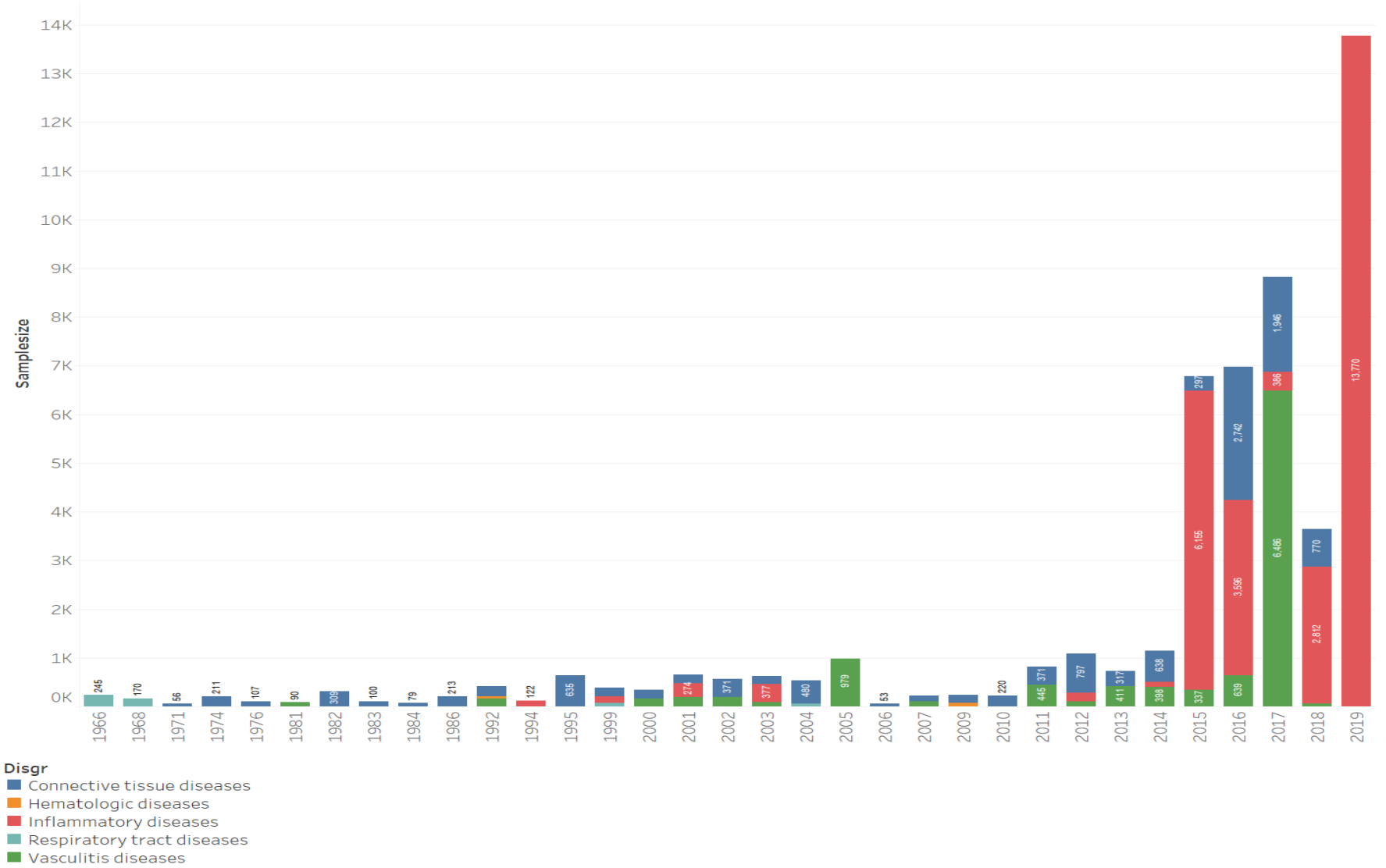


Figure 4- 2. Diseases treated by glucocorticoids presented as the year of publication

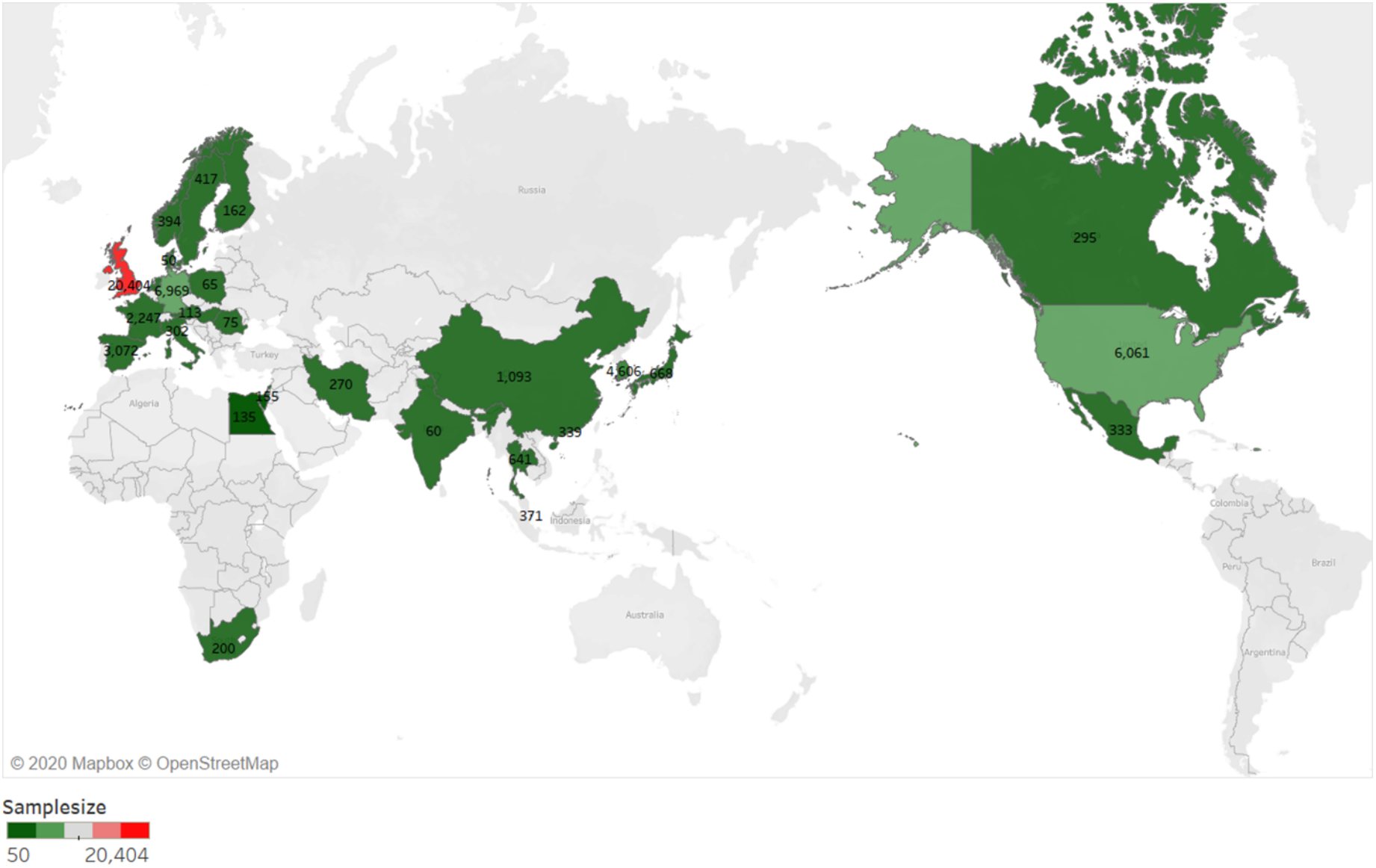


Figure 4- 3. Geographic distribution of outputs reporting the number of patients with chronic glucocorticoid use

4.2.3 The characteristics of glucocorticoid dose reports

The GC dose reports are provided in as prednisolone equivalent (Figure 4-4, Table 4-2 and Appendix 4-3). The dose of GC use varied considerably between studies. Some studies reported the GC usages of more than one regimen. The oral GC used was prednisolone in 57.5% of studies, prednisone for 41.7% of studies and both in 0.8%. GC-usage reports included 1) mean cumulative dose (n=34 studies) ranging from 0.3-36.7 g, 2) median cumulative dose (n=2 studies) ranging from 4.8-8.6 g, 3) mean maintenance dose (n=22 studies) ranging from 3.75-20.0 mg/d, average mean dose (n=19 studies) ranging from 3.1-64.9 mg/d, 4) median average dose (n = 2 studies) ranging from 5.0-6.6 mg/d, 5) initial staring dose (n=35 studies) ranging from 0.1-1.5 mg/kg/d, 6) mean initial starting dose (n=27 studies) ranging from 5.6-258.2mg/d, 7) median initial dose (n= 6 studies) ranging from 25-60mg/d, 8) last follow-up mean dose (n=6 studies) ranging from 5-17.66mg/d and 9) last follow-up median dose (n=3 studies) ranging from 6.0-36.2mg/d.

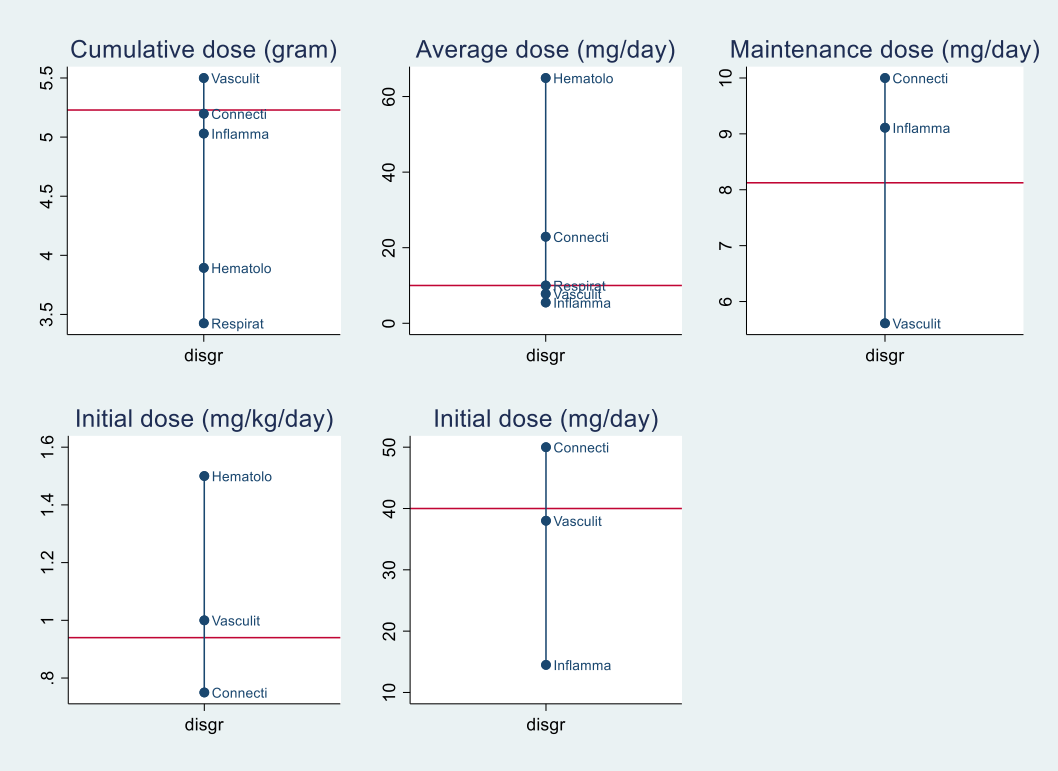


Figure 4- 4. The mean or median dose of glucocorticoid (GC) reports presented as prednisolone equivalent. Five GC-dose reports including mean cumulative dose (g/day), average mean dose (mg/day), mean maintenance dose (mg/day), initial dose (mg/kg/day) and mean initial dose (mg/day) were presented in red line for overall, and black dot for specific subgroup (vasculitis, connective tissue diseases, inflammation diseases, haematologic diseases, and respiratory diseases).

4.2.4 Duration of glucocorticoid use and follow-up period

The mean duration of GC use was reported in 51 studies (40.2% of total) and ranged from 1.5 months to 10 years. The median duration of GCs was reported in 10 studies and ranged from 9 months to 9 years. The mean duration of follow-up was reported in 77 studies with an overall mean follow-up of 5.0 ± 3.6 years (range 1.0 months to 18.1 years). The median duration of follow-up was reported across 40 studies and ranged from 0.05 to 21.9 years.

Table 4- 2. The pattern of glucocorticoid regimens reported in the selected studies

GC regimen reported in studies	GC dose range (prednisolone equivalent) (no. of studies, no. of patients) median (interquatile range)					
	Total	**Vasculitis**	**Connective tissue diseases**	**Inflammatory diseases**	**Haematologic diseases**	**Respiratory diseases**
Cumulative dose (g) mean	0.3 - 36.7 (n=34, N=25637) 5.2 (2.9, 11.1)	2.9 - 15.1 (n=11, N=1824) 5.5 (4.6, 9.4)	1.0 - 36.7 (n=10, N=2293) 5.2 (1.3, 19.6)	0.3 - 17.9 (n=9, N=20969) 5.0 (1.7, 7.3)	3.9 (n=1, N=56) -	2.7 - 18.3 (n=3, N=495) 3.4 (2.7, 18.3)
Cumulative dose (g) median	4.8 - 8.6 (n=2, N=536) 6.7 (4.8, 8.6)	8.6 (n=1, N=150) -	-	4.8 (n=1, N=536) -	-	-
Average dose (mg/d) mean	3.1 - 64.9 (n=19, N= 20936) 10 (7.5, 23.7)	7.5 - 17 (n=3, N=5236) 7.8 (7.5,17)	4.5 - 60.0 (n=6, N=642) 22.9 (10, 30)	3.1 - 10 (n=5, N=14507) 5.5 (4.9, 8.0)	64.9 (n=1, N=56) -	9.1-47.6 (n=3, N=495) 10 (9.1, 47.6)
Average dose (mg/d) median	5.0-6.6 (n=2, N=3846) 5.8 (5.0, 6.6)	-	6.6 (n=1, N=350) -	5.0 (n=1, N=3496) -	-	-
Three-month average dose (mg/d) mean	40.0 (n=1, N=230) -	-	40.0 (n=1, N=230) -	-	-	-
Three-month average dose (mg/d) median	20 (n=1, N=232) -	20 (n=1, N=232) -	-	-	-	-
Six-month average dose (mg/d) mean	8.8 - 25.0 (n=5, N=5) 15.0 (12.7, 17.7)	12.7 - 25.0 (n=2, N=197) 18.8 (12.7, 25.0)	8.8 - 17.7 (n=3, N=227) 15.0 (8.8, 17.7)			
Six-month average dose (mg/d) median	10 - 17.5 (n=2, N=293) 13.8 (10.0, 17.5)	10 - 17.5 (n=2, N=293) 13.8 (10.0, 17.5)	-	-	-	-
GC regimen reported in studies	**GC dose range (no. of studies, no. of patients) median (interquatile range)**					
	Total	**Vasculitis**	**Connective tissue diseases**	**Inflammatory diseases**	**Haematologic diseases**	**Respiratory diseases**
Twelve-month average dose (mg/d) mean	5.0 - 16.0 (n=3, N=697) 8.0 (5.0, 16.0)	5.0 - 16.0 (n=3, N=697) 8.0 (5.0, 16.0)	-	-	-	-

Maintenance dose (mg/d) mean	3.8 - 20.0 (n=22, N=2494) 8.1 (6.2, 12.8)	4.3 - 7.5 (n=6, N=693) 5.6 (5.0, 7.5)	3.8 - 20.0 (n=14, N=1369) 10.0 (7.5, 15.0)	6.2 - 12 (n=2, N=432) 9.1 (6.2, 12.0)	-	-
Maintenance dose (mg/d) median	2.5 - 8.3 (n=2, N=298) 5.4 (2.5, 8.3)	8.3 (n=1, N=164) -	-	2.5 (n=1, N=134) -	-	-
Last follow-up dose (mg/d) mean	5.0 - 17.7 (n=6, N=803) 7.9 (6.3, 17.1)	7.5 - 17.1 (n=3, N=468) 8.3 (7.5, 17.1)	6.3 - 17.7 (n=2, N=255) 12.0 (6.3, 17.7)	-	-	5.0 (n=1, N=80) -
Last follow-up dose (mg/d) median	6.0 - 36.2 (n=3, N=369) 12.4 (6.0, 36.2)	6.0 (n=1, N=150) -	-	12.4 - 36.2 (n=2, N=219) 24.3 (12.4, 36.2)	-	-
Initial dose (mg/d) mean	5.6 - 258.2 (n=27, N=9159) 40.0 (28.1, 55.1)	28.1 – 60.0 (n=9, N=757) 38.0 (33.2, 55.1)	25.0 – 258.2 (n=15, N - 1839) 50.0 (36.7, 60.0)	5.6 - 28.1 (n=3, N=6563) 14.5 (5.6, 28.1)	-	-
Initial dose (mg/d) median	25.0 - 60.0 (n=6, N=1193) 55.0 (40.0, 60.0)	40.0 – 60.0 (n=4, N=647) 60 (50, 60)	25.0 - 50.0 (n=2, N=546) 37.5 (25.0, 50.0)	-	-	-
Initial dose (mg/kg/d)	0.1 - 1.5 (n=35, N=4871) 0.9 (0.6, 1.0)	0.75 - 1.50 (n=9, N=1726) 1.0 (1.0, 1.0)	0.12 - 1.0 (n=25, N=3077) 0.75 (0.5, 1.0)	-	1.5 (n=1, N=68) -	-

4.2.5 Risk of bias assessment and quality of evidence

The quality of studies included in this review varied considerably, from low bias (3.0%) to critical bias (5.2%). 68.1% had serious or high-risk bias, 13.3% moderate bias and in 10.4% bias was undetermined. The contributing causes of bias were commonly from confounding factors (74.4%) and selection bias (40.0%). Seven critical bias articles were excluded before synthesis of the systematic review and meta-analysis [622-628]. The details of the methodological quality and scores from the individual studies are presented in Figure 4-5 and Appendix 4-4.

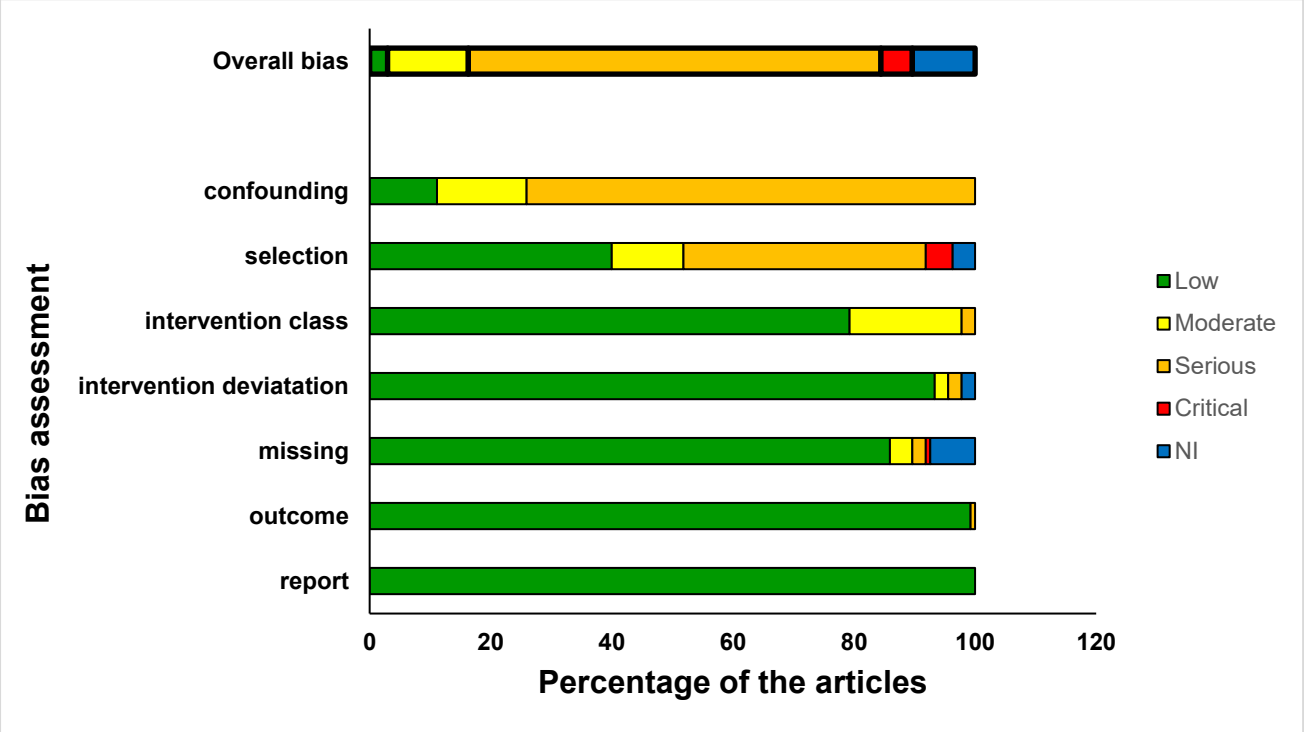


Figure 4- 5. Risk of bias graph: a review of authors' judgements about each risk of bias item presented as percentages across all included studies.

4.2.6 Standardised mortality ratio

Seven studies reported SMR data ranging from 1.03 to 3.37 with an overall pooled SMR of 1.86 (95% CI 1.32, 2.611; I^2 74.3%). SMR was reported in five studies, all of which included patients with rheumatologic illnesses, including vasculitis (medium to small vessel vasculitis, n=5) and connective tissue diseases (SLE, n=1 and autoimmune thrombocytopenia, n=2). The SMR of vasculitis was 1.71 (95% CI 1.23, 2.36, I^2 41.3%), and for the connective tissue diseases were 2.26 (95% CI 1.02, 5.00, I^2 92.2%) (Figure 4-6 and Appendix 4-5).

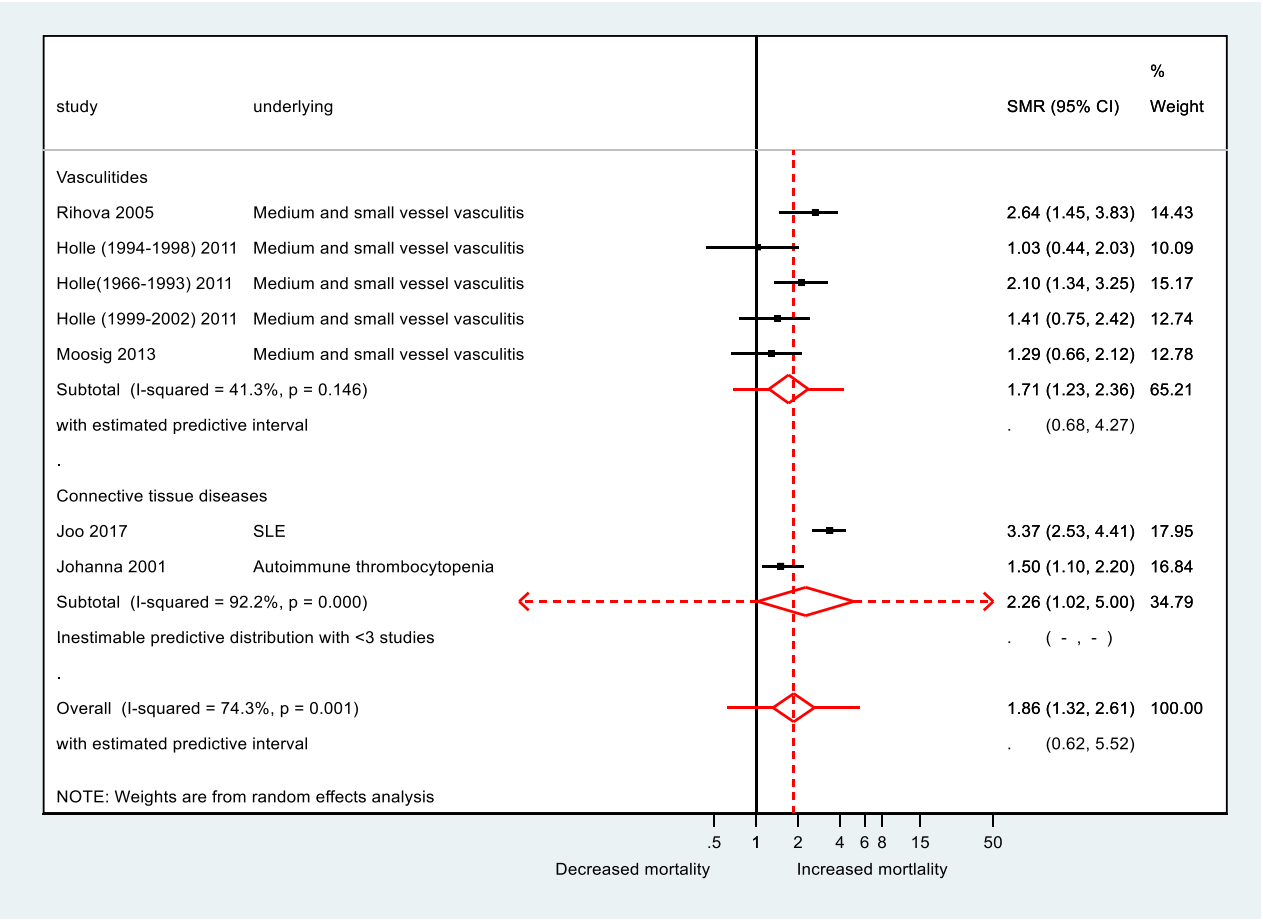


Figure 4- 6. Meta-analysis estimates of SMRs of diseases treated by glucocorticoids. Only two pathogenesis diseases reported standardised mortality ratio (SMR). Meta-analysis analysed by metan program. SLE refers to systemic lupus erythematosus.

4.2.7 The proportion of all-cause mortality

The proportion of deaths across the studies ranged from 0.0 to 0.84. The pooled proportion of all-cause mortality with random-effect analysis was 0.12 (95% CI 0.10, 0.14). Because of the substantial heterogeneity of the results by the I^2 test (89.1%), the logistic regression (LR) test confirmed the heterogeneity and differences using FEM vs REM ($p<0.001$, $\tau 2=1.41$), so the REM model was used for this analysis (details have been

mentioned before in chapter 2 and chapter 3). The underlying condition treated with GC, the severity of the disease, co-morbidity, and co-intervention is all recognised confounding factors and contributors to mortality. To minimise heterogeneity, pre-specified subgroups by disease pathogenesis (as established by all studies) were taken into the analyses (Appendix 4-5). Because data were extracted at the study level rather than the individual patient level, other confounding factors were limited in their application. The Leave-one-out LR test makes it simple to assess the covariates (confounding variables, e.g. disease pathogenesis treated by GC) that might have an effect on the outcome of interest (proportion of death). Two models (with or without covariates) are analysed by mixed-effect logistic regression. Then they are tested by leave-one-out LR under the assumption that the covariate has no effect. If the test between two models is statistically significant ($p < 0.05$), the null hypothesis is rejected, indicating a bias.The meta-analysis with covariates is then chosen because the results will be adjusted by that covariates. This is comparable to meta-regression analysis.

In this phase, the model was fitted using a mixed-effect logistic regression with disease pathogenesis (covariate) to compare the effects of adding or omitting the covariate. The leave-one-out LR test revealed a statistical difference when covariate was included ($p < 0.01$), and hence the outcome was selected using the covariate model. The proportion of mortality was 0.18 (95% CI 0.13, 0.24) in vasculitis (n=40), 0.14 (95% CI 0.08, 0.13) in connective tissue diseases (n=68), 0.3 (95% CI 0.04, 0.12) in inflammatory diseases (n=15), 0.28 (95% CI 0.07, 0.66) in haematologic diseases (n=2), and 0.05 (95% CI 0.01, 0.14) in respiratory diseases (n=3) (Figure 4-7).

Subgroup	No. of study	No. of deaths	No. of patients	Proportion of death	95%CI	Predictive interval	Odds	P value of odds
Vasculitedes	40	1960	10858	0.18	0.13, 0.24	0.02, 0.72	1.00	1.00
Connective tissue diseases	68	1529	11994	0.14	0.08, 0.13	0.01, 0.56	0.55	<0.001
Inflammatory diseases	15	4485	27909	0.07	0.04, 0.12	0.01, 0.48	0.39	<0.001
Hematologic diseases	2	35	124	0.28	0.07, 0.66	0.02, 0.88	1.59	0.46
Respiratory diseases	3	28	495	0.05	0.01, 0.15	0.00, 0.43	0.25	0.04
Overall	128	51380	8037	0.12	0.1, 0.14	0.01, 0.60		

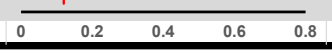


Figure 4- 7. Forest plot demonstrates the proportion of overall mortality across the disease subgroups.

4.2.8 Meta-regression analysis of mortality related to GC dose reports

The overall proportion of deaths in chronic GC use patients was analysed, accounting for the GC dose and disease-pathogenesis. Further analyses of GC doses included mean cumulative dose, average mean daily dose, mean maintenance dose, initial mean dose and underlying diseases. The GC dose reports were taken into account as a

covariate and fitted to a mixed-effect logistic (meta-regression) analysis under the assumption of the binomial distribution.

4.2.8.1 Cumulative dose and mortality

Thirty-four studies, encompassing 25637 patients, reported on the cumulative dose. The overall median cumulative GC dose varied between pathogenesis treatment; vasculitis 5.5g (interquartilerange or IQR 4.6, 9.4), connective tissue disease 5.5 g (IQR 1.3, 19.6), inflammatory disease 5.0 g (IQR 1.7, 7.3), and respiratory diseases 3.4 g (IQR 2.7, 18) (Figure 4-8). There were insufficient studies on patients with haematologic disease to perform a meta-regression of cumulative GC dose. The cumulative dose was fitted to the regression analysis (p=0.02 compared to no covariate) and stratified by tertile, the proportion of death at first (0.3 - 3.89 g), second (3.92 - 7.30 g), and third (8.8-36.7 g) tertile was 0.11 (95% CI 0.02 - 0.20), 0.04 (95% CI 0.02 - 0.06) and 0.16 (95% CI 0.06 - 0.14), respectively (Figure 4-9). The OR of mortality between the 1st and 3rd tertile of the cumulative dose was not statistically different. Conversely, a cumulative dose at 2nd tertile (3.9 to 7.3 g) had a 63% lower proportion of deaths than the cumulative dose of less than 3.9 g (p=0.04).

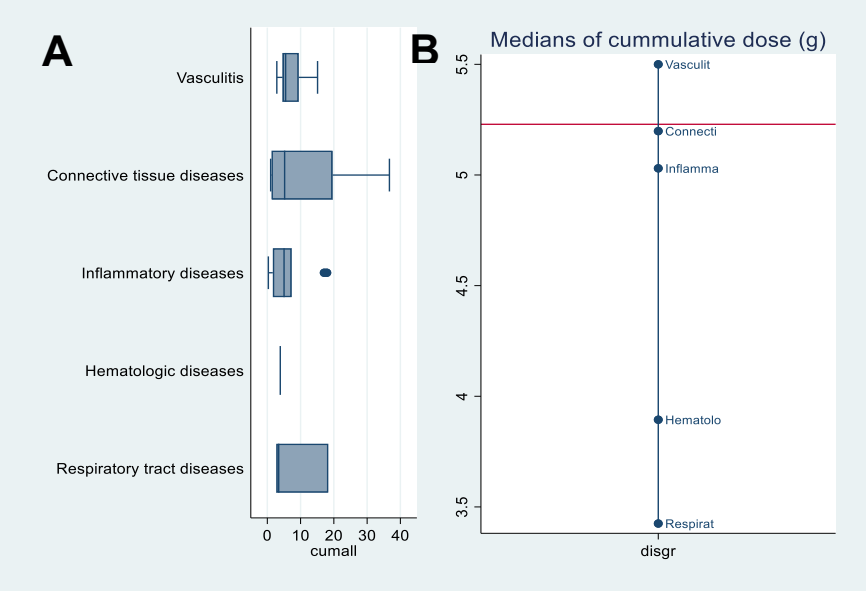


Figure 4- 8. (A) Box-plot graph demonstrating cumulative GC dose in disease subgroups with (B) overall mean cumulative dose (g).

GC reports	covarite	Model selection	No. study	No. patients	No. death		Proportion of death	95%CI	Predictive interval	Odds ratio	P value of odds ratio
Cumulative dose (grams)											
Overall studies (ranged 0.31 - 36.71)	a	0.67†	34	25637	4878		0.09	0.06, 0.14	0.00, 0.78		
Overall studies	b	0.02*	34	25637	4878		0.09	0.06, 0.13	0.01, 0.62		
1sr tertile (0.31-3.89 g)	b		12	18493	4173		0.11	0.06, 0.20	0.01, 0.69	1.00	1.00
2nd tertile (3.92-7.30 g)	b		11	4454	263		0.04	0.02, 0.08	0.01, 0.77	0.37 (0.14, 0.95)	0.04
3rd tertile (8.76-36.71 g)	b		11	2690	442		0.16	0.08, 0.28	0.00, 0.43	1.43 (0.60, 3.40)	0.42
Vasculitis studies (ranged 2.85-15.1)	a	< 0.001*	11	1824	438		0.01	0.07, 0.18			
Ovearll vasculitis studies	b	< 0.001*	11	1824	438		0.11	0.07 , 0.18	0.03, 0.35		
1sr tertile (2.85 - 5.06 g)	b		4	300	8		0.02	0.01, 0.07	0.00, 0.13	1.00	1.00
2nd tertile (5.4 - 8.76 g)	b		4	739	166		0.16	0.08, 0.30	0.04, 0.49	6.74 (2.02, 22.45)	< 0.001
3rd tertile (9.42 - 15.1 g)	b		3	785	264		0.38	0.21, 0.59	0.10, 0.76	15.69 (5.00, 49.21)	< 0.001
Medium to small vessels vasculitis (ranged 1.00 - 36.7)	a	0.02*	7	1271	360		0.18	0.09, 0.31	0.01, 0.77		
Ovearall medium to small vessels vasculitis	b	< 0.001*	7	1271	360		0.18	0.09, 0.31	0.01, 0.83		
1sr tertile (4.6 - 5.4 g)	b		3	647	133		0.06	0.02, 0.19	0.01, 0.69	1.00	1.00
2nd tertile (5.5 - 8.7 g)	b		2	125	32		0.23	0.07, 0.54	0.01, 0.92	3.65 (0.09, 1.09)	0.11
3rd tertile (11.6 - 15.1 g)	b		2	499	195		0.46	0.18, 0.77	0.02, 0.97	7.37 (0.23, 1.84)	< 0.001
Connective tissue diseases (ranged 1.02 -17.89)	a	0.84†	10	2293	183		0.07	0.04, 0.12	0.01, 0.29		
Overall connective tissue diseases	b	< 0.001*	10	2293	183		0.07	0.04, 0.11	0.02, 0.23		
1sr tertile (1.00 - 3.63 g)	b		4	643	86		0.11	0.06, 0.21	0.03, 0.36	1.00	1.00
2nd tertile (4.84 - 11.09 g)	b		3	275	16		0.03	0.01, 0.1	0.01, 0.18	0.31 (0.09, 1.09)	0.07
3rd tertile (19.6 - 36.7 g)	b		3	1375	81		0.07	0.03, 0.15	0.02, 0.28	0.65 (0.23, 1.84)	0.23
Inflammatory diseases (ranged 0.31-17.89)	a	NA	9	20969	4210		NA	NA	NA		
Overall inflammatory diseases	b	0.17†	9	20969	4210		0.09	0.04, 0.19	0.00, 0.80		
1st tertile (0.31-1.65 g)	b		3	17366	4046		0.23	0.07, 0.53	0.01, 0.94	1.00	1.00
2nd tertile (3.92-6.91g)	b		3	3252	101		0.04	0.01, 0.13	0.00, 0.66	0.15 (0.03, 0.84)	0.03
3rd tertile (7.3-17.89g)	b		3	351	63		0.09	0.02, 0.3	0.00, 0.85	0.42 (0.08, 2.16)	0.30
Rheumatoid arthritis (ranged 1.00 - 17.89)$²	a	NA	7	20815	4195		NA	NA	NA		
Overall rheumatoid arthritis	b	< 0.001*	7	20815	4195		0.09	0.04, 0.19	0.00, 0.91		
1st tertile (1.00-3.92 g)	b		3	20078	4125		0.16	0.05, 0.43	0.00, 0.96	1.00	1.00
2nd tertile (6.90-7.30 g)	b		2	503	8		0.01	0.05, 0.07	0.00, 0.72	0.07 (0.01, 0.63)	0.02
3rd tertile (17.1 -17.89 g)	b		2	234	62		0.21	0.05, 0.59	0.00, 0.98	1.30 (0.23, 7.33)	0.76
						0 0.5 1					

Figure 4- 9. Summary of cumulative GC dose reports and proportion of deaths.

4.2.8.1.1 Cumulative dose and mortality in vasculitis

The cumulative dose was further analysed based on underlying pathogenesis across vasculitis, connective tissue diseases, and inflammatory diseases. Strong evidence for an increased proportion of deaths in vasculitis was found with cumulative GC dose (Figure 4-9 and Figure 4-10). The cumulative GC dose in vasculitis was stratified by tertile: OR for a cumulative dose between 2nd tertile (5.4 – 8.8 g) and 3rd tertile (11.6-15.1 g) were 6.7 and 5.7 times higher, respectively, compared to cumulative dose less than 5.1 g (both statistically significant, $p < 0.001$).

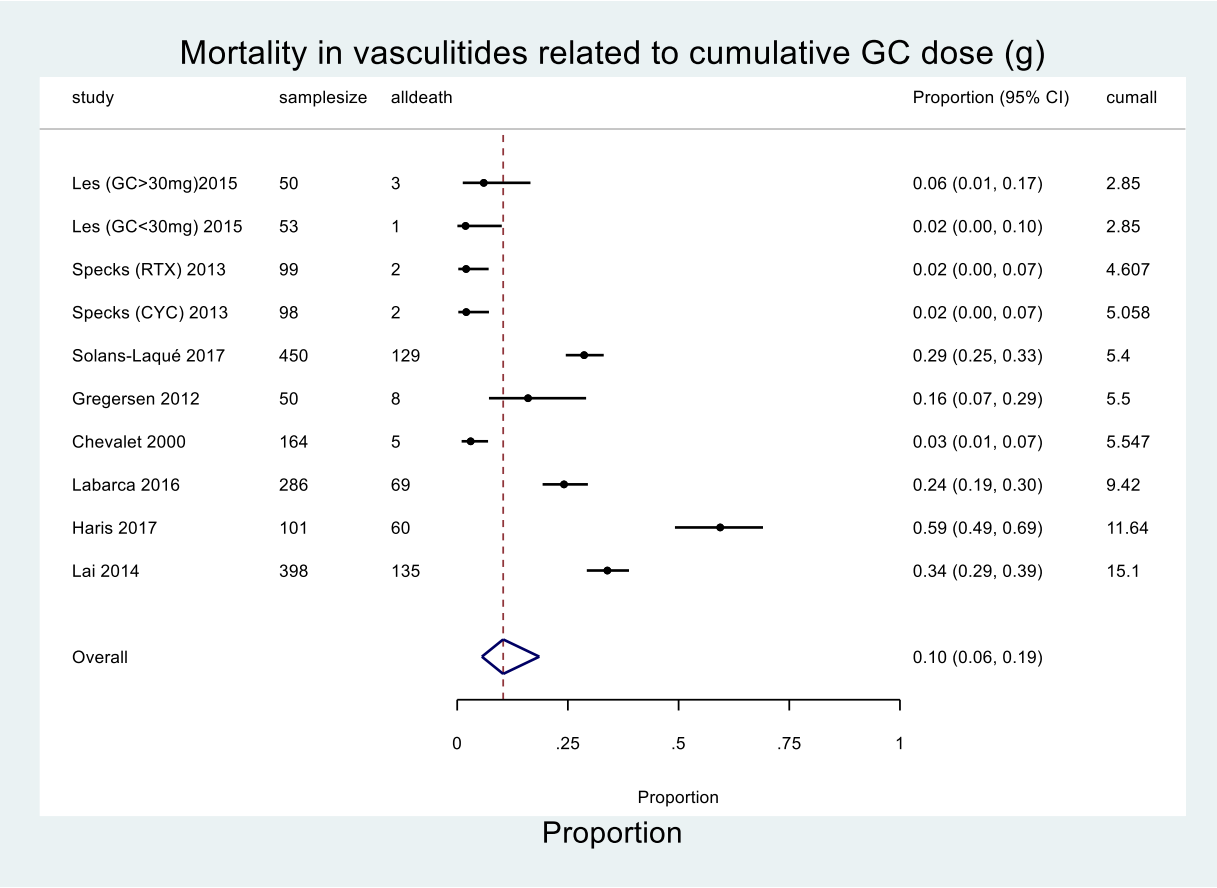


Figure 4- 10. Forest plot demonstrating the proportion of death and cumulative glucocorticoid doses (continuous values) in vasculitis.

4.2.8.1.2 Cumulative dose and mortality in connective tissue diseases

For connective tissue diseases, there was no statistical difference for 2nd (4.8 -11.9 g) or 3rd (19.6 – 36.9 g) tertile (OR 0.31, p = 0.07 and 0.65, p = 0.23) compared to the 1st tertile (1- 3.6 g) (Figure 4-9). The interpretation of cumulative GC dose and mortality in this group, however, should be made with caution; as illustrated in Figure 4-11, the heterogeneity of the underlying disease makes interpreting the effect of the cumulative dose of GC on mortality extremely difficult.

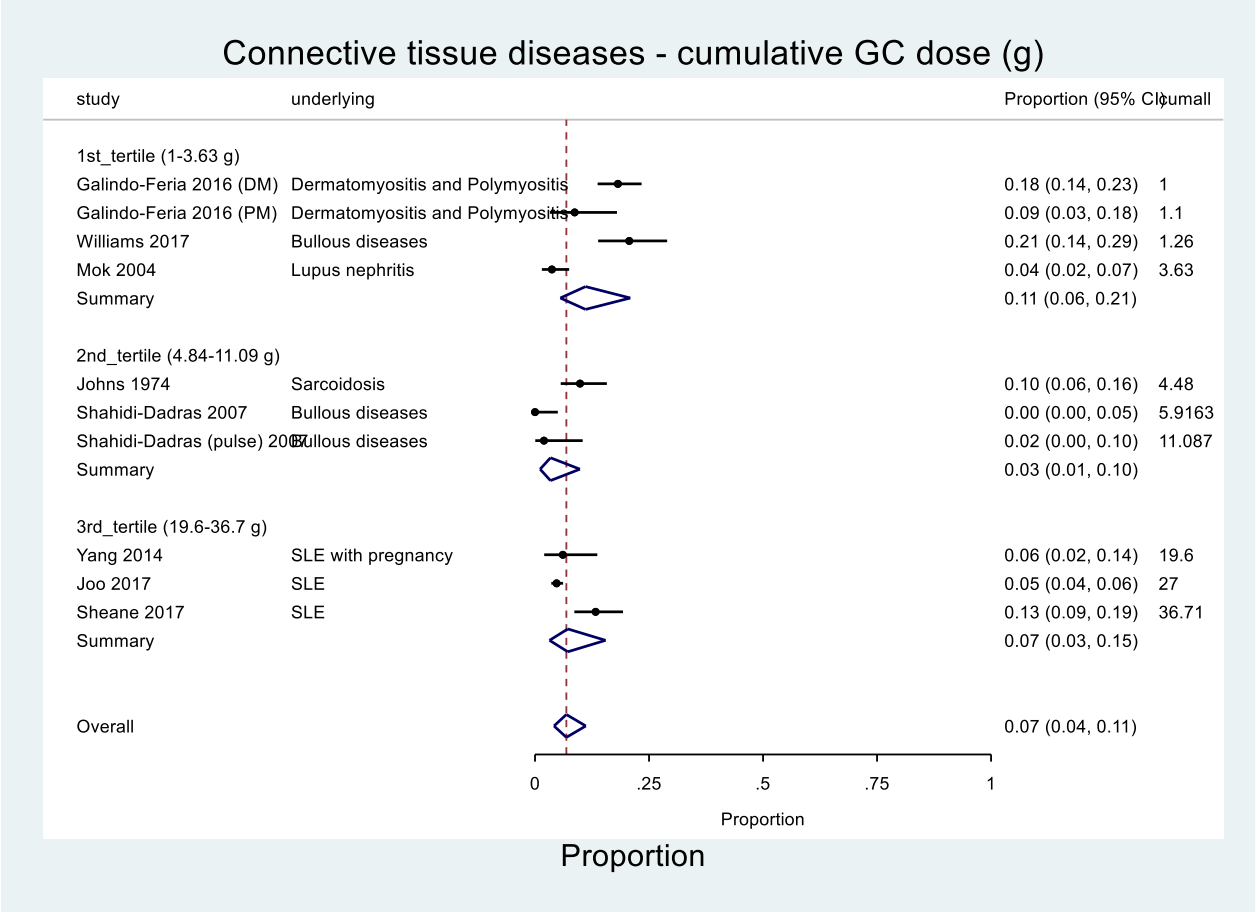


Figure 4- 11. Forest plot demonstrating the proportion of deaths in connective tissue diseases with cumulative GC dose (cumall)

4.2.8.1.3 Cumulative dose and mortality in inflammatory diseases and rheumatoid arthritis

Inflammatory diseases and RA demonstrated the same pattern for all included article analyses. The lowest proportion of deaths was seen in the second tertile of cumulative dose of all studies (OR 0.37, p= 0.04), inflammatory disease OR 0.15, p =0.03), and RA subgroup (OR 0.07, p= 0.02). The supportive reason is that RA patients were the majority of patients enrolled in cumulative dose studies (20,815 patients), all studies (25,637 patients) and in those with inflammatory diseases (20,969 patients). Only two articles in the inflammatory group were not RA (Still's disease, n =150 patients) (Figure 4-12).

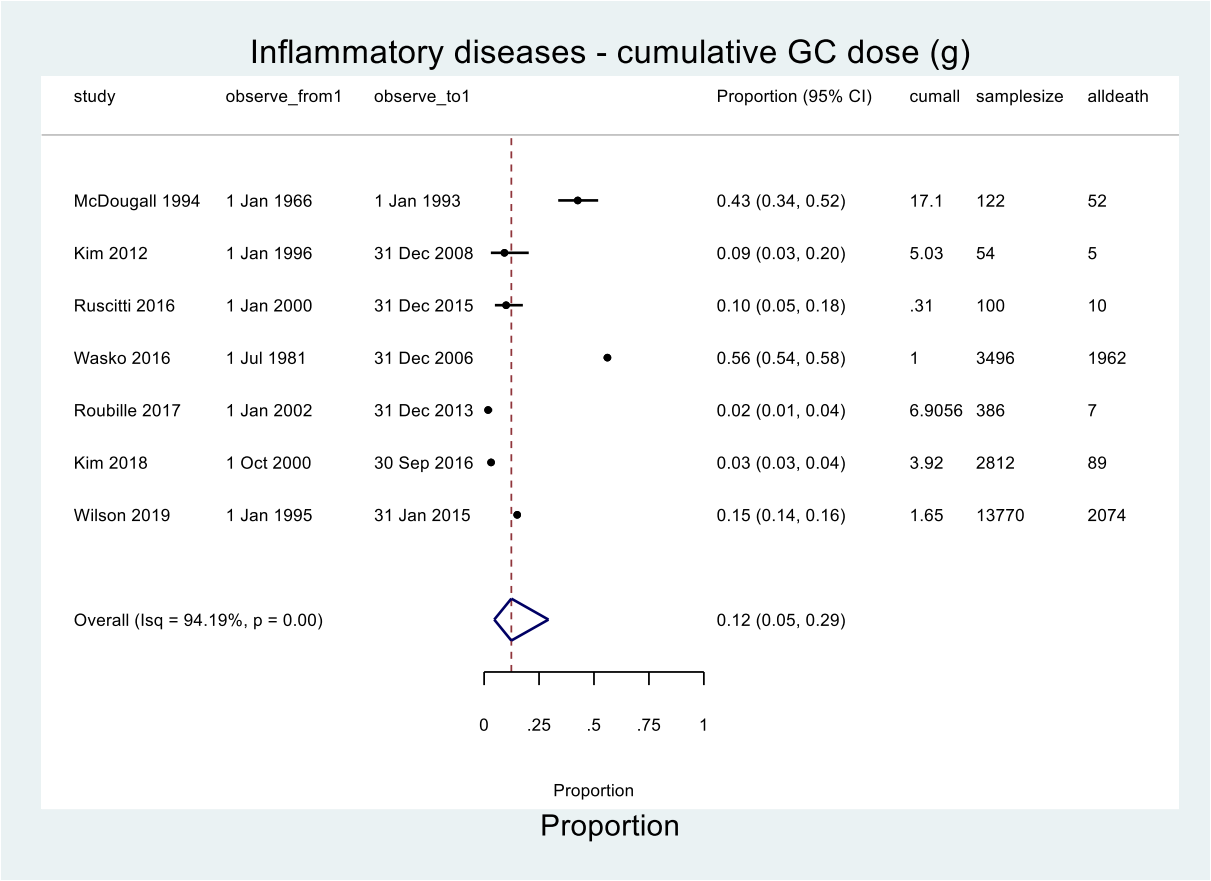


Figure 4- 12. Forest plot demonstrating the proportion of deaths related to cumulative GC dose in inflammatory diseases

Further results highlight that RA patients had a high proportion of deaths with higher cumulative GC doses in studies reporting pre-1990 than more recent publications (Figure 4-13). Other than cumulative GC use, the different period of study demonstrated the different mortality. The study of Wasko 2016 [625] that enrolled 3496 RA with 1g cumulative GC dose during 1981 until 2006 showed the highest proportion of death. In contrast Wilson 2019[629] enrolled 13,770 RA patients during 1995 until 2015 with higher cumulative GC dose, but lower proportion of death.

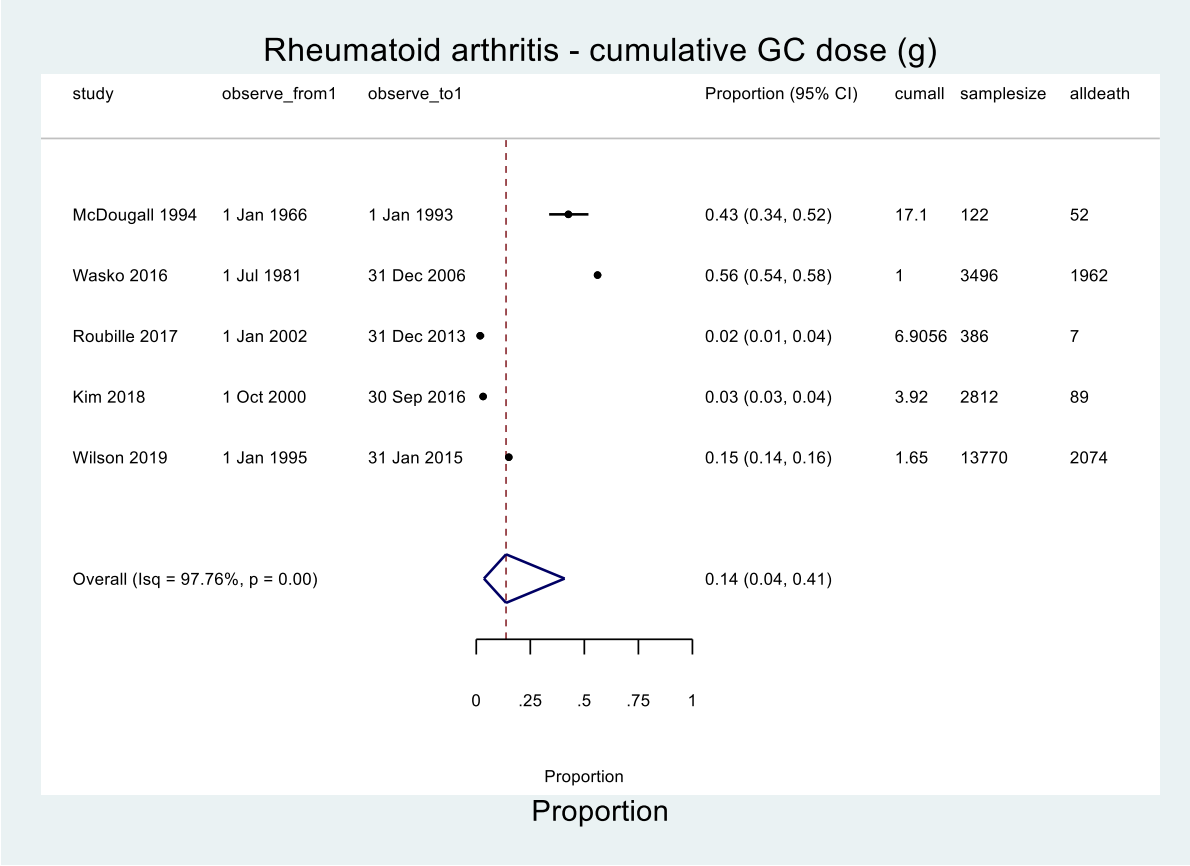


Figure 4- 13. Forest plot demonstrating the proportion of deaths related to cumulative GC dose in rheumatoid arthritis

4.2.8.2 Average daily dose and mortality

Nineteen studies, encompassing 20,936 patients, reported on average daily GC dose. The overall average daily GC dose varied between disease pathogenesis; vasculitis 7.8mg/d (interquartile range 7.5, 17; n=3), connective tissue disease 22.9mg/d (interquartile range 10, 30; n=7), inflammatory diseases 5.5mg/d (interquartile range 4.9, 8.0; n=5), and respiratory diseases 10mg/d (interquartile range 9.1, 47.7; n=3) (Figure 4-14). The haematological diseases group had just one study with an outlier daily dosage of 64.9mg/d and was eliminated from meta-regression.

The average daily GC dose and proportion of deaths were analysed. The regression model without covariates was applied since there was no statistically significant difference between including or excluding average mean dose (covariate) (p= 0.12)[38]. The OR of the proportion of death compared to a baseline GC daily dose ≤ 5 mg/d group was 4.35 times greater for 5 to10 mg/d of GC dose (p= 0.03), and 5.8 times greater for GC daily dose > 10 to 30mg/d (p= 0.02). No significant difference in the proportion of deaths for GC dose > 30 mg/d compared to < 5 mg/d was observed. Four studies documented the use of GC to treat non-rheumatic illnesses (1 study for haematological and 3 studies for respiratory diseases). Sensitivity analysis was conducted by excluding haematologic and respiratory disorders, and there was no effect on the proportion of deaths.

When compared to a dosage of 5 mg/d in rheumatological diseases, a GC dosage of 5 to 10 mg/d and > 10 to 30 mg/d substantially increased the proportion of deaths 5.23 times (p= 0.01) and 5.66 times (p= 0.01), respectively. Inflammatory diseases accounted for the majority of individuals that reported an average daily dosage; all patients had RA and took less than 10mg/d of GC. The OR or proportion of deaths for daily dose > 5-10 mg/d was 2.84 times or 11% higher compared to less than 5 mg/d d (p=0.40).

Furthermore, the dose of GC in vasculitis and connective tissue diseases groups could not be categorised based on data (Appendix 4-6). Vasculitis comprised just 3 articles with average doses equal to 1.5[630], 7.8[631] and 17[632] mg/d, which limited stratification based on GC dose for meta-analysis. Most of the connective tissue diseases were SLE patients; the meta-regression analysis could not demonstrate the effect of differences in mean GC dose (analysed as continuous number) on mortality (p = 0.69).

[38] The reasons for adding or not adding covariate to a regression model fall into 2 categories: 1) to reduce the bias and 2) precision of effect measurement. The main consideration where adding covariate to the regression analysis can make or break resulting GC effect estimate or proportion of death: 1) confounders (by including covariate), 2. downstream outcomes (no covariate), and colliders (no covariate). Then, this is the appropriateness for checking the meta-analysis model with or without covariate.

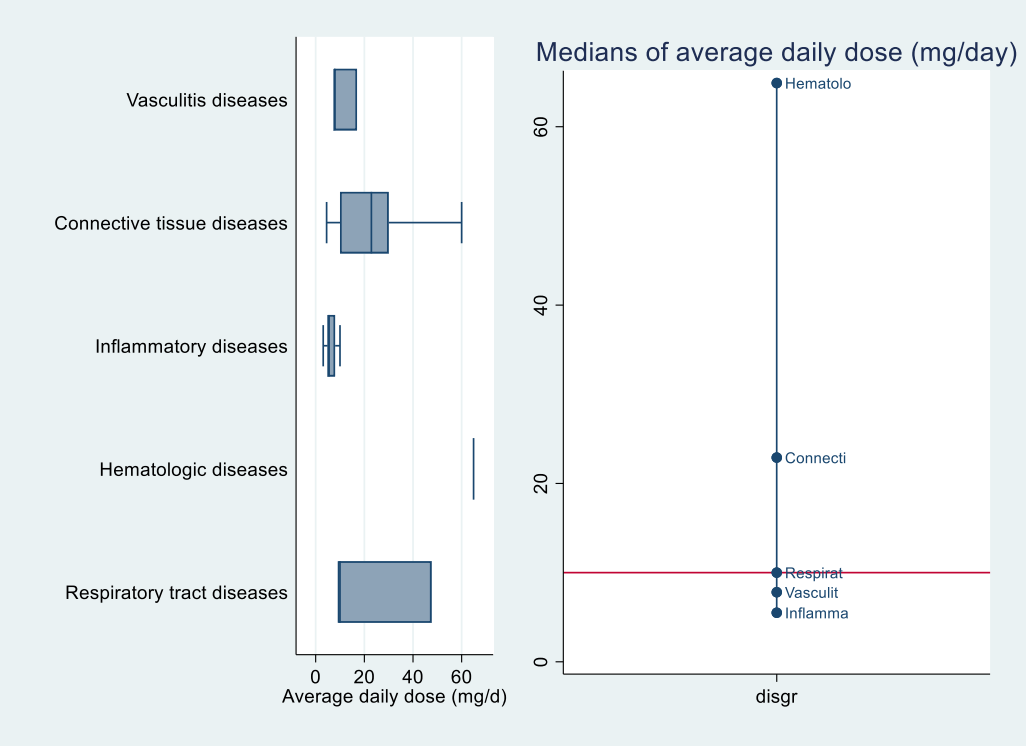


Figure 4- 14. (A) Box-plot graph demonstrating average daily GC dose in variable diseases with (B) overall median average daily dose

GC reports	Model selection with* or without† covriate	No study	No patients	No death		Proportion of death	95%CI	Predictive interval	Odds ratio	P value of odds ratio
Average daily mean dose (mg/day)										
Overall studies (ranged 3.10 -64.9 mg/d)	0.12†	19	20936	2827		0.09	0.06, 0.14	0.01, 0.41		
≤ 5 mg/d		3	562	17		0.03	0.01, 0.08	0.00, 0.21	1.00	1.00
> 5-10 mg/d		8	14699	2194		0.11	0.06, 0.20	0.02, 0.48	4.35 (1.11, 17.04)	0.03
> 10-30 mg/d		5	5457	590		0.15	0.07, 0.28	0.02, 0.58	5.8 (1.40, 24.12)	0.02
> 30 mg/day		3	218	26		0.09	0.03, 0.25	0.01, 0.50	3.72 (0.74, 18.77)	0.11
Rheumatolic diseases studies (ranged 3.10 -30 mg/d)	0.04*	14	20303	2775		0.10	0.06, 0.15	0.02, 0.40		
≤ 5 mg/d		3	562	17		0.03	0.01, 0.08	0.00, 0.19	1.00	1.00
> 5-10 mg/d		6	14284	2168		0.13	0.07, 0.25	0.02, 0.51	5.23 (1.38, 19.75)	0.01
> 10-30 mg/d		5	5457	590		0.15	0.07, 0.28	0.02, 0.54	5.66 (1.46, 21.96)	0.01
Inflammatory diseases: RA (ranged 3.1 - 10 mg/d)	0.43†	5	14507	2144		0.07	0.02, 0.22	0.00, 1.00		
≤ 5 mg/d		2	498	17		0.04	0.01, 0.23	0.00, 1.00	1.00	1.00
> 5-10 mg/d		3	14009	2127		0.11	0.02, 0.39	0.00, 1.00	2.84 (0.26, 31.56)	0.40
Covariate: avaerage dose (continuous number)										
Levae one out LR test with* or without† covariate					0 0.2 0.4 0.6					

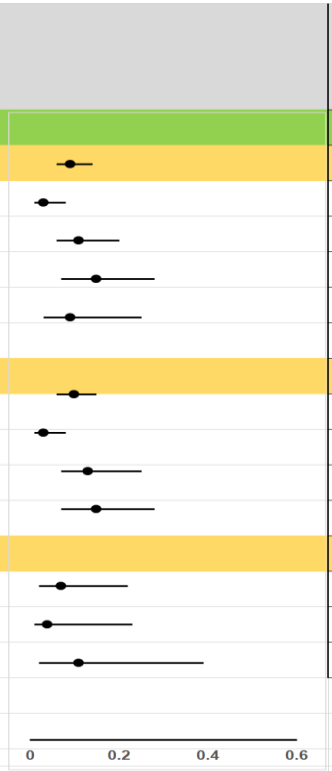


Figure 4- 15. Averge GC dose reports and proportion of deaths. Leave-one-out LR test compared between with-covarite† versus without covariate* analysis- if the p ≤ 0.05 the analysis was fitted with with-covarite† model and the p > 0.05 the analysis was fitted with without-covariate* model.

4.2.8.3 Maintenance dose and mortality

22 studies, comprising 2494 patients reported the maintenance dose in patients with rheumatological diseases. The overall maintenance GC dose ranged between 3.75 to 20 mg/d and was lowest in vasculitis studies at 5.6 mg/d (interquartile range 5.0, 7.5; n=6). For connective tissue diseases the maintenance dose was 10.0 mg/d (interquartile range 7.5, 15; n=14), and for inflammatory diseases 9.11 mg/d (interquartile range 6.22, 12.0; n=2) (Table 4-2, Figure 4-16). Two model of meta-regression analysis (with or without covariate: maintenance dose) was examined. The regression models for maintenance dosage studies were then fitted using the omitted covariate, since no statistically significant difference existed (p = 0.69). The are no differences in mortality across maintenance dosages of 5, > 5-10, or > 10-30 mg/d for overall studies, vasculitis and connective tissue diseases (p > 0.05).

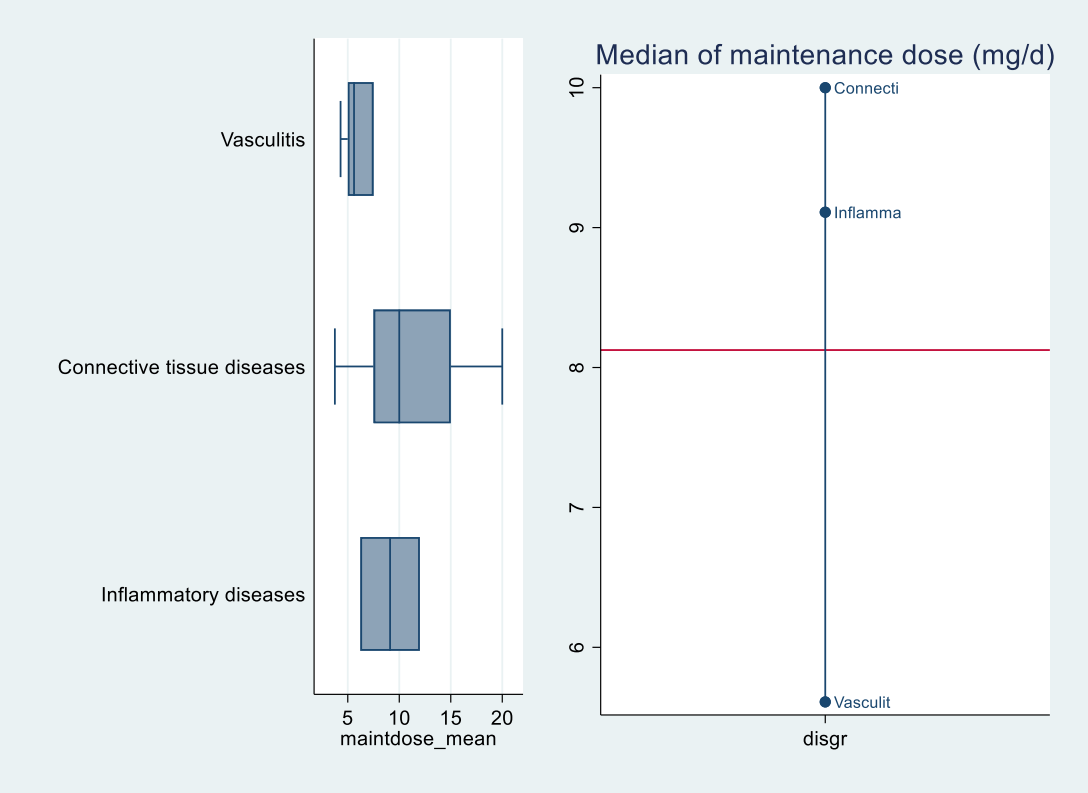


Figure 4- 16. (A) Box-plot graph demonstrating GC maintenance dose in disease cohorts with (B) overall median maintenance dose.

GC reports	Model selection with* or without† covriate	No. of study	no. of patients	no. of death		Proportion of death	95%CI	Predictive interval	Odds ratio	P value of odds ratio
Maintenance dose (mg/day)										
Overall studies (3.75 -20 mg/d)	0.69†	22	2494	352		0.11	0.07, 0.16	0.02, 0.43		
≤ 5 mg/d		4	446	37		0.08	0.03, 0.18	0.01, 0.40	1.00	1.00
> 5-10 mg/d		11	1393	203		0.12	0.07, 0.19	0.07, 0.19	1.53 (0.54, 4.34)	0.43
> 10-30 mg/d		7	655	112		0.12	0.06, 0.22	0.06, 0.22	1.57 (0.51, 4.83)	0.43
Vasculitis studies (4.3-7.5 mg/d)	0.36†	6	693	75		0.11	0.08, 0.15	0.05, 0.21		
≤ 5 mg/d		3	380	34		0.09	0.05, 0.15	0.04, 0.21	1.00	1.00
> 5-10 mg/d		3	313	41		0.13	0.08, 0.20	0.05, 0.28	1.41 (0.70, 2.83)	0.33
Connective tissue diseases (3.75 - 20 mg/d)	0.59†	14	1369	214		0.11	0.06, 0.18	0.01, 0.62		
≤ 5 mg/d		1	562	62		0.04	0.00, 0.32	0.00, 0.64	1.00	1.00
> 5-10 mg/d		7	480	88		0.10	0.05, 0.20	0.01, 0.62	2.48 (0.22, 28.32)	0.46
> 10-30 mg/d		6	327	64		0.14	0.06, 0.28	0.01, 0.71	3.39 (0.29, 39.08)	0.33
Use covariate as maintainant dose (continuous variable					0 0.1 0.2 0.3 0.4					

Figure 4- 17. Maintenance glucocorticoids (GC) dose reports and proportion of deaths.

4.2.8.4 Initial dose and mortality

Initial dose was the most often reported GC regimen. Initial GC dosages were reported as a mean dosage (mg/d) (27 studies with 9159 patients) (Figure 4-18) or as a mg/kg/d (35 studies with 4871 participants) (Figure 4-19). We were unable to establish a correlation between the initial GC dosage (mg/kg/d) and the proportion of deaths ($p > 0.05$). However, for first initial dosage reported as mg/d, higher starting doses resulted in a larger percentage of death ($p < 0.001$). The OR of proportion of deaths in patients receiving an initial mean dosage of > 10-30 mg/d, > 30-60 mg/d, or > 60 mg/d were 9.76 ($p < 0.001$), 8.89 ($p < 0.001$), and 6.32 ($p = 0.03$) times greater, respectively, compared to receiving a GC dosage of > 5-10 mg/d (Figure 4-20).

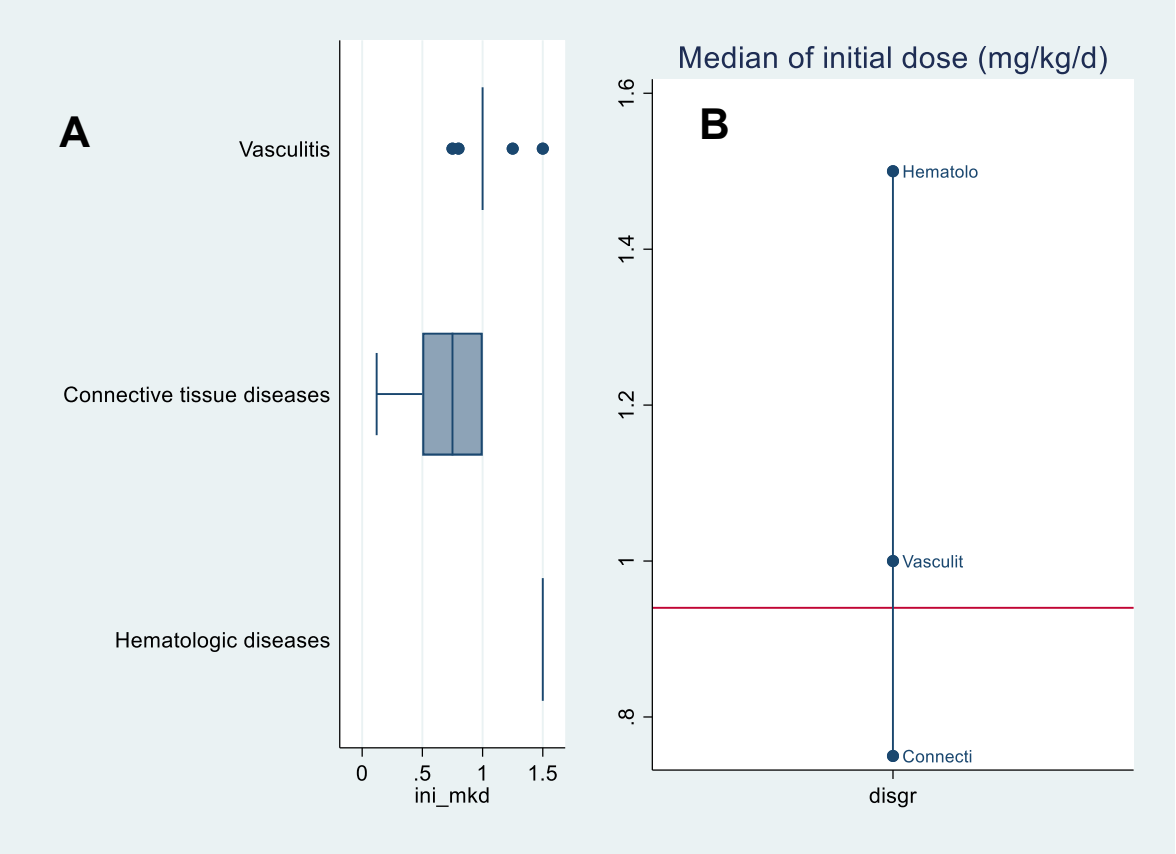


Figure 4- 18. (A) Box-plot graph demonstrating initial GC dose (mg/kg/d) in disease cohorts with (B) overall median initial GC dose (mg/kg/d).

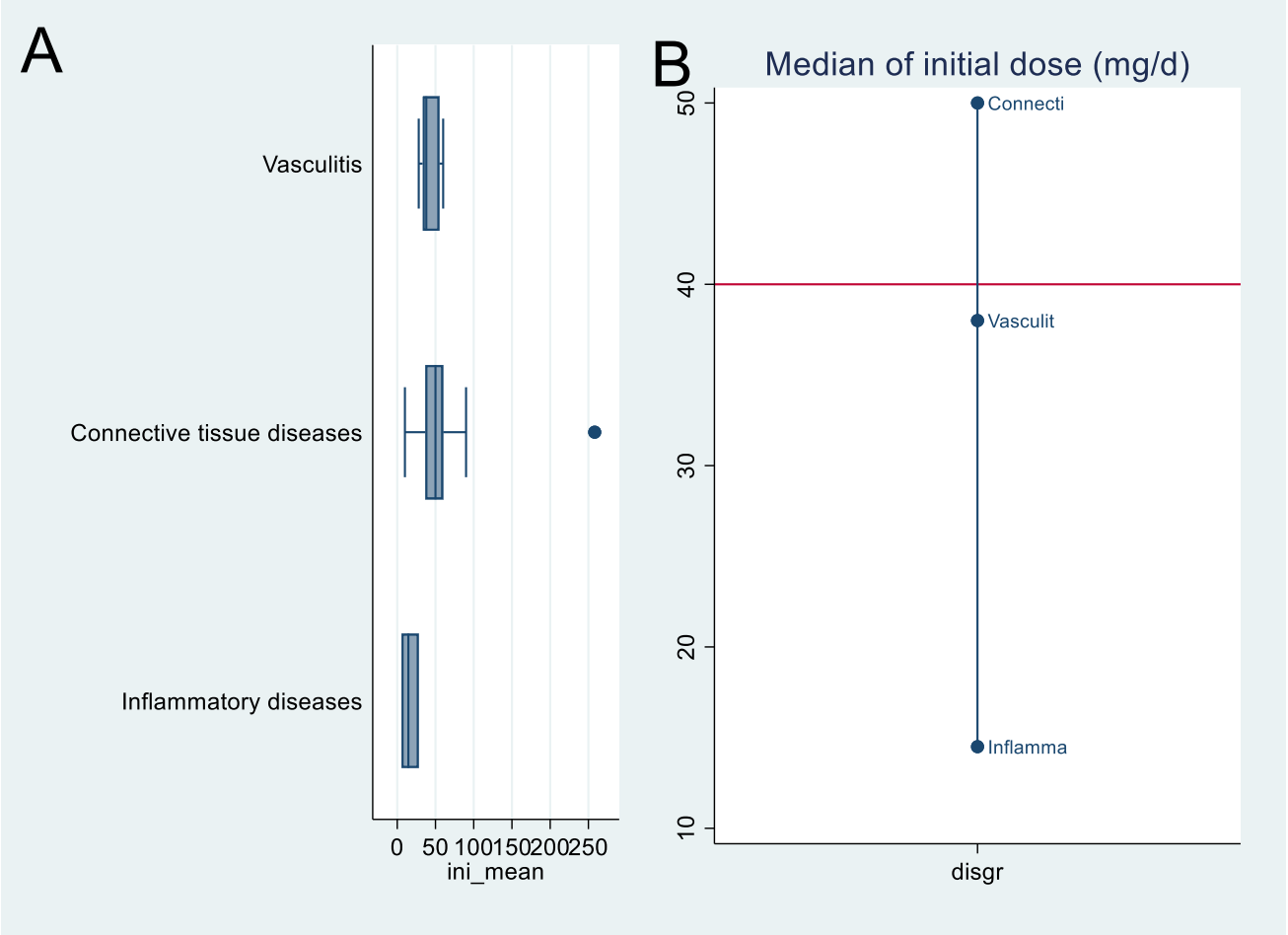


Figure 4- 19. (A) Box-plot graph demonstrating initial mean GC dose (mg/d) in disease cohorts with (B) overall median initial GC dose (mg/d): note: the outlier of initial dose 258.2 mg/d (SLE)

GC reports	covarites (initial dose)	Model selection with* or without† covriate	No. of study	no. of patients	no. of death		Proportion of death	95%CI	Predictive interval	Odds ratio	P value of odds ratio
Initial mean dose (mg/day)											
Overall studies (ranged 5.6 - 258.2 mg/d)	Continuous	0.01*	27	9159	768		0.13	0.10, 0.18	0.04, 0.37		
> 5-10 mg/d[1]	Continuous		2	6241	198		0.02	0.00, 0.06	0.00, 0.11	1.00	1.00
>10-30 mg/d[2]	Continuous		5	987	233		0.17	0.09, 0.30	0.04, 0.48	9.76 (2.3, 41.39)	< 0.001
>30-60mg/d[3]	Continuous		18	1642	311		0.16	0.11, 0.22	0.05, 0.42	8.89 (2.29, 34.48)	< 0.001
>60 mg/d[4]	Continuous		2	289	26		0.11	0.04, 0.29	0.02, 0.43	6.32 (1.18, 33.88)	0.03
Initial dose (mg/kg/day)											
Overall studies (ranged 0.12 - 1.5 mg/kg/d)	tertile	0.82†	35	4871	963		0.12	0.09, 0.18	0.01, 0.70		
1st tertile (0.12-0.75 mg/kg/d)	tertile		15	2017	393		0.12	0.07, 0.21	0.01, 0.71	1.00	1.00
2nd tertile (0.80-1.0 mg/kg/d)	tertile		17	2604	527		0.12	0.07, 0.19	0.01, 0.70	0.94 (0.44, 2.00)	0.87
3rd tertile (1.25-1.5 mg/kg/d)	tertile		3	250	43		0.18	0.05, 0.45	0.01, 0.83	1.42 (0.40, 4.99)	0.58
						0 0.2 0.4 0.6					

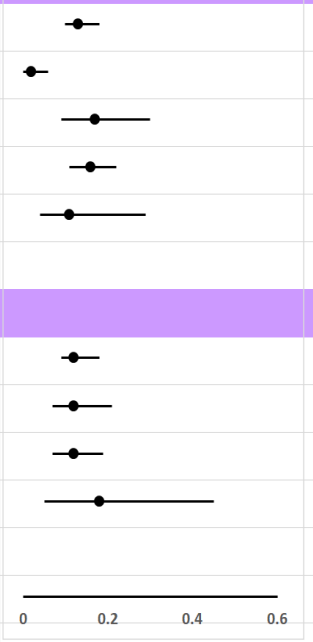


Figure 4- 20. Initial GC dose reports and proportion of deaths

4.2.9 Duration of GC use and mortality

GC exposure linked to the proportion of death was examined; however, the duration of GC exposure was limited at study level. Fifty-one articles reported on the mean duration of GC exposure (ranging between 1.5 months to 10 years), and ten articles reported on the median duration of GC exposure (ranging between 9 months to 9 years). The stratified mean duration of exposure was taken into account for analysis. The analysis was performed under a REM (test of heterogeneity - LR Test: REM vs FEM, $p < 0.001$, $\tau^2 = 1.53$) and without covariate (duration of exposure) model ($p = 0.18$ by leave-one-out LR test for model comparison). The OR for the proportion of deaths during 1-3 years, > 3-5 years and > 5 years compared with the baseline exposure period (≤ 1 year) were 1.33 (95% CI 0.58, 3.02; $p = 0.50$), 2.71 (95% CI 1.13, 6.50; $p = 0.03$), and 1.77 (95% CI 0.67, 4.68; $p = 0.25$), respectively (Figure 4-21). This finding, while preliminary, demonstrated that GC exposure for between 3 to 5 years was associated with significant excess deaths.

The subgroup analysis for the vasculitis that comprised only two exposure periods for analysis, including ≤ 1 year (n= 6 studies) and between 3 to 5 years (n= 4 studies), also supported increased OR of mortality during 3 to 5 years (3.63 times higher (95% CI 1.41, 9.37; p= 0.01). This was also found in medium and small-vessel vasculitis patients where mortality significantly increased 3.81 fold (95% CI 1.26, 11.57; p=0.02) during GC exposure between > 3 to 5 years (36% proportion of death, n=5 studies) compared to ≤ 1 year (9% of the proportion of death, n=3 studies). However, no statistically significant changes in GC exposure length were seen in studies that included participants with connective tissue or inflammatory diseases.

Duration of GC esposures	LR test with* or without† covariate	No. of study	no. of patients	no. of death		Proportion of death	95%CI	Odds	P value of odds
Overall studies	0.18†	51	29154	5085		0.1	0.07, 0.13		
≤1 years		23	15826	2330		0.07	0.04, 0.12	1.00	1.00
> 1 to 3 years		13	1528	171		0.09	0.05, 0.17	1.33 (0.58, 3.02)	0.5
> 3 to 5 years		8	10443	2353		0.19	0.09, 0.36	2.71 (1.13, 6.50)	0.03
> 5 years		7	1357	231		0.12	0.05, 0.27	1.77 (0.67, 4.68)	0.25
Vasculitis studies	0.07†	11	1185	232		0.13	0.08, 0.21		
≤1 years		6	597	55		0.08	0.04, 0.15	1.00	1.00
> 1 to 3 years		1	61	8		0.13	0.06, 0.24	1.62 (0.28, 9.48)	0.59
> 3 to 5 years		4	527	169		0.28	0.14, 0.48	3.63 (1.41, 9.37)	0.01
Medium to small vessels vasculitis	0.15†	8	798	173		0.14	0.08, 0.24		
≤1 years		5	433	50		0.09	0.04, 0.20	1.00	1.00
> 1 to 3 years		1	61	8		0.13	0.02, 0.48	1.35 (0.22, 8.15)	0.75
> 3 to 5 years		2	304	115		0.36	0.14, 0.66	3.81 (1.26, 11.57)	0.02
Connective tissue diseases	0.39†	27	2968	483		0.09	0.06, 0.14		
≤1 years		13	1078	164		0.06	0.03, 0.12	1.00	1.00
> 1 to 3 years		9	830	143		0.13	0.06, 0.26	2.21 (0.80, 6.09)	0.13
> 3 to 5 years		2	265	24		0.09	0.02, 0.35	1.43 (0.25, 8.24)	0.69
> 5 years		3	795	152		0.15	0.04, 0.42	2.50 (0.62, 10.04)	0.20
Inflammatory diseases	0.31†	9	24450	4323		0.08	0.04, 0.18		
≤1 years		1	13770	2074		0.15	0.14, 0.16	1.00	1.00
> 1 to 3 years		3	637	20		0.03	0.01, 0.11	0.17 (0.01, 2.15)	0.17
> 3 to 5 years		2	9651	2160		0.17	0.04, 0.54	1.14 (0.09, 14.11)	0.92
> 5 years		3	392	69		0.13	0.03, 0.39	0.87 (0.08, 9.81)	0.91
					0 0.2 0.4 0.6 0.8				

Figure 4- 21. Forest plot demonstrating the proportion of deaths in different exposure periods, stratified as less ≤ 1 year, > 1 to 3 years, > 3 to 5 years and > 5 years.

4.2.10 Change of proportion of deaths over the duration of follow-up

Although the duration of follow-up and the proportion of deaths did not correlate to GC-related mortalityFigure 4-22 shows that the majority of the studies evaluated outcomes after more than five years of follow-up. The proportion of deaths increased significantly after one year of follow-up, with OR at 1-3 years follow-up of 3.45 (95% CI 1.44, 8.30; p=0.01) compared to less than one year for all studies and for the subset of vasculitis studies 6.26 (95% CI 2.36, 16.59; p=0.01). On the contrary, there was no effect of follow-up duration and the proportion of deaths for studies reporting on patients with connective tissue and inflammatory diseases (p>0.05).

Duration of follow-up	LR test with* or without† covariate	No. of study	no. of patients	no. of death	Proportion of death	95%CI	Odds	P value of odds
Overall studies	0.03*	77	32935	4131	0.12	0.09, 0.12		
≤1 years		12	1111	112	0.05	0.02, 0.09	1.00	1.00
> 1 to 3 years		13	1270	356	0.16	0.09, 0.27	3.45 (1.44, 8.30)	0.01
> 3 to 5 years		19	8487	615	0.15	0.09, 0.23	3.11 (1.37, 7.08)	0.01
> 5 years		33	22067	3048	0.12	0.08, 0.17	2.52 (1.16, 5.46)	0.02
Vasculitis diseases	0.01*	20	2787	710	0.19	0.14, 0.26		
≤1 years		4	369	28	0.06	0.03, 0.14	1.00	1.00
> 1 to 3 years		4	337	141	0.39	0.22, 0.59	6.26 (2.36, 16.59)	< 0.001
> 3 to 5 years		6	678	186	0.24	0.14, 0.38	3.85 (1.45, 10.23)	0.01
> 5 years		6	1403	355	0.19	0.11	3.00 (1.11, 8.10)	0.03
Connective tissue diseases	0.42†	45	6057	895	0.1	0.07, 0.14		
≤1 years		6	504	75	0.05	0.02, 0.13	1.00	1.00
> 1 to 3 years		8	879	210	0.1	0.04, 0.21	2.15 (0.58, 7.88)	0.25
> 3 to 5 years		10	1486	205	0.13	0.06, 0.23	2.69 (0.78, 9.23)	0.12
> 5 years		21	3188	405	0.11	0.07, 0.17	2.35 (0.75, 7.35)	0.14
Inflammatory diseases	0.78†	10	23943	2508	0.09	0.04, 0.17		
≤1 years‡		1	158	7	NA	NA	NA	NA
> 1 to 3 years‡		1	54	5	NA	NA	NA	NA
> 3 to 5 years		2	6255	208	0.05	0.01, 0.22	1.00	1.00
> 5 years		6	17476	2288	0.1	0.04, 0.22	1.85 (0.35, 9.89)	0.47
					0 0.2 0.4 0.6 0.8			

Figure 4- 22. Forest plot demonstrating the proportion of deaths in different duration of follow-up, stratified as less ≤ 1 year, > 1 to 3 years, > 3 to 5 years and > 5 years.

4.2.11 The causes of death

83 of 128 articles reported on the causes of death (Table 4-3 and Figure 4-23. However, in studies that reported the causes of death, 25% of cases had an unclear reason. Cardiovascular disease was the leading cause of mortality (25.6%), followed by malignancy (15.7%), infection (13.4%), respiratory failure (10.8%), and other active illnesses (4.5%). The common causes of deaths were detailed by underlying disease treatment, as shown in Table 4-4 and Figure 4-24. The leading causes of death in vasculitis were infection (22.7%), active underlying disease (14.8%), cardiovascular disease (13.5%), and unknown causes (37.2%). The first and second ranking causes of deaths were the same in the connective tissue diseases, which were infection (32.8%), cardiovascular disease (18.0%), but the third and fourth common causes of death were renal (9.8%) and malignancy (8.9%). Cardiovascular disease was the first leading cause of death in inflammatory diseases, followed by malignancy (22.7%) and respiratory diseases (16.9%). Only one article in the category of the benign haematologic disease reported causes of death, and in 43.8%, this was infection. Unsurprisingly, respiratory (34.6%) and active diseases causes (34.6%) were the major contributors to the causes of death in patients with respiratory diseases. However, it was interesting that cardiovascular disease was the second most common cause of death (26.9%) in respiratory diseases patients. Unfortunately, the unknown cause of death comprised 18.7% in connective tissue diseases, 23.7% in inflammatory diseases, and 12.5% in haematologic diseases. We performed a rigorous analysis of GC dose and causes of death, but there were no conclusive results (Appendix 4-7)

Table 4- 3. Causes of death

Causes of death	No. of deaths (%)
Cardiovascular disease	1104 (25.6)
• Cardiac diseases	1024 (23.8)
• Cerebrovascular diseases	49 (1.1)
• Thromboembolism	31 (0.7)
Malignancy	678 (15.7)
Infection	579 (13.4)
Respiratory	477 (11.1)
• Respiratory failure	457 (10.6)
• Pulmonary haemorrhage	20 (0.5)
Active diseases	195 (4.5)
Renal diseases	108 (2.5)
• Renal failure	81 (1.9)
• Other Renal diseases	27 (0.6)
Gastrointestinal	39 (0.9)
• GI diseases	19 (0.4)
• GI bleeding	16 (0.4)
• Liver failure	3 (0.1)
• Pancreatitis	1 (< 0.1)
Psychiatric	5 (0.1)
• Suicide	4 (0.1)
• Psychiatric	1 (< 0.1)
Other	8 (0.2)
• Trauma	4 (0.1)
• Multi-organ failure	3 (0.1)
• Other hemorrhage	1 (<0.1)
Undetermined	1114 (25.9)
Total	4307 (100)

Table 4- 4. Causes of death by pathogenesis (n=83 articles)

Diseases	No. of studies reported COD	No. of patients	No. of death (%)*	No. of deaths (%)*									
				Cardiovascular diseases	Infection	Active diseases	Malignancy	Gastrointestinal diseases	Respiratory diseases	Renal diseases	Psychiatric diseases	Other causes of death	Undetermined causes
Vasculitis	27	4198	1076 (25.6)	145 (13.5)	244 (22.7)	159 (14.8)	67 (6.2)	15 (1.4)	17 (1.6)	22 (2.0)	3 (0.3)	4 (0.4)	400 (37.2)
Connective tissue diseases	45	6210	863 (13.9)	155 (18.0)	283 (32.8)	24 (2.8)	77 (8.9)	17 (2.0)	57 (6.6)	85 (9.8)	3 (0.3)	161 (18.7)	161 (18.7)
Inflammatory diseases	8	13276	2326 (17.5)	794 (34.1)	44 (1.9)	5 (0.2)	528 (22.7)	7 (0.3)	394 (16.9)	1 (<0.1)	1 (<0.1)	1 (<0.1)	551 (23.7)
Haematologic diseases	1	68	16 (23.5)	3 (18.8)	7 (43.8)	0 (0.0)	4 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (12.5)
Respiratory tract diseases	2	415	26 (6.3)	7 (26.9)	1 (3.8)	7 (26.9)	2 (7.7)	0 (0.0)	9 (34.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	83	24167	4307	1104	579	195	678	39	477	108	5	8	1114

Note: *: weighted percentage deaths

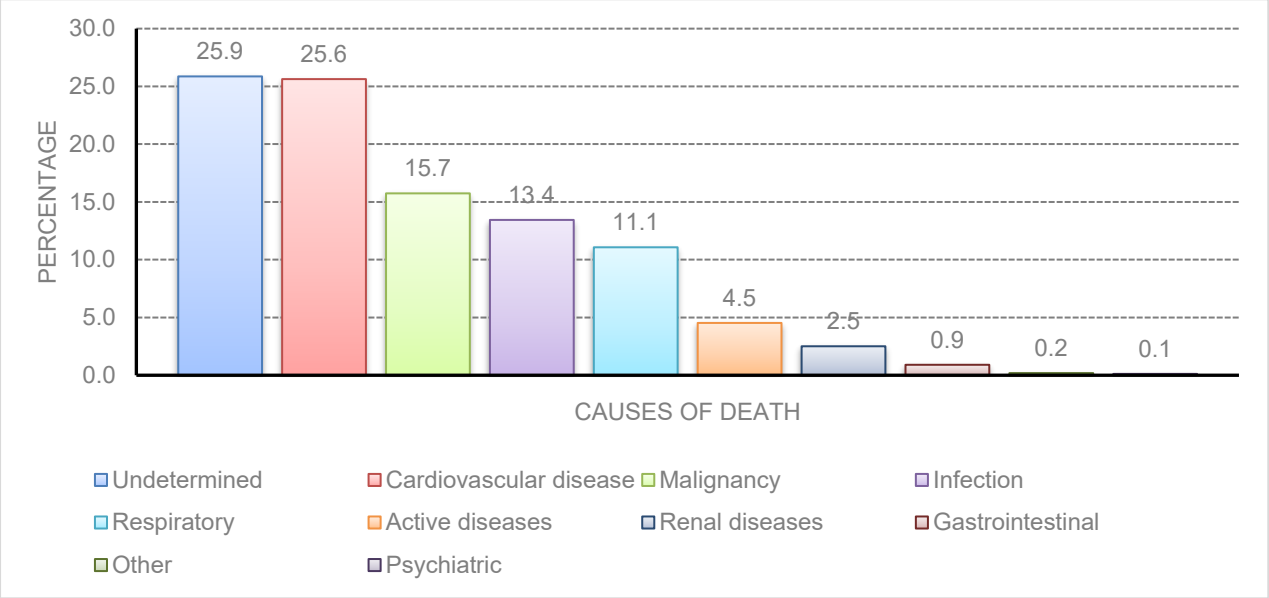


Figure 4- 23. Bar chart plot demonstrating the cause of death in GC treated disease subgroups

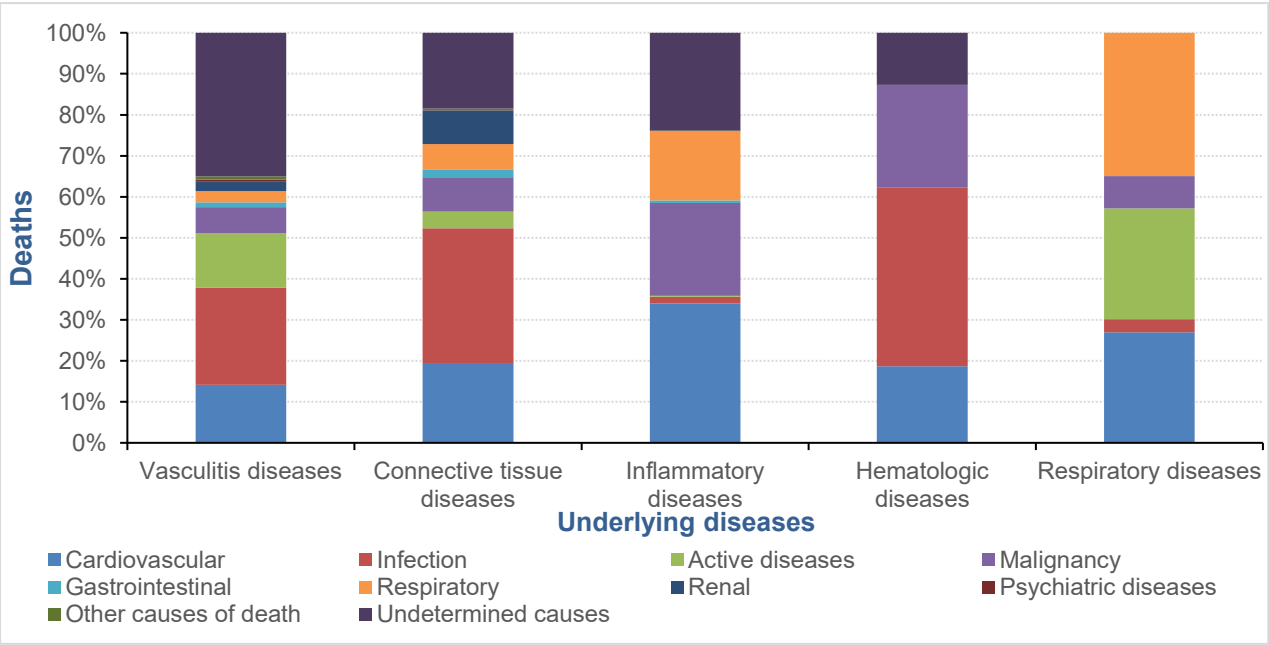


Figure 4- 24. Bar charts demonstrating the causes of deaths in each underlying disease treated by glucocorticoids

4.2.12 Publication bias

Publication bias was assessed using funnel plots by plotting the precision of each study (standard error) on the y-axis versus the estimated effect sizes (proportion of deaths) on x-axis. Each dot corresponds to a single study. The plot would resemble a pyramid or inverted

funnel in an ideal world, with dispersion caused by sample variance. The observed shape was predicted, given the large range of standard errors across the literature. If all studies had the same standard errors, they would all fall on a horizontal line. The asymmetrical funnel plot depicted the inability of a meta-analysis to incorporate some publications, particularly unfavourable outcomes, unpublished publications, or unavailable papers [633]. In general, the Funnel plot was used for the clinical trials[477]. The funnel plot's constraint for proportional data was that the data must be in the range 0 to 1, which could not include a value less than zero. As a consequence, an asymmetrical funnel plot was produced by natural of the data type which also found in Figure 4-25. Thus, an asymmetrical plot could not be used to determine which publication bias existed in this study.

Egger's and Begg's test formal statistical tests was performed to find asymmetry in a funnel plot due to small study effects [634, 635]. The null hypothesis of Egger's test and Begg's test assumed that the symmetrical plot occurs in the funnel plot, with the alternative hypothesis showing that asymmetry occurs [475]. The p-value for Egger's test for this study is 0.27, so there was no evidence to reject the null hypothesis. Therefore no apparent bias existed in the studies that were included in the meta-analysis. Begg's test for small-study effects was also not statistically significant (Kendall's score of 80 ± 97.5, p= 0.24).

In the trim and fill method, there were two stages. First, we trimmed or eliminated studies, starting with the least powerful until we had symmetry in the funnel plot. From the remaining studies, we then derived a new pooled estimate. Second, we filled in the holes we had identified; we reflected them in the pooled estimate line and added new studies for the studies eliminated[636]. Nonparametric trim-and-fill analysis of publication bias was also performed by imputing to the left for linear estimator and we found no difference between observed studies (0.19, 95% CI 0.18, 0.20) and imputed studies (0.19, 95% CI 0.18, 0.20). In summary, there was no publication bias in this study.

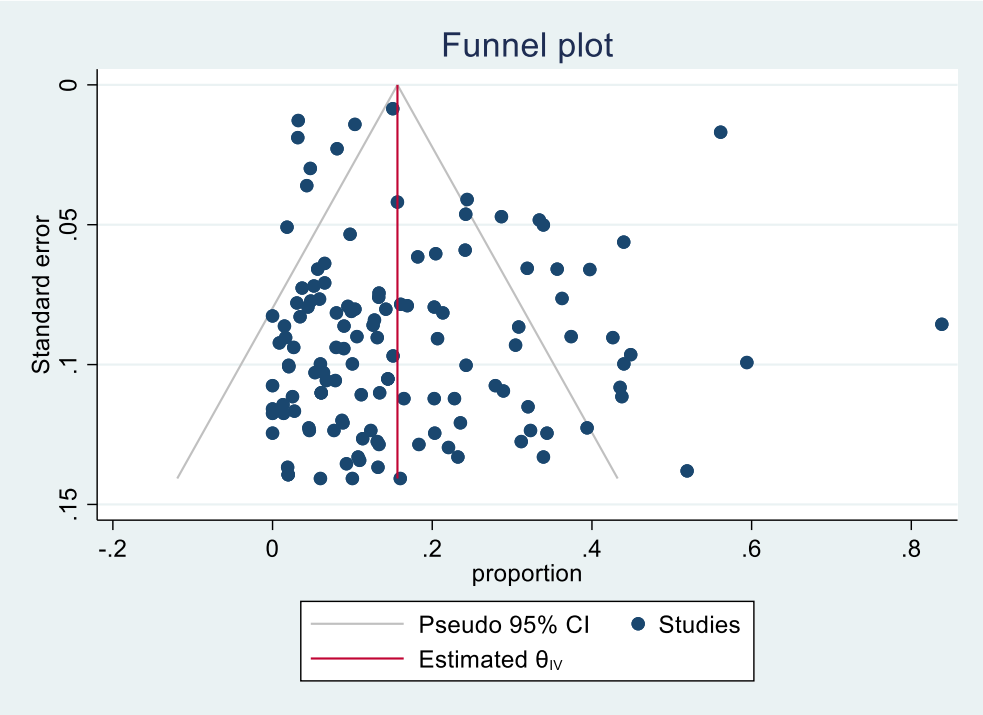


Figure 4- 25. Funnel plots demonstrating the publication bias

4.3 Discussion

We have performed the first large-scale and comprehensive systematic review and meta-analysis that assesses mortality in patients treated with exogenous GC across the globe. We focused on GC dose records and GC exposure duration in different underlying "benign" diseases treated by GC. Additionally, we report the causes of death across the studies and disease subgroups by disease pathogenesis. The significant findings were the incremental effect of cumulative GC dose (g), the average daily dose (mg/d) and the absolute initial dose (mg/d) on increased OR of mortality in specific subgroups of patients. However, relationships between maintenance dose and the proportion of deaths were inconsistent. The proportion of deaths increased by 5.7 to 6.7 fold for cumulative doses higher than 5.0g in patients with vasculitis. An average daily GC dose of more than 5mg/d significantly increased deaths by fivefold in studies that principally comprised patients with RA. An initial mean dose of more than 10mg/d also demonstrated a statistically significant 8.9 to 9.7 fold increased proportion of deaths.

Based on the established genomic and non-genomic effects of GC's, our study aimed to evaluate the impact of GC dose as a predictor of mortality. To date, only a very few studies have explored this relationship[613, 617]. Our study found that the relationship of cumulative GC dose and average mean dose on mortality was also consistent with a population-based cohort study in the UK[613]. In this study, the HR for mortality was 2.05 (95% CI 2.04, 2.06) per 1000mg of cumulative prednisolone-equivalent dose over the past year and 1.26 (95% CI 2.24-1.28) for every increase of GC 5mg per day. There were also supporting data showing that cumulative GC exposure was an independent risk factor for mortality in the British SLE population when compared with age- and sex-matched for 1 SLE to 6 controls [637]. In keeping with our data, the initial dose of GC ≥ 1mg/kg/d increased mortality in the Hong Kong SLE study [638]. The obvious conclusion is that the higher dose of GC may reflect underlying disease activity and disease severity, which remains a challenge in disentangling the GC effects per se or the underlying disease treated by GC [639]. It is accepted that the interpretation of predictors of mortality in GC exposures is subject to many limitations [639]. First, the disease condition and related morbidities may have temporal primacy[640], meaning that during the active phase or early GC exposure, the mortality risk is higher than for inactive disease, which may be subject to a longer duration of follow-up. Second, the longer follow-up mortality may be explained by other factors (age, co-morbidities, concomitant diseases and treatment). Third, the medical care and recommendations/ clinical guidelines and access to GC-sparing therapies have also changed over periods of time. Fourthly, the difficulty in differentiating mortality related to

underlying disease from GC effects. While these possibilities and confounding factors arise from a wide variety of causes, we could not conclude the direct impacts or magnitude of GC on mortality. Our results underline the real world information of GC use, which is high heterogeniety of data collection and reporting, disease-specific complexities in patterns of GC use in patients, that warrants standardised GC data collection and exploration at an individual patient level.

When exploring the duration of follow-up, the findings demonstrated a peak in mortality between 3 to 5 years. The data must be interpreted with caution because there was weak evidence to support GC use throughout the duration of follow-up directly. In all studies and in the subgroup of vasculitis, exposure to GC between 3 to 5 years demonstrated the highest proportion of deaths compared to GC exposure for less than one year. The possible explanation for the time oriented mortality outcome is that is that the proportion of deaths peaked between 3 to 5 years follow-up. While not totally unexpected for increased mortality for the more extended follow-up period, the duration after starting GC at 3 to 5 years may encourage us to pay attention to plausible reasons from either underlying disease treated by GC or specific complications from GC. Additionally, investigating the cause of death during this time frame may help us improve outcomes and avoid patient deaths from treatable causes.

In this meta-analysis, cardiovascular diseases, infection, disease flares and malignancy were the main contributors to deaths across the studies. Causes of deaths also comprised active disease-related complications such as renal failure, respiratory failure, and pulmonary haemorrhage, highlighting the impact of the active underlying disease for which GC is prescribed as the foremost factor in explaining mortality. Contrary to expectations, this study did not find a significant cause of death from adrenal insufficiency.

The accumulation of publications documenting secondary adrenal insufficiency in patients treated with GC might explain a substantial increase in premature deaths than background populations[641-646]. The causes of death in patients with primary adrenal insufficiency were cardiovascular diseases, infection and malignancy [643-645]. These causes of death are similar to those reported in our study, underpinning the potential effect of long-term GC exposure on morbidity and mortality. Further detailed exploration of morbidities in GC treated studies highlighted the increased cardiovascular risk (OR 1.51-3.85), DM (OR 1.73-.81), hypertension (OR 1.53-2.24), hyperlipidaemia (OR 1.51-1.98)[641] which are known to contribute to cardiovascular mortality[647].

Cardiovascular mortality was consistently high across the subgroup of patients using GCs in keeping with the results of previous studies[648]. GCs had a high risk for any

cardiovascular event (HR 4.16, 95% CI 2.98, 5.82), coronary heart disease (HR 2.27, 95% CI 1.48, 3.47), heart failure 3.77 (2.41 to 5.90), and ischaemic cerebrovascular events (2.23, 95% CI 0.96, 5.17)[216]. Cardiovascular mortality also increased in vasculitis, inflammatory diseases or connective tissue diseases in epidemiology studies [216, 252, 617, 620, 629, 630, 648-652]. In the population-based cohort study United Kingdom Clinical Practice Research study, GC-related mortality was seldom reported and confined to cardiovascular risks correlating with traditional risk factors and active inflammatory markers [653, 654]. However, our study further demonstrated the different proportion of causes of death in individual subgroups (excluding haematologic disease because only one article reported causes of death) and the different GC reports (Table 4-5). Interestingly, cardiovascular mortality was highest in inflammatory diseases (the majority patients were RA) yet the average GC dose in inflammatory disease was lower (5.5mg/d) compared to connective tissue diseases (22.9mg/d) or vasculitis (7.8mg/d). However the cumulative dose for all three groups were comparable. The hypothesis is that the natural history of RA increases the chance of long-term exposure to GC and other drugs contributing to CVD (DMARDs, NSAIDS or COX inhibitors)[655]. The inflammatory disease itself is also the main contributing to cardiovascular diseases, supported by increased cardiovascular risk or accelerated atherosclerosis or heart failure approximately 1.5 to 2 fold in cohort studies and meta-analysis of RA [656, 657]. Further analysis also reported increases in both myocardial infarction and cerebrovascular accidents [658]. Inflammation linked to atherosclerosis may underpin plaque formation and prothrombotic states[659]. The advances in RA treatment and early management suppression of inflammation help decrease but cannot eliminate cardiovascular mortality in RA [660, 661]. EULAR's current recommendations for cardiovascular disease treatment are focused on suppressing inflammation and reducing disease activity and conventional cardiovascular risk management [662]. However, for GC, the evidence was inconclusive [654, 662]. Our findings provide insight into the causes of mortality from cardiovascular diseases in GC-use patients (immunological and inflammatory diseases. An increase in cardiovascular HR of 1.08 (95%CI 1.07, 1.10) per 5 mg/day across immune-mediated illnesses, with the greatest HR of 1.30 in SLE (95%CI 1.22, 1.38) highlights the link to GC dose. Increased dose-dependent cardiovascular disease risk variables were seen in this research study regardless of disease activity.

To summarize, both GC and the underlying pathogenesis treated by GC play a significant role in mortality, mainly cardiovascular events. Recommendation for individuals with immune-mediated or inflammatory illnesses or/and chronic GC usage as a risk factor for cardiovascular disease should be comparable to those with diabetes mellitus, hypertension, or dyslipidemia. Additional research into the synergistic effects of GC and inflammation will

demonstrate the critical nature of cardiovascular risk stratification and aggressive cardiovascular risk management in patients taking GC.

Table 4- 5. Glucocorticoid dose reports

GC dose reports (Prednisolone equivalent)	Vasculitis	Connective tissue diseases	Inflammatory diseases	Haematologic diseases	Respiratory diseases
Cumulative dose (g)	5.5 n=11 (IQR 4.6, 9.4)	5.2 n=10 (IQR 1.3, 36.7)	5.03 n=9 (IQR 1.7, 7.3)	3.9 n=1	3.4 n=3 (IQR 2.7, 18.3)
Average dose (mg/d)	7.8 n=3 (IQR 7.5, 17.0)	22.9 n=7 (IQR 10.0, 30.0)	5.5 n=5 (IQR 4.9, 8.0)	No	10.0 n=3 (IQR 9.1, 47.6)
Maintenance dose (mg/d)	5.6 n=6 (IQR 5.0, 7.5)	10.0 n=14 (IQR 7.5, 15.0)	9.1 n=2 (IQR 6.2, 12.0)	64.9 n=1	No
Initila dose (mg/kg/d)	1.0 n=9 (IQR 1.0, 1.0)	0.75 n=25 (IQR 0.5, 1.0)	No	1.5 n=1	No
Initial dose (mg/d)	38.0 n=9 (IQR 33.2, 55.1)	50.0 n=15 (IQR 36.7, 60.0)	14.5 n=3 (5.6, 28.1)	No	No

Infection was one of the leading causes of death in patients using GCs, especially in those with vasculitis (22.7%), connective tissue diseases (32.8%) and haematologic diseases (43.8%), whereas only 1.9% of life-threatening infection led to death in inflammatory diseases. GC are known to suppress immune function and increase susceptibility to infection. Vasculitis patients receive higher GC doses than those with inflammatory diseases or RA (**Error! Reference source not found.**). In current practice, other immunomodulating agents are used in combination, leading to increased susceptibility to severe infections. The earlier publications support our findings that the risk of infection was low in RA patients taking low doses of GC. However, these patients had early active disease with a 2-year follow-up [663]. Unfortunately, there was no information on potential preventative strategies for infections, dose-related associations, concomitant immunosuppressive use, infectious organisms, or target organ involvement.

For malignancy mortality, high rates were observed in patients with inflammatory diseases (22.7%) compared to vasculitis (6.2%) and connective tissue diseases (8.9%). Increased malignancy deaths were reported in RA [664], but not in SLE [665]. Concomitant metabolic syndrome has been postulated to be a major driver of the malignancy risk [666]. As revealed by earlier studies on patients with chronic inflammatory rheumatic diseases and metabolic syndrome, malignancy incidence was higher in patients with co-existing metabolic syndrome [667]. Moreover, a Swedish population-based study of patients with Addison's

disease treated with GC replacement therapy found increased malignancy compared to the background population [643]. Our study did not aim to investigate this association; nevertheless, malignancy in GC users and metabolic syndrome are intriguing areas for further investigation.

4.3.1 Homogeneity of sample size, sample size, study design

The sample size is the key factor that affected the precision of the study. Our research utilizes large sample size studies, which may result in more precise findings. Homogeneity across the studies differed for study design, clinical characteristics, underlying diseases and the dynamics for GC dose ($p < 0.01$). These are the natural 'real world' datasets from cohort studies. The bias assessment helps stratify the quality of study enrolment, which demonstrated a moderate to high bias for the evaluated articles. We were unable to assess the severity of illness or concomitant immunosuppressive therapy at the study level, which are critical confounding variables for mortality. There were different clinical practice guidelines for the entire timeline of data collections; the concomitant treatments, co-morbidities, and individual patient information were not available for further analysis. The GC dose and duration were recorded in different ways and across different sub-groups. The dose for treatment was dynamic and changing across the cohorts and depended on the articles' objectives and reports.

4.3.2 Strengths and Limitations

An important strength of our study is the first meta-analysis and meta-regression of real-world GC use across a broad spectrum of underlying inflammatory and autoimmune diseases. Our methodological approach comprising standard methodology for the literature search, documentation of the duration of the study period, and especially the assumptions made based on the available data allowed analysis of the binary data (single-armed proportion data). The meta-analysis with mixed-effect regression model for binomial distribution data is innovative in terms of an analytical tool. Additionally, a new software programme called *metapreg* was explicitly created for the purpose of performing a single-arm proportion analysis in this investigation. We provided the prediction interval, which indicates the real ranges of the population's proportion of deaths, rather than sample size estimates.

The limitations of the study were the majority of included articles were observational or cohort studies with a general high risk of bias (confounding, selection or severity of diseases). There were also various underlying diseases treated with GC, with high heterogeneity of disease, co-morbidities and co-intervention, which were confounding factors

of mortality. Heterogeneity in baseline risk amongst the studies played a major contribution to clinical application for identifying patients at risk who might benefit or risk from GC. Obtaining the longitudinal epidemiological studies in this analysis improves the yield of patients, but the outcome of this cohort study requires considering of dynamic changing of clinical practice and mortality outcomes. However, using optimal methodology and assumption, the subgroup analysis and meta-regression analysis proceeded.

4.4 Conclusions

Based on our results, three GC determinates, cumulative dose, average mean dose, and absolute initial dose, had a relationship with increased mortality. A cumulative GC dose greater than 3.9 and 5 g increased the proportion of deaths across the studies and subgroups of vasculitis patients, respectively. An increase in average amount to 5 to 30 mg/d increased the OR of death significantly for any rheumatological disease. A starting dose of GC of more than 10mg is associated with an increased OR of mortality. The mortality increased significantly after taking GC for more than one year, reaching a maximum proportion of deaths from 3 to 5 years of GC exposure. The causes of the death overlapped with the causes of death identified for endogenous CS mortality, but also in disease cohorts untreated by GC. The different causes of deaths based on subgroup analysis emphasise disease-specific mortality and the effect of GC treatment.

4.5 Suggestions

Many new therapies have emerged to treat patients with autoimmune, inflammatory or malignancy diseases. Despite this, the class of GC are one of the most effective, rapidly acting and cheap therapeutic agents where use is increasing. The next steps to delineate the effects of GC per se versus underlying disease for exogenous GC studies would be to focus on the GC dose, duration and follow-up period, co-morbidities and mortality. Beyond GC dosage records and monitoring GC adverse effects, genomic and molecular approaches at the pre- and receptor levels for particular patients or specific diseases may offer a more precise approach in prescribing GC.

Further information on the causes of death is required to establish a direct link between GC and mortality. We have limited SMR data on GC treated patients, which is critical for comparisons to the general population of similar age and gender.

Improved techniques for meta-analysis might involve network meta-analysis (NMA) in which all treatments in combination with GC use can be evaluated. Individual patient data (IPD) meta-analysis could also explore disease and GC effect in greater detail. Finally the investigation of GC and mortality in transplant patients would be of considerable interest.

Chapter 5
Pre-receptor Glucocorticoid Metabolism in Human Dermal Fibroblasts: Regulation by Hypoxia and Inflammation

5.1 Introduction and research concept

Delayed WH was found the most in patients with DM[668], ischaemic conditions (e.g. peripheral arterial diseases or PAD, vasculitis, sickle cell diseases[669, 670]), elderly[671, 672], and CS[673]. The tremendous social[674, 675] and economic burden[676, 677] with high morbidity and mortality of delayed WH can be emphasised through diabetic wounds [678-680]. Diabetic wounds remain 10% of the diabetic population (463 million people worldwide in 2019[681]), of which 15-25% of T2DM suffered from diabetic foot ulcer (DFU) once in their lifetime[682]. 5-year mortality of DM was 30.5% of DFU, 46.2% of minor amputation and 56% of major amputation, which was comparable to the overall malignancy of 31%[683]. The time- and resource-consuming diseases require high expenses, such as in the UK, the estimated cost of DFU and amputation was between £837.01m-£962.38 m[684]. Thus, the primary goal of wound management is to enhance wound closure and restore normal function, which requires the sequential organisation of several cell types and molecular processes.

Typically skin findings in DM, elderly or PAD share similar GC excess characteristics, including thinning and dry skin with shiny, paper-thin quality; the fragility of capillary blood vessels leads to bruising easily, susceptibility to infection and delays WH[292, 685-687]. Pioneering works showed systemic or topical GCs disintegrating WH and dermatology cascades, attribute suppressing immunological cells, angiogenesis, fibroblasts proliferation, matrix synthesis, and macrophages chemotactic, bactericidal, and antigen-presenting cells functions[687]. Apart from CS' skin, there also strongly supported local GC excess by increasing 11β-HSD1 activity in ageing skin[145, 146] and specific tissues of wound[688]. The local GC synthesis at the tissue is the exciting part of delayed healing that limits molecular research. Furthermore, the hallmark pathogenesis of a delayed WH is hypoxic; ischemic wounds, commonly found in delayed wounding of PAD or DM or elderly, have not been explored for the interplay between hypoxic tissues and local GC production.

5.1.1 Skin

Skin is the largest organ (~15% of total body weight and covers an area of 1.5 to 2 m^2) which maintains homeostasis via regulation, protection and sensation (Figure 5- 1)[689, 690]. An important role is the ability of the skin's immune system to regulate pro-inflammatory and anti-inflammatory functions during WH[691].

Non-healing and diabetic wounds remain a great social and economic burden (>£800 million in the UK population)[684], with high morbidity and mortality. The prevalence of any wound type in the UK during 2017/2018 was 3.8 million people[692, 693], and chronic wounds attributed 48% of wounds managed at any one time.

5.1.2 Dermal fibroblasts

HDF reside in the skin papillary and reticular dermis and are also associated with hair follicles (Figure 5- 1 5-1)[25, 26]. HDF maintain the structural integrity of the dermis and are a key regulator of WH [694, 695]. They are used to study various molecular, metabolic and pathophysiological states. HDF studies benefit from various advantages, including sample acquisition simplicity, uniformity of cell type, high sample volume, genetic stability, long-term propagation and storage, and ease of maintenance[695, 696]. However, limitations include an oversimplified microenvironment, bias from differences in proliferation, confluence and senescent cells, and lack of *in vivo* skin function physiology. Despite these limitations, they are a useful and practical experimental tool to study skin cell function and regulation mechanisms.

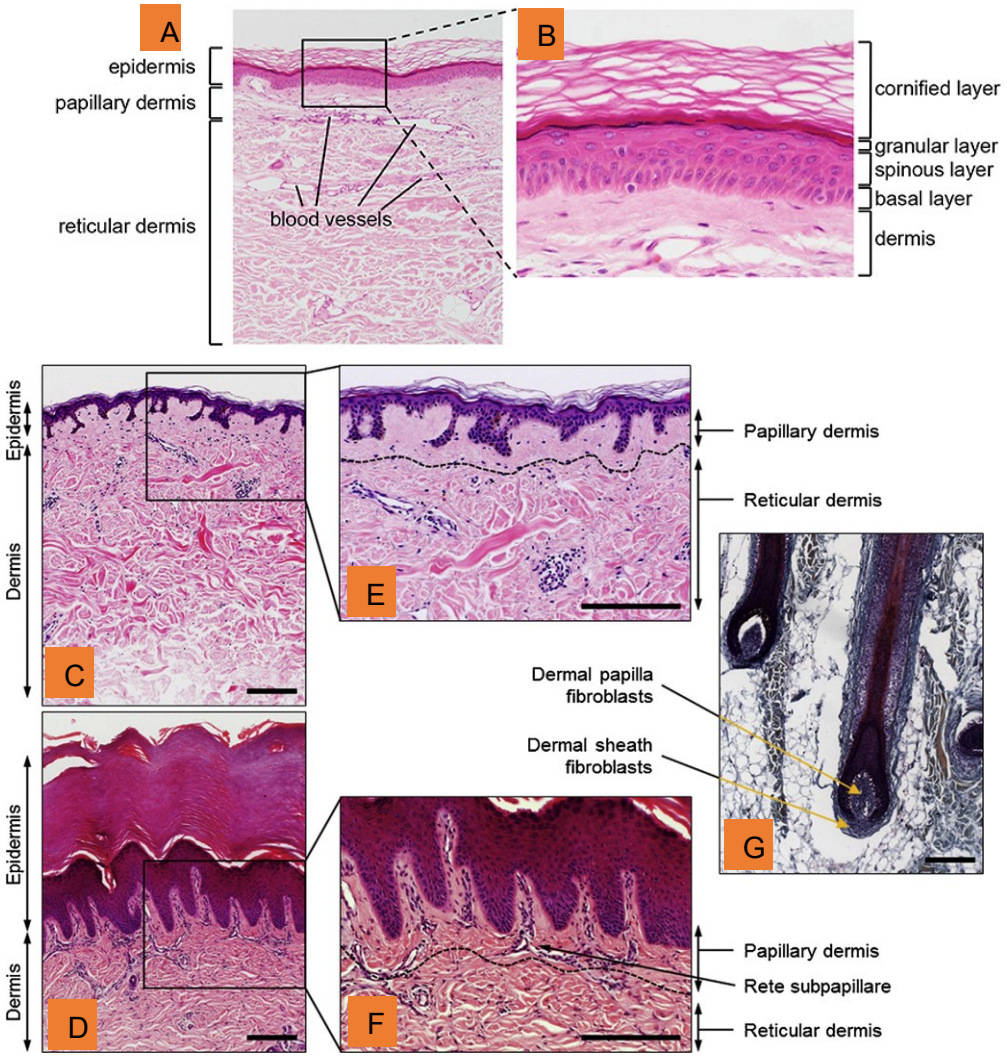


Figure 5- 1. Skin structure

(A) Skin is composed of epidermis, dermis, and hypodermis. (B) The epidermis is composed of epidermal keratinocytes subdivided into cornified, granular, spinous and dermal layers. (C, D) Different thickness of epidermis from back (C) and palm (D) skin. (E, F) Papillary and reticular dermis containing fibroblasts. (G) Fibroblasts associated with hair follicles. Figure modified from [694, 696, 697]. Reproduced with permission from Copyright Clearance Center's RightsLink®European Journal of Cell Biology, Gopu Sriram et al.(2015) with license number 5266870617585, permission granted on March 2022, by email.

5.1.3 Wound healing

5.1.3.1 Normal healing and chronic wounds

Normal WH requires the well-orchestrated interaction between complex biological and molecular events[698, 699] that compose four overlapping stages: haemostasis (coagulation), inflammation (mediated by pro-inflammatory cytokines, e.g. IL-1β and enzymes, e.g. Prostaglandin-Endoperoxide Synthase 2 (PTGS2), proliferation and remodelling[700] (Figure 5-2). Adequate healing requires a healthy microenvironment, good

oxygen delivery[701, 702] and nutrition to the tissue[703, 704]. Defects in any stage, or infection, increase the risk of developing a chronic wound[700, 705].

A major problem of delayed WH is impaired vascular supply leading to hypoxic, ischaemic wounds. However, hypoxia is also involved in normal healing. In the early stages of acute wounds, tissue injury leads to vascular disruption and hypoxia[701]. This activates pathways to restore oxygen supply and modulates cell function, e.g. fibroblast differentiation, cell migration, tissue remodelling and growth factor release[706]. This physiologic response to local wound hypoxia plays a critical role in determining the success of normal healing[707].

A reduced oxygen (hypoxic) environment is a key factor in the aetiology of chronic wounds in T2DM, venous stasis disease or pressure ulcers[708-710]. These conditions share characteristic of impaired healing, including local tissue hypoxia, reduced angiogenesis[708-710] and inappropriate inflammation[711](Figure 5-3).

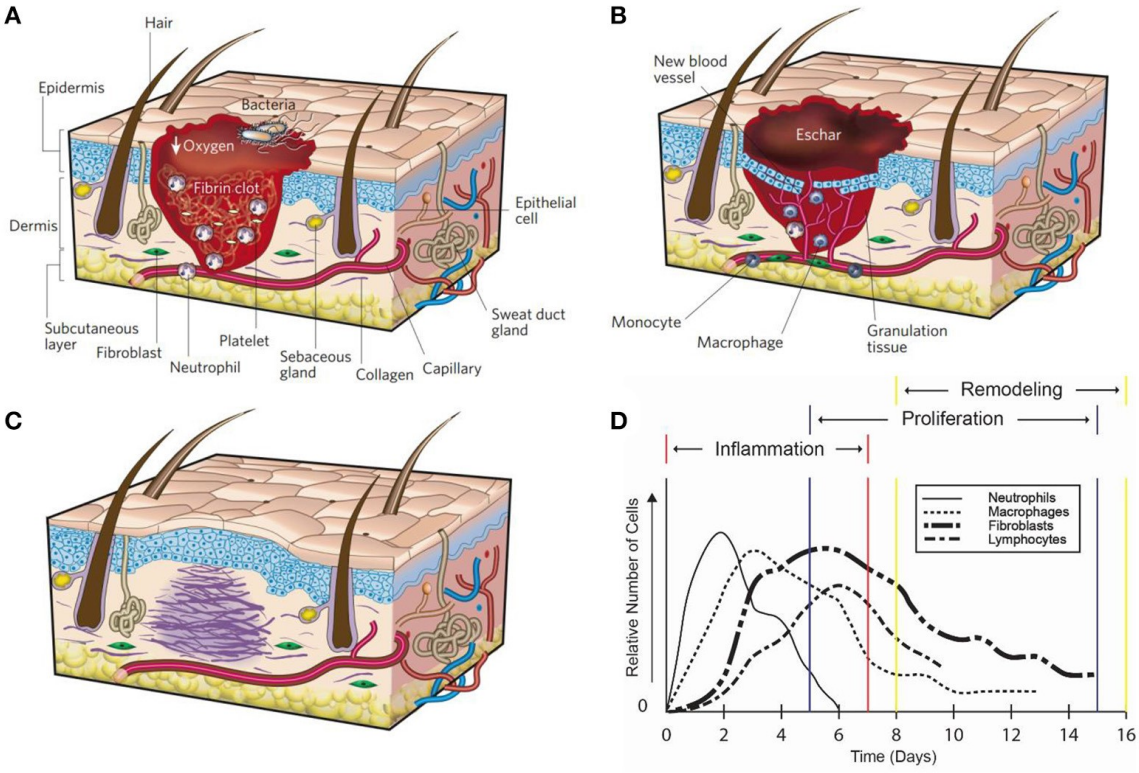


Figure 5- 2. Normal wound healing.

*Haemostasis is the first stage of platelet plug and fibrin clot formation. Platelets and leukocytes release cytokines (e.g. IL-1β / PTGS2) to activate inflmmation, stimulate collagen synthesis, activate differentiation to myofibroblasts, initiate angiogenesis (e.g. VEGF) and start re-epithelialisation[698, 712]. Next,**(A)** inflammation attracts neutrophils to prevent infection and macrophages for debris removal. During the (**B**) proliferative phase, keratinocytes close the wound gap, and fibroblasts / macrophages replace the initial fibrin clot with granulation tissue. Finally, **(C)** remodelling is the stage of wound contraction and extracellular matrix deposition by myofibroblasts with blood vessel regress.*

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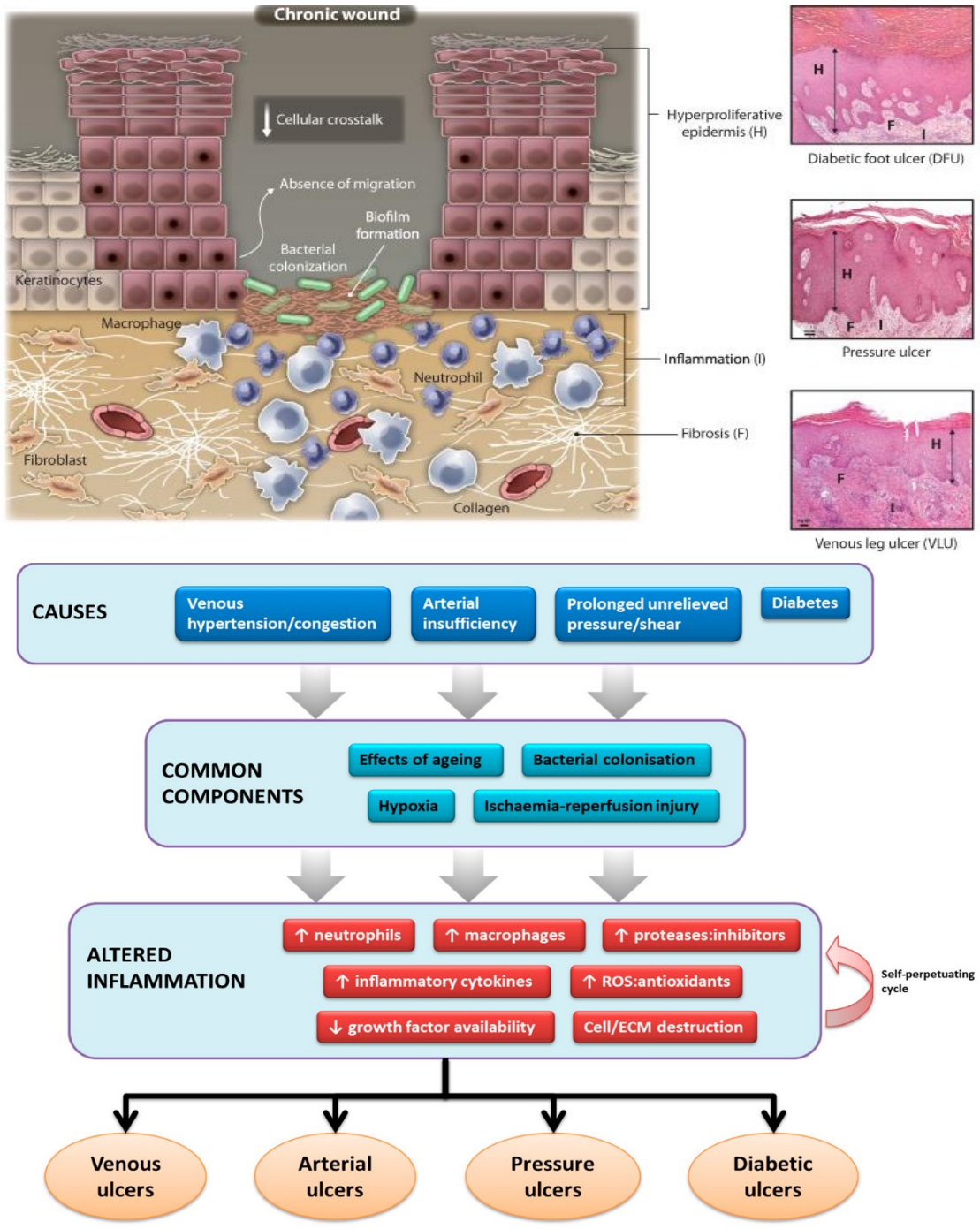

Figure 5- 3. Pathological changes in chronic wounds

Abbreviation H, hyperproliferative epidermis; F, fibrosis; I, increased cellular infiltrate (inflammation); ROS, reactive oxygen sepcies. Reproduced with permission under an open access Creative Common CC BY license from Int. J. Mol. Sci. Ruilong Zero(2016)[714]. And Copyright Clearance Center's

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5.1.3.2 Angiogenesis

Angiogenesis is the process of new capillary formation and is a crucial factor in the WH process. Reduced angiogenesis causes compromised oxygen supply and prolongs tissue hypoxia as observed in ischaemic diabetic wounds[710], peripheral arterial diseases[715], GC use (section 1.4.3.2.8) or the ageing[716-718].

The chronology of angiogenesis involves complex signalling crosstalk between hypoxia, e.g. pro-angiogenic VEGF[719], and inflammatory, e.g. PTGS2[720] pathways, involving a range of biological factors (Table 5-1)[721]. Initially, VEGF and placental growth factor initiate angiogenesis by regulating the differentiation of endothelial progenitor cells, and is a key modulator of fibroblasts and inflammatory cells during WH[722]. VEGF is secreted by a variety of cells, including fibroblasts, macrophages and vascular smooth muscle cells in response to pro-inflammatory signalling and hypoxia[723].

Table 5- 1. Biological factors involved in angiogenesis[719][39]

Angiogenesis phase	Inducing factor
Vessel expansion and vasodilation	Nitric oxide
Increase vessel permeability	VEGF
Migration of plasma protein	VEGF
Endothelial sprouting	Angiopoietin-2
Extracellular matrix degradation	MMP
Growth factors recruitment	Insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor, VEGF
Endothelial proliferation and migration	PDGF
Formation of endothelial layer and lumen	VEGF, Angiopoetin-1
Vascular stabilisation	Plasminogen activator inhibitor-1
Maintenance, differentiation and remodelling of vascular structure	Angiopoetin-1
Inhibition of angiogenesis	Angiostatin, Endostatin, Thrombospondin

5.1.3.3 Dermal fibroblasts and wound healing

HDF play a role in every stage of WH (Figure 5-4)[696]. At 24-48 h after wounding, HDF are actively producing matrix metalloproteinases, degrading the fibrin clot and replacing them with a new granulation tissue[724]. Later, they differentiate into myofibroblasts and deposit ECM which they use for wound contraction and closure[725]. In response to hypoxia

[39] Abbreviation: VEGF refers to vascular endothelial growth factor; PDGF refers to platelet derived growth factor; MMP refers to matrix metalloproteinases

following microvascular injury, HDF release a range of molecular modulators, e.g. transforming growth factor (TGF-β), VEGF and IGF-1, to drive ECM remodelling through regulation of metalloproteases, lysyl oxidases, collagen synthesis and elastin expression[726, 727].

Dysregulation of HDF signalling underpins pathological WH processes including chronic wounds, hyperproliferative scarring and keloids[728-730]. Evidence from chronic leg ulcers highlights the role of HDF in the pathogenesis of these non-healing wounds[731]. Mechanistic studies exploring abnormal HDF function in non-healing wounds describe decreased proliferation and migration, premature fibroblast ageing, abnormal cytokine release and abnormal matrix metalloproteinases activity[727, 732-734]. The dysregulation of HDF are determined by intrinsic mechanisms, including transcriptional regulatory networks and epigenetic processes and extrinsic factors, including cell-cell signalling, soluble signalling mediators (e.g. IL-1β) and ECM components[696, 724].

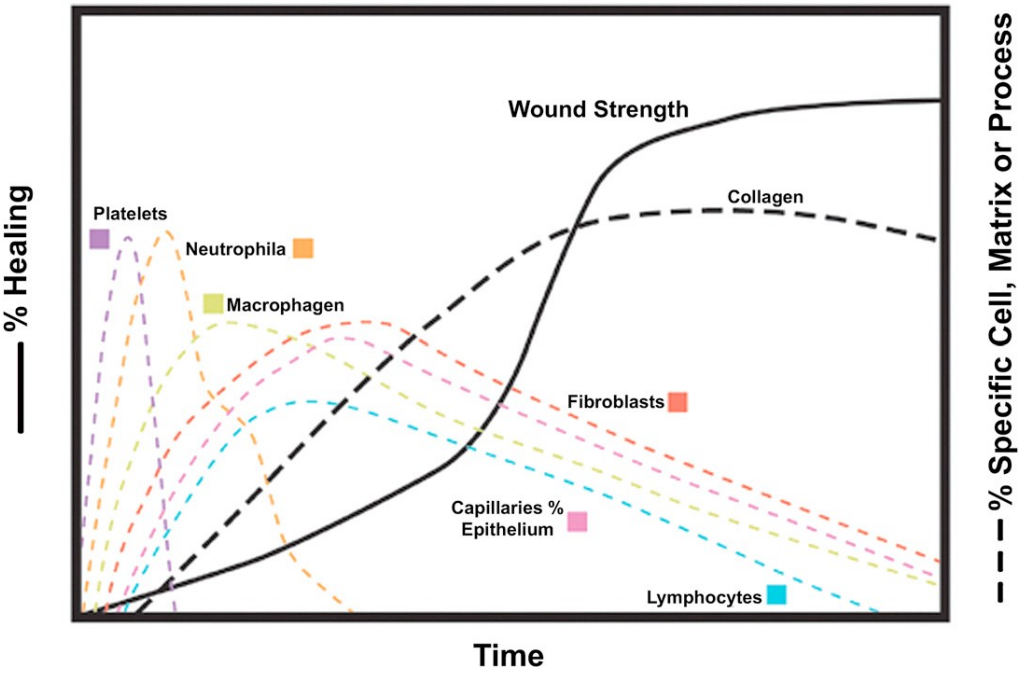

Figure 5- 4. Cellular involvement during the wound healing process

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5.1.3.4 Diabetic wounds

By 2030, 552 million people around the world will have DM [735-738]. Diabetic foot ulcers (DFU), are a common complication in T2DM with a major long-term impact on morbidity, mortality and quality of life[737-740]. In the UK, the prevalence of DFU in diabetes is 5-7%, and 25% of people with diabetes will experience DFU once in their lifetime [737-740]. DFU are a major economic burden; in England, 20% of diabetic healthcare costs are

attributable to foot complications estimated at £650 million per year [741]. DFU are commonly classified as 1) neuropathic, 2) ischaemic, and 3) neuroischaemic, as characterised in Table 5- 2.

Table 5- 2. Etiology and characteristics of chronic wounds.

Feature	Neuropathic	Ischaemic	Neuroischaemic
Sensation	Sensory loss	Painful	Degree of sensory loss
Callus/necrosis	Callus present and often thick	Necrosis common	Minimal callus, prone to necrosis
Wound bed	Pink and granulating, surrounded by callus	Pale and sloughy with poor granulation	Poor granulation
Foot temperature and pulse	Warm with bounding pulse	Cool with absent pulse	Cool with absent pulse
Other	Dry skin and fissuring	Delayed healing	High risk of infection
Typical location	Weight-bearing areas of the foot, such as metatarsal heads, the heel and over the dorsum of clawed toes	Tips of toes, nail edges and between the toes and lateral borders of the foot	Margins of the foot and toes
Prevalence	35%	15%	50%

65% of DFU are ischaemic (hypoxic), double the amputation risk and recurrence rate [742-744]. Without appropriate management, ulcers rapidly deteriorate and require amputation. The 5-year mortality in amputation patients is 50-68%[744]. 85% of lower-extremity amputations begin with DFU[737, 745]. Recent International Working Group on Diabetic Foot guideline indicates that treatment remains limited and improving WH in diabetes represents an unmet clinical need[746, 747]. Pathogenesis of impaired blood supply in DFU includes increased inflammation, excessive matrix metalloproteinase secretion, oxidative stress, free radicals, and a decrease in growth factor expression.

Persistent inflammation and ischaemia are characteristic features of chronic wounds, but their effects on cortisol metabolism and function in human skin are unexplored.

5.1.3.5 11β-HSD1 and skin

Skin expresses both 11β-HSD1 and 11β-HSD2[96]. 11β-HSD1 is predominantly expressed in the suprabasal layers of the epidermis (keratinocytes), but also in HDF, hair follicles and sebaceous glands[3, 146, 688, 748, 749]. In contrast, 11β-HSD2 is expressed predominantly in eccrine sweat glands[146]. 11β-HSD1 mRNA in HDF positively correlated with age, Ultraviolet B and pro-inflammatory cytokines during wounding[145, 146, 688, 750, 751]. Cortisol treatment of HDF increased 11βHSD1 mRNA expression in a dose-dependent manner and decreased 11βHSD2 and GRα expression with no effect on H6PD, suggesting a positive feedback loop to enhance GC activation[146].

GC action in skin modulates inflammation, HDF and keratinocyte proliferation, epidermal turnover and induces an aged skin phenotype[145, 748, 752, 753]. Further, cortisol plays a role in countering the inflammatory response in the skin[754, 755]. Psoriasis[756], allergic contact dermatitis, nummular dermatitis, atopic dermatitis, and other inflammatory skin illnesses or autoimmune processes might be exacerbated by decreased local cortisol production. Antimicrobial and antiviral defences would be compromised if levels of epidermal cortisol were elevated. Elevated local 11β-HSD1 activity and cortisol availability would induce collagen atrophy, ageing skin, decreased keratinocyte proliferation, and delayed wound healing.

5.1.4 11β-HSD1 and wound healing

Intracellular GC excess from overexpression of 11β-HSD1 activity is postulated to delay WH, impaired collagen synthesis and inhibit cell proliferation. This is supported by evidence of increased 11β-HSD1 gene expression and activity during acute wounds in mice[688], accelerated WH in aged 11β-HSD1-knockout mice [145]. Further, topical 11β-HSD1 inhibition reversed skin thinning and delayed WH in mice with systemic GC excess[757].

This pre-clinical evidence of improved healing by 11β-HSD1 inhibition in mouse models led to a recent clinical trial of the effects of the oral selective 11β-HSD1 inhibitor AZD4017 on acute WH in patients with T2DM[758]. However, the underlying mechanisms remain unexplored.

5.1.5 11β-HSD1 and hypoxia

11β-HSD1 expression in relationship to hypoxia is largely unknown. One *in vitro* adipose tissue study demonstrated that hypoxia suppressed 11β-HSD1 mRNA and protein through NF-κB [759] However, the regulation of 11β-HSD1 in response to hypoxia in human skin cells remains to be elucidated.

5.2 Hypothesis

T2DM, CS and exogenous GC excess share common skin characteristics, including decreased or impaired local angiogenesis, impaired cellular and growth factor production, insufficient collagen accumulation, impaired local immune response and skin barrier deficiencies that render skin highly susceptible to infection, reduced epidermal barrier function and impaired healing[292, 685, 686] .

The cutaneous phenotypic overlap between these conditions suggests GC metabolism by 11β-HSD1 may be a common mediator of impaired skin function and WH. Ischaemic skin is one important cause of delayed WH. However, the interplay between GC, hypoxia and inflammation has not been investigated in detail.

Using HDF as a model of GC toxicity, I will explore the hypothesis that hypoxia stimulates 11β-HSD1 expression and activity to increase cortisol availability and de-regulate GC target gene expression.

5.3 Aims

This study aims to explore the effect of hypoxia and inflammation on pre-receptor GC metabolism by 11β-HSD1 and the subsequent impact on GC target genes key to the WH process.

5.4 Materials and methods

5.4.1 Tissue source and reagents

Primary HDF were obtained from the abdominal skin of healthy donors who underwent abdominal surgery (with consent) through Bradford Ethical Tissue bank (REC 17/YH/0086) following tissue bank project approval and guidelines. Fibroblasts were harvested from full-thickness skin specimens cut into 5 mm^2 pieces and explanted onto the surface of Petri dishes containing culture medium: Dulbecco's Modified Eagles Media (DMEM, Life Technologies) with 10% foetal calf serum (FCS) and 100U/ml penicillin/streptomycin. Tissues were cultured in a humidified incubator at 37ºC, under 5% CO_2. After reaching 70-80% confluence, cells were prepared for passaging or storage.

5.4.2 HDF preparation

Frozen vials were thawed at 37ºC and the cells were transferred immediately to 5ml phosphate-buffered saline (PBS, Sigma Chemical Co., Saint Louis, USA), mixed by inversion and centrifuged at 1100g for 5 min. Following removal of PBS, pellets were resuspended in 10 ml pre-warmed culture medium, seeded in 75 cm^2 flasks and incubated at 37ºC and 5% CO_2. Media was replaced every two days until cells reached 70-90% confluence.

5.4.3 HDF passaging

HDF were passaged when the cellular confluence reached 70-90%. Culture medium was removed, followed by rinsing with 3 ml of PBS. After removing the PBS, cells were trypsinised with 3 ml of 0.5g/l trypsin and 0.2g/l ethylenediaminetetraacetic acid in PBS and incubated at 37ºC, under 5% CO_2 for 5 minutes. 7 ml of DMEM was added immediately to neutralise the trypsin. The cells were centrifuged (1100g at room temperature) for 5 minutes and the cell pellet was passaged 1:3 by suspending in 30 ml of DMEM and dividing into three 75 cm^2 flasks.

5.4.4 Freezing down HDF cells

Upon reaching 70-90% confluence, HDF were trypsinised and resuspended in 10 ml of complete media before being centrifuged at 1100g for 10 minutes. Media was aspirated to leave the pellet, which was resuspended in 3 ml of FCS supplemented with 10% DMSO. Cells were then aliquoted into 1.5 ml cryovials and frozen at -80ºC in a cryo-freezing container (Nalgene, Hereford, UK).

5.4.5 HDF experiments

For all experiments, cells between passages 3 to 9 were used to maintain consistency and avoid the influence of senescence on biological function. HDF were plated at 100,000 cells per well (6-well plate) or 40,000 cells per well (12-well plate) and incubated overnight at 37°C, under 5% CO_2 to adhere. Treatments were initiated at 70% confluence.

5.4.6 Cell culture models of normoxia and hypoxia

In a normoxic cell culture experiment, cells are maintained in incubators with 37°C, ambient air (21% volume fraction O2) enriched with 5% CO2, and humidity provided by spontaneous water evaporation.

Oxygen deficiency impairs cellular functions and interferes with a variety of biological processes. The cellular response to hypoxia varies between organs or tissues such as the trachea (19.7%), brain (4.4%), lung (5.6%), liver (5.4%), intestinal tissue (7.6%), and skin (1.1%)[760, 761]. Generally, the hypoxic state model is 1-2%. As a result of these considerations, the hypoxic experiment for HDF is optimized for 1% O2.

5.4.7 Treatment

For normoxia/hypoxia experiments, cells were either maintained at 21% O_2 or 1% O_2 in an H35 Hypoxystation® (Don Whitley Scientific) for 24 or 96 h. Primary HDF were treated in triplicate with 100% ethanol (vehicle), IL-1β (10 ng/ml), IL-1β with 200 nM cortisone (E) or IL-1β with E and 1 μM of selective 11β-HSD1 inhibitor (AZD4017) as in Figure 5-5. The limitation is the absence of cortisol and AZD4017 treatment in isolation, which would have provided an appropriate control.

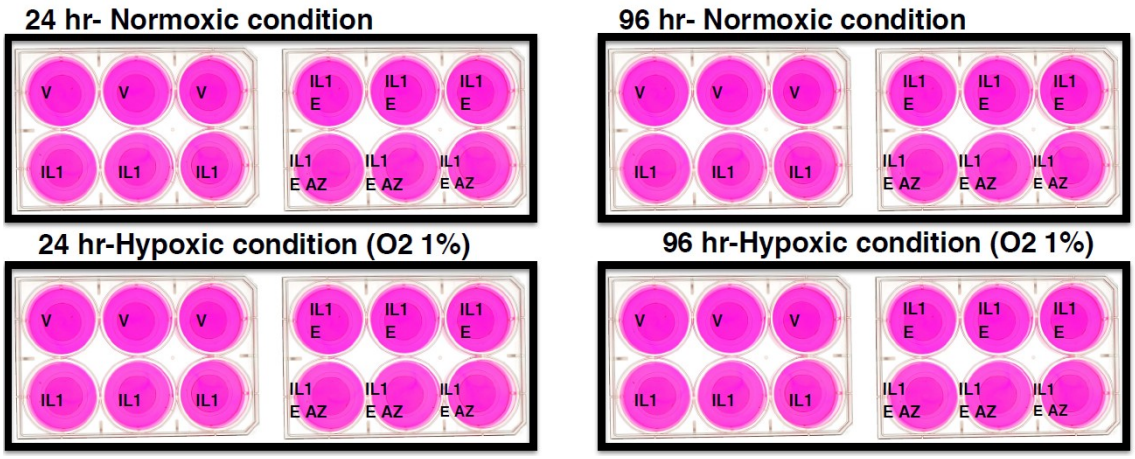

Figure 5- 5. Experiment model. V = vehicle, IL1 = IL-1β, E = cortisone, AZ = AZD4017.

5.4.8 AZD4017

AZD4017 is a novel orally bioavailable small molecule inhibitor of 11β-HSD1 enzyme activity. It is potent and highly selective *in vitro* and *in vivo*. The half-maximal inhibitory concentration (IC50) for inhibition of 11β-HSD1 activity is 2 nM. AZD4017 is selective (> 2000x) for 11β-HSD1 over human recombinant 11β-HSD2. AZD4017 acquired through the AstraZeneca Open Innovation platform following appropriate processes and procedures

5.4.9 Gene expression analysis

Quantitative real-time polymerase chain reaction (real-time qPCR) was conducted in accordance with recommended guidelines[762].

5.4.10 RNA extraction

The principles of ribonucleic acid (RNA) extraction involve 1) cell lysis and inhibition of ribonuclease (RNase) activity, 2) removal of DNA, and 3) solation of total RNA (of which 1-5% is mRNA). RNA was extracted using the Qiagen RNeasy® Plus Mini (Qiagen, Maryland, USA) following the manufacturer's protocol. Samples were kept on ice throughout, and the eluted RNA stored at -20^{o}C.

5.4.11 RNA quantification and quality assessment

RNA quantity and quality was assessed by Qubit® (Thermo Scientific, Loughborough, UK), following the manufacturer's guidance. The tubes for RNA measurement were set up as two assay tubes for standards and one assay tube for each sample. The preparation method is described in Table 5-3. Where necessary, samples were diluted to 100 ng/μl with distilled water.

Table 5- 3. The mixture preparation for Qubit assay

Agents	***Standard assay tubes***	***User sample assay tubes***
Volume of working solution or master-mix (prepare from 1: 199 of Qubit™ reagent: Qubit™ buffer)	190 μl	198 μl
Volume of Standard 1 and standard 2 (from kit)	10 μl	—
Volume of user sample	—	2 μl
Dye		
Total Volume	200 μl	200 μl

5.4.12 cDNA synthesis

Complementary DNA (cDNA) synthesis was conducted by adding 12 µl of mRNA to a final concentration of 10-60 ng/µl and 8 µl of master mix (Tetro cDNA synthesis Kit, Thermo Scientific, Loughborough, UK) containing 4 µl of 5X RT Buffer, 1 µl of dNTP Mix, 1 µl of random hexamer primer mix, 1 µl of ribosafe RNAase inhibitor and 1 µl of reverse transcriptase, following the manufacturer's protocol.

Samples underwent cDNA synthesis using a DNA Engine Tetrad^{R} thermal cycler (Bio Rad, Watford, UK) using the following parameters: 25°C for 10 minutes, followed by 45°C for 30 minutes, and terminated incubating at 85°C for 5 minutes. cDNA samples were diluted to a 10 ng/ul final concentration with distilled water.

5.4.13 Real-time qPCR

Fluorescence-based real-time qPCR is used to detect and quantify nucleic acids and mRNAs[763-765]. 11β-HSD1, cyclooxygenase-2 (COX-2 or PTGS2), GRα, vascular endothelial growth factor A (VEGFA) and H6PD were quantified by real-time qPCR using an ABI7500 system with QuantStudio^{TM} software (Applied Biosystems, Warrington, UK) and standard TaqMan® primers and probes. 18S rRNA was used as the reference gene. Polymerase chain reactions (PCRs) were performed in 384-well plates with a total volume of 10 µl for each reaction, containing 1 µl of cDNA (1 ng/µl final concentration), 5µl TaqMan® Universal PCR mastermix (2x), 0.5 µl FAM-labelled target gene or 0.15 µl VIC-labelled 18S ribosomal rRNA primers and probe and nuclease-free water. PCR reactions were conducted as follows: 95°C for 5 minutes, 95°C for 10 seconds and 40 cycles of RT-qPCR (60°C for 50 seconds).

The relative amplification of each sample was measured. After each cycle, the probe signal strength indicated the quantity of cDNA. The threshold level was set at 0.01 (typically within or slightly above this exponential phase), and cycle threshold (*C*t) values were obtained for the cycle number during which amplification occurred in the exponential reaction phase (Figure 5-6). The more cycles the sample has to complete to reach this phase, the lower the initial expression. This method assumes that amplification efficiency is identical in all samples.

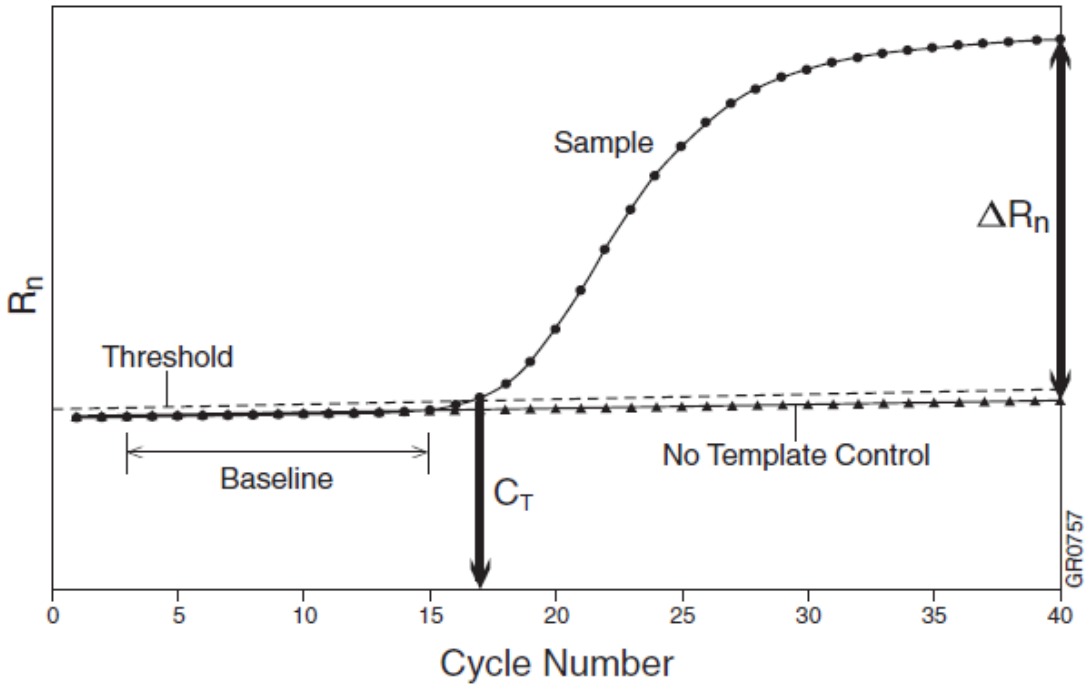

Figure 5- 6. ***Real-time PCR principle***

Baseline; initial cycles in which there is little change in fluorescence signal, Rn; normalized reporter or the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye. ΔRn; The magnitude of the signal generated by the specified set of PCR conditions (ΔRn=Rn−baseline); threshold; level of ΔRn—automatically determined by the software or manually set—used for Ct determination (threshold level set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve).

Both target gene and 18S primer and probes were run in parallel on the same qPCR plate. Levels of gene expression were reported relative to 18S RNA in arbitrary units (AU). The difference between genes of interest and 18S were determined ($\Delta Ct=Ct_{\text{gene of interest}} - Ct_{18s}$). Each sample was run in duplicate and the mean values of the duplicates (ΔCt) were used to calculate transcript levels. Data were reported as the mean ΔCt±S.E. of replicates. For display purposed, mRNA expression data were transformed and expressed graphically as $AU = 1{,}000{,}000 \times 2^{-\Delta Ct}$. Negative controls, replacing cDNA with DNAse treat water, were used to confirm the lack of genomic DNA contamination.

5.4.14 11β-HSD1 activity assay

11β-HSD1 activity was inferred from the % conversion of cortisone (11β-HSD1 substrate) to cortisol. Cell culture supernatants were used to calculate cortisol concentration using a commercial cortisol enzyme-linked immunosorbent assay or ELISA (R&D Systems, Abingdon, UK) following the manufacturer's instructions. This assay employs the competitive sandwich enzyme immunoassay technique in which cortisol present in a sample competes with a fixed amount of horseradish peroxidase-labelled cortisol for sites on a mouse

monoclonal antibody. The monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate during the incubation. Following a wash to remove excess conjugate and unbound sample, a chromogenic substrate solution was employed to determine the bound enzyme activity. The colour development is stopped, and the absorbance is read at 450 nm. The intensity of the colour is inversely proportional to the concentration of cortisol in the sample. The limitation of ELISA technique is relative novel, however there was a moderate correlation with the “gold-standard” radioactivity. method[766].

5.4.15 Statistical analysis

Analyses were performed on untransformed data. STATA 16.0 was used for statistical analysis. Means and standard deviations were used to summarize continuous data (or standard error of the mean). Box plots were used to visualize the data, and $p<0.05$ was considered statistically significant. We employed a multilevel mixed-effects linear regression model.

5.5 Results

Unless otherwise indicated, all expression data are expressed as ΔCt±SE.

5.5.1 Hypoxia

5.5.1.1 11β-HSD1 expression

Baseline levels of 11β-HSD1 in HDF were very low (Appendix 5-1). The ability of hypoxia to regulate 11β-HSD1 in HDF was investigated (Figure 5-7 and Appendix 5-2). At 24 h, results showed a trend towards increased 11β-HSD1 expression in hypoxia vs normoxia, but this was not statistically significant (21.8±1.1 vs 22.3±0.8, p=0.35, n=5). At 96 h, the expression of 11β-HSD1 in both normoxia and hypoxia was not significantly different (20.6±0.8 vs 20.9± .6, p=0.90, n=5). However, the 11β-HSD1 expression increased considerably in a time-dependent manner for normoxia (24 h vs 96 h, p<0.001) and hypoxia (24 h vs 96 h, p=0.01).

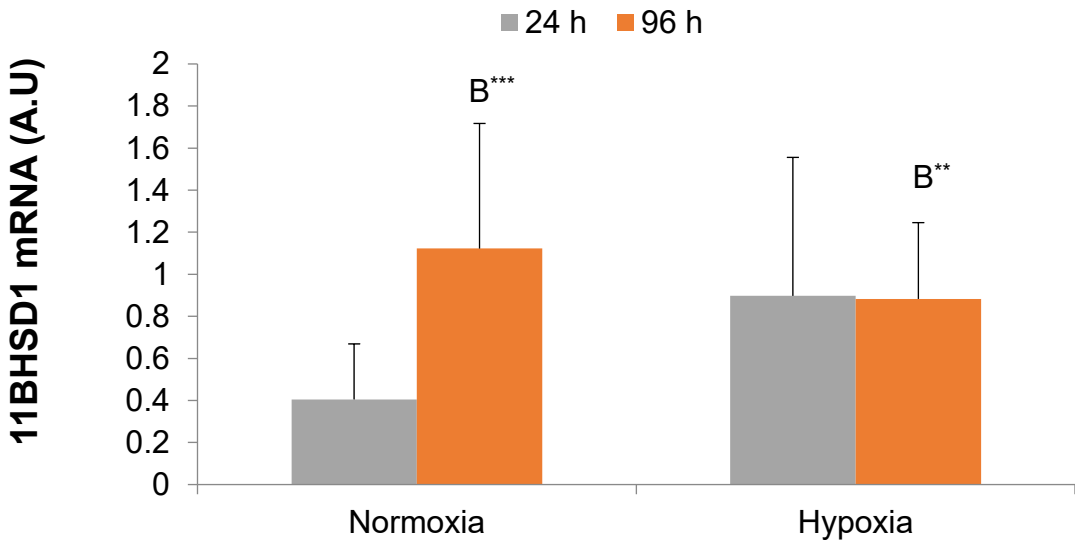

Figure 5- 7. 11β-HSD1 mRNA expression in vehicle.

*Comparison: A: normoxia vs hypoxia B: 24 h vs 96 h (n=5). Significant level: **=p<0.01, *** =p<0.001*

5.5.5.2 GR-α expression

We explored the effect of hypoxia on GC receptor GR-α expression in HDF (Figure 5-8 and Appendix 5-5). At 24 h, GR-α mRNA increased ~40% in hypoxia compared to normoxia (12.9±0.3 vs 13.3±0.3, respectively, $p<0.05$, n=5). At 96 h, GR expression was also ~ 40% higher in hypoxia than normoxia (12.8±0.4 vs 13.3±0.4, respectively, $p<0.05$, n=5). GR-α expression was unaffected by time for hypoxia ($p=0.6$) or normoxia ($p=0.5$).

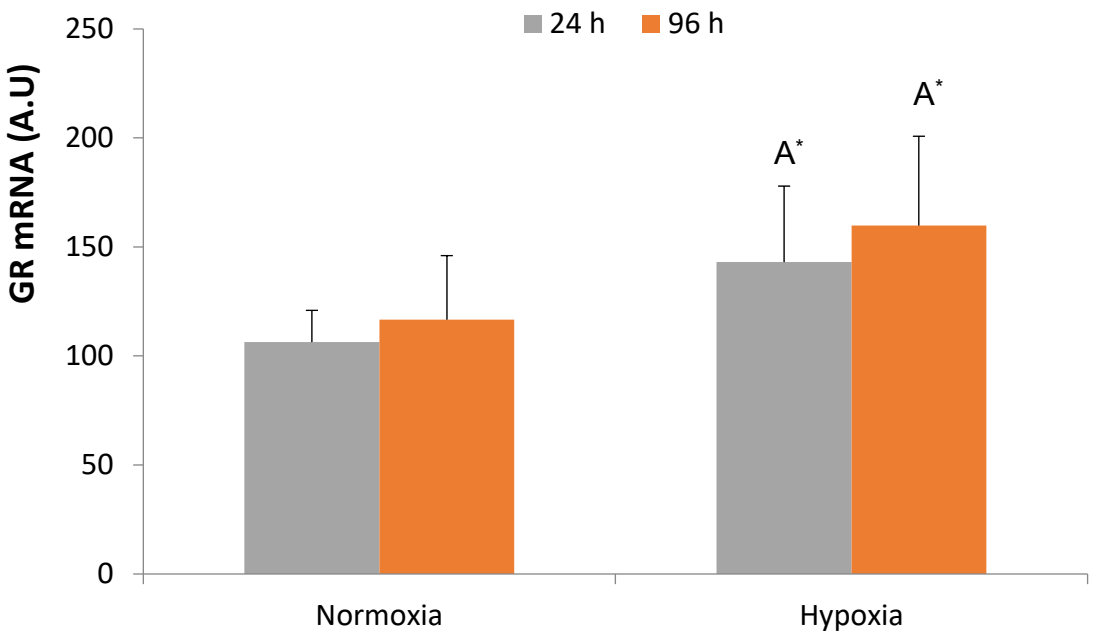

Figure 5- 8. GR-α mRNA expression.

*Comparison: A: normoxia vs hypoxia B: 24 h vs 96 h (n=5). Significant level: *= p<0.05*

5.5.5.3 H6PD expression

Similarly to 11β-HSD1, H6PD (11β-HSD1 co-factor enzyme) mRNA gene expression increased at 96 h in both hypoxia ($p<0.001$) and normoxia ($p<0.01$) (Figure 5- 9, Appendix 5-6) and was comparable between normoxia and hypoxia at both 24 h (18.3±0.7 vs 18.2±1.0, $p=0.68$, $n=5$) and 96 h (17.6±0.9 vs 17.4±0.9, $p=0.25$, $n=5$).

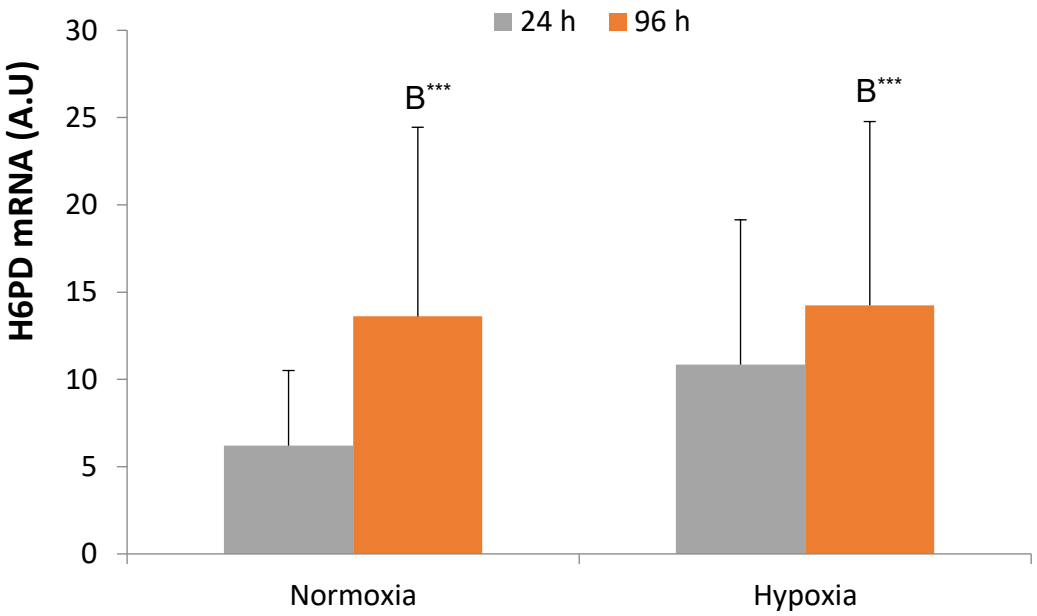

Figure 5- 9. H6PD mRNA expression.

*Comparison: A: normoxia vs hypoxia B: 24 h vs 96 h (n=5). Significant level: *** = p<0.001*

5.5.5.4 PTGS2 expression

PTGS2 expression was significantly increased in hypoxia compared to normoxia by 5.7-fold at 24 h (17.5±1.2 vs 19.4±1.8, respectively, p=0.001, n=5) and by 4.8-fold at 96 h (16.6±1.0 vs 18.5±1.0, respectively, p<0.001, n=5). Under hypoxic conditions, PTGS2 expression was comparable at 96 h vs 24 h (p=0.066), and this was also observed in normoxia (Figure 5-10 and Appendix 5-7)

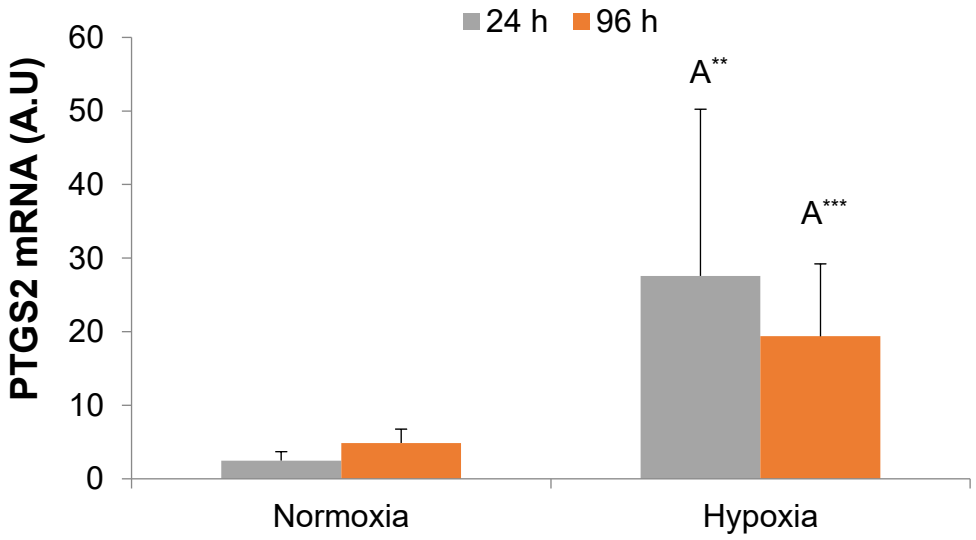

Figure 5- 10. PTGS2 mRNA expression.

*Comparison: A: normoxia vs hypoxia B: 24 h vs 96 h (n=5). Significant level: ** = p<0.01*** = p<0.001*

5.5.5.5 VEGFA expression

As anticipated, hypoxia substantially elevated VEGFA gene expression by 4.7-fold at 24 h ($p<0.001$, $n=5$) and 6.2-fold at 96 h ($p<0.001$, $n=5$). Duration of exposure did not affect gene expression (Figure 5-11). All gene expression statistical comparisons for vehicle-treated HFD are presented in Appendix 5-8

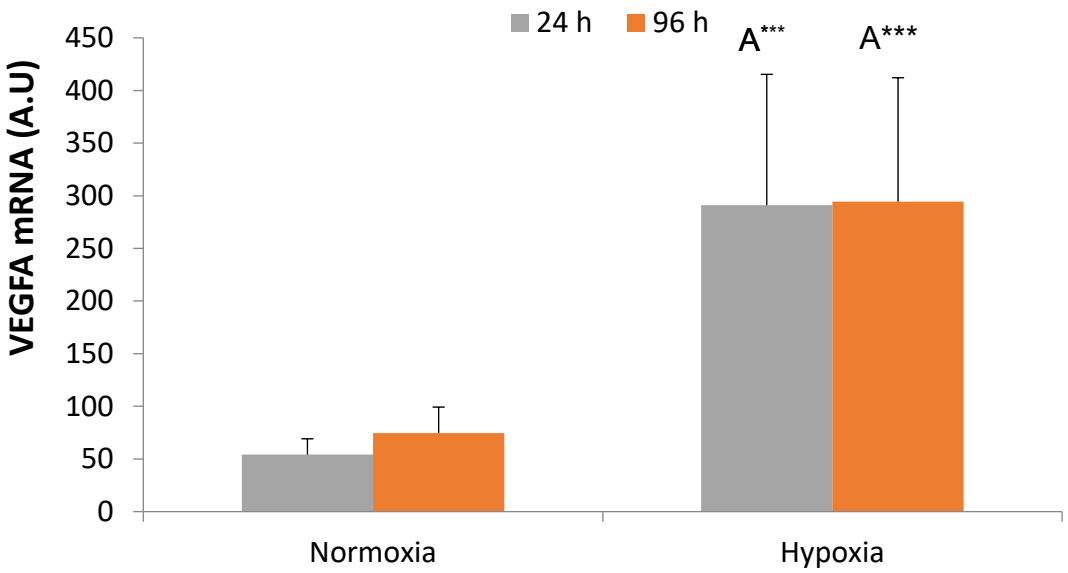

Figure 5- 11. VEGFA mRNA expression.

*Comparison: A: normoxia vs hypoxia B: 24 h vs 96 h (n=5). Significant level: ***= p<0.001*

5.5.2 Hypoxia and IL-1β

5.5.2.1 1β-HSD1 expression

Similarly to vehicle-treated cells (Figure 5- 7), 11β-HSD1 expression remained greater at 96 h vs 24 h across all treatments and O_2 conditions ($p<0.001$, Appendix 5-2).

After 24 h, treatment with the pro-inflammatory cytokine IL-1β induced 11β-HSD1 gene expression by 38-fold and 42-fold (relative to vehicle) in normoxia and hypoxia, respectively ($p<0.001$, Figure 5-12, Appendix 5-1, Appendix 5-2, Appendix 5-3). This was unaffected by the addition of cortisone (11β-HSD1 substrate) or the selective 11β-HSD1 inhibitor AZD4017.

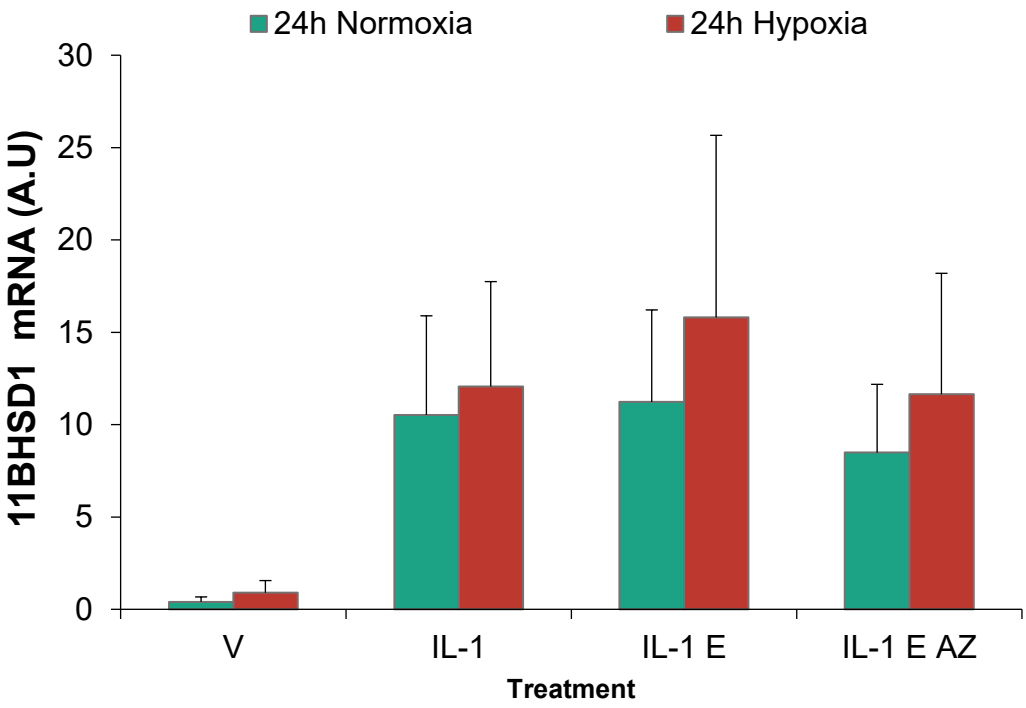

Figure 5- 12. 11β-HSD1 mRNA expression at 24 h (n=5)

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance *** = p<0.001*

IL-1β also induced 11β-HSD1 expression at 96 h by 92-fold and 76-fold in normoxia and hypoxia, respectively (p<0.001Figure 5-13, Appendix 5-1). Under hypoxic conditions, there was a trend towards a further increase by cortisone (~ 73%, p=0.11) and a trend towards reversal by AZD4017 (~ 37.5%, p=0.11). There was also a trend towards increased expression in hypoxia compared to normoxia in cortisone-treated cells (p=0.17). This suggests the forward-feedback mechanism for amplification of local cortisol levels in hypoxia, in response to inflammation also exists in skin.

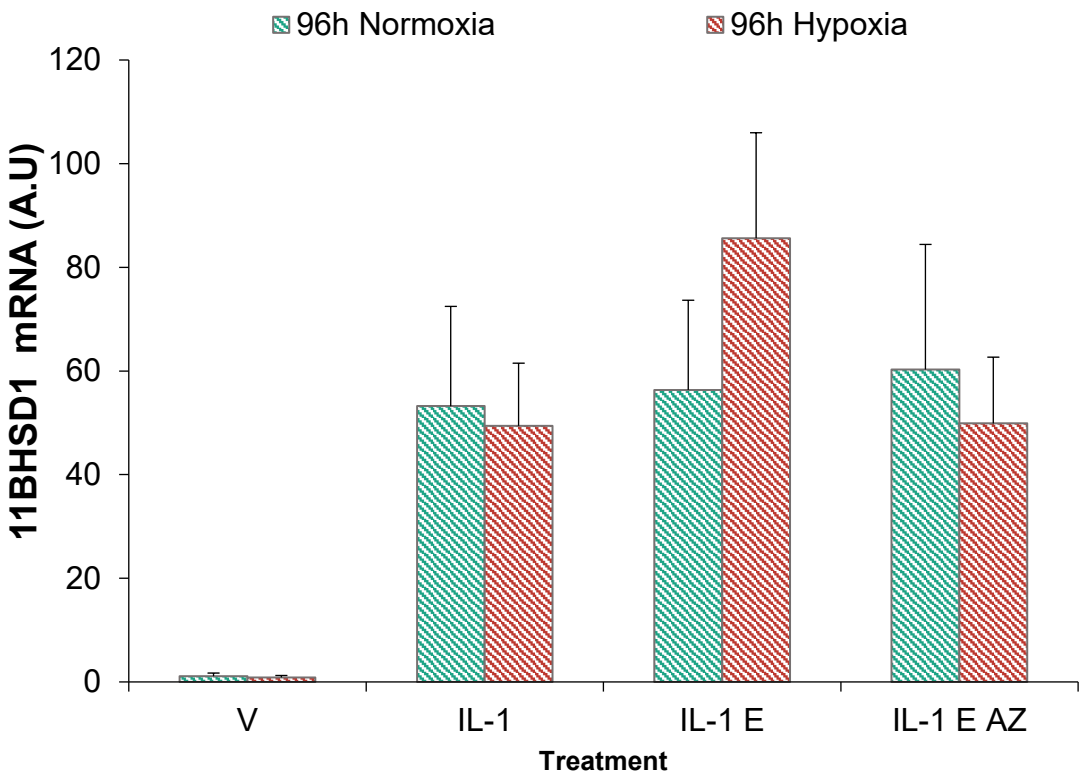

Figure 5- 13. 11β-HSD1 mRNA expression at 96 h (n=5)

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance *** = p<0.001*

5.5.2.2 11β-HSD1 activity

In agreement with 11β-HSD1 mRNA data, 11β-HSD1 activity was 2-fold greater in hypoxia compared to normoxia ($p<0.001$, Figure 5-14, Appendix 5-4). Activity was reduced by 90% following 11β-HSD1 inhibitor co-treatment under both normoxia ($p<0.05$) and hypoxia ($p<0.01$), demonstrating AZD4017 efficacy.

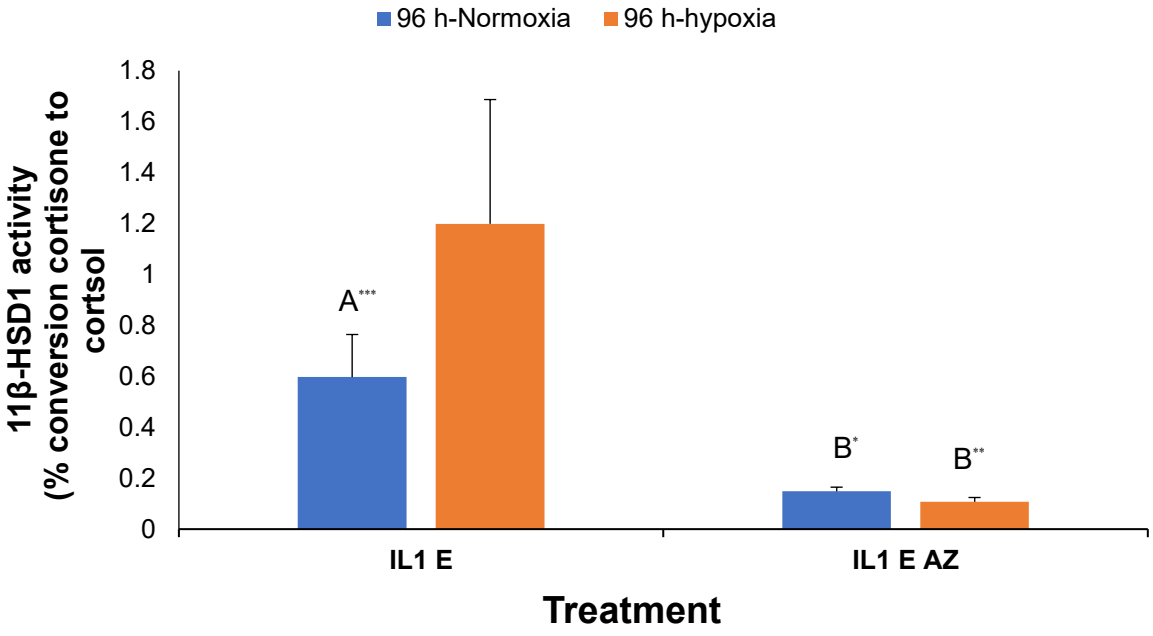

Figure 5- 14. 11β-HSD1 activity (n=3).

*Comparison: A: normoxia vs hypoxia, B: IL1 E AZ vs IL-1 E. IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance *= p<0.05, ** = p<0.01, ***= p<0.001*

5.5.2.3 GR-α expression

GC receptor GR-α expression was modestly suppressed by IL-1β in hypoxia at both 24 h ($p<0.01$, Figure 5-15) and 96 h ($p<0.05$, Figure 5-16, Appendix 5-6), but expression remained comparable to baseline levels.

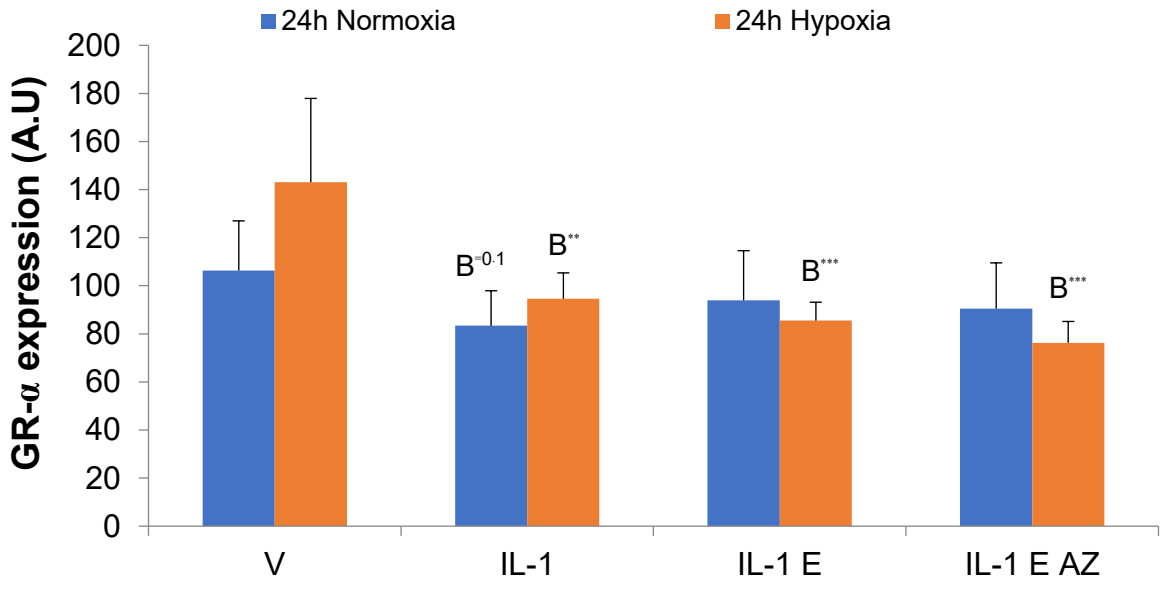

Figure 5- 15. GR-α mRNA expression at 24 h (n=5).

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance ** = p<0.01, *** = p<0.001*

GR-α mRNA levels were further suppressed by cortisone treatment at 96 h (but not 24 h) in both hypoxia ($p<0.001$) and normoxia ($p<0.01$), with trends towards reversal by 11β-HSD1 inhibition. This suggests a modest negative feedback regulation of cortisol receptor expression.

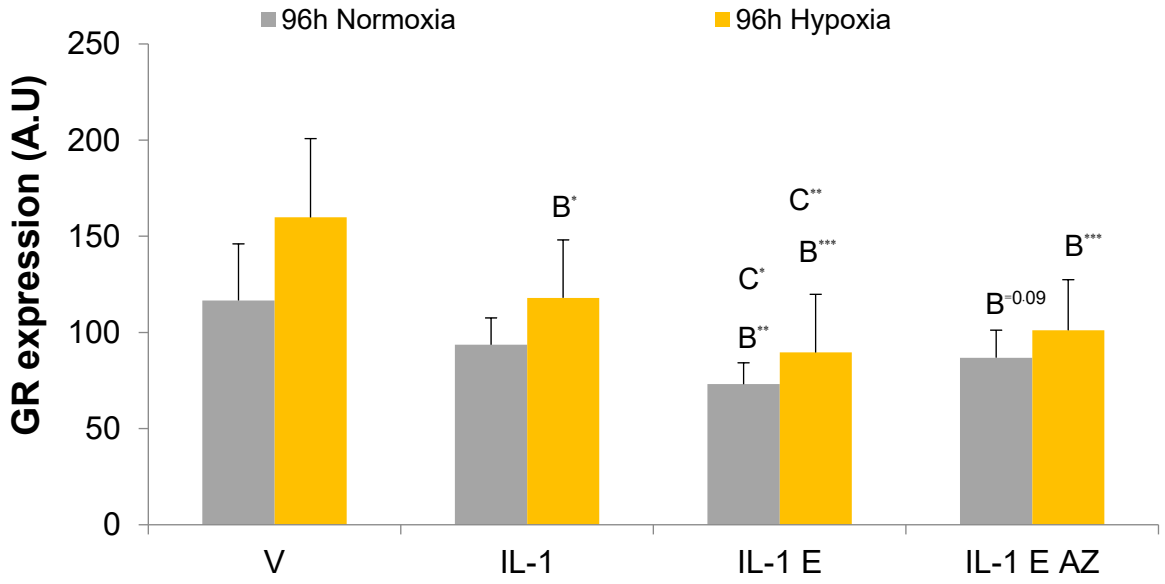

Figure 5- 16. GR-α mRNA expression at 96 h (n=5).

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance * = p<0.05, ** = p<0.01, *** = p<0.001*

5.5.2.4 H6PD expression

H6PD (the enzyme providing co-factor to 11β-HSD1) was unaffected by hypoxia, IL-1β or cortisone at either 24 or 96 h (Figure 5-17, Figure 5-18 and Appendix 5-6).

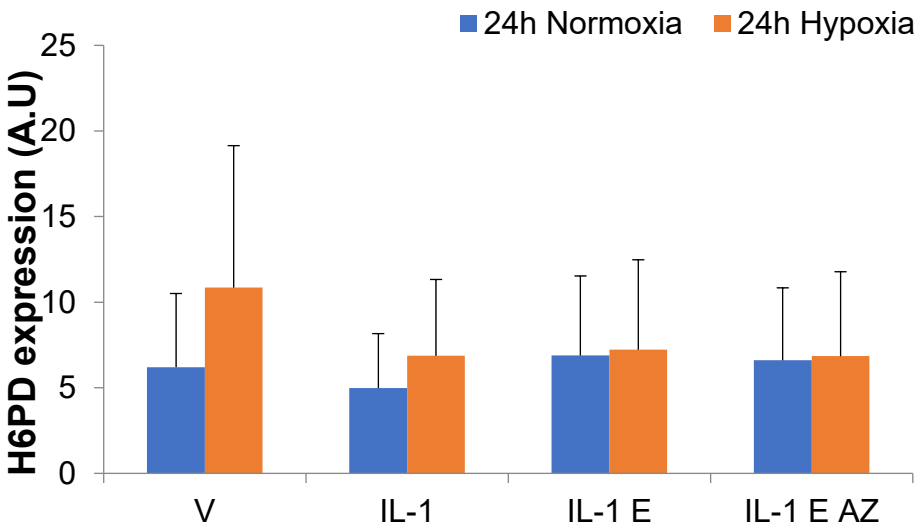

Figure 5- 17. H6PD mRNA expression at 24 h (n=5).

Abbreviation: IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017.

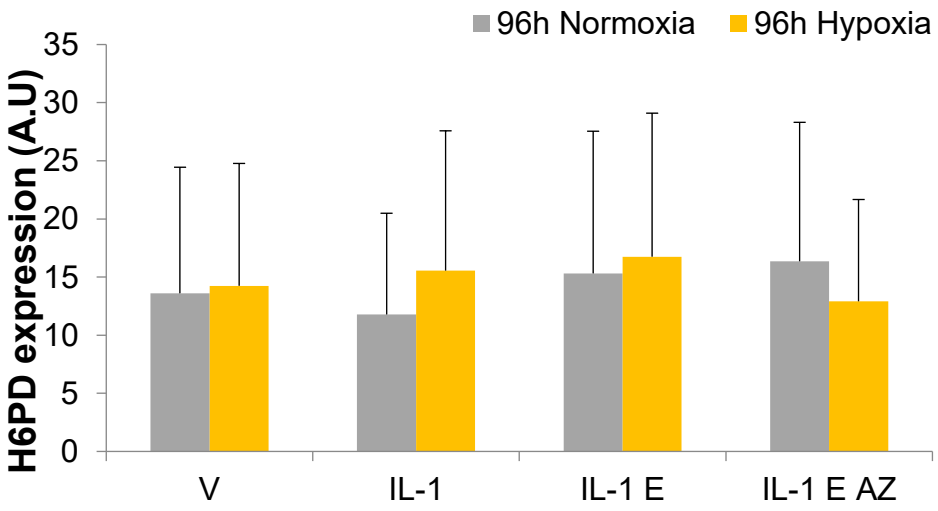

Figure 5- 18. H6PD mRNA expression at 96 h (n=5).

Abbreviation: IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017.

5.5.2.5 PTGS2 expression

PTGS2 is integral to inflammation and WH. PTGS2 mRNA was induced 50-fold and 106-fold by IL-1β at 24 and 96 h in normoxia, respectively ($p<0.001$) and 4-fold further by hypoxia at 24 h ($p<0.001$, Figure 5-19, Figure 5-20, Appendix 5-7). A similar synergistic induction of PTGS2 by IL-1β and hypoxia was observed across all treatments at 24 h, although this effect was less apparent at 96 h (possibly due to higher baseline PTGS2).

Similarly to VEGFA, cortisone and 11β-HSD1 inhibition had no effect on PTGS2 expression in normoxia or hypoxia at 24 h (Figure 5-21). At 96 h, PTGS2 mRNA was suppressed 60% and 70% by cortisone in normoxia and hypoxia, respectively ($p<0.001$) and these were both reversed by 11β-HSD1 inhibition ($p<0.001$).

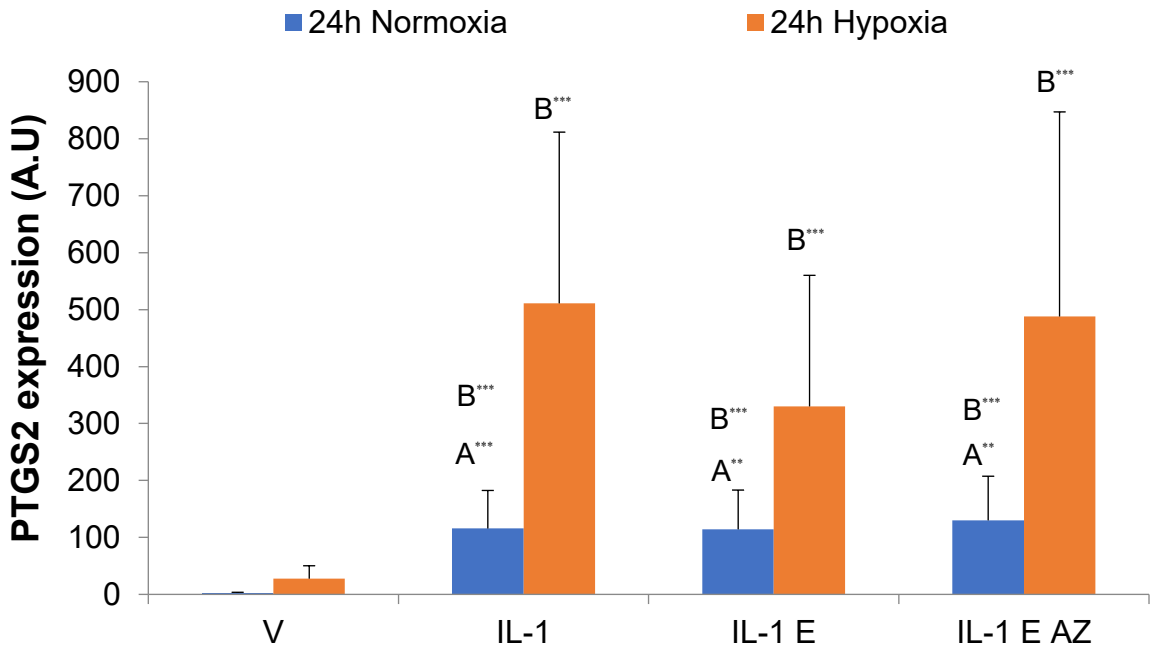

Figure 5- 19. PTGS2 mRNA expression at 24 h (n=5).

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance ** = p<0.01, *** = p<0.001*

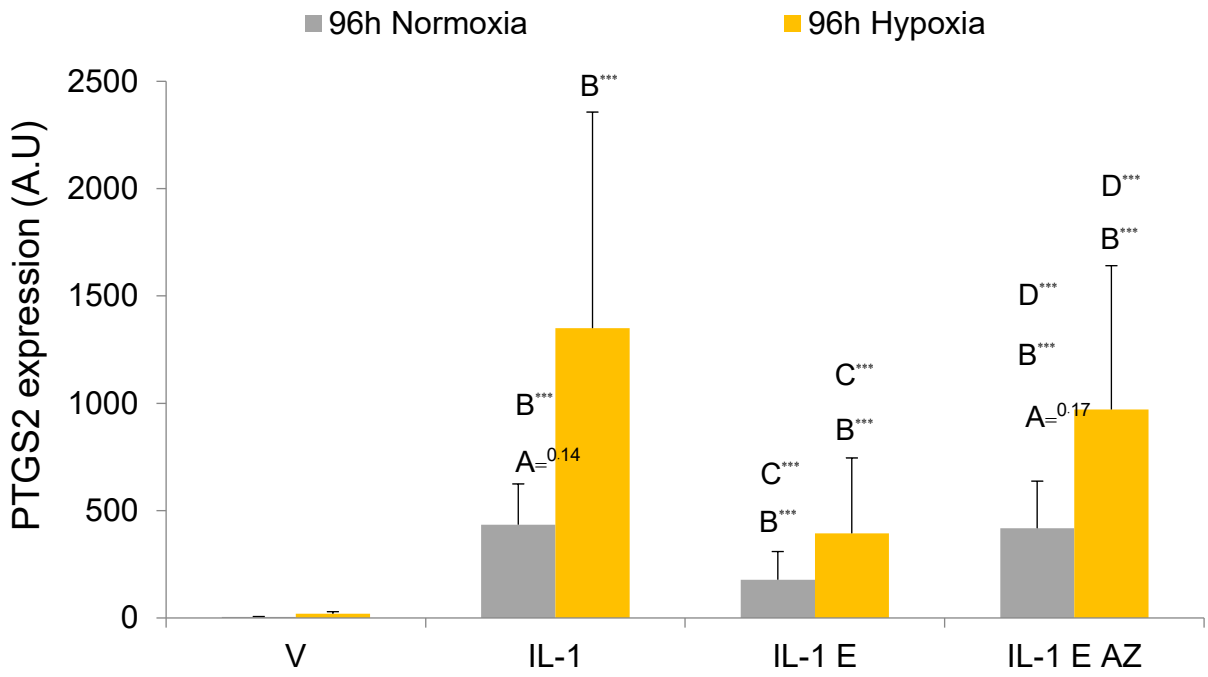

Figure 5- 20 PTGS2 mRNA expression at 96 h (n=5).

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance ** = p<0.01, *** = p<0.001*

5.5.2.6 VEGFA expression

Expression of VEGFA mRNA was induced 4-fold by IL-1β at 24 in normoxia (n=5, p<0.001) and 3-fold further in synergy with hypoxia at 24 h (p<0.01) (Figure 5-21 and Appendix 5-8). A comparable effect was observed at 96 h (Figure 5-22) Cortisone and 11β-HSD1 inhibition had no effect at 24 h under either oxygen concentration. At 96 h, VEGFA gene was suppressed by 49% by cortisone treatment (p<0.05) under hypoxia (but not normoxia) and this was reversed by 11β-HSD1 inhibition (p<0.05). These data suggest that induction of 11β-HSD1 by hypoxia causes regulation of GC target genes such as VEGFA in a time-dependent manner. Further, these findings indicate that VEGFA is less sensitive to regulation by GC than PTGS2.

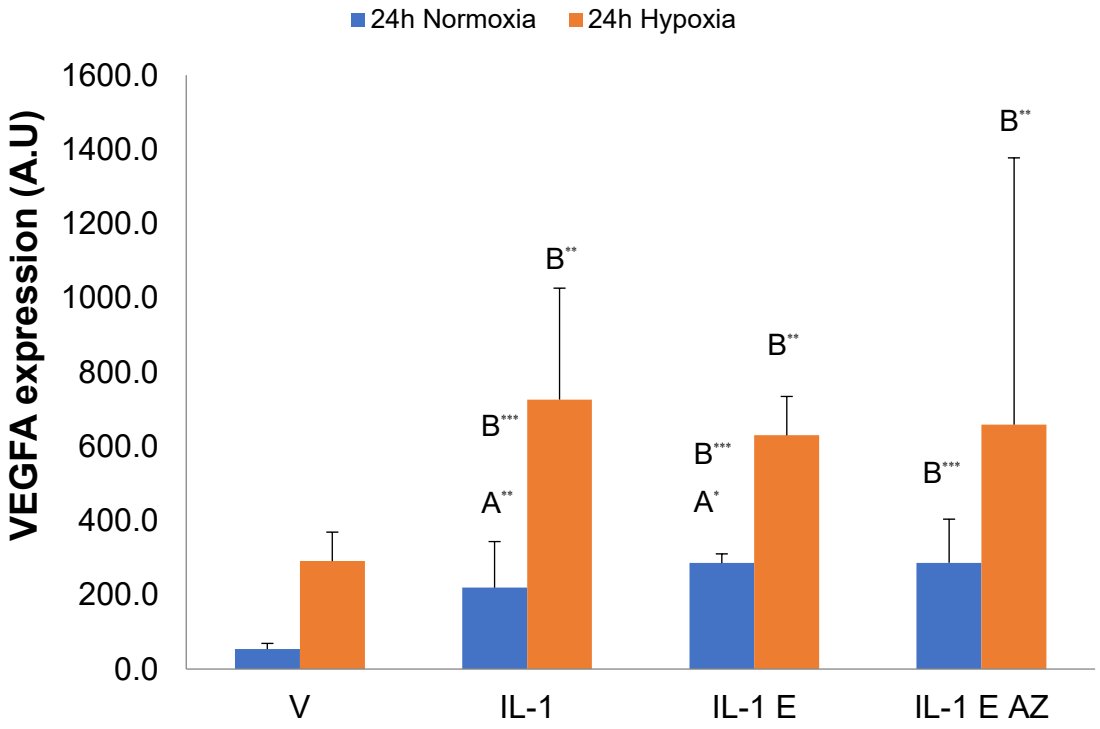

Figure 5- 21. VEGFA mRNA expression at 24 h (n=5).

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance * = p<0.05, ** = p<0.01, *** = p<0.001*

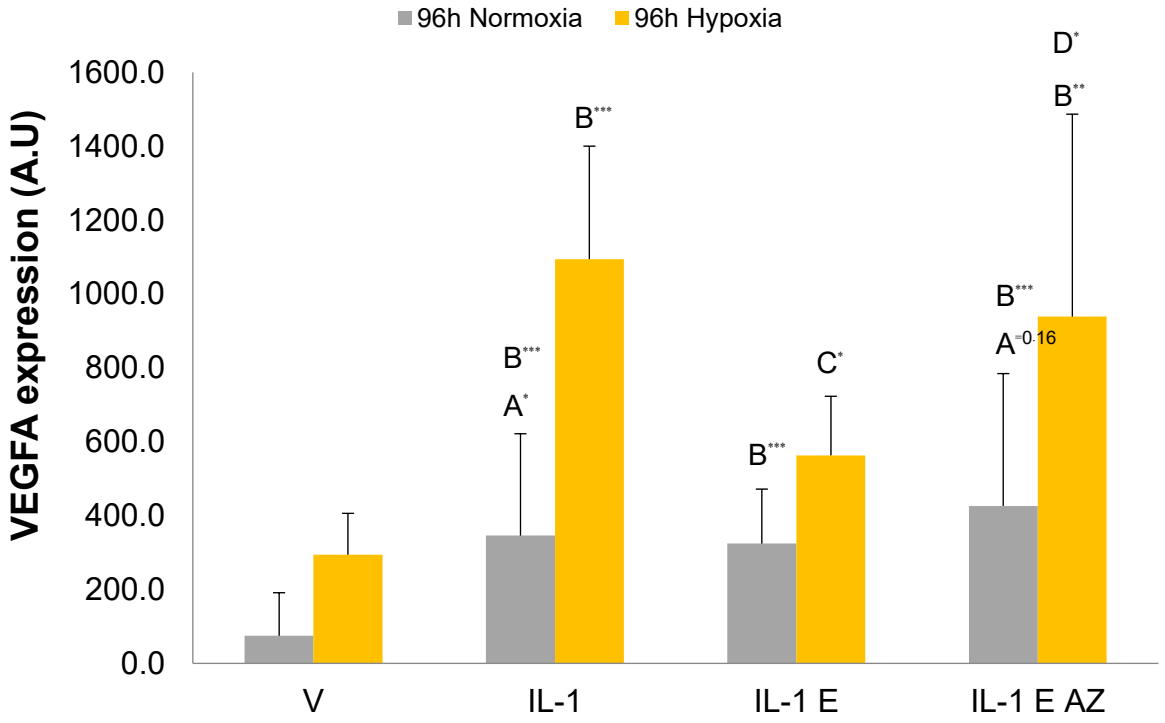

Figure 5- 22. VEGFA mRNA expression at 96 h (n=5).

*Comparison: A: normoxia vs hypoxia, B: V vs IL-1, C: IL-1 E vs IL-1, D: IL1 E AZ vs IL-1 E. V = vehicle, IL-1 = IL-1β, IL-1 E= IL-1β + cortisone and IL-1 E AZ = IL-1β + cortisone + 11β-HSD1 inhibitor AZD4017. Significance * = p<0.05, ** = p<0.01, *** = p<0.001*

5.5.4 11β-HSD1 substrate titration

5.5.4.1 VEGFA expression

To explore the effect of hypoxia on PTGS2 and VEGFA expression in more detail, experiments were repeated with different cortisone concentrations. As previously shown (Figure 5-22), 200 nM cortisone suppressed VEGFA expression under 96 h hypoxic ($p<0.01$) but not normoxic conditions ($p=0.14$, Figure 5-23, Appendix 5-9, Appendix 5-10).

At lower cortisone concentrations, the influence of hypoxia on 11β-HSD1 becomes apparent. To suppress VEGFA expression, a minimum effective cortisone concentration of ≥25 nM ($p<0.01$) was observed for hypoxia, whereas in normoxia there was only a trend at 200 nM ($p=0.14$). This suggests hypoxia sensitises VEGFA to regulation by cortisol (through 11β-HSD1) by approximately 8-fold. VEGFA expression was up to 63% lower (with 50 nM cortisone) in hypoxia vs. normoxia ($p<0.01$). This effect was also significant at 25 nM ($p<0.01$), 100 nM ($p<0.05$) and 200 nM ($p<0.01$) cortisone.

These results suggest that under pro-inflammatory and hypoxic conditions (such as in DFU), HDF are sensitised to the effects of cortisol though increased 11β-HSD1 activity and suppress VEGFA to a greater extent, which may impede wound repair. However, this preliminary analysis was conducted in two biological replicates and requires further validation.

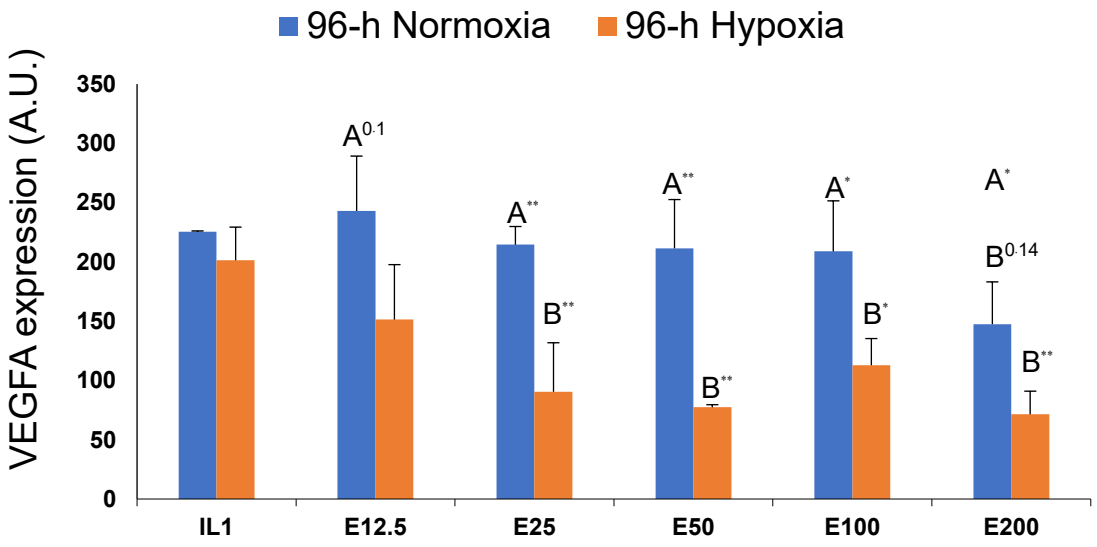

Figure 5- 23. VEGFA mRNA expression in different cortisol concentration at 96 h (n=2). *Comparisons: A = normoxia vs hypoxia, B = IL-1 vs IL-1 E. IL1 = IL-1β with 12.5, 25, 50, 100 or 200nM cortisone (E). Significance * = p<0.05, ** = p<0.01, *** = p<0.001*

5.5.4.2 PTGS2 expression

In agreement with previous data (Figure 5-20), 200 nM cortisone suppressed PTGS2 to a comparable degree in both normoxia and hypoxia, although due to the large variability with IL-1β treatment between the two biological replicates, this was not statistically significant (Figure 5-24, Appendix 5-9, Appendix 5-10). For the same reason, the minimum effective cortisone concentration required to suppress PTGS2 was inconclusive.

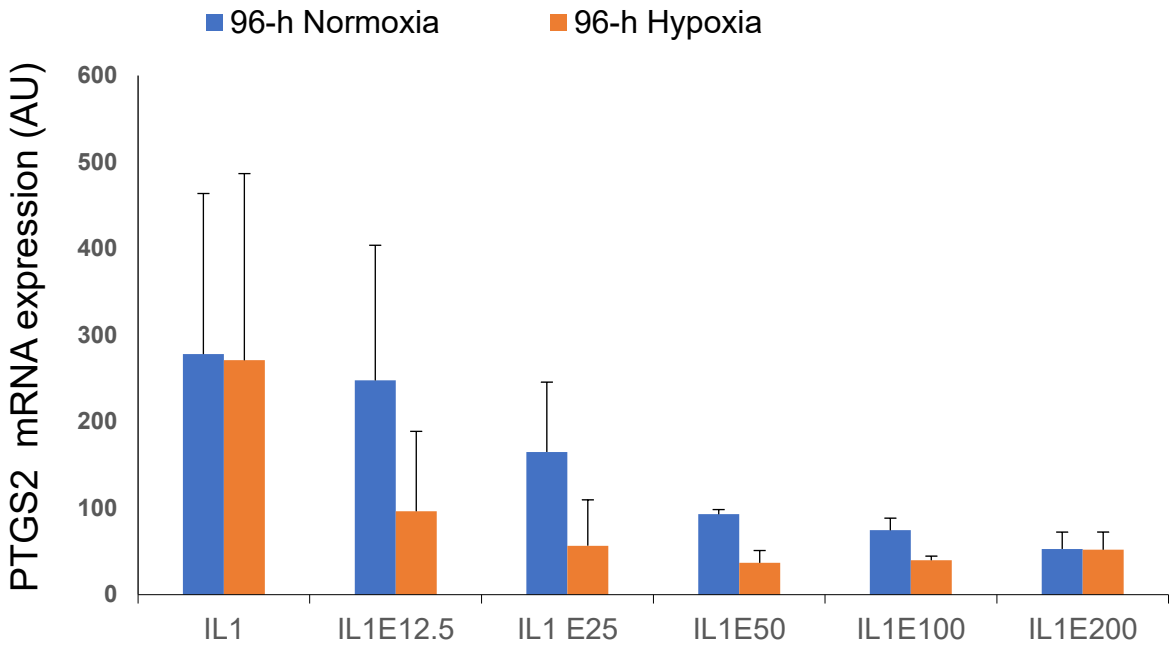

Figure 5- 24. PTGS2 mRNA expression in different cortisol concentration at 96 h (n=2). *Comparisons: A = normoxia vs hypoxia, B = IL-1 vs IL-1 E. IL1 = IL-1β with 12.5, 25, 50, 100 or 200nM cortisone (E). Significance * = p<0.05, ** = p<0.01, *** = p<0.001*

5.6 Discussion

The interplay between inflammation, hypoxia, and cortisol activation by 11β-HSD1 in human skin cells has not been explored to date. Local hypoxia and inflammation often develop concurrently in acute or delayed wounds and work in synergy, but the effect of pre-receptor GC metabolism on gene regulation in a pro-inflammatory and hypoxic setting is unknown.

IL-1β is known to induce 11β-HSD1 expression, and 11β-HSD1 is highly expressed in active inflammatory sites to limit and resolve inflammation[626-631]. Here, we show the first evidence that 11β-HSD1 expression and activity are enhanced by hypoxia in the presence of IL-1β and cortisone. Importantly, 11β-HSD1 gene expression was induced by IL-1β in synergy with hypoxia and amplified tissue cortisol by a forward-feedback mechanism. However, previous studies were conducted under normoxic conditions. Only one experiment performed in adipose tissue found that increased local GC amplification exacerbates the metabolic insult caused by hypoxia[17].

Conversely, we found GR-α, in the presence of IL-1β and cortisone, was suppressed, despite hypoxia alone increasing expression. This is in agreement with known negative feedback by GC on GR expression [146], although the suppression was modest and not sufficient to prevent regulation of GC target genes (e.g. VEGFA and PTGS2). H6PD gene expression (supplying 11β-HSD1 co-factor) was unaffected by hypoxia, IL-1β or cortisone, also as previously found[146].

Additionally, the expression of *11β-HSD1* and *H6PD* were time dependent manner. This phenomenon raised the possibility that cell stress increased local cortisol production over time, resulting in forward-feedback mechanism. This was supported by incresed 11-oxo-reductase activity in HDF that was 5- to 10- fold higher than dehydrogenase, and by the experiment of dexamthasone induced significantly more 11β-HSD1 activity at 48 h compared to 0 h[767].

The effect of hypoxia alone on pro-inflammatory PTGS2 or pro-angiogenic VEGFA gene expression was investigated and confirmed that expression of both genes was elevated under hypoxic conditions. In acute skin injury, IL-1β is first released from keratinocytes and initiates autocrine and paracrine signalling to amplify cytokine release and fibroblast responses [768]. Our findings are the first evidence of a novel cellular mechanism that may explain how hypoxia drives impaired WH.

Hypoxia, in synergy with IL-1β, induced PTGS2 and VEGFA gene expression. This induction was suppressed by cortisone through 11β-HSD1 (prevented by selective 11β-HSD1 inhibition with AZD4017). This was found to be time-dependent (at 24 hours, insufficient cortisol was generated by 11β-HSD1 to suppress gene expression) and 11β-

HSD1 substrate dose-dependent (by inducing 11β-HSD1, hypoxia sensitises VEGFA and PTGS2 to suppression by much lower cortisone concentrations).

Most importantly, cortisol generated by 11β-HSD1 following induction by hypoxia (under inflammatory conditions) was able to override the induction of PTGS2 and VEGFA by hypoxia. This is the first evidence of hypoxia disrupting pro-inflammatory and pro-angiogenic signalling by inducing local GC activation and may be a key factor in the pathogenesis of chronic wounds. Subsequently, 11β-HSD1 inhibition may represent a novel therapy for the treatment of hypoxic wounds. However, further *in vivo* studies with 11β-HSD1 inhibitors under hypoxic conditions and in patients with ischaemic wounds (e.g. venous leg ulcers) are required to generate proof-of-concept. Indeed, preclinical evidence supports the use of 11β-HSD1 inhibitors to improve WH in animal models of ageing and GC excess [145, 688], but this remains to be explored in animal models of diabetes (and hypoxia). Further, increased 11β-HSD1 activity in diabetic mice was detected [769], but there was no exploration of 11β-HSD1 activity in diabetic human skin.

Our finding that hypoxia sensitises HDF to lower GC concentrations by inducing 11β-HSD1 activity may be particularly relevant to patients suffering from stress or on low-dose systemic GC therapy, who may suffer more pronounced adverse effects in the presence of hypoxia and tissue inflammation.

Our results are limited to effects in HDF. Skin is a complex organ, and WH is dependent on many cell types and mediators interacting in a highly sophisticated temporal sequence. Therefore, our findings should be extended to full- thickness skin, together with studying effects on cell function (e.g. effects of conditioned media from our HDF experiments on *in vitro* angiogenesis / tubule differentiation), *in vivo* animal models of healing under hypoxic conditions, expression of 11β-HSD1 (and correlation with markers of hypoxia) in normal human skin and wounds compared to diabetic skin wounds and skin / wounds from patients with peripheral arterial disease.

In conclusion, our *in vitro* study demonstrates that 11β-HSD1 gene expression and activity in HDF was increased in hypoxia under inflammatory conditions, and this led to sensitiation of HDF to supression of VEGFA and PTGS2 by cortisone. 11β-HSD1 inhibition reversed this effect, suggesting 11β-HSD1 blockade may represent a novel mechanism, and therapy, to maintain adequate pro-angiogenic and pro-inflammatory signalling in hypoxic wounds such as DFU and venous leg ulcers

Chapter 6
General discussion

The rationale for this thesis was based on my exploration of the deleterious effects of GC on patients, and I chose to address this through a combination of epidemiological based studies and cell-based studies, the latter using skin as a target tissue. Fundamentally this has provided me with the privileged opportunity to be trained in a variety of research skills that will be invaluable for my ongoing career, from molecular biology/ discovery science to applied health research- systematic review and metanalysis and evidence synthesis.

The first section investigated the impact of endogenous and exogenous CS on mortality through the most comprehensive and largest systematic review and meta-analysis ever undertaken in this field. Specifically, I evaluated the effect of a hypoxic environment upon the pre-receptor regulation of GC in HDFs. The second section is laboratory based, broadly speaking evaluating GC action in the skin and modulation by 11β-HSD. This molecular-based research raises the awareness of local GC regulation independent of systemic GC levels, especially in the setting of hypoxia in skin cells with translational relevance for the management of ischaemic WH.

Importantly, the methodology used in the meta-analyses was innovative and ensured a rigorous approach to my statistical analyses. While each chapter contains a focused discussion on future areas for research, it is appropriate here to summarise my principal findings, set these in context of current thinking and set out my plans for future research direction.

6.1 The difference in mortality between endogenous and exogenous CS

Meta-analyses of endogenous and exogenous CS were conducted independently; for endogenous CS, I demonstrated an improvement in mortality compared to historical series where up to 50% of all patients died within five years. Since the discovery of endogenous CS in 1912 or the first use of GC in RA patients in the late 1940s, advancements in our knowledge and treatment of CS have significantly improved outcomes. However, mortality remains unacceptably high across all forms of GC excess states and this requires rigourous exploration to address prevention. Here we would like to demonstrate an important point and gap in the evidence addressing the association between CS or GC use and mortality.

6.1.1 Similarities and differences in the long-term complications of endogenous vs exogenous CS; molecular mechanism remain unknown.

Regardless of the characteristics, endogenous CS and chronic exogenous GC usage have a number of similarities in terms of presentation, morbidity, and death. However, treating and, ideally, preventing mortality are major challenges because of a lack of understanding of the differences between endogenous and exogenous CS in terms of molecular complexity and contributing variables. For instance, in classical endogenous CS, cortisol is continuously supraphysiologic, spontaneously released throughout the day, and mediates its effects through both MR and GR transactivation. For high affinity synthetic GC, effects are mostly mediated via GR rather than MR. Serum concentrations of exogenous CS are also dependent on the dosage, potency and duration of GC use, which are continuously changing in response to underlying disease activity. We do not fully understand if endogenous CS works through genomic (transrepression vs transactivation) or non-genomic pathways, as suggested for exogenous CS.

Additionally, there is the confounding issue of underlying disease; underlying illnesses and inflammatory processes undoubtedly contribute to exogenous CS-related problems and contribute to mortality. It is impossible to distinguish between fatalities caused directly by GC or illnesses. Endogenous CS is also heterogeneous in its subtypes; pituitary and ACS have many differences in the pattern and levels of cortisol secretion, treatments (transsphenoidal surgery vs adrenalectomy vs medical therapy) and the consequent associated endocrine disorders, for example panhypopituitarism in CD. Ongoing tissue-specific characterisation of the molecular actions of the natural hormone cortisol together with synthetic GC's will be important in identifying contributors to excess mortality.

6.1.2 Differences in acquired data between endogenous and exogenous CS

This became apparent as I conducted my meta-analysis research. The majority of patients with endogenous CS are treated by a multidisciplinary team of endocrinologists and surgeons who manage the illness ideally through early CS diagnosis, provide specialised CS treatment, and monitor for cure or remission. Since endogenous CS was pathognomonic, active treatment of associated co-morbidities (for example metabolic, cardiovascular disorders) is likely to be much less than in patients receiving exogenous GC, because the priority is to cure the CS first. Contrast this with exogenous CS where the physicians engaged in disease-directed GC use are rheumatologists, immunologists, organ transplantation teams, haematologists, pulmonologists, cardiologists, or oncologists. Their main focus is on treating the underlying disease for which GC are prescribed, as well as the

implications and preventions of GC side effects. All of these factors contribute to the discrepancy in data gathering for endogenous and exogenous CS. Direct relationships between GC levels and mortality are difficult to establish for both forms of CS.

6.2 Causes of death highlight the possibility to improve mortality

The mortality in endogenous and exogenous CS share some common patterns in terms of causes of deaths notably for ischaemic cardiovascular disease and infection. This highlights how reducing long-term cardiovascular disease risk factors in CS is a critical concern. For exogenous CS, it is more complicated; dependant on the underlying disease it may be possible to use another biological therapy rather than GC. Usually this is not possible and the emphasis becomes to use GC at the lowest possible dosage to manage the illness, for the shortest possible period of time. Future advances might involve new GC medications with greater transrepression versus transactivation effects but to date the development of such "selective" GC has been disappointing despite much effort.

Prospective cohorts are now urgently required focusing on GC-treated patients in particular disease groups with well documented GC data entry, incidence records of co-morbidities and mortality, with control groups comprising non-GC-treated patients or the general population. If followed up in large numbers death rate at 3 to 5 years should be provide the required data.

Equally contentious is the early detection of CS and how this can be achieved. As we know, CS is diagnosed based on pathognomonic clinical features or in some specific groups of patients with secondary illness such as uncontrolled diabetes, resistant HT or osteoporosis. By the time it becomes clinically apparent, it is likely that end-organ damage has already occurred and it may be too late to cure for example cardiovascular disease. Further understanding of this aspect of CS can be gained by evaluating the lower levels of autonomous cortisol secretion occuring in a significant proportion of patients with adrenal incidentalomas and its link to metabolic and cardiovascular complications. There is a need is to reconsider and further develop screening tools for better early detection of CS, for example technological applications for face detection, changes to visceral fat, skin thickness evaluation, and of crucial importance better biochemical tools that accurately measure circulating and/or urinary GC's but also tissue-specific markers of GC excess.

CS should be considered as a disease of high cardiovascular risk that requires complete surveillance for cardiovascular complications. Recommendation for follow-up of CS should be life long and certainly requires more than ten years after stopping GC or disease remission in endogenous CS.

6.3 Physician and patient education of GC adverse effects needs to be emphasised

Although adverse effects associated with GCs are well-known to healthcare providers, some causes of mortality compel us to rethink why certain avoidable diseases persist. For example, my research highlights how particular attention should be paid to improving mortality within the first 30 days of operation in endogenous CS, where many causes appeared "preventable". VTE is a particular case in point, where despite renewed interest in treating CS patients prophylactically, increased mortality remains. Infection prevention is a further area that demands more attention. Finally, the importance of adrenal insufficiency, either in in the post-operative endogenous CS patient or when caused by suppression of the normal hypothalamo-pituitary-adrenal axis in exogenous CS is now a major recognised cause of reversible death in these patients.

In each case the issue is not physician knowledge as to their existence or treatment following presentation, but a better appreciation of, and appropriate management by physician and patient alike to prevention.

6.4 Dealing with mortality data

Mortality data is a significant resource for improving healthcare systems. The data are acquired from various sources, including medical records, ongoing longitudinal demographic and health surveillance, civil registration systems, and other data sources such as census or household surveys. Data quality is of fundamental importance if accurate mortality outcomes are to be understood. Clear case definition, precision, relevance, completeness of the data records, timelines, coverage (our studies enrolled for more than 90% of CS reported the mortality at the censor period), accessibility to the information, the original purpose of data collections, the homogeneity for overall causes of death are all factors that I tried to address in my analyses. As I discovered data are also variable from country to country. Despite my firm belief that I was as rigorous as possible with the presentation by systematic review and meta-analysis accessing studies in all aspects, including transparency with bias and quality assessment, some issues were not controllable.

There are many measures and reports of death in epidemiological studies. SMR is one of the most appropriate for death measurement as it is refers to a comparable agea and gender matched background population. The SMR reported in our datasets are from CD and small numbers from ACS. However they are minority when compared to the overall number of articles. The SMR related to specific risk stratification or causes of deaths were scanty and confined only CD. We could not analyse the SMR in exogenous CS despite its greater

prevalence because the SMR data reported was solely based on disease pathogenesis. Some articles were excluded form our studies because of indequate data such as GC use, mortality reported only as hazard ratios, or presentation as only p values and relative risk rather than raw numbers. In many cases the percentage or numbers of deaths were not reported and obtaining further data even from personal requests was not possible.

Patient risk identification also requires further investigation and improvement. Most of CS characteristics or co-morbidities are those that are identified and reported for the first enrollement, but not across the censor period, yet it is reasonable to propose that the changes in medical diagnosis and management along the follow-up period impact upon mortality. Our analysis involved longitudinal data extraction and meta-analysis which allows us to see the effect of study period. However, clinical features during this period, disease severity or causes of death or risk associated mortality outcomes are a difficult part, especially when making comparison across the studies.

Regrettably, it remains a concern that in too many cases the cause of death was "unknown". Underlying factors here included discrepancy with death certificate records, a death registry, or patients being lost outside the hospital. This was more of an issue for exogenous CS. The cause of death in endogenous CS is reasonably well established, but sadly gaps remain in exogenous CS. The proportion of deaths due to unknown reasons reaches 30% in exogenous CS, with not a single death reported as adrenal insufficiency. Clearly this is far from complete.

This highlights the constraint in obtaining data for a systematic review and meta-analysis of mortality. However, the study's findings indicate the direction for a more precise CS registry or, a GC registry with longitudinal surveillance of risk-associated causes of death, such as cardiovascular disease, infection or cancer. This restriction, however, is the most advantageous acquisition for future medical publishing in terms of gathering and presenting the data set of CS patients, details of GC exposure or co-morbidities. This should emphasise the dynamics of GC use and disease activity for both endogenous and exogenous CS that are required for several time points reporting, including at the censor time. Additionally, it is critical to consider the dimensions of study design, population enrolment, including the severity of diseases (endogenous CS) or the severity of underlying conditions treated with GC, consistency of management, the measurement and methods used in individual studies, the frequency and point of time used to measure GC exposure and mortality outcomes.

6.5 The novelty in statistical analysis and methodology

The statistical analysis is one of the novelties of this thesis for performing the meta-analysis of proportion data that are not comparable. Meta-analysis is research tool that requires a solid methodology to yield rigourous conclusions. The prior well-known statistical methods available to me while undertaking my PhD had significant limitations. I began my analysis using the Revman program developed by Cochrane or the popular meta-analysis package (meta or metan) available in STATA. All of the tools apply the inverse variant method for generating the estimated effect size. I found some anomalies and aberrant results - for example studies in which mortality was equal to zero were automatically excluded by the method. Cochrane recommended adding arbitrary numbers to all results to eliminate zero problems, for example, 0.5. This is inappropriate if the sample size is small and I was always mindful that endogenous CS is also a rare disease. Taking arbitrary numbers was not ideal for my analysis. Perhaps more importantly, these programs assume the data are normally distributed (as discussed in chapter 2), whereas the proportion data had a binomial distribution.

The metaprop package is recommended for single-armed proportion meta-analysis within both STATA and R programs. Metaprop generates the effect size using Freeman-Tukey double arcsine transformations, and consequently, all studies with zero mortality could be included without transformation. However, during my analysis in 2019, the Freeman-Tukey double arcsine transformations showed seriously mislead single proportion results [421]. The generalised linear mixed models seemed to be the most appropriate alternative methods, but no program had been developed for this purpose. At this stage in my PhD, I could not write the coding to develop the command from first principles, yet I realised the inappropriateness of my ongoing analysis for generating results especially for the secondary analysis of data we extracted from published articles.

To undetake the correct analysis, I explored local expertise at the University of Leeds who have experience in meta-analysis. I found that there was limited expertise in single-armed proportion meta-analysis, even across the Cochrane and Joanna Briggs Institute. I purchased many meta-analysis textbooks and reviewed the journals, purchased online software called "comprehensive meta-analysis" created by Dr Michael Borenstein and Cooper, experts in meta-analysis, and contacted the business to inquire about the program's approach; however, I could not find a program to generate effect size assuming a binomial distribution and analysis of the data by generalised linear mixed models.

I sent an email to the developer of *metaprop* inquiring about the limitations of Freeman Turky Double Arcsine. Through this route, I discovered a software programme (*metapreg*)

under development that I could use for this analysis, under the premise of a biomial distribution. I then sent an email to the *metapreg* developer, this being Victoria Nyagan in Belgium. I am endebted to her expertise and for allowing me to use her software during development and for enabling my rigourous analyses to continue. Since then, I've been confident in my analytic approach, but there were many issues with employing a developing software that required multiple revisons.

Additionally after developing and publishing the meta-analysis protocol; screening over 100,000 articles; rigorously extracting full-text from British library and historical sources; emailing authors for data extraction and questions; I wrote my own ACCESS program for data extraction because of the limitations of using Cochrane's recommended program within excel that can result in incorrect data entry.

6.6 Excellent research has informed my clinical practice

Through my epidemiological and laboratory methods, I have acquired a wealth of wisdom. Before starting my PhD, I was working as a single handed clinical endocrinologist with personal values to provide the best possible care through clinical care informed by my reading, clinical guidelines and literature review. However, my PhD experience has expanded my horizons; I now have a greater understanding of disease and the power that research can bring in informing my future clinical practice and hopefully that of others.

6.7 My future work: the marriage of molecular research, epidemiology and clinical translation

Overt CS remains a serious condition with excess mortality that requires urgent treatment, but this is just "the tip of the iceberg" for our knowledge. Under the iceberg, there remains a plethora of mystery and intrigue to be explored.

Specific future studies for endogenous or exogenous CS and 11β-HSD1 have been discussed in specific chapters. Improving patient care or making in roads into reducing mortality in CS mortality requires in-depth understanding of the effects of GC's working across multi-disciplinary teams. For rare diseases such as CS, this requires the concerted efforts of many countries with worldwide networks contributing the research to help delivery of better patient outcomes. As I return to my homeland of Thailand, my future work aims to add to this synergistic effort by developing a national/ south east Asia CS registry. For sure there will be cultural/ ethnicity differences in the presentation of exogenous and endogenous CS that will enhance this global effort. My key publications (one submitted, one in final prepration) will build on the presentations of my thesis data at international meetings, to help give me the credibility to undertake this collaborative work. Such databases will not only

assimilate clinical or outcome data; precision medicine should be the next step for GC treatment and effective approach. Here I will utilise todays technologies (for example advances in steroid biochemistry, genomics) to undertake deeper phenotyping of patients to gain a greater insight into the link between GC excess, co-morbidities and mortality.

I also have a greater comprehension as to the pathophysiology behind the appearance of CS. One illustration of my intriguing research is the local control of GC by 11β-HSD systems. Here I postulate a link to cardiovascular mortality with obstructive sleep apnea (OSA) in both endogenous CS and exogenous GC used in hypoxic prone diseases (e g., primary or secondary pulmonary fibrosis). The CS phenotype significantly increases the likelihood of having OSA (hypoxic condition) by 50% when compared to age-, gender-, and body mass index-matched controls (23%, $p = 0.003$) [770], while the prevalence of OSA in the general population is between 2% and 5% [771]. As shown in this thesis, hypoxia increases 11β-HSD1 gene expression and activity, particularly in inflammatory conditions. Further study of 11β-HSD1 expression and activity in hypoxic conditions is promising when these jigsaw pieces are combined. Additionally, the impact of hypoxia in the skin and local GC excess needs additional research, particularly in patients with DM (both type 1 and type 2) with varying duration of diabetes, levels of insulin resistance, and varying degrees of micro- or macrovascular problems. Hypoxic skin is not limited to diabetic wounds, but also occurs in other low-perfusion states such as vasculitis or peripheral arterial disease, as well as systemic hypoxic states such as chronic obstructive pulmonary disease, pulmonary fibrosis, obstructive sleep apnea (at any stage mild, moderate, or severe), chronic heart failure, left-to-right shunt heart diseases, or severe systemic hypoxia such as Eisenmenger syndrome.

Over 100 years after Harvey Cushing's initial work, and equipped with the skills acquired through this PhD, the future is bright in terms of my ongoing career development, establishing myself as a National and International authority in Endocrinology. There is still a great deal we don't know, and I hope to continue to make a significant contribution in my own part of the world to further our understanding of GC action and the fascinating disease - Cushing's syndrome.

Appendix

Appendix 2- 1. Prospero registration(CRD42017067530)

NIHR | National Institute for Health Research

PROSPERO
International prospective register of systematic reviews

Citation

Padiporn Limumpornpetch, Nadia Othonos, Mar Pujades-Rodriguez, Paul Stewart, Ann Morgan, Ana Tiganescu. The effect of exogenous and endogenous Cushing's syndrome on all-cause and cause-specific mortality: protocol for a systematic review. PROSPERO 2017 CRD42017067530 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42017067530

Review question

What is the overall standardized all-cause mortality ratio (SMR) or overall mortality in patients with Cushing's syndrome?
What are the specific causes of death in Cushing's syndrome?

Searches

This systematic review covers the following databases: PubMed, EMBASE, CINAHL, and the Cochrane Library. The search will be restricted by language (English).
Reference lists of key papers (review articles) will be browsed to identify missing studies, and authors in the field will be contacted by email.

Types of study to be included

Cohort studies reporting the SMR, death or moratlity rate will be included.

Condition or domain being studied

Endogenous and exogenous Cushing's syndrome.

Participants/population

Adults (18 years of age or older), who have been diagnosed with endogenous Cushing's syndrome or use exogenous glucocorticoids (exogenous Cushing's syndrome).

Intervention(s), exposure(s)

The exposure is exogenous Cushing's syndrome, which will be defined as patients who are treated with synthetic forms of glucocorticoids such as oral prednisolone or dexamethasone or corticosteroid; at a dose equivalent to > 5 mg of prednisolone, for at least 3 weeks within one year.

Comparator(s)/control

Not applicable.

Main outcome(s)

SMR, Death, mortality rate.

Additional outcome(s)

Specific causes of death (ratio of all-cause mortality).

Data extraction (selection and coding)

The studies identified by the search strategy will be collated and duplicates will be removed. Two investigators will screen the titles and abstracts in a parallel manner. Studies identified as meeting the eligibility criteria or which do not provide sufficient information to determine inclusion eligibility will be retrieved for further review. The two investigators will independently determine eligibility on the basis of the study protocol examining the full text of each article and will extract the information using a standardised extraction form. Any disagreements over eligibility will be resolved through discussion and with the intervention of a third investigator.
Types of data that will be extracted: Study name/article title; Authors; Year of publication; Journal and full reference details; Country and Continent; Study type; Year of the study; Study populations: endogenous or exogenous; and Level of care.

Risk of bias (quality) assessment

Two review authors will independently assess the risk of bias in the included studies by using the Newcastle-

Ottawa Quality Assessment Scale (NOS), a star-based rating system made up of three parts (selection, comparability, and outcome).

Strategy for data synthesis

The following variables will be collected as exposure variables from each study:
Age at study: age range and mean or median
Gender (proportion of female to male)
Duration of observation (year)
Study period (range)
Country
What about the site and the description of the population (e.g. tertiary hospital) and the study design?
Type of Cushing's syndrome (exogenous or endogenous)
- Exogenous Cushing's syndrome
o Sample size
o Underlying disease/s
o Type of glucocorticoids use
o Dose and duration of glucocorticoid exposure
- Endogenous Cushing's syndrome
o Sub-type of endogenous glucocorticoids exposure (ACTH-dependent Cushing's syndrome; microadenoma, macroadenoma, invisible adenoma or Cushing's disease, adrenal or ectopic Cushing's syndrome)
o Disease activity for endogenous Cushing's syndrome (remission or active disease)
o Type of treatment (adrenalectomy, radiation, pituitary surgery or medication)
Co-morbidities and psychiatric diseases (Proportion of people without death)
Standard Mortality Ratio
Cause of death

Analysis of subgroups or subsets

Exogenous and endogenous Cushing's syndrome.

Contact details for further information

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Type and method of review

Prognostic, Systematic review

Anticipated or actual start date
21 August 2017

Anticipated completion date
31 December 2018

Funding sources/sponsors
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Conflicts of interest
None known

Language
English

Country
England

Stage of review
Review Ongoing

Subject index terms status
Subject indexing assigned by CRD

Subject index terms
Cause of Death; Cushing Syndrome; Humans

Date of registration in PROSPERO
20 August 2017

Date of first submission
23 September 2018

Stage of review at time of this submission

Stage	Started	Completed
Preliminary searches	Yes	No
Piloting of the study selection process	No	No
Formal screening of search results against eligibility criteria	No	No
Data extraction	No	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

The record owner confirms that the information they have supplied for this submission is accurate and complete and they understand that deliberate provision of inaccurate information or omission of data may be construed as scientific misconduct.
The record owner confirms that they will update the status of the review when it is completed and will add publication details in due course.

Appendix 2- 2. Search Term

PubMed (1996 to 31 Mar 2019)

https://www.ncbi.nlm.nih.gov/pubmed

1. **Exogenous Cushing's syndrome (N=31,695)**

((((((((((Prednisone[MeSH Terms])) OR Prednison*) OR Prednisolone[MeSH Terms])) OR glucocorticoid*[MeSH Terms]) OR glucocorticoid*) OR ((corticosteroid*) OR corticosteroid[MeSH Terms])))) AND ((((death) OR death[MeSH Terms]) OR mortality) OR mortality[MeSH Terms])))

2. **Endogenous Cushing's syndrome (N=4,637)**

((((((((((Adrenal tumor*) OR Adrenal tumor[MeSH Terms]) OR Adrenal tumour*)) OR (((Adrenal adenoma) OR Adrenal adenoma*) OR Adrenal adenoma[MeSH Terms])) OR (((adrenocortical adenoma) OR adrenocortical adenoma*) OR adrenocortical adenoma[MeSH Terms])) OR ((glucocorticoid producing adenoma) OR glucocorticoid producing adenoma[MeSH Terms]) OR (glucocorticoid producing tumor) OR glucocorticoid producing tumor[MeSH Terms]) OR glucocorticoid producing tumor*) OR glucocorticoid producing tumour*))) OR (((adrenal carcinoma) OR adrenal carcinoma[MeSH Terms]) OR adrenal carcinoma*) OR ((((((((((Cushing's syndrome[MeSH Terms]) OR Cushing's syndrome) OR Cushing*)) OR (((ACTH secreting tumor) OR ACTH secreting tumor[MeSH Terms]) OR ACTH secreting tumo*)) OR ((ACTH producing tumor) OR ACTH producing tumor[MeSH Terms])) OR (((ACTH secreting adenoma[MeSH Terms])) OR ACTH secreting adenoma*)) OR ((ACTH producing adenoma[MeSH Terms]) OR ACTH producing adenoma)) OR (((Pituitary adenoma) OR Pituitary adenoma[MeSH Terms]) OR Pituitary adenoma*)) OR ((Cushing's disease) OR Cushing's disease[MeSH Terms])) OR ((pituitary tumor) OR (pituitary tumor*) OR pituitary tumor[MeSH Terms])))) OR ((Cushing's disease) OR Cushing's disease[MeSH Terms]) AND ((((death) OR death[MeSH Terms]) OR mortality) OR mortality[MeSH Terms])))

Web of Science (1900 to 31 Mar 2019)
http://isiknowledge.com/wos

1. **Exogenous Cushing's syndrome (N=12,197)**

#1 N=141,636

(TS=(Predniso* or corticosteroid* or glucocorticoid*)) *AND* **LANGUAGE:** (English) *AND* **DOCUMENT TYPES:** (Article)

Indexes=SCI-EXPANDED, ESCI Timespan=All years

#2 N=1,125,226

(TS=(death or mortality)) *AND* **LANGUAGE:** (English) *AND* **DOCUMENT TYPES:** (Article)

Indexes=SCI-EXPANDED, ESCI Timespan=All years

#3 N=12,197

#1 AND #2

Indexes=SCI-EXPANDED, ESCI Timespan=All years

2. **Endogenous Cushing's syndrome (N=2548)**

#1 N= 37,838

(TS=((Adrenal tumor*) OR (Adrenal tumour*) OR (Adrenal adenoma*) OR (adrenocortical adenoma*) OR (glucocorticoid producing adenoma*) OR (glucocorticoid producing tumor*) OR (glucocorticoid producing tumour*) OR (Cushing's syndrome) OR (Cushing*) OR (ACTH secreting tumo*) OR (ACTH secreting adenoma*) OR (ACTH producing adenoma*) OR (Pituitary adenoma*) OR (Cushing's disease) OR (pituitary tumour*) OR (pituitary tumor*))

Indexes=SCI-EXPANDED, ESCI Timespan=All years

#2 N=1,125,226

(TS=(death or mortality)) *AND* **LANGUAGE:** (English) *AND* **DOCUMENT TYPES:** (Article)

Indexes=SCI-EXPANDED, ESCI Timespan=All years

#3 N= 2,548

#1 AND #2

Indexes=SCI-EXPANDED, ESCI Timespan=All years

EMBASE (1974 to 31 Mar 2019)
http://ovidsp.dc1.ovid.com/sp-4.03.0b/ovidweb.cgi

1. **Exogenous Cushing's syndrome (N=57002)**

(Predniso* or corticosteroid* or glucocorticoid*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] AND (death or mortality).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] limit to english language

2. **Endogenous Cushing's syndrome (N=3586)**

(Adrenal tumor* or Adrenal tumour* or Adrenal adenoma* or adrenocortical adenoma* or glucocorticoid producing adenoma* or glucocorticoid producing tumor* or glucocorticoid

producing tumour* or Cushing's syndrome or Cushing* or ACTH secreting tumo* or ACTH secreting adenoma* or ACTH producing adenoma* or Pituitary adenoma* or Cushing's disease or pituitary tumour* or pituitary tumor*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word] AND (death or mortality).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]

limit to english language

EBSCO (CINAHL) (1981 to 31 Mar 2019)

1. **Exogenous Cushing's syndrome (N=3466)**

(Predniso* or corticosteroid* or glucocorticoid*) AND (death or mortality)

2. **Endogenous Cushing's syndrome (N=378)**

((Adrenal tumor) or (Adrenal tumour) or (Adrenal adenoma) or (adrenocortical adenoma) or (glucocorticoid producing adenoma) or (glucocorticoid producing tumor) or (glucocorticoid producing tumour) or (Cushing's syndrome) or Cushing or (ACTH secreting tumor) or (ACTH secreting adenoma) or (ACTH producing adenoma) or (Pituitary adenoma) or Cushing's disease) or (pituitary tumour) or (pituitary tumor) (MesH) OR (Adrenal tumor* or Adrenal tumour* or AdrenPubal adenoma* or adrenocortical adenoma* or glucocorticoid producing adenoma* or glucocorticoid producing tumor* or glucocorticoid producing tumour* or Cushing's syndrome or Cushing* or ACTH secreting tumo* or ACTH secreting adenoma* or ACTH producing adenoma* or Pituitary adenoma* or Cushing's disease or pituitary tumour* or pituitary tumor*)) AND (death or mortality)

Cochrane (1992 – 31Mar 2019)
https://www.cochranelibrary.com/advanced-search

title/abstract/keyword

1. **Exogenous Cushing's syndrome (N=5150)**

(Predniso* or corticosteroid* or glucocorticoid*) AND (death or mortality)

2. **Endogenous Cushing's syndrome (N=378)**

((Adrenal tumor) or (Adrenal tumour) or (Adrenal adenoma) or (adrenocortical adenoma) or (glucocorticoid producing adenoma) or (glucocorticoid producing tumor) or (glucocorticoid producing tumour) or (Cushing's syndrome) or Cushing or (ACTH secreting tumor) or (ACTH secreting adenoma) or (ACTH producing adenoma) or (Pituitary adenoma) or Cushing's disease) or (pituitary tumour) or (pituitary tumor) (MesH) OR (Adrenal tumor* or Adrenal tumour* or AdrenPubal adenoma* or adrenocortical adenoma* or glucocorticoid producing

adenoma* or glucocorticoid producing tumor* or glucocorticoid producing tumour* or Cushing's syndrome or Cushing* or ACTH secreting tumo* or ACTH secreting adenoma* or ACTH producing adenoma* or Pituitary adenoma* or Cushing's disease or pituitary tumour* or pituitary tumor*)) AND (death or mortality)

Appendix 2- 3. Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) assessment tool

At protocol stage Specify the review question Endogenous CS

Participants	**Endogenous CS** Definition: Adults (≥18 years of age) who have been diagnosed with Endogenous CS (excluding carcinoid/neuroendocrine tumour and ectopic CS).
Experimental intervention	No
Comparator	No (If use SMR comparator is normal population)
Outcomes	Primary: SMR or numbers of deaths Secondary: Specific causes of death

List the competing exposure domains relevant to all or most studies

Comorbidity or Complication of CS: Diabetes, hypertension, obesity, cardiovascular diseases, Duration of GC exposure or active diseases, Duration of follow-up, Age, Sex
List co-interventions that could be different between intervention groups and that could impact on outcomes

Treatment

Design Cohort

Participants	Endogenous CS: adrenal, pituitary, mixed
Experimental intervention	-
Comparator	No

At protocol stage Specify the review question Exogenous CS

Participants	**Exogenous CS** Definition: Adults (≥18 years of age) use exogenous oral glucocorticoids (GCs) at a dose equivalent to > 5 mg of prednisolone for at least 3 weeks within one year.
Experimental intervention	Chronic GC exposure (defined term as the definition of CS participants)
Comparator	No (If use SMR comparator is normal population withage- and gender- match)
Outcomes	Primary: SMR or Numbers of death Secondary: Specific causes of death

List the confounding domains relevant to all or most studies

Co-morbidity or Complication of CS: Diabetes, hypertension, obesity Underlying disease treated (Rheumatoid arthritis, connective tissue diseases, autoimmune vasculitis group) Duration of disease, Duration of follow-up, Patients' immunity, Age: Giant cell arteritis, Sex, Chronic renal failure: Lupus nephritis, ANCA, Disease activity and inflammatory process, GC doses, organ involvement, Adverse of other drug events

List co-interventions that could be different between intervention groups and that could impact on outcomes

DMARDS, Immunomodulator, Plasmapheresis, Splenectomy in ATP, Cardiovascular drugs: Statin, aspirin (vasculitis), ACE-I (lupus), Vaccination

Assess risk of bias

Responses underlined in green are potential markers for low risk of bias, and responses in red are potential markers for a risk of bias. Where questions relate only to sign posts to other questions, no formatting is used.

No	Signalling questions	Response options
1. Bias due to confounding/competing exposure		
B1	Is there potential for confounding/competing exposure in this study?	Y / PY / PN / N

	If N/PN to B1: the study can be considered to be at low risk of bias due to confounding and no further signalling questions need be considered (go to B6) If Y/PY to B1: determine whether there is a need to assess time-varying confounding	
B2	Was the analysis based on splitting participants' follow up time according to CS status, GCs use or other treatment use?	NA / Y / PY / PN / N / NI
B3	Endogenous CS, were other medications or intervention discontinued or switched and this was the cause of death? Exogenous CS, were other medications discontinued or switched and this was the cause of death?	NA / Y / PY / PN / N / NI
B4	Did the authors use an appropriate analysis method that controlled for all the important confounding/competing exposures domains and for time-varying confounding/competing exposures?	NA / Y / PY / PN / N / NI
B5	If Y/PY to B4: Were confounders that were controlled for, measured validly and reliably by the variables available in this study?	NA / Y / PY / PN / N / NI
B6	Risk of bias judgement due to confounding/competing exposure	Low / Mod / Serious / Critical / NI
B7	What is the predicted direction of bias due to confounding/ competing exposure?	Higher mortality / Lower mortality /More statistically significant / Unpredictable
2. Bias in selection of participants into the study		
B8	Was participant selection making the study population more or less severe (so less representative of all the patients with that disease) and this makes people more or less likely die? e.g. if patients recruited from A&E, they will be more likely to die because of other diseases, medication, high severity. If N/PN go to B11	Y / PY / PN / N/NI
B9	If Y/PY to B8: Was the selection bias making more or less likely that the patient has CS? E.g. if recruited from A&E, patients are not necessarily more or less likely to have CS	NA / Y / PY / PN / N / NI
B10	If Y/PY toB9 : Was the selection bias likely to be influenced by the death or a cause of the death? E.g. if recruited from A&E, patients would be more likely to die	NA / Y / PY / PN / N / NI
B11	Do start of follow-up and start of CS (or GC exposure) coincident for most participants?	Y / PY / PN / N/NI
B12	If Y/PY to B9 and B10, or N/PN to B11: Were adjustment techniques used that are likely to correct for the presence of selection biases?	NA / Y / PY / PN / N / NI
B13	Risk of bias in selection of participants into the study	Low / Mod / Serious / Critical / NI

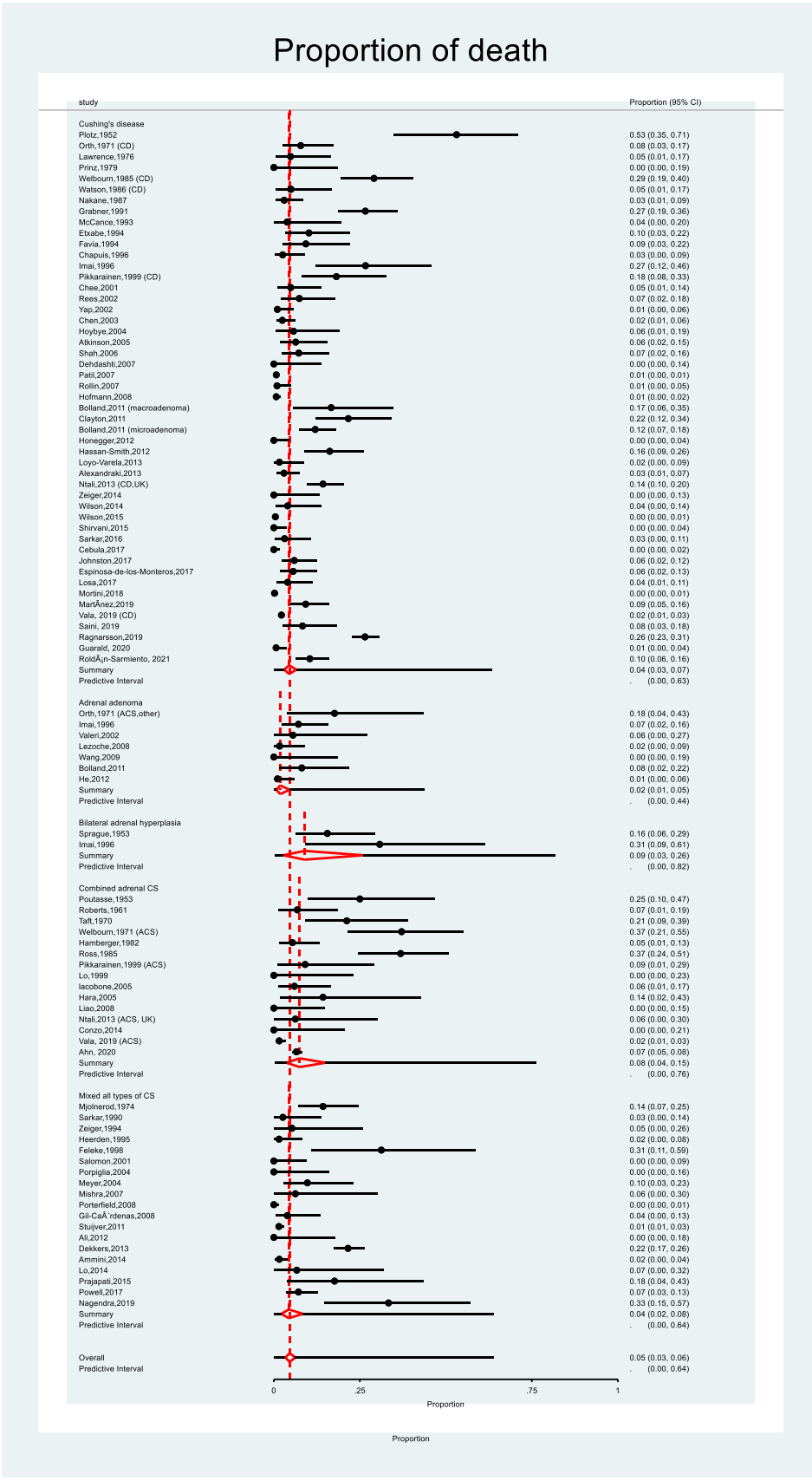
B14	What is the predicted direction of bias due to selection of participants into the study?	Higher mortality / Lower mortality /More statistically significant / Unpredictable
3. Bias in classification of interventions		
B15	Was CS (or GC exposure)clearly defined?	Y / PY / PN / N / NI
B16	Was the information used to define CS (or GC exposure) recorded at diagnosis/ enrollment?	Y / PY / PN / N / NI
B17	Could classification of CS status have been affected by knowledge of the outcome or risk of the outcome? Endogenous CS: Would people who die be more likely to be diagnosed with CS or vice versa)? Exogenous CS: Incorrect assignment of GC exposure status	Y / PY / PN / N / NI
B18	Risk of bias in classification of interventions	Low / Mod / Serious / Critical / NI
B19	What is the predicted direction of bias due to classification of patients as having or not CS (or GC exposure)?	Higher mortality / Lower mortality /More statistically significant / Unpredictable
Bias due to deviations from intended interventions: Endogenous CS: treatment of CS; Exogenous CS: GC exposure or treatment		
B20	Were there deviations from the intended CS status (GC exposure) ascertainment beyond what would be expected in usual practice?	Y / PY / PN / N / NI
B21	Were important co-interventions balanced across intervention groups?	Y / PY / PN / N / NI
B22	Was the intervention implemented successfully for most participants?	Y / PY / PN / N / NI
B23	Did study participants adhere to the assigned intervention regimen?	Y / PY / PN / N / NI
B24	If N/PN to B22-244.3: Was an appropriate analysis used to estimate the effect of starting and adhering to the intervention?	NA / Y / PY / PN / N / NI
B25	Risk of bias due to deviations from intended interventions	Low / Mod / Serious / Critical / NI
B26	What is the predicted direction of bias due to deviations from the intended interventions?	Higher mortality / Lower mortality /More statistically significant / Unpredictable
5. Bias due to missing data		
B27	Were outcome data (death or SMR) available for all, or nearly all, participants?	Y / PY / PN / N / NI

B28	Were participants excluded due to missing data on CS status or GC exposure?	Y / PY / PN / N / NI
B29	Were participants excluded due to missing data on other variables needed for the analysis?	Y / PY / PN / N / NI
B30	If PN/N B27, or Y/PY to B28, B29: Are the proportion of participants and reasons for missing data similar across CS?	NA / Y / PY / PN / N / NI
B31	If PN/N toB27, or Y/PY to B28, B29: Is there evidence that results were robust to the presence of missing data?	NA / Y / PY / PN / N / NI
B32	Risk of bias of missing data	Low / Mod / Serious / Critical / NI
B33	What is the predicted direction of bias due to missing data?	Higher mortality / Lower mortality /More statistically significant / Unpredictable
6. Bias in measurement of death or SMR		
B34	Could death or SMR ascertainment have been influenced by knowledge of the CS status (GC exposure)?	Y / PY / PN / N / NI
B35	Were death or SMR assessors aware of the CS status (GC exposure) of study participants?	Y / PY / PN / N / NI
B36	Were any systematic errors in measurement of the death related to CS status (GC exposure)?	Y / PY / PN / N / NI
B37	Risk of Bias in measurement of deaths or SMR	Low / Mod / Serious / Critical / NI
B38	What is the predicted direction of bias due to measurement of death?	Higher mortality / Lower mortality /More statistically significant / Unpredictable
7. Bias in selection of the reported result		
B39	Risk of Bias in selection of the reported result	Low / Mod / Serious / Critical / NI
8. Overall bias		
B40	Overall risk of bias judgement	Low / Mod / Serious / Critical / NI
B41	What is the overall predicted direction of bias for this outcome?	Higher mortality / Lower mortality /More statistically significant / Unpredictable

Appendix 3- 1. Results of the bias assessment for included studies


Study
Loyo-Varela,2013
Martínez,2019
McCance,1993
Meyer,2004
Mishra,2007
Mjolnerod,1974
MortiNo information,2018
Nagendra,2019
Nakane,1987
Ntali,2013 (CD,UK)
PatilL,2007
Pikkarainen,1999
Plotz,1952
Porpiglia,2004
Porterfield,2008
Poutasse,1953
Powell,2017
Prajapati,2015
Prinz,1979
Ragnarsson,2019
Rees,2002
Roberts,1961
Roldán-Sarmiento, 2021
Rollin,2007
Ross,1985
SaiNo information, 2019
Salomon,2001
Sarkar,1990
Sarkar,2016
Shah,2006
ShirvaNo information,2015
Sprague,1953
Stuijver,2011
Swearingen, 1999
Taft,1970
Vala, 2019
Valeri,2001 (CD)
Valeri,2002 (ACS)
Wang,2009
Watson,,1986
Welbourn,1971 (ACS)
Welbourn,1985 (CD)
Wilson,2014
Wilson,2015
Yaneva,2013
Yap,2002
Zeiger,1994
Judgement
Serious
Moderate
Low
No information
Domains:
D1: Bias due to confounding.
D2: Bias due to selection of participants.
D3: Bias in classification of interventions.
D4: Bias due to deviations from intended interventions.
D5: Bias due to missing data.
D6: Bias in measurement of outcomes.
D7: Bias in selection of the reported result.

Appendix 3- 2. Meta-regression analysis for the proportion of death with subtypes



****

Effect	Logit	SE	z	P>|z|	Lower	Upper
endogr						
Cushing's disease	-3.07	0.21	-14.74	0.00	-3.47	-2.66
Adrenal adenoma	-3.97	0.47	-8.37	0.00	-4.90	-3.04
Bilateral adrenal hyperplasia	-2.32	0.65	-3.57	0.00	-3.59	-1.04
Combined adrenal CS	-2.51	0.40	-6.33	0.00	-3.28	-1.73
Mixed all types of CS	-3.09	0.36	-8.52	0.00	-3.80	-2.38
Overall	-3.03	0.16	-18.47	0.00	-3.36	-2.71

****
Marginal summary: Absolute measures
****

Effect	Proportion	SE(logit)	z(logit)	P>|z|	Lower	Upper
endogr						
Cushing's disease	0.04	0.21	-14.74	0.00	0.03	0.07
Adrenal adenoma	0.02	0.47	-8.37	0.00	0.01	0.05
Bilateral adrenal hyperplasia	0.09	0.65	-3.57	0.00	0.03	0.26
Combined adrenal CS	0.08	0.40	-6.33	0.00	0.04	0.15
Mixed all types of CS	0.04	0.36	-8.52	0.00	0.02	0.08
Overall	0.05	0.16	-18.47	0.00	0.03	0.06

NOTE: H0: P = 0.5 vs. H1: P != 0.5

****

Test of heterogeneity - LR Test: RE model vs FE model

	DF	Chisq	p	tau2	isq
Overall	1.00	1259.48	0.00	1.81	

****
Marginal summary: Relative measures
****

Effect	Rel Ratio	SE(lor)	z(lor)	P>|z|	Lower	Upper
endogr						
Adrenal adenoma	0.42	0.46	-1.90	0.06	0.17	1.03
Bilateral adrenal hyperplasia	2.01	0.59	1.19	0.23	0.64	6.37
Combined adrenal CS	1.69	0.42	1.27	0.21	0.75	3.82
Mixed all types of CS	0.98	0.40	-0.06	0.95	0.45	2.13

****

Model comparison(s): Leave-one-out LR Test(s)

Excluded Effect	chi2	df	p
endogr	9.40	4.00	0.05

Appendix 3- 3. Peri-operative vs long-term proportion of death (pre-and post- 2000*)*

Group*	No. of study	No. of CS	No. of death	Proportion of death	95% CI		τ^2	P value
All CS cohort							1.52	<0.01
Peri-operative death	21	10274	77	0.01	0.01	0.03		
Long-term	71	8907	698	0.06	0.05	0.09		
All CS cohort (Before 2000)							0.69	<0.001
Peri-operative death	8	379	19	0.04	0.02	0.08		
Long-term death	23	1031	183	0.15	0.11	0.21		
All CS cohort (since 2000-2021)							1.28	<0.001
Peri-operative death	13	9895	58	0.01	0.00	0.02		
Long-term death	48	7876	515	0.04	0.03	0.06		
CD cohorts (Before 2000)							0.84	<0.001
Peri-operative death	3	136	4	0.02	0.01	0.1		
Long-term death	11	617	105	0.14	0.08	0.23		
CD cohorts (2000-2021)							1.43	<0.001
Peri-operative death	6	9484	56	0.01	0.00	0.02		
Long-term death	29	4734	312	0.04	0.02	0.06		
Adrenal adenoma cohort (Before 2000)							NA	NA
Peri-operative death	0	0	0	NA	NA	NA		
Long-term death	2	87	8	0.09	0.05	0.17		
Adrenal adenoma cohorts (2000-2021)							0.19	0.62
Peri-operative death	3	95	2	0.02	0.00	0.09		
Long-term death	2	130	4	0.03	0.01	0.1		
Bilateral adrenal hyperplasia cohort (Before 2000)							NA	NA
Peri-operative death	1	45	7	0.19	0.11	0.31		
Long-term death	1	13	4	0.09	0.03	0.26		
Combinded ACS report cohort (Before 2000)							0.06	<0.001
Peri-operative death	3	132	7	0.05	0.02	0.11		
Long-term	5	171	49	0.28	0.20	0.37		
Combinded ACS report cohort (2000-2021)							NA	NA
Peri-operative death	1	23	0					
Long-term death	6	1608	86	0.04	0.02	0.08		
Combined reports of all types of CS cohort (Before2000)							NA	NA
Peri-operative death	1	66	1	0.02	0.00	0.08		
Long-term death	4	143	17	0.11	0.05	0.23		
Combined reports of all types of CS cohort (2000 -2021)							<0.001	1.3
Peri-operative death	3	293	0	0	0.00	1		
Long-term death	11	1404	113	0.06	0.03	0.12		

Appendix 4- 1. Characteristics of the 116 articles (128 study group) included in the systematic review of exogenous Cushing's syndrome.

1st Author (year)	Country	Study design Level of care]	No. patients	Observation period	Mean age at diagnosis [Median]	Data source	No. of women (%)	No. of deaths (%)	Mean follow-up in years [Median]	GC type	Mean GC use duration in months [Median]
Vasculitis group: Giant cell arteritis											
Bengtsson 1981 [772]	Sweden	Retro [S]	90	NR	71.0	Med	67 (74.4)	13 (14.4)	4.9	PRN	59.0
Chevalet 2000 [773]	France	Prospective cohort [C]	164	1992 -NR	73.3	Med	116 (70.7)	5 (3)	NR	PRL	12.0
Gran 2001 [774]	Norway	Retro [S]	64	1987 -1997	NR	Med	49 (76.6)	13 (20.3)	5.3	PRL	NR
Hachulla 2001 [775]	France	Retro [S]	133	1977 -1995	72.0	Registry	95 (71.4)	41 (30.8)	5.6	PRL & PRN	40.0
Uddhammar 2002 [776]	Sweden	Retro [S]	136	1973 -1995	70.4	Med	NR	114 (83.8)	[10]	PRL	[35]
Les 2015 [777]-medium dose	Spain	Retro [S]	53	2004 -2012	74.7	Med	37 (69.8)	1 (1.9)	[2.9]	PRN	NR
Les 2015 [777]-high dose	Spain	Retro [S]	50	2004 -2012	73.3	Med	31 (62.0)	3 (6)	[2.9]	PRN	NR
Labarca 2016 [778]	US	Retro [S]	286	1998 -2014	75.0	Med	213 (74.5)	69 (24.1)	[5.1]	NR	NR
Wilson 2017 [632]	UK	Retro [C]	5011	1987 -2013	72.9	Med	3713 (74.1)	517 (10.3)	[1.8]	PRL	NR
Ly 2017 [779]	France	Prospective cohort [S]	428	1976 -2014	75.0	Med	274 (64.0)	143 (33.4)	5.1	PRL	NR
Vasculitis group: Takayasu arteritis											
Park 2005 [780]	Korea	Retro [S]	94	1991 -2003	[29.5]	Med	NR	6 (6.4)	5.1	PRL	NR
Vasculitis group: ANCA assoiciated vasculitis											
Hoffman 1992 [781]	US	Retro [S]	158	NR	[41.0]	Med	79 (50)	32 (20.3)	NR	PRN	[12]
Koldingsnes 2002 [782]	Norway	Retro [S]	56	1984 -2000	[50.3]	Registry	21 (37.5)	13 (23.2)	[4.7]	PRL	[24]
Slot 2003 [783]	Netherlands	Retro [S]	85	1982 -2001	56.0	Meds	30 (35.3)	37 (43.5)	5.0	PRN	NR
Harper 2005 [784]	UK	Retro [S]	229	1990 -2000	[65]	Med	105 (45.9)	91 (39.7)	5.0	PRL	60.0

Rihova 2005 [785]	Prague, Czech Republic	Retro [S]	61	1986 -1997	[54]	Med	24 (39.3)	19 (31.1)	NR	PRN	NR
Holle 2011 [786]	Germany	Retro [S]	155	1966 -1997	[48]	Meds	79 (51)	22 (14.2)	[6.6]	PRL	NR
Holle 2011 [786]	Germany	Retro [S]	167	1999 -2005	[55]	Med	82 (49.1)	8 (4.8)	[3.9]	PRL	NR
Holle 2011 [786]	Germany	Retro [S]	123	1994 -2005	[52]	Med	61 (49.6)	13 (10.6)	[7.3]	PRL	NR
McGregor 2012 [787]	US	Retro [S]	61	2000 -2009	[63]	Registry	23 (37.7)	8 (13.1)	2.9	PRL	20 [13]
Gregersen JW, 2012 [788]	Denmark	Retro [S]	50	1999 -2007	[66]	Med	47 (94)	8 (16)	1.0	PRL	12.0
Specks 2013 [789]	US	Retro [S]	98	NR	[51.5]	Med	46 (46.9)	2 (2)	0.5	PRN	5.5
Specks 2013 [789]	US	Retro [S]	99	NR	54.0	Med	54 (54.5)	2 (2)	0.5	PRN	5.5
Nakaya 2013 [790]	Japan	Retro [S]	64	2000 -2010	69.0	Med	25 (39.1)	22 (34.4)	3.3	PRL	6.0
Moosig 2013 [631]	Germany	Retro [S]	150	1990 -2009	49.1	Med	74 (49.3)	12 (8)	7.7	PRL	53.0
Lai 2014 [791]	China	Retro [S]	398	1997 -2011	[66]	Med	205 (51.5)	135 (33.9)	[2.2]	PRN	NR
Andreiana 2015 [630]	Romania	Retro [S]	75	2000 -2014	[60]	Med	39 (52)	24 (32)	[3.2]	PRL	38.4
Yamagata 2016 [792]	Japan	Retro [S]	150	2002 -2012	70.0	Med	89 (59.3)	32 (21.3)	[2.0]	PRL	NR
Fukui 2016 [793]	Japan	Retro [S]	81	2000 -2015	71.0	Med	47 (58.0)	9 (11.1)	NR	PRL	NR
Haris 2017 [794]	Hungary	Retro [S]	101	1998 -2013	61.4	Med	61 (60.4)	60 (59.4)	2.6	PRL	NR
Pu 2017 [795]	China	Retro [S]	123	2004 -2012	61.9	Med	59 (48)	46 (37.4)	1.4	PRN	NR
Abe 2017 [796]	Japan	Retro [S]	52	2002 -2014	73.2	Med	28 (53.8)	27 (51.9)	2.1	PRL	NR
Judge 2017 [797]	UK	Retro [S]	232	1990 -2011	[64]	Med	86 (37.1)	74 (31.9)	[1.0]	PRN	NR
Solans-Laqué 2017 [798]	Spain	Retro [C]	450	1990 -2014	[54.2]	Med	223 (49.6)	129 (28.7)	[82]	PRL	NR
Shobha 2018 [799]	India	Retro [S]	60	2002 -2012	44.0	Med	25 (41.7)	11 (18.3)	4.7	PRL	NR
Vasculitis group: Anti - glomerular basement membrane disease											

Huart 2016 [800]	France	Retro [C]	122	1983 -2006	[31.0]	Registry	45 (36.9)	16 (13.1)	1.0	PRL	12.0
Vasculitis group: Central Nervous System Vasculitis											
Salvaran 2015 [801]	US	Retro [S]	159	1983 -2011	[48.0]	Med	91 (57.2)	15 (9.4)	[1.0]	PRN	[9]
Vasculitis group: Medium and small vessel vasculitis											
Bourgarit 2005 [802]	France	Retro [S]	595	1953 -1999	52.2	Med	243 (40.8)	145 (24.4)	6.4	PRL	NR
Mathew 2007 [803]	UK	Retro [S]	106	1976 -2004	[58.7]	Med	NR	16 (15.1)	NR	PRL	NR
Alibaz-Oner 2017 [804]	US	Retro [S]	89	1980 -2014	[51.1]	Med	46 (51.7)	7 (7.9)	6.5	PRN	NR
Connective tissue diseases: Systemic lupus erythematosus (SLE)											
Wallace 1982 [805]	US	Retro [S]	230	1950 -1980	27.0	Med	200 (87.0)	82 (35.7)	10.0	PRL	NR
Harisdangkul 1984 [806]	US	Retro [S]	79	1977 -1981	34.1	Med	71 (89.9)	16 (20.3)	3.5	PRL	NR
Hashimoto 1992 [807]	Japan	Retro [S]	141	NR	[28.7]	Med	NR	18 (12.8)	6.0	PRN	NR
Shayakul 1995 [808]	Thailand	Retro [S]	569	1984 -1993	28.0	Med	515 (90.5)	89 (15.6)	3.2	PRL	NR
Huong 1999 [809]	France	Retro [S]	180	1980 -1993	27.0	Med	147 (81.7)	24 (13.3)	9.1	other	NR
Illei 2001 [810]	US	Clinical trial [S]	82	1986 -1999	NR	Med	68 (82.9)	11 (13.4)	11.0	PRN	NR
Illei 2002 [811]	US	Retro [S]	145	1981 -1990	[29.2]	Med	NR	5 (3.4)	[10.1]	PRN	NR
Badsha 2002 [812]	Singapore	Retro [S]	55	1989 -2000	[35.2]	Med	46 (83.6)	6 (10.9)	0.5	PRL	6.0
Liang 2004 [813]	China	Retro [S]	162	1991 -2001	28.8	Med	131 (80.9)	26 (16)	NR	PRN	NR
Mok 2004 [814]	Hong Kong	Retro [S]	189	1988 -2001	31.1	Med	167 (88.4)	7 (3.7)	8.0	PRL	NR
Mikdashi 2004 [815] NPDI=0	US	Retro [S]	64	1992 -2003	37.0	Med	121 (93.1)	0(0)	7.0	PRN	NR
Mikdashi 2004 [815] NPDI≥ 1	US	Retro [S]	66	1992 -2003	37.0	Med	121 (93.1)	8 (12.1)	7.0	PRN	NR
Tang 2009 [816]	China	Retro [S]	94	1985 -2004	27.9	Med	84 (89.4)	5 (5.3)	3.2	PRN	NR
Patel 2011 [817]	US	Prospective cohort [S]	86	NR	32.0	Med	72 (83.7)	24 (27.9)	10.0	PRN	NR

Lopez 2012 [818]	UK	Retro [S]	350	1991 -NR	[36.0]	Med	322 (92)	34 (9.7)	[9]	PRN	[108]
Arends 2012 [819]	Netherlands	Observational study [S]	50	1995 -2009	NR	Med	NR	5 (10)	[9.6]	PRL	NR
Ayodele 2013 [820]	South Africa	Retro [S]	66	1995 -2009	[30.2]	Med	61 (92.4)	26 (39.4)	4.7	NR	NR
Moroni 2013 [821]	Italy	Retro [S]	89	1968 -2012	28.8	Med	84 (94.4)	6 (6.7)	[21.9]	PRL	NR
Fatemi 2013 [822]	Iran	Retro [S]	82	1994 -2010	32.3	Med	65 (79.3)	5 (6.1)	[8]	PRL	NR
Mok 2014 [810]	Hong Kong	Clinical trial [S]	74	2005 -2012	36.2	Med	70 (94.6)	0 (0)	0.5	PRN	6.0
Mok 2014 [810]	Hong Kong	Clinical trial [S]	76	2005 -2012	36.1	Med	68 (89.5)	1 (1.3)	0.5	PRL	6.0
Mahmoud 2015 (LN) [823]	Egypt	Retro [S]	135	1999 -2011	24.4	Med	129 (95.6)	17 (12.6)	4.6	PRN	NR
Koo 2016 [824]	Korea	Retro [S]	193	1980 -2008	31.2	Med	167 (86.5)	10 (5.2)	13.2	PRL	26.0
Jung 2016 [825]	South Korea	Retro [S]	230	1997 -2015	[41.8]	Med	194 (84.3)	13 (5.7)	5.5	PRL	NR
Pego-Reigosa 2016 [826]	Spain	Cross sectional study [C]	1918	2011 -2012	[35.0]	Med	NR	155 (8.1)	[8.8]	PRL	NR
Joo 2017 [827]	Korea	Prospective cohort [S]	1120	1998 -2012	[27.4]	Med	1031 (92.1)	53 (4.7)	NR	PRL	NR
Sheane 2017 [828]	Canada	Retro [S]	173	1970 -2015	33.8	Med	147 (85)	23 (13.3)	15.1	PRL	96.0
Mahmoud 2018 [829]	Eygypt	Retro [S]	770	2002 -2015	[22.1]	Med	707 (91.8)	33 (4.3)	6.1	NR	NR
Wei 2011 [830] *SLE with pregnancy	China	Retro [S]	86	2005 -2010	28.2	Med	86 (100)	0 (0)	[0.7]	PRN	8.7
Yang 2014 [831] *SLE with pregnancy	China	Retro [S]	82	1992 -2012	NR	Med	NR	5 (6.1)	1.3	PRN	9.0
Connective tissue diseases: Bullous diseases											
Krain 1974 [832]	US	Retro [S]	59	1955 -1973	[64.5]	Med	23 (39.0)	13 (22)	5.0	PRL	36.0
Rosenberg 1976 [833]	US	Retro [S]	107	1955 -1970	NR	Med	53 (49.5)	48 (44.9)	NR	PRL	3.0

Joly 2002 [834]	France	Clinical trial [S]	171	NR	[81]	Med	111 (64.9)	62 (36.3)	1.0	NR	12.0
Seo 2003 [835]	Korea	Retro [S]	51	1993 -2001	46.8	Med	29 (56.9)	1 (2.0)	2.0	PRL	32.1
Shahidi-Dadras 2007 [836]	Iran	Clinical trial [S]	51	1997 -2003	46.9	Med	25 (49.0)	1 (2)	1.4	PRL	12.0
Shahidi-Dadras 2007 [836]	Iran	Clinical trial [S]	72	1997 -2003	42.6	Med	36 (50.0)	0 (0)	1.0	PRN	12.0
Mimouni 2010 [837]	Israel	Retro [S]	155	1976 -2004	53.5	Med	94 (60.6)	16 (10.3)	NR	PRN	72.0
Kim 2011 [838]	Korea	Retro [S]	199	1993 -2008	[46.1]	Med	102 (51.3)	13 (6.5)	3.9	PRL	NR
Zhang 2013 [839]	China	Retro [S]	80	2005 -2010	[71.0]	Med	41 (51.3)	35 (43.8)	2.7	PRN	NR
Cai 2014 [839]	Singapore	Retro [S]	316	2004 -2009	[75.7]	Med	NR	139 (44)	3.0	PRL	NR
Bai 2016 [840]	China	Retro [S]	68	2008 -NR	[52.7]	Med	NR	6 (8.8)	3.1	PRL	NR
Kalinska-Bienias 2017 [841]	Poland	Retro [S]	65	2000 -2013	[76.2]	Med	NR	21 (32.3)	2.3	PRN	NR
Williams 2017 [842]	UK Germany	Clinical trial [S]	121	NR	77.2	Med	57 (47.1)	25 (20.7)	[1.0]	PRL	1.5
Connective tissue diseases: Dermatopolymyositis											
Henriksson 1982 [843]	Sweden	Retro [S]	79	1967 -NR	52.2	Med	45 (57.0)	25 (31.6)	5.2	PRL	27 [19]
Agarwal 2006 [844]	US	Retro [S]	53	1991 -2002	48.8	Med	38 (71.7)	7 (13.2)	5.3	PRN & other	NR
Naji 2010 [845]	Iran	Retro [S]	65	-NR	34.5	Med	44 (67.7)	3 (4.6)	NR	PRL	7.9
Uchino 2012 [846]	Japan	Retro [S]	115	1970 -2009	55.5	Med	82 (71.3)	19 (16.5)	14.3	PRL	NR
Schiopu 2012 [847]	US	Retro [S]	160	1997 -2003	48.4	Med	116 (72.5)	27 (16.9)	[4.6]	NR	NR
Taborda 2014 [848]	UK	Retro [S]	90	1976 -2007	[38.5]	Med	64 (71.1)	13 (14.4)	[11.5]	PRN	NR
Johnson 2015 [849]	US	Retro [S]	100	1990 -2011	[50.1]	Med	65 (65.0)	6 (6.0)	3.0	PRN	36.0
Galindo-Feria 2016 [850]	Mexico	Retro [S]	69	1985 -2012	[46]	Med	51 (73.9)	6 (8.7)	[5.8]	PRL	NR
Galindo-Feria 2016 [850]	Mexico	Retro [S]	264	1985 -2012	[40]	Med	201 (76.1)	48 (18.2)	[2.9]	PRL	NR
Nuño-Nuño 2017 [851]	Spain	Retro [S]	467	1980 -2014	[41.1]	Med	348 (74.5)	113 (24.2)	[9.7]	PRL	76.9

Connective tissue diseases: Glomerulonephritis											
Rose 1971 [852]	UK	Clinical trial [S]	56	1967 -1970	[34.2]	Med	NR	6 (10.7)	0.5	PRN	6.0
Connective tissue diseases: IgA nehropathy											
Goumenosl 1995 [853]	UK	Retro [S]	66	NR	[40]	Med	20 (30.3)	3 (4.5)	[3.8]	PRL	24.0
Connective tissue diseases: IgM nehropathy											
Kuthong 2000 [854]	Thailand	Retro [S]	72	1978 -1996	24.5	Med	35 (48.6)	1 (1.4)	5.0	PRL	NR
Connective tissue diseases: Nephrotic syndrome											
Nolasco 1986 [855]	UK	Retro [S]	79	1963 -1982	[42]	Med	39 (49.4)	13 (16.5)	7.6	PRL	NR
du Buf-Vereijken 2004 [856]	Netherlands	Retro [S]	56	1991 -2002	50.0	Med	10 (15.4)	5 (8.9)	4.3	PRN	NR
Funabik 1992 [857]	Japan	Retro [S]	65	-NR	[42]	Med	NR	8 (12.3)	8.0	PRL	NR
Shin 2012 [858]	Korean	Retro [S]	122	1990 -2009	49.5	Med	50 (41.0)	2 (1.6)	5.2	PRL	NR
Connective tissue diseases: Idiopathic pulmonary fibrosis											
Tukiainen 1983 [859]	Finland	Prospective cohort [S]	100	1967 -1979	53.0	Med	51 (51.0)	44 (44)	6.8	PRL	34.8
Park 2009 [860]	Korea	Retro [S]	83	1991 -2006	[54.4]	Med	56 (67.5)	24 (28.9)	[4.4]	PRL	17.4
Connective tissue diseases: Myasthenia gravis											
Evol 2000 [861]	Italy	Retro [S]	113	1978 -1998	[68.5]	Med	NR	9 (8)	NR	PRN	54.6
Connective tissue diseases: Sarcoidosis											
Johns 1974 [862]	US	Retro [S]	152	1962 -1972	30.2	Med	112 (73.7)	15 (9)	4.0	PRN	41.6
Kandolin 2015 [863]	Finland	Retro [S]	62	2010 -2014	48.6	Med	48 (77.4)	7 (11.3)	[1.4]	PRN	12.0
Connective tissue diseases: Autoimmune thrombocytopenia											
Jacobs 1986 [864]	South Africa	Retro [S]	134	1971 -1981	NR	Med	NR	2 (1.5)	3.0	PRN	NR
Portielje 2001 [865]	Netherlands	Retro [S]	99	1974 -1994	[41]	Med	96 (97.0)	24 (24.2)	10.5	PRN	24.0
Sailer 2003 [866]	Austria	Retro [S]	113	1991 -2001	[[49.8]	Med	55.4 (49.0)	3 (2.7)	[4.4]	PRL	NR
Inflammatory diseases: Inflammatory bowel disease											

Bruewer 2003 [867]	Germany	Retro [S]	73	1982 -2000	[32.9]	Med	33 (45.2)	2 (2.7)	[0.4]	PRL	NR
Bruewer 2003 [867]	Germany	Retro [S]	146	1982 -2000	[34]	Med	75 (51.4)	0 (0)	[0.1]	PRL	NR
Longo 2003 [868]	US	Retro [C]	158	1997 -2001	59.0	Med	2 (1.3)	7 (4.4)	0.1	PRL	78.0
Inflammatory diseases: Polymyalgia rheumatica											
Gonzalez-Gay 1999 [869]	Spain	Retro [S]	134	1987 -1998	70.5	Med	85 (63.4)	12 (9)	NR	PRN	20.2
Gran 2001 [774]	Norway	Retro [S]	274	1987 -1997	NR	Med	181 (66.1)	56 (20.4)	5.3	PRL	NR
Inflammatory diseases: Rheumatoid arthritis											
McDougall 1994 [870]	Canada	Case control study [S]	122	1966 -1993	41.7	Med	85 (69.7)	52 (42.6)	18.1	PRN	82.8
Bakker 2012 [871]	Netherlands	Prospective cohort [S]	117	NR	54.0	Med	70 (59.8)	1 (0.9)	[2.1]	PRN	24.0
Ajeganova 2014 [872]	Sweden	Clinical trial [S]	112	1995 -2009	50.6	Registry	77 (68.8)	10 (8.9)	10.0	PRL	120.0
Listing 2015 [616]	Germany	Prospective cohort [S]	6155	2001 -2011	[55.8]	Registry	5113 (83.1)	198 (3.2)	3.5	PRL	42.0
Chester 2016 [873]	US	Prospective cohort [S]	3496	1981 -2006	56.9	Med	2662 (76.1)	1357 (38.8)	[5]	PRN	42.8 [24]
Roubille 2017 [874]	France	Retro [S]	386	2002 -2013	47.5	Med	200 (51.8)	7 (1.8)	NR	PRL	33.0
Kim 2018 [875]	Korea	Retro [S]	2812	2000 -2016	[51.5]	Med	2330 (82.9)	89 (3.2)	7.8	PRL	NR
Wilson 2019 [629]	UK	Retro [C]	13770	1995 -2015	56.2	Med	NR	2074 (15.1)	8.1	PRN	{9.5]
Inflammatory diseases: Still's disease											
Kim 2012 [876]	Korea	Retro [S]	54	1996 -2008	[37.3]	Med	39 (72.2)	5 (9.3)	2.2	PRL	NR
Ruscitti 2016 [877]	Italy	Retro [S]	100	2000 -2015	45.4	Med	66 (66)	10 (10)	3.5	PRN	NR
Haematologic diseases: Aplastic anaemia											
Gluckman 1992 [878]	france Belgium Switzerlands	Clinical trial [S]	56	NR	NR	Med	NR	19 (33.9)	[1.7]	PRL	2.0
Haematologic diseases: Evan's syndrome											
Michel 2009 [879]	France	Prospective cohort [S]	68	2005 -NR	56.4	Med	41 (61.3)	16 (23.5)	4.8	PRL	NR

Respiratory tract diseases: Asthma											
Maunsell 1968 [880]	UK	Retro [S]	170	1952 -1962	NR	Med	101 (59.4)	10 (5.9)	NR	PRN	60.3
Walsh 1966 [601]	UK	Retro [S]	245	1953 -1965	[48.0]	Med	152 (62.0)	16 (6.5)	NR	PRL	12.0
Respiratory tract diseases: COPD											
Niewoehner 1999 [881]	US	Clinical trial [S]	80	1994 -1996	68.1	Med	3 (3.8)	2 (2.5)	0.3	PRN	2.0

Appendix 4- 2. Number of chronic GC use patients reported across the world

Country	Sample size
Europe	
UK	20404
Germany	6969
Spain	3072
France	2247
Sweden	417
Netherlands	416
Norway	394
Italy	302
Finland	162
UK and Germany (in 1 study)	121
Austria	113
Hungary	101
Romania	75
Poland	65
Prague and Czech Republic	61
France, Belgium and Switzerlands	56
Denmark	50
America	
US	6061
Mexico	333
Canada	295
Asia	
Korea	4958
China	1093
Eygypt	770
Japan	668
Thailand	641
Singapore	371
Hong Kong	339
Iran	270
India	60
Africa	
South Africa	200
Israel	155
Egypt	135

Appendix 4- 3. Prednisolone equivalent dose

The following figures were used to convert glucocorticoid doses into prednisolone equivalent doses. In the case of budesonide, an estimate of the potential to suppress plasma cortisol levels was used as this was deemed more relevant than the therapeutic equivalence.

5mg Prednisolone is equivalent to1-3:

Prednisone 5mg

Hydrocortisone 20mg

Dexamethasone 750µg

Methylprednisolone 4mg

Triamcinolone 4mg

Fluocortolone 5mg

Paramethasone 2mg

Budesonide 7.25 mg

Appendix 4- 4. Risk of bias summary of exogenous CS articles: review authors' judgements about each risk of bias item for each included study.

1st Author, Year	Risk of bias assessment							
	Confounding	selectionion	Intervention classification	Intervention deviation	Missing data	Outcome	Report	Overall
Rose,1971	Serious	Serious	Low	Low	Low	Low	Low	Serious
Krain,1974	Serious	NI	Low	Low	Low	Low	Low	NI
Johns,1974	Low	Serious	Low	Low	Low	Low	Low	Serious
Rosenberg,1976	Serious	Low	Low	Low	Low	Low	Low	Serious
Bengtsson,1981	Low	Low	Low	Moderate	Low	Low	Low	Moderate
Henrikss,1982	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Wallace,1982	Serious	Serious	Moderate	Low	Low	Low	Low	Serious
Tukiainen,1983	Low	NI	Moderate	Low	Low	Low	Low	NI
Harisdangkul,1984	Serious	Serious	Moderate	Low	Low	Low	Low	Serious
Nolasco,1986	Serious	Low	Low	Low	Low	Low	Low	Serious
Hoffman,1992	Serious	Low	Low	Low	Low	Low	Low	Serious
Hashimoto,1992	Serious	Serious	Serious	Low	Low	Low	Low	Serious
Funabiki,1992	Serious	Low	Low	Low	Low	Low	Low	Serious
McDougall,1994	Serious	Serious	Low	Low	Low	Low	Low	Serious
Shayakul,1995	Serious	Low	Moderate	Low	Low	Low	Low	Serious
Goumenosl,1995	Serious	Serious	Moderate	Low	Low	Low	Low	Serious
Huong,1999	Serious	Serious	Low	Low	Moderate	Low	Low	Serious
Gonzalez-Gay,1999	Low	Low	Low	Low	Low	Low	Low	Low
Kurathong,2000	Low	Moderate	Low	Low	Low	Low	Low	Moderate
Chevalet,2000	Low	NI	Low	Serious	NI	Low	Low	NI
Evoli,2000	Serious	Serious	Low	Low	Low	Low	Low	Serious
Hachulla,2001	Low	Low	Low	Low	Low	Low	Low	Low
Gran,2001	Low	Low	Low	Low	Low	Low	Low	Low
Gran,2001	Low	Low	Low	Low	Low	Low	Low	Low
Gabor,2001	Serious	Serious	Low	Low	Low	Low	Low	Serious
Uddhammar,2002	Serious	Low	Low	Low	Low	Low	Low	Serious
Koldingsnes,2002	Moderate	Low	Low	Low	Moderate	Low	Low	Moderate
Joly,2002	Serious	Serious	Low	Low	Low	Low	Low	Serious
Badsha,2002	Serious	Moderate	Low	Low	Low	Low	Low	Serious
Illei,2002	Serious	Serious	Low	Low	Low	Low	Low	Serious
Bruewer,2003	Serious	Serious	Low	Low	Low	Low	Low	Serious
Slot,2003	Serious	Serious	Low	Low	Low	Low	Low	Serious
Longo,2003	Serious	Serious	Low	Moderate	Low	Low	Low	Serious
Bruewer,2003	Serious	Serious	Low	Low	Low	Low	Low	Serious
Seo,2003	Serious	Low	Low	Low	Low	Low	Low	Serious
Mok,2004	Serious	Serious	Low	Low	Low	Low	Low	Serious
Liang,2004	Serious	Serious	Low	Low	Serious	Low	Low	Serious

du Buf-Vereijken,2004	Serious	Serious	Low	Low	Low	Low	Low	Serious
Mikdashi,2004	Serious	Moderate	Moderate	Low	Low	Low	Low	Serious
Rihova,2005	Serious	Serious	Low	NI	Low	Low	Low	NI
Bourgarit,2005	Serious	Low	Low	Low	Low	Low	Low	Serious
Park,2005	Serious	Moderate	Low	Low	Low	Low	Low	Serious
Harper,2005	Serious	Low	Low	Low	NI	Low	Low	NI
Agarwal,2006	Serious	NI	Low	Low	NI	Low	Low	NI
Mathew,2007	Serious	Low	Low	Low	NI	Low	Low	NI
Shahidi-Dadras,2007	Serious	Low	Low	Low	Low	Low	Low	Serious
Shahidi-Dadras,2007	Serious	Low	Low	Low	Low	Low	Low	Serious
Park,2009	Serious	Low	Low	Low	Low	Low	Low	Serious
Tang,2009	Serious	Serious	Low	Low	Low	Low	Low	Serious
Naji,2010	Serious	Low	Low	Low	Low	Low	Low	Serious
Mimouni,2010	Moderate	Low	Moderate	Low	Low	Low	Low	Moderate
Holle,2011	Serious	Low	Low	Low	Low	Low	Low	Serious
Wei,2011	Low	Serious	Low	Low	Low	Low	Low	Serious
Patel,2011	Serious	Serious	Low	Low	Low	Low	Low	Serious
Holle,2011	Serious	Low	Low	Low	Low	Low	Low	Serious
Kim,2011	Serious	Low	Low	Low	Low	Low	Low	Serious
Holle,2011	Serious	Low	Low	Low	Low	Low	Low	Serious
McGregor,2012	Serious	Low	Low	Low	Low	Low	Low	Serious
Bakker,2012	Moderate	Moderate	Low	Low	Low	Low	Low	Moderate
Arends,2012	Serious	Serious	Low	Low	NI	Low	Low	NI
Uchino,2012	Serious	Serious	Low	Low	Low	Low	Low	Serious
Lopez,2012	Serious	Moderate	Low	Low	Low	Low	Low	Serious
Kim,2012	Serious	Low	Low	Low	Low	Low	Low	Serious
Shin,2012	Moderate	Serious	Low	Low	Low	Low	Low	Serious
Gregersen,2012	Serious	Serious	Low	Low	Low	Low	Low	Serious
Schiopu,2012	Moderate	Low	Serious	Low	Low	Low	Low	Serious
Moroni,2013	Serious	Moderate	Low	Low	Low	Low	Low	Serious
Fatemi,2013	Serious	Serious	Low	Low	Low	Low	Low	Serious
Zhang,2013	Serious	Serious	Low	Low	Low	Low	Low	Serious
Nakaya,2013	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Specks,2013	Serious	Serious	Low	Serious	Low	Low	Low	Serious
Specks,2013	Serious	Serious	Low	Serious	Low	Low	Low	Serious
Olugbenga,2013	Serious	Serious	Low	Low	Low	Low	Low	Serious
Moosig,2013	Serious	Serious	Low	Low	NI	Low	Low	NI
Cai,2014	Moderate	Moderate	Low	Low	Low	Low	Low	Moderate
Mok,2014	Serious	Serious	Low	Low	Low	Low	Low	Serious
Mok,2014	Serious	Serious	Low	Low	Low	Low	Low	Serious
Yang,2014	Serious	Serious	Low	Low	Low	Low	Low	Serious
Taborda,2014	Serious	Low	Moderate	Low	Moderate	Low	Low	Serious

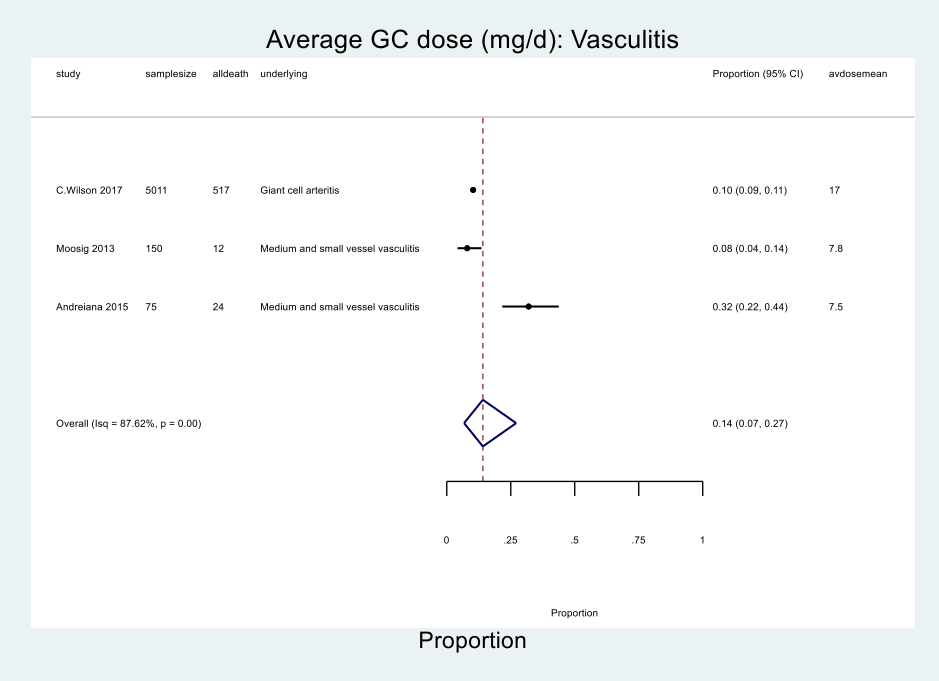
Lai,2014	Serious	Serious	Low	Low	Low	Low	Low	Serious
Ajeganova,2014	Serious	Low	Low	Low	Low	Low	Low	Serious
Les,2015	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Salvarani,2015	Serious	Low	Moderate	Low	Low	Low	Low	Serious
Johnson,2015	Moderate	Moderate	Low	Low	Low	Low	Low	Moderate
Listing,2015	Moderate	Serious	Low	Moderate	NI	Low	Low	NI
Kandolin,2015	Serious	Serious	Moderate	Low	Low	Low	Low	Serious
Andreiana,2015	Serious	NI	Low	Low	Low	Low	Low	NI
Mahmoud,2015	Serious	Serious	Low	Low	Low	Low	Low	Serious
Les,2015	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Galindo-Feria,2016	Serious	Low	Moderate	Low	Low	Low	Low	Serious
Yamagata,2016	Serious	Low	Low	Low	Low	Low	Low	Serious
Ruscitti,2016	Serious	Low	Low	Low	Low	Low	Low	Serious
Bai,2016	Serious	Moderate	Moderate	Low	Low	Low	Low	Serious
Huart,2016	Serious	Low	Low	Low	Low	Low	Low	Serious
Pego-Reigosa,2016	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Koo,2016	Serious	Serious	Low	Low	Low	Low	Low	Serious
Jung,2016	Serious	Serious	Moderate	Low	Low	Low	Low	Serious
Labarca,2016	Serious	Serious	Low	Low	Low	Low	Low	Serious
Galindo-Feria,2016	Serious	Low	Moderate	Low	Low	Low	Low	Serious
Fukui,2016	Serious	Moderate	Moderate	Low	Low	Low	Low	Serious
Wasko,2016	Moderate	Low	Moderate	Low	Low	Low	Low	Moderate
Williams,2017	Low	Low	Low	Low	Serious	Low	Low	Serious
Abe,2017	Serious	Serious	Low	Low	Low	Low	Low	Serious
Nuño-Nuño,2017	Moderate	Low	Moderate	Low	Low	Low	Low	Moderate
Sheane,2017	Low	Low	Low	Low	NI	Low	Low	NI
Wilson,2017	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Haris,2017	Serious	Low	Low	Low	Low	Low	Low	Serious
Pu,2017	Serious	Serious	Low	Low	Low	Low	Low	Serious
Solans-Laqué,2017	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Ly,2017	Serious	Low	Low	Low	Low	Low	Low	Serious
Roubille,2017	Serious	Low	Low	Low	Low	Low	Low	Serious
Alibaz-oner,2017	Serious	Moderate	Moderate	Low	Serious	Low	Low	Serious
Joo,2017	Moderate	Serious	Moderate	Low	Low	Low	Low	Serious
Judge,2017	*Serious*	*Serious*	*Low*	*Low*	*Low*	*Low*	*Low*	*Serious*
Kalinska-Bienias,2017	*Serious*	*Low*	*Low*	*Low*	*Low*	*Low*	*Low*	*Serious*
Shobha,2018	*Serious*	*Low*	*Low*	*Low*	*Moderate*	*Low*	*Low*	*Serious*
Mahmoud,2018	*Serious*	*Low*	*Moderate*	*Low*	*NI*	*Low*	*Low*	*NI*
Kim,2018	*Moderate*	*Moderate*	*Moderate*	*NI*	*Low*	*Low*	*Low*	*NI*
Wilson,2019	*Moderate*	*Moderate*	*Moderate*	*Low*	*Low*	*Low*	*Low*	*Moderate*

Appendix 4- 5. Articles reported standardised mortality ratio

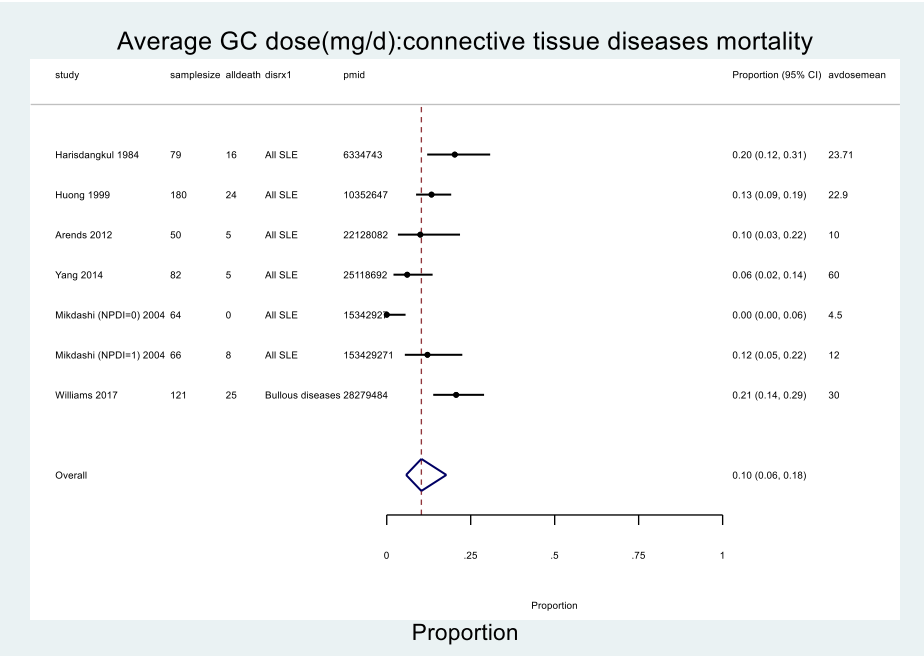
1st Author	Disease	No. of patients	No. of deaths	SMR (CI)	Duration of follow-up in years Median [mean]	GC measure reported and dose	Mean duration of GC use in months
Moosig (2013)	Medium to small vessel vasculitis	150	12	1.3 (0.7-2.1)	[7.6]	Last followed-up dose 8.3 mg/d	53 months
Julia U. Holle (2011)	Medium to small vessel vasculitis	155	22	2.1 (1.3-3.3)	6.6	Maintenance dose 7 mg/d	NR
Zuzana Rihova (2005)	Medium to small vessel vasculitis	61	19	2.7 (1.5-3.8)	NR	At start of enrolment 60 mg/d	NR
Julia U. Holle (2011)	Medium to small vessel vasculitis	123	13	1.4 (0.8-2.4)	7.3	Maintenance dose 5 mg/d	NR
Julia U. Holle (2011)	Medium to small vessel vasculitis	167	8	1.0 (0.4-2.03)	3.9	Maintenance dose 5 mg/d	NR
Joo (2017)	SLE	1120	53	3.4 (2.5-4.4)	NR	Cumulative dose 27 g	NR
Johanna (2001)	ATP	99	24	1.5 (1.1-2.2)	9.4 [10.5]	NR	24 months

Appendix 4- 6. Average mean dose and proportion of death

Vasculitis

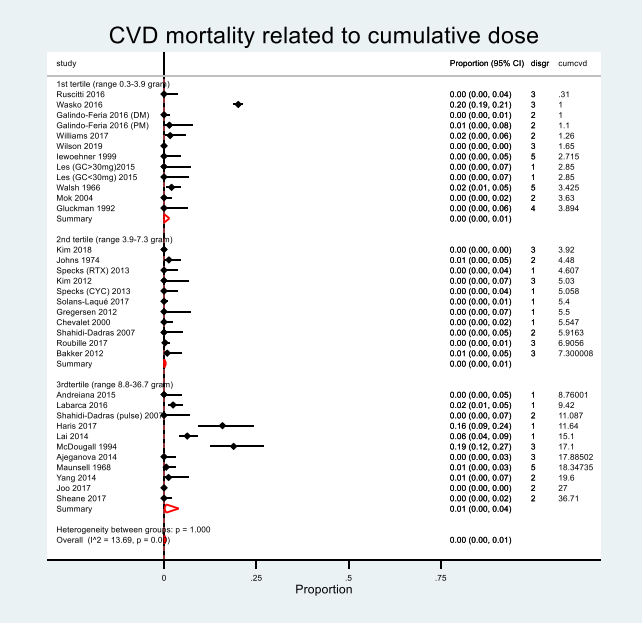


Connective tissue diseases

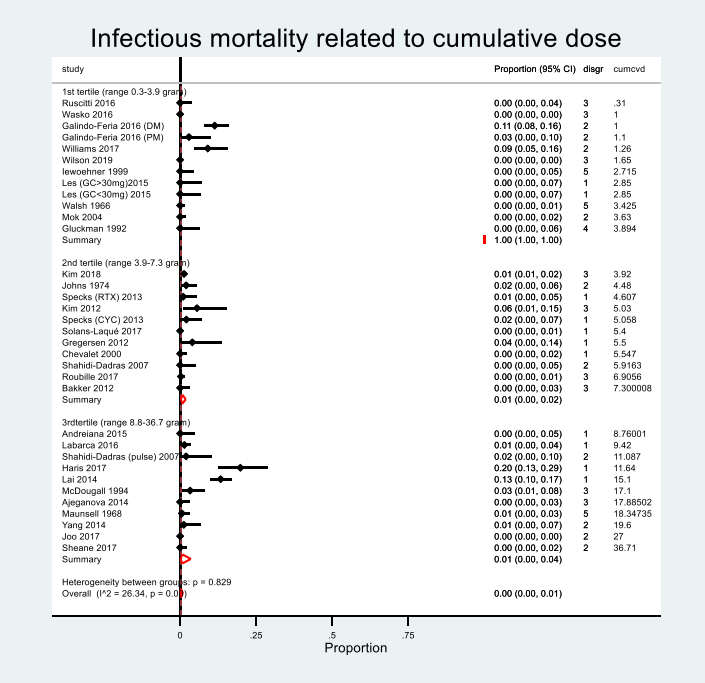


Appendix 4- 7. Analysis information for causes of deaths and GC dose records

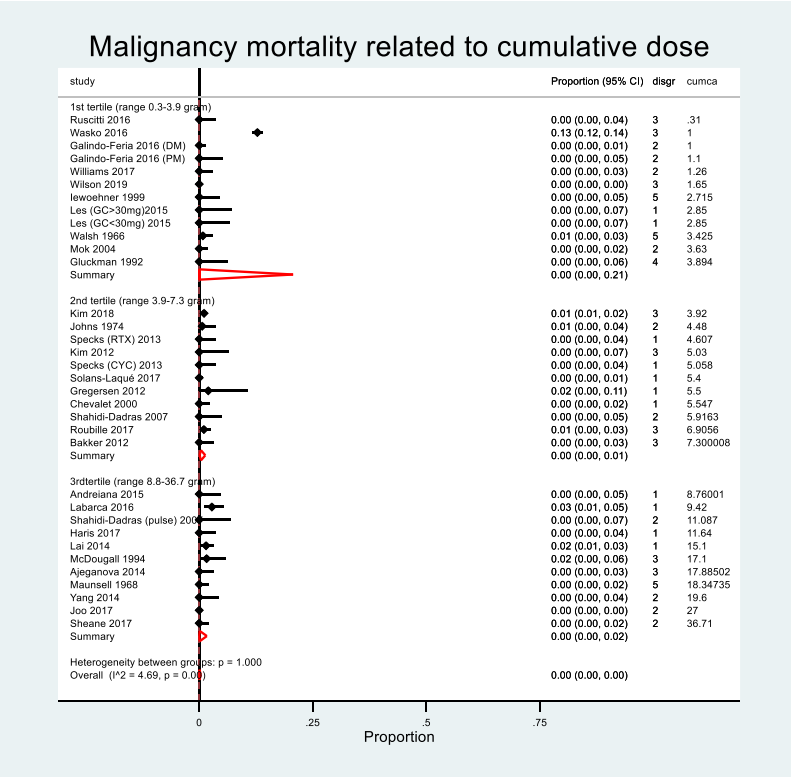
Cumulative dose and cardiovascular mortality



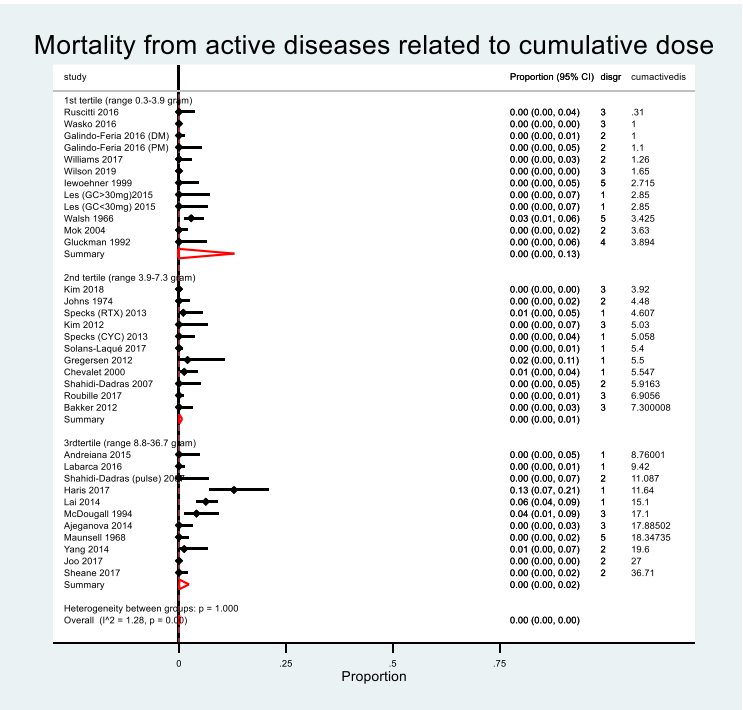
Cumulative dose and infectious mortality



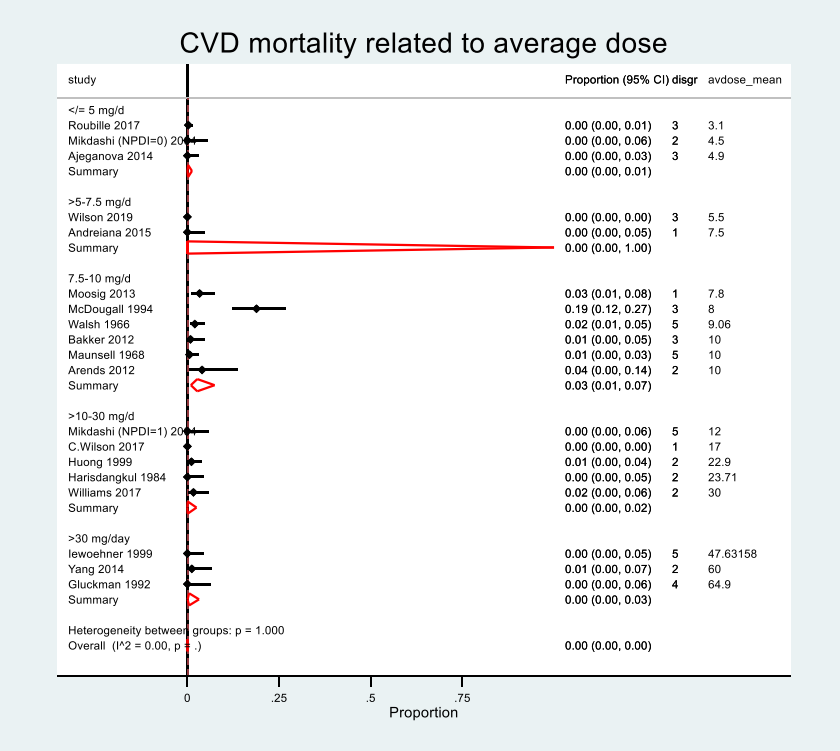
Cumulative dose and malignancy



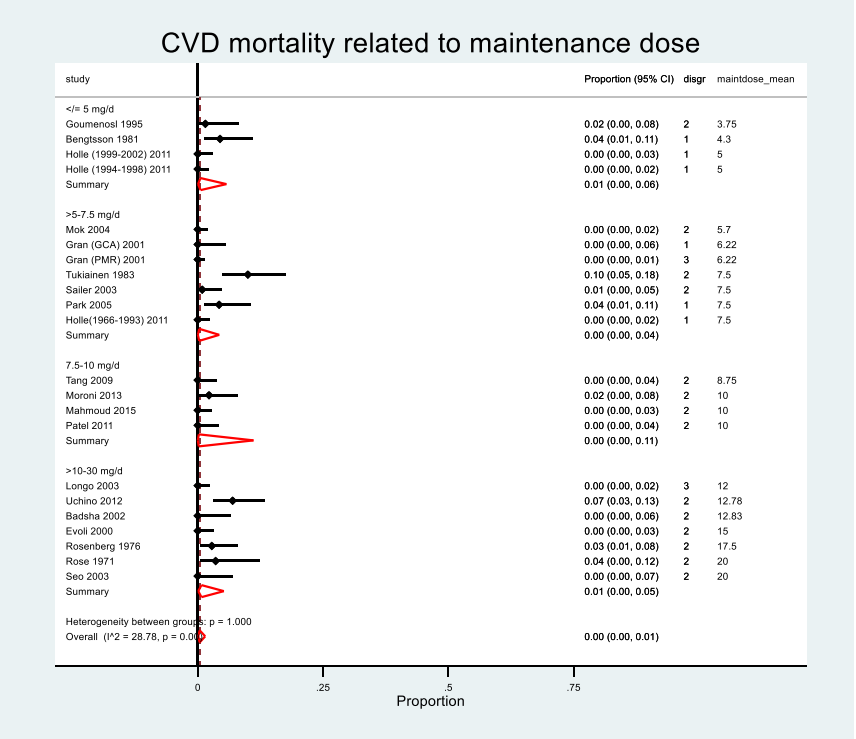
Active underlying diseases



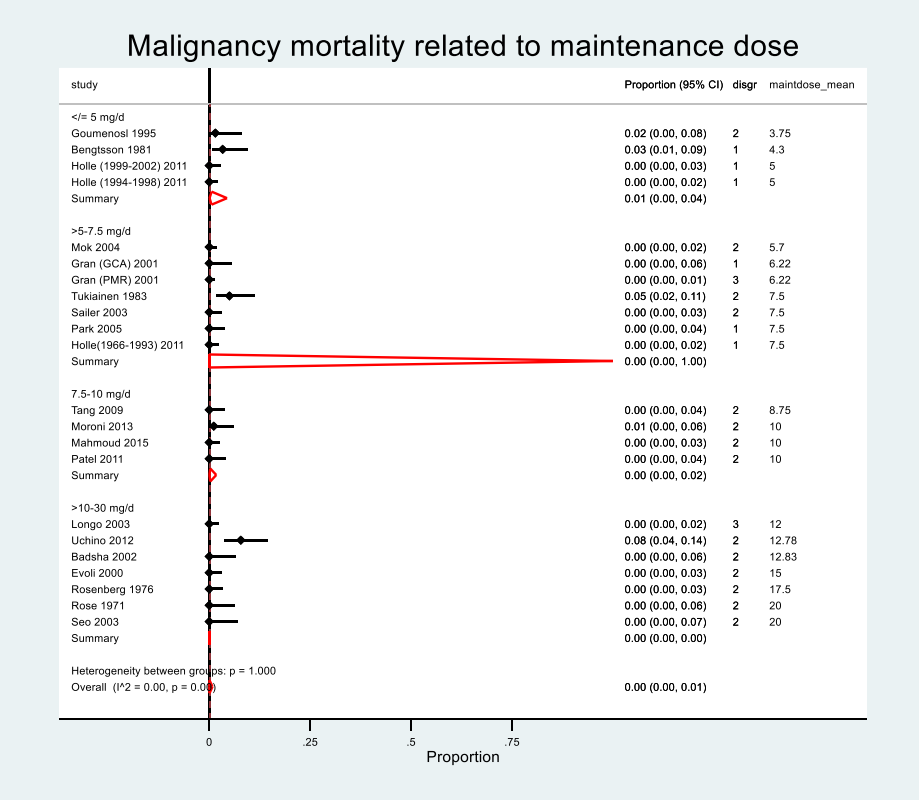
Average GC dose (mg/d) and cardiovascular deaths



Maintenance doses and cardiovascular deaths



Maintenance doses and malignancy deaths



Appendix 5- 1. Mean ΔCt values of gene expression

mRNA gene expression	Treatment =V				Treatment =IL1				Treatment =IL1 + E				Treatment =IL1 + E			
	Mean	Std. Err.	95% Conf.		Mean	Std. Err.	95% Conf.		Mean	Std. Err.	95% Conf.		Mean	Std. Err.	95% Conf.	
BHSD deltaCT value																
24 hr Normoxia	22.30	0.83	20.57	24.02	17.62	0.99	15.54	19.70	17.52	1.00	15.44	19.60	17.70	0.86	15.90	19.50
24 hr Hypoxia	21.83	1.05	19.62	24.03	17.12	0.75	15.55	18.70	17.07	0.91	15.16	18.98	17.23	0.77	15.63	18.84
96 hr Normoxia	20.63	0.82	18.90	22.35	14.53	0.48	13.53	15.54	14.37	0.43	13.48	15.27	14.39	0.49	13.37	15.41
96 hr Hypoxia	20.58	0.59	19.34	21.81	14.49	0.37	13.72	15.26	13.69	0.36	12.93	14.45	14.48	0.37	13.71	15.25
H6PD deltaCT value																
24 hr Normoxia	18.29	0.73	16.76	19.82	18.44	0.67	17.03	19.85	18.33	0.81	16.64	20.02	18.25	0.76	16.66	19.83
24 hr Hypoxia	18.20	0.97	16.16	20.23	18.21	0.76	16.63	19.80	18.20	0.78	16.57	19.84	18.25	0.77	16.64	19.87
96 hr Normoxia	17.60	0.88	15.76	19.44	17.54	0.79	15.88	19.19	17.46	0.90	15.58	19.33	17.07	0.81	15.38	18.77
96 hr Hypoxia	17.35	0.85	15.57	19.12	17.34	0.88	15.50	19.17	17.07	0.82	15.35	18.78	17.26	0.77	15.65	18.87
GR deltaCT value																
24 hr Normoxia	13.33	0.33	12.64	14.03	13.64	0.25	13.12	14.15	13.52	0.31	12.88	14.16	13.56	0.29	12.95	14.18
24 hr Hypoxia	12.93	0.32	12.26	13.61	13.41	0.17	13.05	13.77	13.54	0.12	13.28	13.79	13.73	0.19	13.32	14.13
96 hr Normoxia	13.25	0.36	12.49	14.01	13.45	0.22	12.99	13.90	13.81	0.22	13.34	14.27	13.55	0.20	13.13	13.98
96 hr Hypoxia	12.82	0.40	11.98	13.66	13.21	0.32	12.53	13.88	13.70	0.40	12.87	14.53	13.42	0.31	12.78	14.07
PTGS2 deltaCT value																
24 hr Normoxia	19.37	0.78	17.74	21.01	14.18	0.95	12.19	16.17	14.39	1.00	12.29	16.50	14.13	0.95	12.15	16.11
24 hr Hypoxia	17.54	1.21	15.02	20.07	12.19	1.01	10.08	14.29	12.75	0.88	10.90	14.60	12.41	0.98	10.36	14.45
96 hr Normoxia	18.51	0.96	16.50	20.52	12.07	0.97	10.04	14.10	14.04	1.12	11.69	16.38	12.24	1.00	10.15	14.34
96 hr Hypoxia	16.58	0.96	14.58	18.58	11.30	1.22	8.76	13.85	13.88	1.37	11.00	16.76	11.53	1.15	9.13	13.93
VEGFA deltaCT value																
24 hr Normoxia	14.42	0.44	13.50	15.35	12.65	0.66	11.28	14.02	12.46	0.75	10.88	14.04	12.35	0.67	10.95	13.76
24 hr Hypoxia	12.76	0.99	10.69	14.83	11.52	1.05	9.32	13.71	11.60	0.99	9.53	13.67	11.63	1.03	9.47	13.78
96 hr Normoxia	14.10	0.57	12.91	15.29	11.75	0.43	10.85	12.65	12.09	0.58	10.88	13.30	11.61	0.55	10.45	12.76
96 hr Hypoxia	12.34	0.73	10.81	13.86	10.95	0.88	9.10	12.79	11.82	0.84	10.07	13.57	11.04	0.88	9.20	12.87

Appendix 5- 2. Statistic test for 11β-HSD1 gene expression

11BHSD1 n=5	Mean diff	95% CI of diff	**P value**
Treatment			
V			
24h normoxia vs. 24h hypoxia	0.5	-0.3, 1.3	**0.3502**
24h normoxia vs. 96h normoxia	1.7	0.9, 2.45	**<0.001**
24h normoxia vs. 96h hypoxia	1.7	0.9, 2.5	**0.0006**
24h hypoxia vs. 96h normoxia	1.2	0.4, 2.0	**0.0167**
24h hypoxia vs. 96h hypoxia	1.3	0.5, 2.0	**0.0127**
96h normoxia vs. 96h hypoxia	0.0	-0.7, 0.8	**0.9214**
IL1			
24h normoxia vs. 24h hypoxia	0.5	-0.3, 1.3	**0.3203**
24h normoxia vs. 96h normoxia	3.1	2.3, 3.9	**<0.001**
24h normoxia vs. 96h hypoxia	3.1	2.3, 3.9	**<0.001**
24h hypoxia vs. 96h normoxia	2.6	1.8, 3.4	**<0.001**
24h hypoxia vs. 96h hypoxia	2.6	1.8, 3.4	**<0.001**
96h normoxia vs. 96h hypoxia	0.0	-0.7, 0.8	**0.9312**
IL1 + E			
24h normoxia vs. 24h hypoxia	0.4	-0.3, 1.2	**0.3723**
24h normoxia vs. 96h normoxia	3.1	2.4, 3.9	**<0.001**
24h normoxia vs. 96h hypoxia	3.8	3.0, 4.6	**<0.001**
24h hypoxia vs. 96h normoxia	2.7	1.9, 3.5	**<0.001**
24h hypoxia vs. 96h hypoxia	3.4	2.6, 4.2	**<0.001**
96h normoxia vs. 96h hypoxia	0.7	-0.1, 1.5	**0.1716**
IL1 + E + AZ			
24h normoxia vs. 24h hypoxia	0.5	-0.3, 1.3	**0.3516**
24h normoxia vs. 96h normoxia	3.3	2.5, 4.1	**<0.001**
24h normoxia vs. 96h hypoxia	3.2	2.4, 4.0	**<0.001**
24h hypoxia vs. 96h normoxia	2.8	2.1, 3.6	**<0.001**
24h hypoxia vs. 96h hypoxia	2.8	2.0, 3.5	**<0.001**
96h normoxia vs. 96h hypoxia	0.1	-0.9, 0.7	**0.8589**

11BHSD1 n=5	Mean Diff.	95% CI of diff.	**Mixed model (P value)**

24 h normoxia			
V vs. IL1	4.7	3.9, 5.5	**<0.001**
V vs. IL1 + E	4.8	4.0, 5.6	**<0.001**
V vs. IL1 + E + AZ	4.6	3.8, 5.4	**<0.001**
IL1 vs. IL1 + E	0.1	-0.7, 0.9	**0.8375**
IL1 vs. IL1 + E + AZ	-0.1	-0.9, 0.7	**0.8745**
IL1 + E vs. IL1 + E + AZ	-0.2	-1.0, 0.6	**0.7165**
24 h hypoxia			
V vs. IL1	4.7	3.9, 5.5	**<0.001**
V vs. IL1 + E	4.8	4.0, 5.5	**<0.001**
V vs. IL1 + E + AZ	4.6	3.8, 5.4	**<0.001**
IL1 vs. IL1 + E	0.1	-0.7, 0.8	**0.9175**
IL1 vs. IL1 + E + AZ	-0.1	-0.9, 0.7	**0.8256**
IL1 + E vs. IL1 + E + AZ	-0.2	-0.9, 0.6	**0.746**
96 h normoxia			
V vs. IL1	6.1	5.3, 6.9	**<0.001**
V vs. IL1 + E	6.3	5.5, 7.0	**<0.001**
V vs. IL1 + E + AZ	6.2	5.5, 7.0	**<0.001**
IL1 vs. IL1 + E	0.2	-0.6, 0.9	**0.7519**
IL1 vs. IL1 + E + AZ	0.1	-0.6, 0.9	**0.7775**
IL1 + E vs. IL1 + E + AZ	0.0	-0.8, 0.8	**0.9732**
96 h hypoxia			
V vs. IL1	6.1	5.3, 6.9	**<0.001**
V vs. IL1 + E	6.9	6.1, 7.7	**<0.001**
V vs. IL1 + E + AZ	6.1	5.3, 6.9	**<0.001**
IL1 vs. IL1 + E	0.8	0.01, 1.6	**0.1103**
IL1 vs. IL1 + E + AZ	0.0	-0.8, 0.8	**0.9853**
IL1 + E vs. IL1 + E + AZ	-0.8	-1.6, -0.01	**0.1144**

Appendix 5- 3. Mean fold-change of gene expression

mRNA gene expression	Treatment =IL1/V				Treatment =IL1 E200/V				Treatment =IL1 E200 AZ/V			
	Mean	Std. Err.	95% Conf.		Mean	Std. Err.	95% Conf.		Mean	Std. Err.	95% Conf.	
BHSD												
24 hr Normoxia	38.3	20.4	-2.6	79.2	45.8	27.7	-9.6	101.2	30.1	10.9	8.3	51.9
24 hr Hypoxia	42.4	22.8	-3.1	87.9	39.2	20.2	-1.2	79.6	34.1	15.7	2.7	65.5
96 hr Normoxia	92.2	27.4	37.5	146.9	104.5	32.0	40.4	168.6	109.7	35.8	38.1	181.4
96 hr Hypoxia	75.9	16.6	42.7	109.1	129.1	24.6	79.8	178.4	80.7	19.3	42.1	119.2
H6PD												
24 hr Normoxia	0.9	0.1	0.7	1.2	1.0	0.1	0.7	1.3	1.1	0.1	0.9	1.2
24 hr Hypoxia	1.1	0.2	0.6	1.5	1.1	0.2	0.7	1.4	1.0	0.2	0.7	1.4
96 hr Normoxia	1.1	0.1	0.9	1.3	1.1	0.1	0.9	1.3	1.5	0.3	0.9	2.1
96 hr Hypoxia	1.0	0.1	0.8	1.2	1.2	0.1	1.1	1.4	1.1	0.1	0.9	1.2
GR												
24 hr Normoxia	0.9	0.2	0.5	1.3	1.0	0.2	0.6	1.3	0.9	0.1	0.7	1.1
24 hr Hypoxia	0.7	0.1	0.6	0.9	0.7	0.1	0.5	0.9	0.6	0.1	0.4	0.8
96 hr Normoxia	0.9	0.1	0.7	1.1	0.7	0.2	0.4	1.0	0.9	0.2	0.5	1.2
96 hr Hypoxia	0.8	0.2	0.5	1.1	0.6	0.1	0.4	0.7	0.7	0.1	0.5	0.9
PTGS2												
24 hr Normoxia	50.2	20.0	10.1	90.3	41.2	14.3	12.5	69.8	44.9	10.5	24.0	65.8
24 hr Hypoxia	67.0	30.5	5.9	128.0	35.9	12.0	11.8	59.9	48.0	17.9	12.2	83.9
96 hr Normoxia	106.3	25.6	55.1	157.5	34.7	18.2	-1.7	71.0	94.8	28.4	38.0	151.6
96 hr Hypoxia	47.8	15.0	17.9	77.9	10.1	5.4	-0.7	20.8	38.3	10.0	18.4	58.2
VEGFA												
24 hr Normoxia	3.8	0.9	2.0	5.7	4.8	1.7	1.4	8.2	4.8	1.5	1.9	7.7
24 hr Hypoxia	2.4	0.3	1.9	3.0	2.2	0.1	2.0	2.5	2.2	0.1	2.0	2.4
96 hr Normoxia	5.9	1.8	2.2	9.5	5.7	2.6	0.4	11.0	8.1	4.0	0.1	16.2
96 hr Hypoxia	2.9	0.7	1.5	4.3	1.6	0.3	0.9	2.2	2.6	0.5	1.7	3.5

Appendix 5- 4. Summary of 11β-HSD1 activity at 72 h and statistical analysis

Oxygen condition	Treatment	Mean cortisol production (ng/mL)	Std. Err.	[95% Conf.Interval]	
72 Normoxia	IL1	0.1	0.01	0.07	0.10
	IL1E12.5	0.6	0.11	0.38	0.82
	IL1E200	12.1	2.71	6.71	17.54
	IL1E12.5AZ	0.2	0.01	0.13	0.17
	IL1F12.5	7.9	1.19	5.56	10.31
72 Hypoxia	IL1	0.1	0.01	0.06	0.11
	IL1E12.5	1.2	0.31	0.57	1.82
	IL1E200	23.5	5.23	13.03	33.88
	IL1E12.5AZ	0.1	0.01	0.08	0.13
	IL1F12.5	8.4	1.12	6.12	10.59

% conversion (n=3)	P value
Hypoxia VS normoxia	
IL1 E 200	0.0015
IL1 E12.5	0.0002
IL1 E12.5 AZ	0.823
Normoxia	
IL1 E12.5 vs. IL1 E200	0.40
IL1 E12.5 vs. IL1 E12.5 AZ	0.02
IL1 E200 vs. IL1 E12.5 AZ	0.001
96h hypoxia	
IL1 E12.5 vs. IL1 E200	0.16
IL1 E12.5 vs. IL1 E12.5 AZ	< 0.001
IL1 E200 vs. IL1 E12.5 AZ	< 0.001

Appendix 5- 5. Statistic test for GR gene expression

GR n=5	Mean Diff.	95.00% CI	P value	Summary
Treatment				
V				
24h normoxia vs. 24h hypoxia	0.4	-0.1, 0.9	0.03	ns
24h normoxia vs. 96h normoxia	0.1	-0.4, 0.6	0.63	ns
24h normoxia vs. 96h hypoxia	0.5	0.03, 1.0	0.0048	**
24h hypoxia vs. 96h normoxia	-0.3	-0.8, 0.2	0.08	ns
24h hypoxia vs. 96h hypoxia	0.1	-0.4, 0.6	0.54	ns
96h normoxia vs. 96h hypoxia	0.4	-0.1, 0.9	0.02	*
IL1				
24h normoxia vs. 24h hypoxia	0.2	-0.3, 0.7	0.21	ns
24h normoxia vs. 96h normoxia	0.2	-0.3, 0.7	0.30	ns
24h normoxia vs. 96h hypoxia	0.4	-0.1, 0.9	0.02	*
24h hypoxia vs. 96h normoxia	0.0	-0.5, 0.4	0.83	ns
24h hypoxia vs. 96h hypoxia	0.2	-0.3, 0.7	0.26	ns
96h normoxia vs. 96h hypoxia	0.2	-0.2, 0.7	0.18	ns
IL1 + E				
24h normoxia vs. 24h hypoxia	0.0	-0.5, 0.5	0.93	ns
24h normoxia vs. 96h normoxia	-0.3	-0.8, 0.2	0.11	ns
24h normoxia vs. 96h hypoxia	-0.2	-0.7, 0.3	0.32	ns
24h hypoxia vs. 96h normoxia	-0.3	-0.8, 0.2	0.13	ns
24h hypoxia vs. 96h hypoxia	-0.2	-0.6, 0.3	0.37	ns
96h normoxia vs. 96h hypoxia	0.1	-0.4, 0.6	0.54	ns
IL1 + E + AZ				
24h normoxia vs. 24h hypoxia	-0.2	-0.6, 0.3	0.36	ns
24h normoxia vs. 96h normoxia	0.0	-0.5, 0.5	0.97	ns
24h normoxia vs. 96h hypoxia	0.1	-0.3, 0.6	0.44	ns
24h hypoxia vs. 96h normoxia	0.2	-0.3, 0.7	0.34	ns
24h hypoxia vs. 96h hypoxia	0.3	-0.2, 0.8	0.09	ns
96h normoxia vs. 96h hypoxia	0.1	-0.3, 0.6	0.47	ns

O2 condition and time	Mean diff of ΔCt	95.00% CI of diff.	P Value	Summary
24 h normoxia				
V vs. IL1	-0.3	-0.8, 0.2	0.10	ns
V vs. IL1 + E	-0.2	-0.7, 0.3	0.31	ns
V vs. IL1 + E + AZ	-0.2	-0.7, 0.3	0.21	ns
IL1 vs. IL1 + E	0.1	-0.4, 0.6	0.52	ns
IL1 vs. IL1 + E + AZ	0.1	-0.4, 0.6	0.68	ns
IL1 + E vs. IL1 + E + AZ	0.0	-0.5, 0.4	0.82	ns
24h hypoxia				
V vs. IL1	-0.5	-1.0, 0.004	0.009	**
V vs. IL1 + E	-0.6	-1.1, -0.1	<0.001	***
V vs. IL1 + E + AZ	-0.8	-1.3, -0.3	<0.001	***
IL1 vs. IL1 + E	-0.1	-0.6, 0.4	0.49	ns
IL1 vs. IL1 + E + AZ	-0.3	-0.8, 0.2	0.08	ns
IL1 + E vs. IL1 + E + AZ	-0.2	-0.7, 0.3	0.29	ns
96h normoxia				
V vs. IL1	-0.2	-0.7, 0.3	0.27	ns
V vs. IL1 + E	-0.6	-1.0, -0.1	0.002	**
V vs. IL1 + E + AZ	-0.3	-0.8, 0.2	0.09	ns
IL1 vs. IL1 + E	-0.4	-0.8, 0.1	0.047	*
IL1 vs. IL1 + E + AZ	-0.1	-0.6, 0.4	0.56	ns
IL1 + E vs. IL1 + E + AZ	0.3	-0.2, 0.7	0.16	ns
96h hypoxia				
V vs. IL1	-0.4	-0.9, 0.1	0.03	*
V vs. IL1 + E	-0.9	-1.4, -0.4	<0.001	***
V vs. IL1 + E + AZ	-0.6	-1.1, -0.1	<0.002	***
IL1 vs. IL1 + E	-0.5	-1.0, -0.01	0.007	**
IL1 vs. IL1 + E + AZ	-0.2	-0.7, 0.3	0.23	ns
IL1 + E vs. IL1 + E + AZ	0.3	-0.2, 0.8	0.13	ns

Appendix 5- 6. Statistic test for H6PD gene expression

H6PD n=5	Mean diff of ΔCt	95%CI of diff.	p-value	Summary
Treatment				
V				
24h normoxia vs. 24h hypoxia	0.1	-0.3, 0.5	0.68	ns
24h normoxia vs. 96h normoxia	0.7	0. 3, 1.1	<0.001	***
24h normoxia vs. 96h hypoxia	0.9	0.5, 1.4	<0.001	***
24h hypoxia vs. 96h normoxia	0.6	0.2, 1.0	0.01	**
24h hypoxia vs. 96h hypoxia	0.9	0.4, 1.3	<0.001	****
96h normoxia vs. 96h hypoxia	0.3	-0.2, 0.7	0.25	ns
IL1				
24h normoxia vs. 24h hypoxia	0.2	-0.2, 0.7	0.29	ns
24h normoxia vs. 96h normoxia	0.9	0.5, 1.3	<0.001	***
24h normoxia vs. 96h hypoxia	1.1	0.7, 1.5	<0.001	***
24h hypoxia vs. 96h normoxia	0.7	0.2, 1.1	<0.001	***
24h hypoxia vs. 96h hypoxia	0.9	0.4, 1.3	<0.001	***
96h normoxia vs. 96h hypoxia	0.2	-0.2, 0.6	0.36	ns
IL1 + E				
24h normoxia vs. 24h hypoxia	0.1	-0. 3, 0.6	0.55	ns
24h normoxia vs. 96h normoxia	0.9	0.4, 1.3	<0.001	***
24h normoxia vs. 96h hypoxia	1.3	0.8, 1.7	<0.001	***
24h hypoxia vs. 96h normoxia	0.7	0.3, 1. 2	<0.001	***
24h hypoxia vs. 96h hypoxia	1.1	0.7, 1.6	<0.001	***
96h normoxia vs. 96h hypoxia	0.4	-0.03, 0.8	0.07	ns
IL1 + E + AZ				
24h normoxia vs. 24h hypoxia	0.0	-0.4, 0.4	0.97	ns
24h normoxia vs. 96h normoxia	1.2	0.7, 1.6	<0.001	***
24h normoxia vs. 96h hypoxia	1.0	0.6, 1.4	<0.001	***
24h hypoxia vs. 96h normoxia	1.2	0.8, 1.6	<0.001	***
24h hypoxia vs. 96h hypoxia	1.0	0.6, 1.4	<0.001	***
96h normoxia vs. 96h hypoxia	-0.2	-0.6, 0.2	0.39	ns

H6PD n=5	Mean diff of ΔCt	95% CI of diff.	p-value	Summary
Treatment				
24h normoxia				
V vs. IL1	-0.2	-0.6, 0.3	0.49	ns
V vs. IL1 + E	0.0	-0.5, 0.4	0.84	ns
V vs. IL1 + E + AZ	0.0	-0.4, 0.5	0.84	ns
IL1 vs. IL1 + E	0.1	-0.3, 0.5	0.62	ns
IL1 vs. IL1 + E + AZ	0.2	-0.2, 0.6	0.37	ns
IL1 + E vs. IL1 + E + AZ	0.1	-0.3, 0.5	0.69	ns
24h hypoxia				
V vs. IL1	0.0	-0.4, 0.4	0.95	ns
V vs. IL1 + E	0.0	-0.4, 0.4	0.99	ns
V vs. IL1 + E + AZ	-0.1	-0.5, 0.4	0.81	ns
IL1 vs. IL1 + E	0.0	-0.4, 0.4	0.96	ns
IL1 vs. IL1 + E + AZ	0.0	-0.56, 0.4	0.85	ns
IL1 + E vs. IL1 + E + AZ	-0.1	-0.5, 0.4	0.81	ns
96h normoxia				
V vs. IL1	**0.1**	**-0.4, 0.5**	**0.77**	**ns**
V vs. IL1 + E	0.1	-0.3, 0.6	0.52	ns
V vs. IL1 + E + AZ	0.5	0. 1, 1.0	0.01	*
IL1 vs. IL1 + E	0.1	-0. 4, 0.5	0.72	ns
IL1 vs. IL1 + E + AZ	0.5	0.04, 0.9	0.03	*
IL1 + E vs. IL1 + E + AZ	0.4	-0.04, 0.8	0.07	ns
96h hypoxia				
V vs. IL1	0.0	-0.4, 0.4	0.96	ns
V vs. IL1 + E	0.3	-0.1, 0.7	0.19	ns
V vs. IL1 + E + AZ	0.1	-0.3, 0.5	0.67	ns
IL1 vs. IL1 + E	0.3	-0.2, 0.7	0.21	ns
IL1 vs. IL1 + E + AZ	0.1	-0.37, 0.5	0.71	ns
IL1 + E vs. IL1 + E + AZ	-0.2	-0.6, 0.2	0.38	ns

Appendix 5- 7 Statistic test for PTGS2 gene expression

PTGS2 n=5(Mixed model)	Mean diff of ΔCt	95% CI of diff.	p-value	Summary
Treatment				
V				
24h normoxia vs. 24h hypoxia	**1.8**	**0.8, 2.9**	**0.001**	***
24h normoxia vs. 96h normoxia	**0.9**	**-0.2, 1.9**	**0.098**	**ns**
24h normoxia vs. 96h hypoxia	2.8	1.7, 3.9	<0.001	***
24h hypoxia vs. 96h normoxia	-1.0	-2.0, 0.1	0.065	ns
24h hypoxia vs. 96h hypoxia	**1.0**	**-0.1, 2.0**	**0.066**	**ns**
96h normoxia vs. 96h hypoxia	**1.9**	**0.9, 3.0**	<0.001	***
IL1				
24h normoxia vs. 24h hypoxia	**2.0**	**0.9, 3.1**	<0.001	***
24h normoxia vs. 96h normoxia	**2.1**	**1.0, 3.2**	<0.001	***
24h normoxia vs. 96h hypoxia	2.9	1.8, 3.9	<0.001	***
24h hypoxia vs. 96h normoxia	0.1	-0.9, 1.2	0.824	ns
24h hypoxia vs. 96h hypoxia	**0.9**	**-0.2, 1.9**	**0.092**	ns
96h normoxia vs. 96h hypoxia	**0.8**	**-0.3, 1.8**	**0.144**	ns
IL1 + E				
24h normoxia vs. 24h hypoxia	**1.6**	**0.6, 2.76**	**0.002**	**
24h normoxia vs. 96h normoxia	**0.4**	**-0.76, 1.4**	**0.496**	**ns**
24h normoxia vs. 96h hypoxia	0.5	-0.5, 1.6	0.325	**ns**
24h hypoxia vs. 96h normoxia	-1.3	-2.4, -0.24	0.014	*
24h hypoxia vs. 96h hypoxia	**-1.1**	**-2.2, -0.1**	**0.031**	*
96h normoxia vs. 96h hypoxia	**0.2**	**-0.9, 1.2**	**0.762**	**ns**
IL1 + E + AZ				
24h normoxia vs. 24h hypoxia	**1.7**	**0.7, 2.8**	**0.001**	**
24h normoxia vs. 96h normoxia	**1.9**	**0.8, 3.0**	<0.001	***
24h normoxia vs. 96h hypoxia	2.6	1.5, 3.7	<0.001	***
24h hypoxia vs. 96h normoxia	0.2	-0.9, 1.2	0.756	ns
24h hypoxia vs. 96h hypoxia	**0.9**	**-0.2, 1.9**	**0.093**	ns
96h normoxia vs. 96h hypoxia	**0.7**	**-0.3, 1.8**	**0.171**	ns

O2 condition and time	Mean diff of ΔCt	95% CI of diff.	p-value	Summary
24h normoxia				

V vs. IL1	**5.2**	**4.1, 6.3**	<0.001	***
V vs. IL1 + E	5.0	3.9, 6.0	<0.001	***
V vs. IL1 + E + AZ	5.2	4.2, 6.3	<0.001	***
IL1 vs. IL1 + E	**-0.2**	**-1.3, 0.8**	**0.680**	**ns**
IL1 vs. IL1 + E + AZ	0.0	-1.0, 1.1	0.928	**ns**
IL1 + E vs. IL1 + E + AZ	**0.3**	**-0.8, 1.3**	**0.615**	**ns**
24h hypoxia				
V vs. IL1	**5.4**	**4.3, 6.4**	<0.001	***
V vs. IL1 + E	4.8	3.7, 5.9	<0.001	***
V vs. IL1 + E + AZ	5.1	4.1, 6.2	<0.001	***
IL1 vs. IL1 + E	**-0.6**	**-1.6, 0.5**	**0.283**	**ns**
IL1 vs. IL1 + E + AZ	-0.2	-1.3, 0.8	0.672	**ns**
IL1 + E vs. IL1 + E + AZ	**0.3**	**-0.7, 1.4**	**0.515**	**ns**
96h normoxia				
V vs. IL1	**6.4**	**5.4, 7.5**	<0.001	***
V vs. IL1 + E	4.5	3.4, 5.5	<0.001	***
V vs. IL1 + E + AZ	6.3	5.2, 7.3	<0.001	***
IL1 vs. IL1 + E	**-2.0**	**-3.0, -0.9**	<0.001	***
IL1 vs. IL1 + E + AZ	-0.2	-1.2, 0.9	0.737	ns
IL1 + E vs. IL1 + E + AZ	**1.8**	**0.7, 2.9**	**0.001**	**
96h hypoxia				
V vs. IL1	**5.3**	**4.2, 6.3**	<0.001	***
V vs. IL1 + E	2.7	1.6, 3.8	<0.001	***
V vs. IL1 + E + AZ	5.1	4.0, 6.1	<0.001	***
IL1 vs. IL1 + E	**-2.6**	**-3.6, -1.5**	<0.001	***
IL1 vs. IL1 + E + AZ	-0.2	-1.3, 0.8	0.667	ns
IL1 + E vs. IL1 + E + AZ	**2.4**	**1.3, 3.4**	<0.001	***

Appendix 5- 8. Statistic test for VEGFA gene expression

VEGFA n=5	Mean diff of ΔCt	95% CI of diff.	P value	Summary
Treatment				
V				
24h normoxia vs. 24h hypoxia	1.7	1.0, 2.3	<0.001	***
24h normoxia vs. 96h normoxia	0.3	-0.3, 1.0	0.42	ns
24h normoxia vs. 96h hypoxia	2.1	1.4, 2.7	<0.001	***
24h hypoxia vs. 96h normoxia	-1.3	-2.0, -0.7	0.0008	***
24h hypoxia vs. 96h hypoxia	0.4	-0.2, 1.1	0.29	ns
96h normoxia vs. 96h hypoxia	1.8	1.1, 2.4	<0.001	***
IL1				
24h normoxia vs. 24h hypoxia	1.1	0.5, 1.8	0.005	**
24h normoxia vs. 96h normoxia	0.9	0.2, 1.6	0.03	*
24h normoxia vs. 96h hypoxia	1.7	1.0, 2.4	<0.001	***
24h hypoxia vs. 96h normoxia	-0.2	-0.9, 0.4	0.56	ns
24h hypoxia vs. 96h hypoxia	0.6	-0.1, 1.2	0.15	ns
96h normoxia vs. 96h hypoxia	0.8	0.14, 1.5	0.04	*
IL1 + E				
24h normoxia vs. 24h hypoxia	0.9	0.2, 1.5	0.03	*
24h normoxia vs. 96h normoxia	0.4	-0.3, 1.0	0.35	ns
24h normoxia vs. 96h hypoxia	0.6	-0.01, 1.3	0.11	ns
24h hypoxia vs. 96h normoxia	-0.5	-1.1, 0.12	0.23	ns
24h hypoxia vs. 96h hypoxia	-0.2	-0.9, 0.4	0.59	ns
96h normoxia vs. 96h hypoxia	0.3	-0.4, 0.9	0.50	ns
IL1 + E + AZ				
24h normoxia vs. 24h hypoxia	0.7	0.06, 1.4	0.07	ns
24h normoxia vs. 96h normoxia	0.7	0.9, 1.4	0.06	ns
24h normoxia vs. 96h hypoxia	1.3	0.7, 2.0	0.001	**
24h hypoxia vs. 96h normoxia	0.02	-0.6, 0.7	0.96	ns
24h hypoxia vs. 96h hypoxia	0.6	-0.1, 1.3	0.14	ns
96h normoxia vs. 96h hypoxia	0.6	-0.1, 1.2	0.16	ns

O2 condition and time	Mean diff of ΔCt	95% CI of diff.	P Value	Summary
24h normoxia				

V vs. IL1	1.8	1.1, 2.4	<0.001	***
V vs. IL1 + E	2.0	1.3, 2.6	<0.001	***
V vs. IL1 + E + AZ	2.1	1.4, 2.7	<0.001	***
IL1 vs. IL1 + E	0.2	-0.5, 0.8	0.64	ns
IL1 vs. IL1 + E + AZ	0.3	-0.4, 1.0	0.46	ns
IL1 + E vs. IL1 + E + AZ	0.1	-0.6, 0.8	0.79	ns
24h hypoxia				
V vs. IL1	1.2	0.6, 1.9	0.002	**
V vs. IL1 + E	1.2	0.5, 1.8	0.004	**
V vs. IL1 + E + AZ	1.1	0.5, 1.8	0.005	**
IL1 vs. IL1 + E	-0.1	-0.7, 0.6	0.83	ns
IL1 vs. IL1 + E + AZ	-0.1	-0.8, 0.6	0.78	ns
IL1 + E vs. IL1 + E + AZ	-0.03	-0.7, 0.6	0.95	ns
96h normoxia				
V vs. IL1	2.4	1.7, 3.0	<0.001	***
V vs. IL1 + E	2.0	1.4, 2.7	<0.001	***
V vs. IL1 + E + AZ	2.5	1.8, 3.2	<0.001	***
IL1 vs. IL1 + E	-0.3	-1.0, 0.3	0.40	ns
IL1 vs. IL1 + E + AZ	0.1	-0.5, 0.8	0.71	ns
IL1 + E vs. IL1 + E + AZ	0.5	-0.2, 1.1	0.23	ns
96h hypoxia				
V vs. IL1	1.4	0.7, 2.1	0.0005	***
V vs. IL1 + E	0.5	-0.1, 1.2	0.19	ns
V vs. IL1 + E + AZ	1.3	0.6, 2.0	0.001	**
IL1 vs. IL1 + E	-0.9	-1.5, -0.2	0.03	*
IL1 vs. IL1 + E + AZ	-0.1	-0.8, 0.6	0.82	ns
IL1 + E vs. IL1 + E + AZ	0.8	0.1, 1.4	0.05	*

Appendix 5- 9. PTGS2 and VEGFA gene expression in variable cortisone concentration (n=2)

Oxygen condition	Treatment	PTGS2		VEGFA	
		Δ Ct	Std. Err.	Δ Ct	Std. Err.
96 Normoxia	IL1	12.3	1.2	12.1	0.0
	IL1E12.5	12.4	1.1	12.1	0.3
	IL1E25	12.8	0.8	12.2	0.1
	IL1E 50	13.4	0.1	12.3	0.3
	IL1 E100	13.8	0.8	12.3	0.1
	IL1E200	14.3	0.6	12.8	0.4
96 Hypoxia	IL1	12.6	1.6	12.3	0.2
	IL1E12.5	15.1	2.8	12.8	0.5
	IL1E25	15.7	2.5	13.6	0.7
	IL1E 50	15.6	1.4	13.7	0.0
	IL1 E100	14.6	0.2	13.2	0.2
	IL1E200	14.5	0.5	13.9	0.3

Appendix 5- 10. Statistical analysis of PTGS2 and VEGFA mRNA expression in different cortisone concentration (n=2)

Dose titration PTGS2 (n=2),	PTGS2 gene	VEGFA gene
Hypoxia vs normoxia	p value	p value
IL1	>0.99	0.96
IL1E12.5	0.32	0.10
IL1E25	0.27	0.006
IL1E 50	0.52	0.006
IL1E100	0.98	0.032
IL1E200	>0.99	0.016
96h Normoxia		
IL1 vs. IL1E12.5	>0.99	0.99
IL1 vs. IL1 E25	0.99	0.99
IL1 vs. IL1E50	0.89	0.98
IL1 vs. IL1E100	0.77	0.96
IL1 vs. IL1E200	0.54	0.14
IL1E12.5 vs. IL1 E25	0.99	0.97
IL1E12.5 vs. IL1E50	0.93	0.92
IL1E12.5 vs. IL1E100	0.82	0.88
IL1E12.5 vs. IL1E200	0.59	0.10
IL1 E25 vs. IL1E50	0.99	0.99
IL1 E25 vs. IL1E100	0.94	0.99
IL1 E25 vs. IL1E200	0.75	0.20
IL1E50 vs. IL1E100	0.99	>0.99
IL1E50 vs. IL1E200	0.96	0.27
IL1E100 vs. IL1E200	0.99	0.30
96h hypoxia		
IL1 vs. IL1E12.5	0.36	0.34
IL1 vs. IL1 E25	0.23	0.009
IL1 vs. IL1E50	0.24	0.008
IL1 vs. IL1E100	0.54	0.038
IL1 vs. IL1E200	0.61	0.004
IL1E12.5 vs. IL1 E25	0.99	0.058
IL1E12.5 vs. IL1E50	0.99	0.046
IL1E12.5 vs. IL1E100	0.99	0.34
IL1E12.5 vs. IL1E200	0.99	0.017
IL1 E25 vs. IL1E50	>0.99	0.99
IL1 E25 vs. IL1E100	0.93	0.52
IL1 E25 vs. IL1E200	0.87	0.70
IL1E50 vs. IL1E100	0.94	0.41
IL1E50 vs. IL1E200	0.89	0.82
IL1E100 vs. IL1E200	>0.99	0.12

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