Investigating Antisense Oligonucleotides for Reducing ACTH Production in an *In Vitro* Model of Cushing's Disease

Hanan Gaber Ali Eltumi

A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

The University of Sheffield
Faculty of Medicine, Dentistry and Health
Department of Oncology and Metabolism

July, 2023

Summary

Background: Cushing's disease (CD) is caused by high levels of blood cortisol resulting from excess secretion of adrenocorticotropic hormone (ACTH) from a corticotroph adenoma in the anterior pituitary gland. Clinical features include hypertension, diabetes, osteoporosis, and depression. If untreated CD has an increased mortality of five-fold owing to cardiovascular comorbidities, stroke and raised vulnerability to infection. Transsphenoidal surgery is considered the first-line treatment, but remission is achieved in only 65% of cases and the relapse rate is high. Furthermore, medical treatments are often accompanied by unpleasant side-effects. Antisense therapy is a technique for suppressing gene expression at the level of translation using antisense oligonucleotides (ASOs) against the mRNA of interest.

Aims: Using an *in vitro* AtT-20 mouse cell model, the overall aim of the project was to investigate antisense therapy as a treatment for CD by targeting ASOs against ACTH-encoding *Pomc* mRNA thereby reducing production of the hormone.

Methods: Computer-aided design of POMC ASOs, transfection of ACTH-hypersecreting AtT-20 cells with POMC ASOs, measurement of ACTH by immunoassay and the innate immune response by ELISA, qualitative and quantitative assessment of ASO nuclease degradation, and statistical analysis using ANOVA and t tests.

Results: Following ASO design guidelines and using ASO design programs, four POMC ASOs targeted at different *Pomc* exon sites were selected for experimentation. The ASOs were used unmodified, with a phosphorothioate-modified backbone, or with 2'-*O*-methyl- or locked nucleic acid (LNA)-modified end-nucleotides. All POMC ASOs significantly reduced ACTH secretion from AtT-20 cells when compared with untreated cells or control ASOs (14-59% of normal ACTH levels; ANOVA, $P < 0.05$). LNA-modified POMC ASOs were the most effective when used at lower concentrations (1 nM) and over time (five days). Modified ASOs were more stable to nuclease degradation. None of the ASOs appeared to stimulate the expression of cytokines associated with the innate immune response.

Conclusions: The results indicated that POMC ASOs reduce ACTH secretion from mouse AtT-20 cells. With further investigation *in vivo*, the ASOs may be useful as a new therapy for CD.

Declaration

I hereby declare that this thesis has been written by myself and has not been accepted previously in any publication for a higher degree. The work reported in this thesis was performed by myself, except where specifically acknowledged in the text. All information sources have been specifically acknowledged by means of references.

Hanan Gaber Ali Eltumi

July, 2023

Acknowledgements

Praise to the Almighty Allah who gave me the opportunity and strength to undertake this research and to pursue a PhD. I am ever so grateful for his generosity. I would also like to express my appreciation to the Ministry of Health of Libya and the Libyan Cultural Affairs in London, UK, for all their help and assistance.

I would like to first express my deep gratitude to my supervisors Dr Helen Kemp and Professor John Newell-Price for their help and guidance throughout the period of this project. Without their patience and dedication, I could not have completed this thesis.

I am grateful for the kind help and support of my colleagues in the Department of Oncology and Metabolism at the University of Sheffield; Professor Richard Ross, Dr Ian Wilkinson, Mrs Susan Justice, Ms Julie Porter, Ms Susan Smith, and Mrs Susan Clark. I also wish to extend my thanks to Dr Ahmed Alzahrani for his encouragement.

Finally, and most importantly, I am indebted to my mother whose encouragement and support were the driving force behind this work. I think words will never be enough to express how grateful I am to my husband and children who gave me their everlasting love and stood by me during the most difficult of times during my studies. I would like to extend my thanks to my brothers and sisters.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	Full Version of Word
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ASO	Antisense oligonucleotide
ATCC	American Tissue Culture Collection
Bp	Base pair/s
cAMP	Cyclic AMP
CD	Cushing's disease
cDNA	Complementary deoxyribose nucleic acid
CLIP	Corticotropin-like intermediate lobe peptide
CMV	Cytomegalovirus
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor 1
CT	Computerised tomography
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribose nucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Fragment antigen-binding region
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
HPA	Hypothalamus-pituitary-adrenal
LNA	Locked nucleic acid
MC2R	Melanocortin-2 receptor
MOE	2'-*O*-methoxy-ethyl
mRNA	Messenger ribose nucleic acid
MSH	Melanocyte-stimulating hormone
OMe	2'-*O*-methyl
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMO	Phosphorodiamidate morpholino oligomer
PNA	Peptide nucleic acid
POMC	Proopiomelanocortin
PS	Phosphorothioate
RNA	Ribose nucleic acid
RNase H	Ribonuclease H
Rpm	Revolutions per minute
RT	Reverse transcriptase
siRNA	Small-interfering RNA
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron
TSH	Thyroid-stimulating hormone
UORF	Upstream open-reading frame
UTR	Untranslated region

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List of Publications

Eltumi H, Whatmore J, Kemp EH, Newell-Price J (2021). Antisense oligonucleotides as a novel therapy for Cushing's disease. ENDO 2021 (Online). **Journal of the Endocrine Society, 2021**; 5 (Issue Supplement 1): A532–A533.

Kemp EH, Eltumi H, Whatmore J, Newell-Price J (2021). Use of antisense oligonucleotides as a therapy for Cushing's disease. 23rd European Congress of Endocrinology 2021 (Online). **Endocrine Abstracts, 2021**; 73: PEP13.8.

Chapter 1

Introduction

1 Introduction

Cushing's disease (CD) was first described in 1912 by Harvey Cushing (Cushing, 1912). The clinical features in his female patient were truncal obesity, buffalo hump, severe abdominal striae, hypertension, and erythrocytosis. Twenty years later, he linked these clinical features to a basophilic pituitary adenoma that hyper-secreted adrenocorticotropic hormone (ACTH) leading to excessive levels of blood cortisol (Cushing, 1932). The following introduction includes a discussion of the hypothalamic pituitary adrenal (HPA) axis, the clinical manifestations and current management of CD, and the concept of antisense oligonucleotides (ASO) and their potential application as therapeutic agents for CD.

1.1 The Hypothalamic-Pituitary-Adrenal Axis

The HPA axis, along with the sympathetic nervous system, constitutes the major system accountable for controlling the stress response (Figure 1.1). The role of the HPA axis is controlled by tight hormonal interactions between the hypothalamus, the pituitary gland, and the adrenal glands. In general, the HPA axis acts to cope with the stress demands, principally by producing and secreting corticotropin-releasing hormone (CRH), ACTH, and glucocorticoids (Miller and O'Callaghan, 2002). Following stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), which binds to the G protein-coupled corticotropin-releasing hormone receptor type 1 (CRHR1) (Vale et al., 1981). The CRHR1 is located on the surface of the anterior pituitary, mainly on corticotroph cells (Dore et al., 2017). The binding of CRH to CRHR1 results in the secretion of ACTH. On reaching the adrenal cortex, ACTH binds to the melanocortin-2 receptor (MC2R) on the surface of the zona fasciculate, triggering the production and secretion of the glucocorticoid cortisol (Vale et al., 1981). Cortisol acts in a negative feedback fashion to reduce the production of both CRH and ACTH from the hypothalamus and anterior pituitary, respectively. The effect of this is to reduce the secretion of cortisol from the adrenal gland and maintain its blood concentration (Miller and O'Callaghan, 2002). The elements of the HPA axis are discussed in more detail in the following sections.

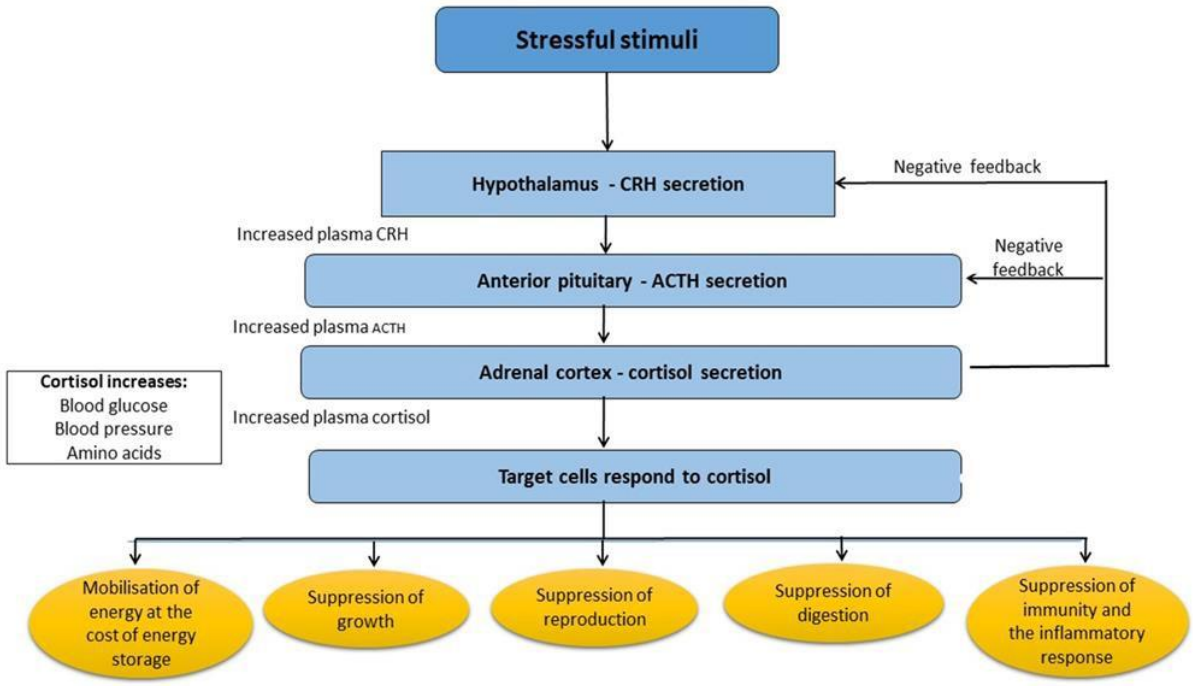


Figure 1.1: The hypothalamic pituitary adrenal axis.

The HPA axis connects three endocrine glands, namely, the hypothalamus, the pituitary gland, and the adrenals. The hormonal interaction between the glands regulates the response to stress by controlling the release of cortisol from the adrenals. ACTH, adrenocorticotropic hormone; CRH, corticotrophin-releasing hormone.

1.1.1 The hypothalamus

The hypothalamus is a small structure, constituting four grams of the 1400-gram of the brain weight. Nevertheless, it is involved heavily in the control of multiple basic life functions including thermoregulation, digestion, fluid and electrolyte balance, the circadian rhythm, and emergency responses to stress (Saper and Lowell, 2014). The parvocellular neurosecretory cells localised within the hypothalamic paraventricular nucleus are responsible for the release of hormones such as CRH and thyrotropin-releasing hormone, which act upon the anterior lobe of the pituitary gland to trigger the secretion of other hormones into the circulation (Figure 1.2) (Smith and Vale, 2006). In addition, the hormones oxytocin and vasopressin are released from magnocellular neurosecretory cells in the paraventricular and supraoptic nuclei to the posterior pituitary (Figure 1.2) (Smith and Vale, 2006).

1.1.2 The pituitary gland

The pituitary gland is a small endocrine gland localised within a fossa of the sphenoid bone named the sella turcica. The pituitary gland lies outside of the dura mater, and consequently, it sits exterior to the blood-brain barrier, and so is vascularised. The gland comprises two functionally discrete lobes, the anterior lobe and the posterior lobe, which are separated by an intermediate lobe that is almost absent in adult humans (Figure 1.2) (Ritchie and Balasubramanian, 2014).

The anterior lobe of the pituitary gland is responsible for the regulation of multiple physiological processes such as homeostasis, growth, metabolism, development, and reproduction. These processes are controlled by five highly differentiated cell types that secrete six diverse hormones. The corticotrophs secrete ACTH, thyrotrophs secrete thyroid-stimulating hormone (TSH), lactotrophs secrete prolactin, somatotrophs secrete growth hormone, and gonadotrophs secrete both follicle-stimulating hormone and luteinising hormone (Yeung et al., 2006). Each of these anterior pituitary cell types have unique surface G protein-coupled receptors for specific hypothalamic hormones. In the case of corticotrophs, they express the CRHR1, which binds CRH released from the hypothalamus.

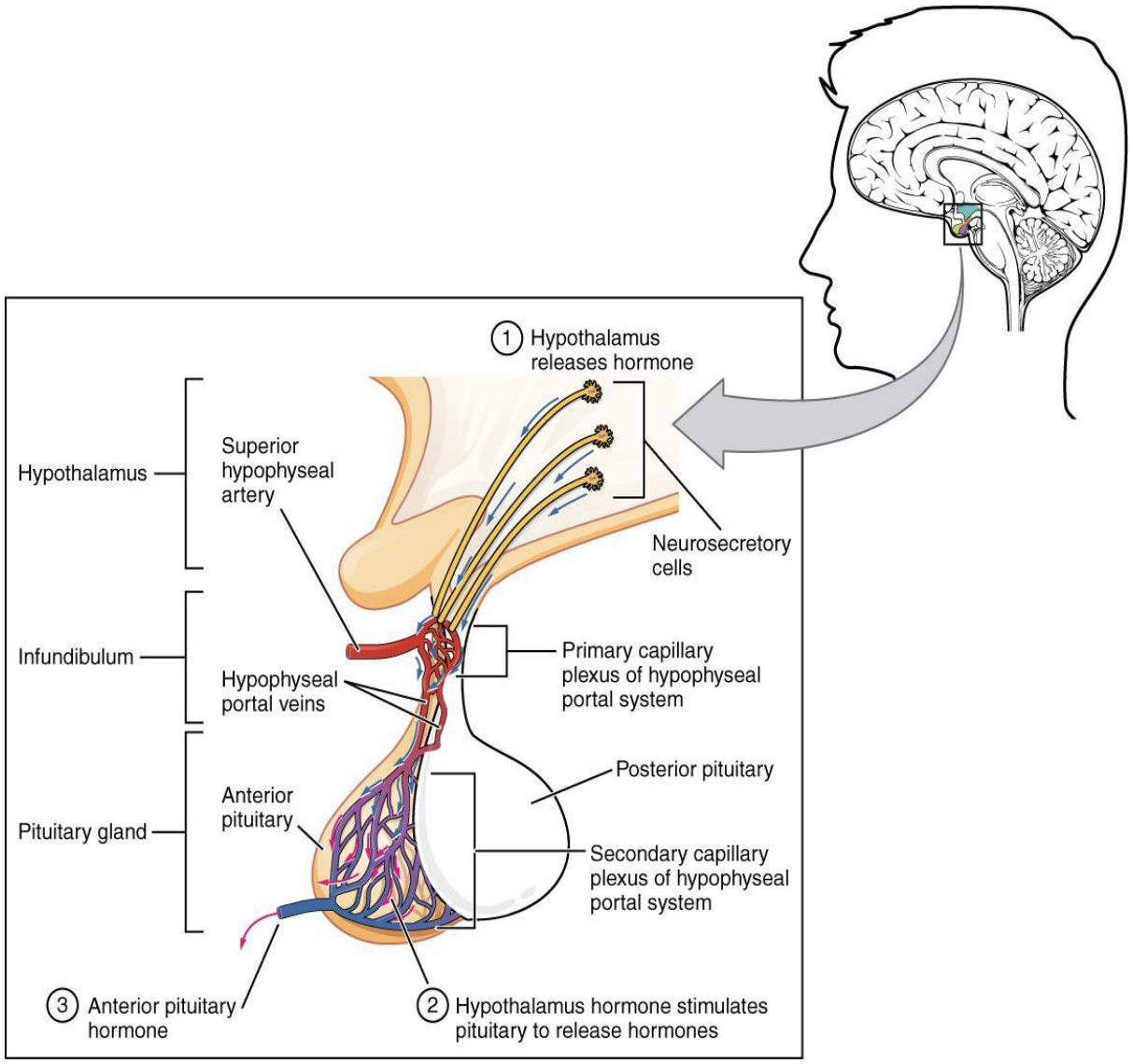


Figure 1.2: Anatomy and physiology of the hypothalamus and pituitary gland.

The hypothalamic hormones such as corticotrophin-releasing hormone are secreted into the portal venous system and stimulate the anterior pituitary gland to secrete hormones such as adrenocorticotropic hormone. Hormones such as oxytocin are secreted directly from the hypothalamus to be stored and secreted by the posterior pituitary. The image, from http://philschatz.com/anatomy-book/contents/m46699.html, is used with kind permission from David Harris, Editor-in-Chief at OpenStax (Rice University, Houston, Texas, USA).

1.1.2.1 Corticotrophin-releasing hormone receptor

The corticotrophin-releasing hormone receptor 1 (CRHR1) is the main CRH-receptor subtype expressed in the anterior pituitary. It is activated when bound to its ligand CRH and is responsible for activating the expression of the proopiomelanocortin (POMC) gene, which encodes ACTH. The receptor is also found in the brain cortical areas, limbic system, and cerebellum, with limited expression in the periphery (Grammatopoulos and Chrousos, 2002, Potter et al., 1994). The CRHR1 is coded for by the CRHR1 gene that is located at 17q21.31 in the genome (Hillhouse and Grammatopoulos, 2006). Upon activation by CRH binding, CRHR1 undergoes conformational changes and couples to a stimulatory G protein. This triggers the adenylyl cyclase-cyclic adenosine monophosphate (cAMP) signalling pathway, resulting in elevation of intracellular cAMP levels with the subsequent downstream effects of ACTH production and secretion (Hillhouse and Grammatopoulos, 2006, Aguilera et al., 2004, Grammatopoulos and Chrousos, 2002, Aguilera et al., 1986).

1.1.2.2 Proopiomelanocortin and ACTH

The *POMC* gene has a key role in the HPA axis because it encodes regulatory hormones including ACTH (Newell-Price, 2003). The human gene is situated on chromosome 2p23.3. The transcribed region of the gene comprises three exons interspersed with two introns (Figure 1.3) (Raffin-Sanson et al., 2003, Whitfeld et al., 1982). The mouse gene has a similar, but not identical, structure and is located on chromosome 12 (Uhler et al., 1983). The main sites of *POMC* expression are corticotrophs in the anterior pituitary, the arcuate nucleus of the hypothalamus, and melanocytes (Raffin-Sanson et al., 2003). This tissue-specific expression is explained by the activity of different transcription factors and the methylation patterns of the *POMC* promoter (Drouin, 2016, Jenks, 2009, Newell-Price, 2003, Drouin et al., 1989). In corticotrophs, the active promoter is located upstream of exon 1 (Figure 1.3). It is activated by several transcription factors including T box factor (TPIT), pituitary homeobox 1 (PITX), neurogenic differentiation factor 1 (NEUROD1), and orphan nuclear receptor of the Nur subfamily (NUR77/NGFIB) (Drouin, 2016, Lamolet et al., 2001, Lamonerie et al., 1996), which themselves allow the tight control and tissue-specific expression of POMC (Jenks, 2009).

Transcription of human *POMC,* and the subsequent splicing of the primary RNA transcript, results in the production of the major mature *POMC* mRNA of 1150 nucleotides with a poly-A tail (Figure 1.3) (Drouin, 2016, Jenks, 2009, Newell-Price, 2003). A further upstream promoter gives a 1350-nucleotide transcript, which can be present at low levels in the anterior pituitary as well as in ACTH-secreting tumours that are not located in the pituitary gland (Jenks, 2009, Newell-Price, 2003). In other tissues, an 800-nucleotide transcript is produced from a third promoter in intron B (Figure 1.3). However, this transcript lacks the signal peptide and the function of it remains unknown (Newell-Price, 2003, Raffin-Sanson et al., 2003).

On translation of mature human *POMC* mRNA, exon 1 remains untranslated while exon 2 encodes the 26-amino acid signal peptide, required for transport of the POMC peptide through the endoplasmic reticulum (Newell-Price, 2003), and a short segment of the amino-terminal peptide (Figure 1.3). Exon 3 comprises all the *POMC*-encoded peptide hormones (Figure 1.3) (Raffin-Sanson et al., 2003, Chang et al., 1980).

The 267-amino acid human POMC peptide undergoes intracellular trafficking through the endoplasmic reticulum and Golgi apparatus during which time it undergoes enzymatic cleavage to generate cell type-specific peptide hormones (Figure 1.3) (Newell-Price, 2003, Raffin-Sanson et al., 2003). For example, pituitary corticotrophs express propeptide convertase (PC) 1, which cleaves POMC to generate the amino-terminal peptide, ACTH, β-lipotrophin, and β-endorphin (Newell-Price, 2003, Raffin-Sanson et al., 2003). In melanotrophs, α-melanocyte stimulating hormone (α-MSH) and β-endorphin are formed by cleavage of POMC with PC1, PC2, carboxypeptidase E, and amino-acetylating and amidating enzymes (Newell-Price, 2003, Raffin-Sanson et al., 2003). The peptide hormones are stored within secretory granules and released by exocytosis in response to stimulation (Cawley et al., 2016).

A variety of functions are carried out by the POMC peptide hormones. Secreted from corticotrophs, ACTH (amino acids 1-39) (Figure 1.3) plays a key role in the stress response and homeostasis by controlling cortisol release from the zona fasciculata of the adrenal glands via the MC2R (Gallo-Payet, 2016, Spiga and Lightman, 2015, Yeung et al., 2006).

α-MSH is produced from the vestigial lobe in humans and the intermediate lobe in rodents (Trifanescu et al., 2011). It is a cleavage product from ACTH (amino acids 1-13) (Figure 1.3) and has a local effect on controlling melanogenesis and thermoregulation in the skin. Its receptors are melanocortin receptors MC1R and MC5R. Furthermore, α-MSH produced from neurons in the arcuate nucleus has an effect on sexual behaviour and appetite (Wessells et al., 2000).

γ-MSH, which is derived from the amino-terminal peptide of POMC (Figure 1.3), has a restricted biological role and stimulates via the MC3R.

Corticotrophin-like immediate peptide (CLIP) (Figure 1.3), derived from ACTH cleavage (amino acids 18-39) is thought not to circulate nor have any biological action in humans (Cowley et al., 2001).

β-lipotrophin lies at the carboxy-terminus of POMC (amino acids 42-134) (Figure 1.3), and is cleaved to γ-lipotrophin (amino acids 42-101) and β-endorphin (amino acids 104-134). The endorphins, which are endogenous opioid peptides affecting human brain, are known to have a role in behaviour regulation as well as the onset of some psychiatric diseases, diabetes, and obesity (Dalayeun et al., 1993, Goldfarb et al., 1991).

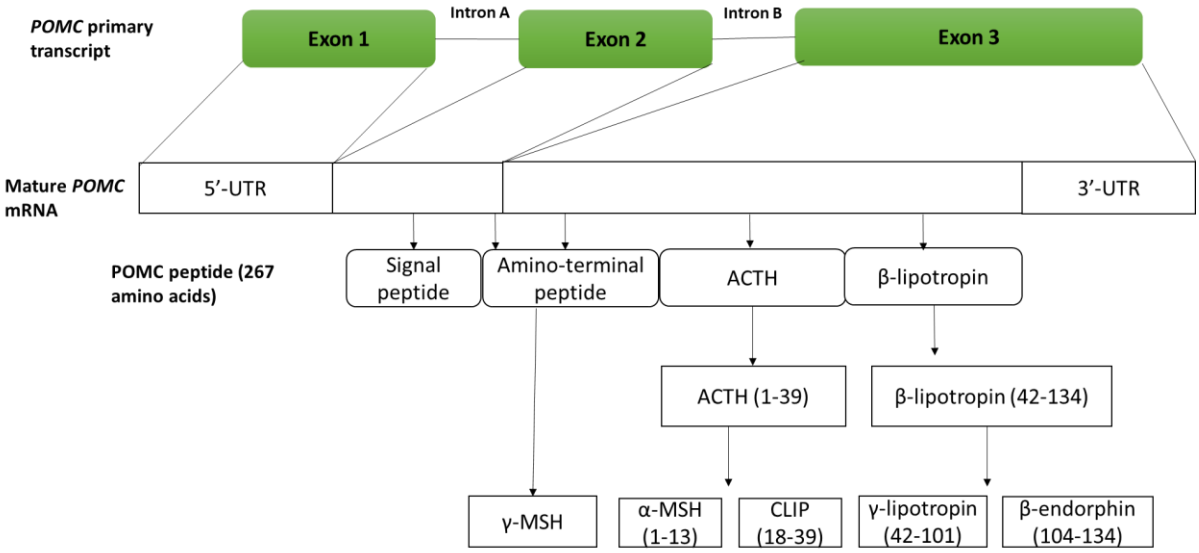


Figure 1.3: Overview of the structure of the human *POMC* RNA transcript and posttranslational modification of the proopiomelanocortin peptide.

The human *POMC* gene comprises three exons. Exons 2 and 3 are translated into the 267-amino acid POMC peptide that includes a 26-amino acid signal sequence. The signal peptide directs POMC to the secretory system of the cell. The POMC molecule is then cleaved into several peptide hormones including ACTH. ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate peptide; α-MSH, α-melanocyte-stimulating hormone; γ-MSH, γ-melanocyte-stimulating hormone; POMC, proopiomelanocortin; UTR, untranslated reg

1.1.3 The adrenals and cortisol

Once released into the circulation and reaching the adrenal cortex, ACTH binds to MCR2 triggering steroidogenesis and cortisol secretion (Figure 1.1) (Cuevas-Ramos and Fleseriu, 2014, Smith and Vale, 2006, Wikberg et al., 2000, Simpson and Waterman, 1988). Initially, activation of the MC2R results in cholesterol transport into the mitochondria. Here, cholesterol is then converted to glucocorticoids by several enzymatic reactions (Cuevas-Ramos and Fleseriu, 2014, Smith and Vale, 2006). Glucocorticoids (corticosterone, cortisol, and cortisone) are involved in multiple metabolic activities, comprising the stress response, anti-inflammatory responses, behaviour (Gallo-Payet, 2016, Chan et al., 2011, Corander and Coll, 2011), elevating blood sugar levels by triggering gluconeogenesis, and stimulating lipid and protein catabolism (Gallo-Payet, 2016, Arlt and Stewart, 2005). Chronically increased glucocorticoids levels raise visceral adiposity, change body fat distribution, and are responsible for a number of metabolic abnormalities (Gallo-Payet, 2016, Dallman et al., 2004), as well as immune system suppression (Katsu and Iguchi, 2016).

Circulating cortisol has a negative feedback effect on the HPA axis, and ultimately suppresses its own production, by acting on both the hypothalamus and the anterior pituitary to inhibit the secretion of CRH (Dallman et al., 1987a, Dallman et al., 1987b) and ACTH (Zhang et al., 2016), respectively. Cortisol binds to the glucocorticoid receptor type 2 (NR3C1) forming a NR3C1/cortisol complex, which enter the nucleus, here the complex binds to glucocorticoid response elements of the genes encoding CRH and POMC (Smith and Vale, 2006) causing a reduction in their expression. Subsequently, glucocorticoid synthesis is downregulated by this negative feedback mechanism. The HPA axis is very tightly regulated, such that alterations in the feedback mechanism described lead to very high serum and urinary cortisol concentrations, this being the basis of ACTH-dependent Cushing's syndrome

1.2 Cushing's Syndrome and Cushing's Disease

1.2.1 Cushing's syndrome

Cushing's syndrome is a devastating disease affecting about 1-3 individuals per million per year. If untreated, it has an increased mortality of five-fold owing to cardiovascular comorbidities, stroke or raised vulnerability to infection (Lacroix et al., 2015, Castinetti et al., 2012). Cushing's syndrome refers to the distinctive clinical appearances of chronic lengthy exposure to highly increased concentration of glucocorticoids, which can be from endogenous cortisol or exogenous corticosteroid use. Iatrogenic exposure to exogenous steroids is recognised as the commonest cause of Cushing's syndrome (Lau et al., 2015), while endogenous Cushing's syndrome is very infrequent with an estimated incidence ranging from 0.2-5 per million individuals per year (Lacroix et al., 2015).

Endogenous Cushing's syndrome is more frequent in females than males and can be of an ACTH-dependent or ACTH-independent aetiology. In 80-85% of cases, they are ACTH-dependent. Eighty percent of these cases result from a pituitary corticotroph adenoma and this is termed CD. The remaining 20% are due to ectopic ACTH secretion from sources such as small cell lung carcinomas, bronchial carcinoids and phaeochromocytomas (Lacroix et al., 2015, Newell-Price et al., 2006). Adrenal adenomas and adrenal carcinomas, primary adrenal conditions that cause ACTH suppression, are found to be the predominant sources of ACTH-independent Cushing's syndrome at 60% and 40%, respectively. Furthermore, rare adrenal causes are due to primary pigmented nodular adrenal disease, macronodular adrenal hyperplasia, and McCune Albright syndrome (Newell-Price et al., 2006).

1.2.2 Cushing's disease

Cushing's disease is caused by unregulated increased secretion of ACTH by a pituitary corticotroph adenoma, which results in cortisol over-secretion from the adrenals. Normally, a rise in blood cortisol levels causes a downregulation of ACTH release and consequently a downregulation of cortisol production. However, corticotroph tumour cells are relatively resistant to the negative feedback mechanism and persist in releasing ACTH in spite of the increase in the blood level of cortisol

(Lacroix et al., 2015). Although CD is recognised as an uncommon cause of increased cortisol levels with a prevalence of about 40 cases per million (Buliman et al., 2016, Castinetti et al., 2012), it represents the most frequent form of endogenous Cushing's syndrome.

Corticotroph tumours are sporadic with only a few cases clarified in hereditary endocrine syndromes (Albani et al., 2018). The genes involved in the formation of these tumors are listed in Table 1.1. Mutations in the ubiquitin-specific peptidase 8 (*USP8*) gene have recently been identified in 23-60% of corticotroph tumours (Losa et al., 2019, Wanichi et al., 2019). Limited to corticotrophic adenomas, these mutations cause a rise in expression of the *POMC* gene and the gene encoding epidermal growth factor receptor (*EGFR*) (Hayashi et al., 2016).

Table 1.1: Mutations in corticotroph tumours and Cushing's disease

Gene	Mutation	Mutation type	Disease	Reference
AIP	c.696G>C/p.P232P	Silence	CD	(Georgitsi et al., 2007)
CYP21A2	-	Deletion	ACTH-producing PA	(Boronat et al., 2004)
	-	Splicing	ACTH-producing PA	(Haase et al., 2011)
	p.V281L	Missense	CD with CAH	(Haase et al., 2011)
DICER1	c.3046delA/p.S1016VfsX1065	Frameshift	Pituitary blastoma presenting with CD	(Sahakitrungruang, 2014)
	c.5538>T/p.E1813V>>	Missense		
GNAS	p.Q227H	Missense	Corticotroph adenomas	(Riminucci et al., 2002)
	p.R179G	Missense	Corticotroph adenomas	(Williamson et al., 1995)
	p.R2201H	Missense	CD	(Riminucci et al., 2002)
	p.Q227R	Missense	Corticotroph adenomas	(Riminucci et al., 2002)
MEN1	p.R460X	Nonsense	MEN1 with CD	(Matsuzaki et al., 2004)
	p.R415X	Nonsense	Paediatric familial/syndromic CD	(Stratakis et al., 2010)
	-	Deletion	Paediatric familial/syndromic CD	(Stratakis et al., 2010)
NROB1	g.259_260insAGCG	Insertion	ACTH-secreting PA and X-linked adrenal hypoplasia congenita	(De Menis et al., 2005)
NR3C1	p.1559N	Missense	CD	(Karl et al., 1996)
TP53	p.L145R	Missense	Atypical PA causing CD	(Kawashima et al., 2009)
	c.308A>G /p.K103R	Missense	Recurrent CD	(Stratakis et al., 2010)
USP8	p.P720Q	Missense	CD	(Ma et al., 2015)
	p.P720R	Missense	CD	(Reincke et al., 2015)
	p.5718P	Missense	CD	(Ma et al., 2015)
	p.5718del	Deletion	CD	(Perez-Rivas et al., 2015)
	p.5718C	Missense	CD	(Reincke et al., 2015)

ACTH, adrenocorticotropic hormone; AIP, aryl hydrocarbon receptor-interacting protein; CAH, congenital adrenal hyperplasia; CD, Cushing's disease; CYP21A2, cytochrome P450 family 21 subfamily A member 2; DICER1, Dicer 1, ribonuclease III; GNAS, guanine nucleotide binding protein, α-stimulating activity polypeptide; MEN1, multiple endocrine neoplasia type 1; NR3C1, nuclear receptor subfamily 3 group C member 1; NROB1, nuclear receptor subfamily 0 group B member 1; PA, pituitary adenomas; TP53, tumour protein p53; USP8, ubiquitin-specific peptidase 8; -, not detailed.

1.2.2.1 Clinical features

The characteristic clinical signs of CD are those accompanying chronic lengthy exposure to increased cortisol, involving obesity, abdominal striae, osteoporosis, and easy bruising (Figure 1.4) (Wardlaw, 2001). The stereotypical features of a patient with CD was described by the neurosurgeon Harvey Cushing in his patient Minnie G “The moon shaped plethoric face, accompanied by a buffalo hump, wasted limbs, and purple striae are classical, and are due to the catabolic effects of excess and prolonged glucocorticoids" (Cushing, 1932). Abnormal fat deposition associated with excessive cortisol level is due to the significant effect of cortisol on fat metabolism (Djurhuus et al., 2002), resulting in the most distinctive features of CD comprising ‘moon face’, truncal adiposity and ‘buffalo hump’ (Arasiewicz et al., 2016, Bertagna et al., 2009).

The signs of cutaneous striae, easy bruising, proximal weakness, and osteoporosis, are the result of protein wasting (Castinetti et al., 2012). In addition, patients with CD often have an increased rate of cardiovascular and metabolic complications comprising hypertension and diabetes, psychological and psychiatric complications, as well as infections, all leading to high mortality and morbidity rates (Newell-Price et al., 2006). Since these are a result of endogenous hyper-cortisolism that is accompanied by an increased morbidity rate, it is essential to diagnose CD as early as possible and apply a management plan in order to assure a successful prognosis, to improve the quality of life of patients, and to minimise complications (Buliman et al., 2016).

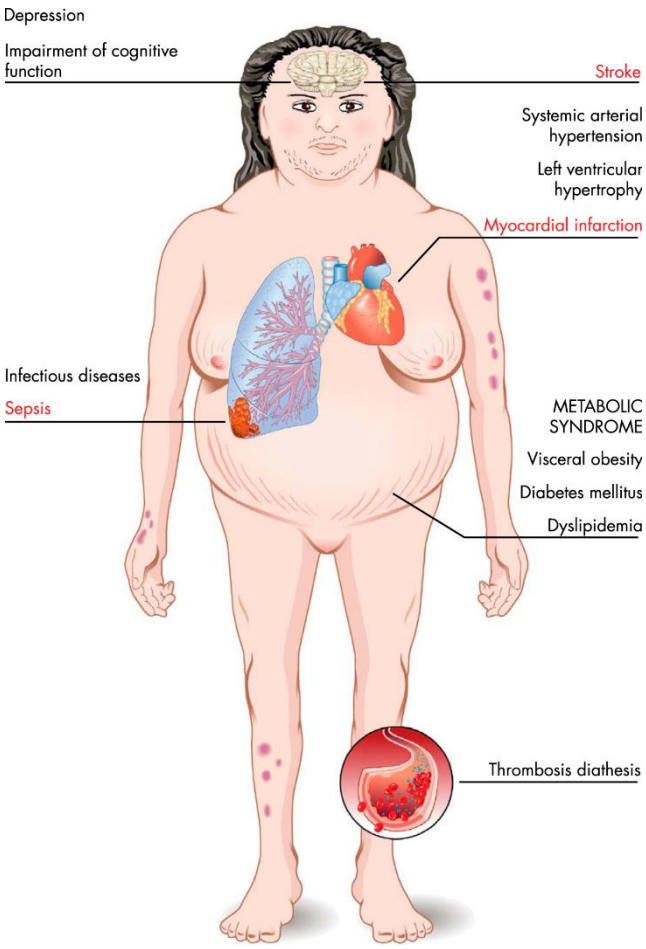


Figure 1.4: Clinical complications associated with Cushing's syndrome.

Excessive cortisol gives rise to the clinical manifestations of Cushing's syndrome that may comprise truncal and facial fat deposition, plethoric facial appearance, muscle atrophy, easy bruising, and skin thinning. Clinical complications and comorbidities such as myocardial infarction, stroke, and sepsis are accompanied by excessively increased mortality. The image, from a paper by (Pivonello et al., 2015), was used with kind permission from Oxford University Press (Oxford, UK).

1.2.2.2 Diagnosis

The clinical diagnosis of CD is difficult since several signs and symptoms caused by chronic exposure to increased cortisol, for example obesity, psychiatric disorders, and irregular menstrual cycle, are common (Nieman et al., 2008). For a definitive and accurate diagnosis, biochemical evaluation of autonomous hypercortisolaemia is required, followed by careful assessment to discover the driving source and localise the lesion. The differential diagnosis of CD is given in Figure 1.5.

1.2.2.3 Management

The treatment of CD aims to normalise the circulating cortisol levels, improve the clinical features and complications accompanying hypercortisolism, and reduce the rate of recurrence (Nieman et al., 2015). The current management and treatment of the CD is multimodal, including surgery, radiation, and medical drugs (Figure 1.6). Surgery is the most commonly used treatment modality, which usually includes adenomectomy, the resection of the pituitary tumour itself and, rarely, hemi- or total hypophysectomy. If ineffective or in patients where surgery is contraindicated, other modalities of treatment are employed and may involve, repeating the pituitary surgery, bilateral adrenalectomy, medical treatment, and radiotherapy.

1.2.2.3.1 Surgery

About 90% of the pituitary adenomas can be resected via a transsphenoidal surgical approach (Buliman et al., 2016). It is the only curative therapy currently used and, unless otherwise contraindicated, considered the first-line treatment for most CD cases (Buliman et al., 2016, Lau et al., 2015, Nieman et al., 2015, Tritos and Biller, 2014). Remission is achieved in of 60–90% for microadenomas and < 65% for macroadenomas (Buliman et al., 2016), although there is around a 30% recurrence at a 30-year follow-up (Buliman et al., 2016, Nieman et al., 2015). Further treatment for these cases involves repeating the surgical eradication, which is considered the gold standard in managing any remaining pituitary tumour, or alternatively second-line managements are used (Nieman et al., 2015).

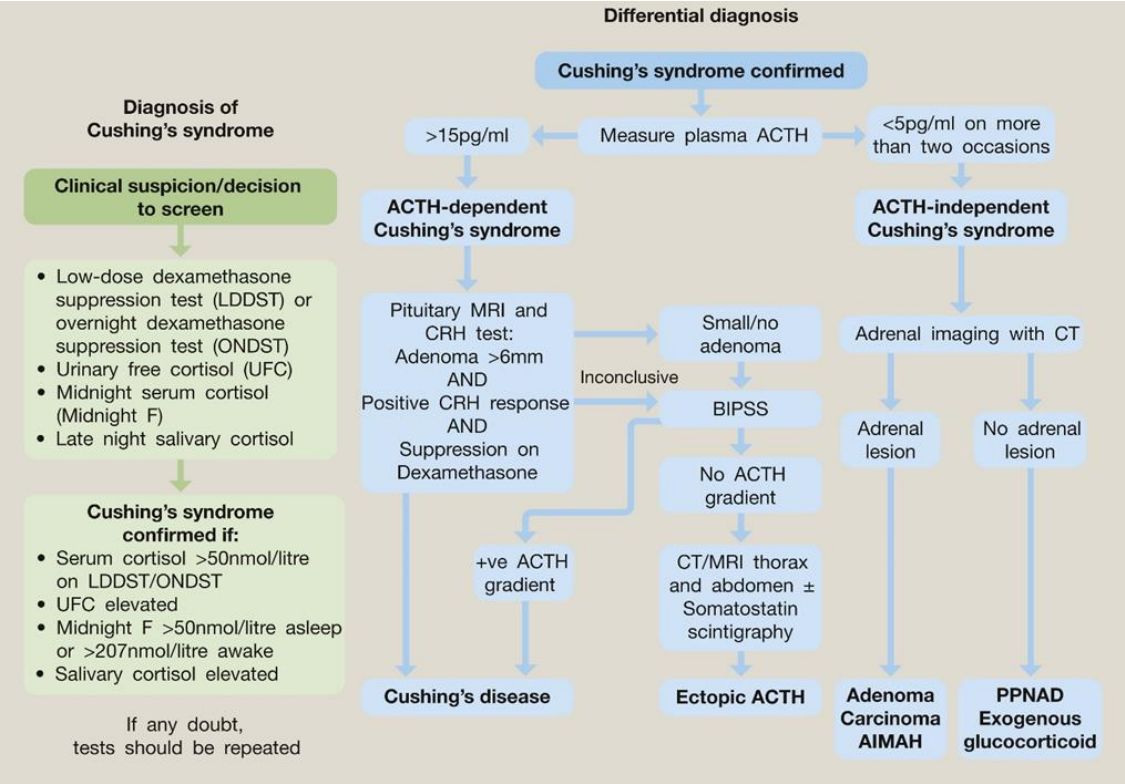


Figure 1.5: Diagnosis and differential diagnosis of Cushing's syndrome.

The diagnosis and differential diagnosis of Cushing's syndrome are outlined. ACTH, adrenocorticotrophic hormone; AIMAH, ACTH-independent macronodular adrenal hyperplasia; BIPSS, bilateral inferior petrosal sinus sampling; CT, computerised tomography; CRH, corticotrophin-releasing hormone; LDDST, low-dose dexamethasone suppression test; MRI, magnetic resonance imaging; ONDST, overnight dexamethasone suppression test; PPNAD, primary pigmented nodular adrenal disease; UFC, urinary free cortisol. The image, from a paper by (Daniel and Newell-Price, 2017) was used with kind permission from Elsevier Limited (Cambridge, UK).

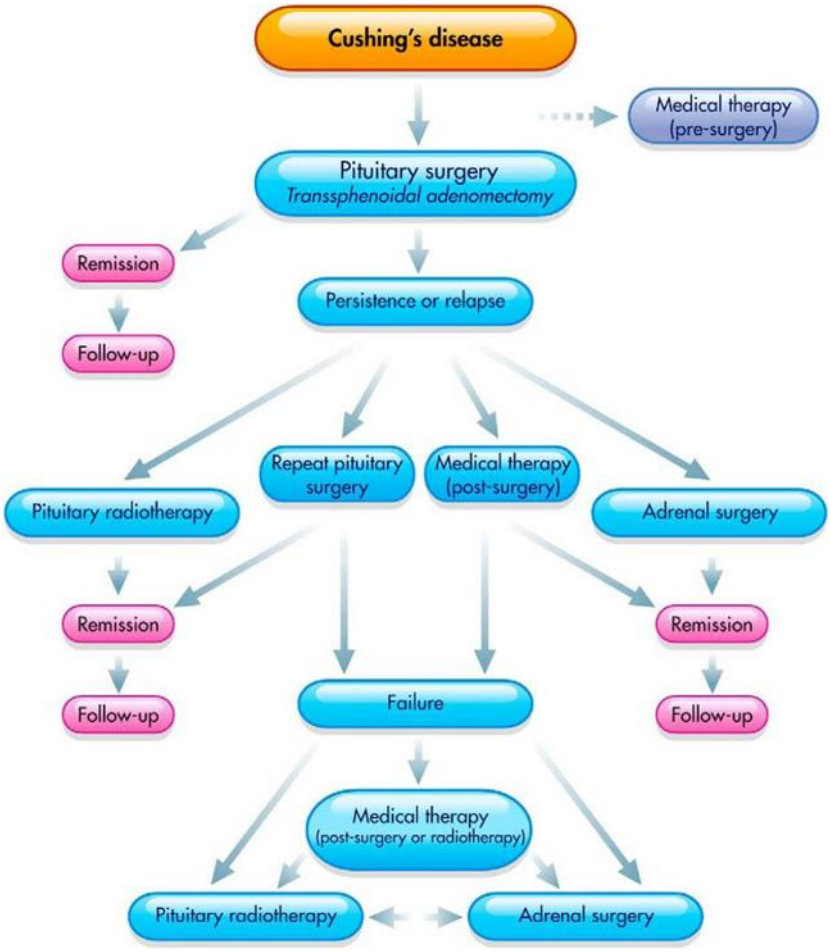


Figure 1.6: Treatment algorithm for Cushing's disease.

The treatment algorithm for Cushing's disease is outlined. The image, from a paper by (Pivonello et al., 2015), was used with kind permission from Oxford University Press (Oxford, UK).

Alternative treatment involves total bilateral adrenalectomy. Although this leads to instant correction of increased cortisol, those patients are at risk of developing acute adrenal insufficiency and therefore need long-life therapy with mineralocorticoid and glucocorticoid replacement therapy (Du et al., 2013, Bertagna et al., 2009). Moreover, 8-47% of these techniques result in Nelson's syndrome since the suprarenal gland resection eliminates the negative feedback effect of cortisol on the anterior pituitary resulting in quick enlargement of the ACTH-producing pituitary adenoma (Cawley et al., 2016).

1.2.2.3.2 Radiotherapy

Radiotherapy is recommended for cases where surgical treatment is contraindicated or where there is the possibility that the tumour is aggressive or non-eradicable (Nieman et al., 2015). Nevertheless, the advantages of radiation therapy are not noticed directly, and months to years may be needed for ACTH levels to be normalised (Vance, 2009). Thus, adjunctive medical treatment is generally given with radiation to help in lowering the production of cortisol while awaiting the radiation effects.

1.2.2.3.3 Medical treatments

Medical treatment has an adjunctive role in managing CD. It is applied to patients who have had unsuccessful surgical treatment and are waiting for useful outcomes of radiotherapy. Furthermore, medical treatment can also be used in cases where surgery and radiation therapy are contraindicated. Finally, medical therapy might be used to control the high levels of cortisol before pituitary surgery or in those cases with an indeterminate source of excessive ACTH (Tritos and Biller, 2014).

Present medical treatments for CD are grouped into three types according to their site of action. Some drugs directly affect the pituitary ACTH-secreting tumours inhibiting the release of ACTH, others are steroidogenesis inhibitors acting on the adrenals to prevent the synthesis of cortisol, and the remainder are glucocorticoid receptor antagonists (Langlois et al., 2017, Lau et al., 2015, Cuevas-Ramos and Fleseriu, 2014). The chief disadvantage of these medications is that although increased cortisol levels might be controlled, patients are still not cured and need a long-term treatment period (Castinetti et al., 2012).

For centrally acting agents, some success has been reported for these medications (Tritos and Biller, 2014, Fleseriu et al., 2012). Cabergoline is a D2-specific dopamine receptor agonist that was initially trialled because there are an elevated number of D2 receptors on ACTH-secreting tumour cells. A review of five small clinical studies (Ferriere et al., 2017) revealed a sustained reduction in urinary free cortisol in about 40% of managed cases. Side-effects were reported in 29% of cases. The efficacy of cabergoline is still controversial because of the lack of data, (Ferriere et al., 2017, Tritos and Biller, 2014).

Pasireotide, another centrally-acting agent, is a somatostatin analogue that interacts with subtypes 4 and 5 of the somatostatin receptor on corticotroph adenomas. The drug suppresses secretion of ACTH and cell proliferation, decreases urinary free cortisol, and improves the clinical manifestations (Colao et al., 2012). Nevertheless, a 12-month study analysing CD management with pasireotide revealed an increased rate of diabetes mellitus and hyperglycaemia owing to insulin secretion suppression. Of 162 cases, 118 (73%) had a hyperglycaemia-related side-effect. In summary, this study revealed that pasireotide decreases the level of cortisol in CD patients and is a probable treatment, but careful controlling of blood glucose levels is necessary (Lacroix et al., 2018, Colao et al., 2012).

There are several drugs available that can inhibit one or more stages in cortisol synthesis. Ketoconazole was initially designed as an antifungal but has also been utilised to treat CD patients (Nieman et al., 2015). A retrospective study on the utilisation of ketoconazole by Castinetti and colleagues reported that 49% of CD cases achieved normalisation of urinary free cortisol (Castinetti et al., 2014). Ketoconazole not only inhibits adrenal steroidogenesis, it can also inhibit gonadal steroidogenesis leading to gynaecomastia and hypogonadism in men if applied for long time (DeFelice et al., 1981). Moreover, possible hepatotoxicity can accompany prolonged utilisation of ketoconazole, so careful monitoring of liver function is required (Castinetti et al., 2014, Cuevas-Ramos and Fleseriu, 2014).

Metyrapone, a frequently utilised steroidogenesis inhibitor, significantly decreases the level of cortisol with common but mild adverse effects (Daniel et al., 2015). Nevertheless, decreased cortisol results in a compensatory elevation in ACTH levels,

which cause additional steroidogenesis and might overwhelm the blocking-effect of the medication (Daniel and Newell-Price, 2015). Mineralocorticoid effects like hypertension, oedema, and hypokalaemia may also occur as a result of mineralocorticoid precursor accumulation (Tritos and Biller, 2014).

Mifepristone is the only presently available glucocorticoid receptor antagonist acting via competitive inhibition of the glucocorticoid receptor (Castinetti et al., 2012). The drug is contraindicated in pregnant women as it antagonises the progesterone receptor. It improves diastolic blood pressure and also normalises the abnormal glucose level in hyperglycaemic patients. It is therefore approved for the treatment of those CD cases that are hyperglycaemic and not considered suitable for surgical therapy (Fleseriu et al., 2012).

Medical treatment has a significant adjunctive role in the therapy of CD but cannot be deemed an ultimate therapy. This demonstrates an unmet clinical need for those patients who are not candidates for surgical therapy and not satisfied by the current alternatives. An international consensus statement “Treatment of Adrenocorticotropin-Dependent Cushing’s Syndrome: A Consensus Statement” was released in 2008, stating that “a medical therapy that acts directly on the pituitary tumour to normalise ACTH secretion would represent a major non-surgical advance in the treatment of this disease [Cushing’s disease]” (Biller et al., 2008).

In conclusion, the long-term safety and efficacy of medical treatment for CD must be assessed and the role of combination treatment need to be better characterised. Since CD pathogenesis becomes better understood at the molecular level, it is probable that targeted, new medical treatments for CD will be discovered. The *POMC* gene is over-expressed in corticotroph adenomas in CD patients. Knock-down of *POMC* expression would lead to a specific downstream decrease in the levels of ACTH and therefore cortisol. One means to achieve this is by utilising antisense oligonucleotide (ASO) technology and this is discussed in the next sections.

1.3 Antisense Oligonucleotide Therapy

Over the past 30 years, there have been many published papers demonstrating applications of antisense nucleic acids for specific suppression of gene expression. Antisense oligonucleotides are short, single-stranded DNA molecules typically 15–20 nucleotides in length that act by binding to their target mRNA through complementary Watson-Crick base-pairing to inhibit translation (Scherer and Rossi, 2003). Several mechanisms of ASO action have been described and these are discussed in the following sections.

1.3.1 Mechanisms of antisense oligonucleotide action

Hybridisation of ASOs to their target mRNA can cause suppression of gene expression by various mechanisms according to the location of hybridisation and the chemical make-up of the ASO. Formation of an ASO–mRNA heteroduplex can either induce RNase H activity resulting in degradation of mRNA, sterically block the ribosomal translation machinery, or interfere with mRNA maturation by altering splicing. All of the mechanisms result in down-regulation of the synthesis of the target protein (Figure 1.7) (Chan et al., 2006).

1.3.1.1 RNase H cleavage of mRNA

The RNase H-dependent cleavage of mRNA targets (Figure 1.7) is the most commonly utilised ASO mechanism, and is the most effective knock-down technique (Crooke, 2017, Bilanges and Stokoe, 2005). RNase H acts independently of nucleotide sequence and causes degradation of the ASO-mRNA heteroduplex while leaving the ASO intact. Consequently, the ASO is free to hybridise with further mRNA target molecules and is, therefore, considered to be a catalytic process (Rinaldi and Wood, 2018). The end-products of the degraded mRNAs are processed by the normal cellular degradation pathways (Rinaldi and Wood, 2018). Antisense oligonucleotides with phosphorothioate (PS)-linked or phosphodiester-linked backbones are susceptible to RNase H (Rinaldi and Wood, 2018).

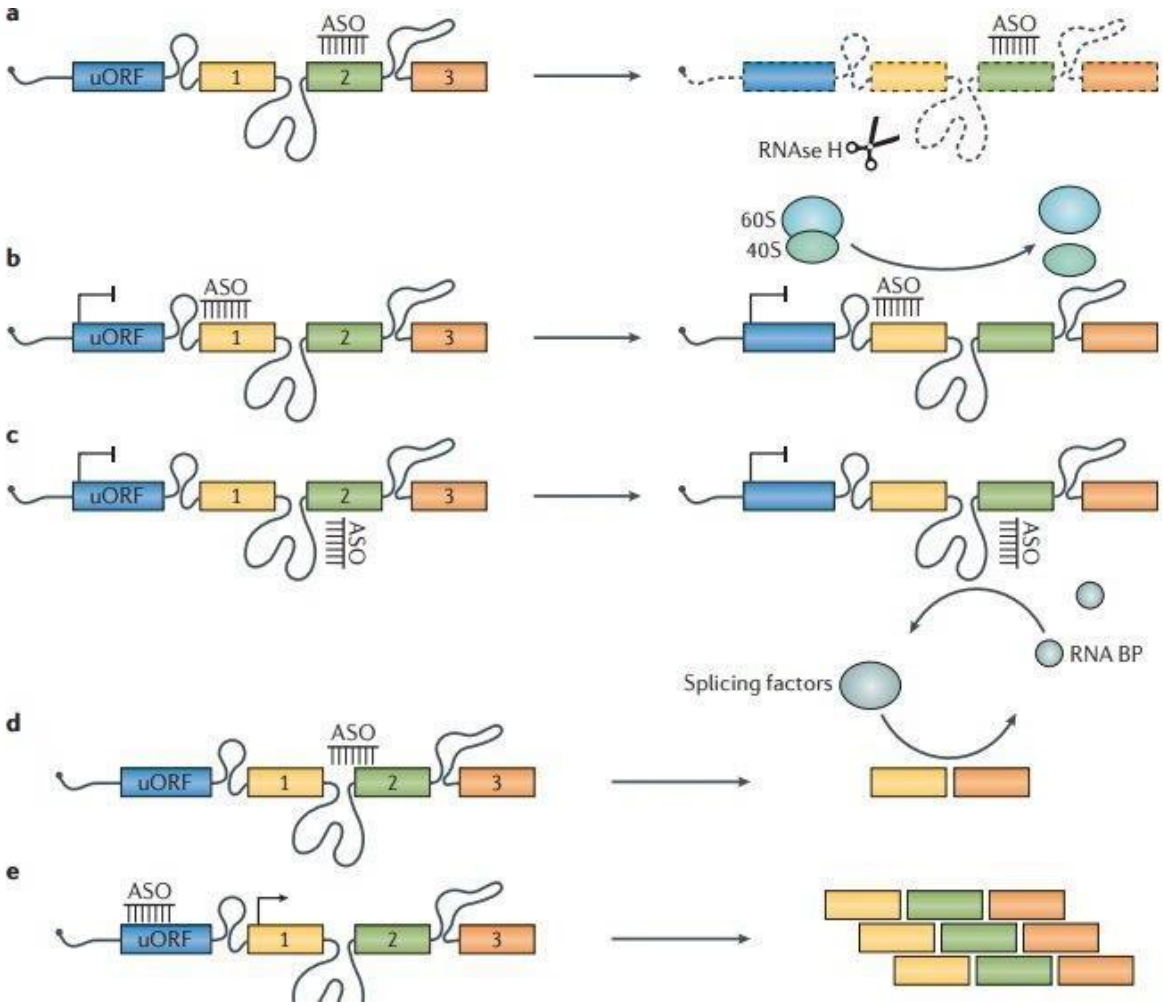


Figure 1.7: The mechanisms of action of antisense oligonucleotides.

Antisense oligonucleotides (ASOs) can act by (a) eliciting RNase H-cleavage of mRNA; (b) sterically blocking translation; (c) obstructing the binding of RNA-binding proteins; (d) modifying mature mRNA by modulating splicing factor action; or (e) elevating expression of the primary open reading frame by suppressing expression of the upstream open reading frame (uORF). The image, from a paper by (Rinaldi and Wood, 2018), was used with kind permission from Springer Nature (Heidelberg, German).

1.3.1.2 Steric blocking of translation

Some chemical modifications that have been designed to increase the affinity and stability of ASOs result in ASO-mRNA heteroduplexes that are no longer a substrate for RNase H. However, the ASOs can sterically block the ribosomal machinery to inhibit mRNA translation (Figure 1.7). Steric blocking of translation is generally achieved by designing ASOs that bind at or nearby the mRNA initiation codon which then prevents the ribosomal subunit from binding (Chery, 2016). In the case of treating myotonic dystrophy, ASOs have been designed to untranslated sections of mRNA. These stop the sequestration and binding of critical RNA-binding proteins (Figure 1.7), resulting in decreased translation (Rinaldi and Wood, 2018).

1.3.1.3 Modulation of mRNA splicing

Some ASOs have been designed to cause modulation of pre-mRNA splicing by hybridisation to exon-intron junctions (Figure 1.7). In terms of therapeutic development, ASOs have been developed for inducing exon-skipping in the dystrophin-encoding Duchene muscular dystrophy gene (Rinaldi and Wood, 2018, Popplewell et al., 2010). By modulating splicing to reframe mutated dystrophin transcripts, a partly functional dystrophin protein can be synthesised (Popplewell et al., 2010). To avert RNase H-cleavage of ASO-mRNA hybrids in this mechanism, ASOs are commonly phosphorodiamidate morpholino oligomers (PMO) or have 2'-*O*-methyl (OMe) modifications (Aartsma-Rus and van Ommen, 2009).

1.3.2 Challenges facing the therapeutic use of antisense oligonucleotides

Initial research into ASO therapy was expected to rapidly deliver new disease treatments. However, there have been several obstacles to overcome which are outlined in the next sections.

1.3.2.1 Susceptibility to degradation

The first main challenge in developing ASO therapies was the high susceptibility of nucleic acids to rapid degradation by endonucleases and exonucleases resulting in increased turnover and inadequate intracellular concentrations to inhibit the expression of the target genes (Dagle et al., 1991, Eder et al., 1991). This resulted in

poor pharmacokinetic characteristics, which meant ASOs generally failed to fulfil the therapeutic requirements in clinical experimentations (Rinaldi and Wood, 2018). To overcome problems with degradation, both chemical modifications and delivery systems have been used to improve ASO resistance to nucleases. These are discussed in Sections 1.3.3 and 1.3.4, respectively.

1.3.2.2 Intracellular delivery

The second main challenge is intracellular delivery. Synthetically produced ASOs are large, negatively-charged molecules of about 30 kD such that their delivery is very difficult as they often cannot cross cell membranes to reach their mRNA target. Again, ASO modifications and delivery strategies have been developed to enhance cellular uptake of ASOs (Sections 1.3.3 and 1.3.4, respectively) In addition, the release of ASOs from endosomal vehicles within the cell is a rate-limiting stage for ASO activity (Juliano, 2018). Small molecular enhancers such as dioleylphosphatidylethanolamine have been used to improve the escape of ASOs from the endosomes and into the cytosol (Juliano, 2018).

1.3.2.3 Toxicity

The third main challenge in developing ASO therapies is their potential toxicity, which falls into two classes (Table 1.2). One class is that of sequence-specific toxicity, mainly resulting from off-target influences of the ASO, but also including immunogenic effects relating to the nucleotide composition of the ASO (Frazier, 2015). The second class is that of non-sequence-specific toxicity. Such effects can result in thrombocytopenia and the activation of complement and are largely due to the non-specific binding properties of ASOs to serum proteins (Frazier, 2015). Furthermore, ASO delivery systems themselves may result in adverse reactions (Table 1.2). Lower ASO doses administered locally, rather than systemically, can alleviate some of these issues. In addition, several chemical modifications of ASOs have been established that decrease non-sequence specific toxicity (Section 1.3.3) (Dias and Stein, 2002).

Table 1.2: Potential toxic effects of antisense oligonucleotide therapy

Type of toxic effect	Mechanism of effect	Result of effect
Sequence-specific	Hybridisation of ASO to off-target sequences	Affects the expression of other non-target genes
	Activation of RNAse H related to other genes	Affects the expression of other non-target genes
	Four contiguous guanines can form higher-order structures	Non-specific biological effects
	Unmethylated cytosine-phosphorus-guanine (CpG) motifs that are immunostimulatory	Immunoactivation, systemic cytokine release, hepatotoxicity
Non-sequence specific:		
Phosphorothioate backbone modification	Binding to heparin-binding proteins	Thrombocytopenia
Polyanionic nature of ASO	Binding of various proteins	Activation of complement
Adenosine	Activation of adenosine receptors	Bronchoconstriction
Delivery system	Cationic liposomes enhance immunostimulatory properties of ASOs	Release of cytokines
	Cationic liposomes affect cellular function	Interactions with cellular receptors and plasma proteins can cause various effects

1.3.3 Chemical modifications to improve antisense oligonucleotide technology

Chemical modifications of ASOs have been used to increase nuclease resistance and ASO-mRNA heteroduplex stability, as well as to decrease non-sequence specific toxicity (Dias and Stein, 2002). The modifications of ASOs are commonly categorised as first, second, or third generation (Figure 1.8).

1.3.3.1 First generation modifications

The main first generation modification was the PS-modified backbone, in which a sulphur atom replaced one of the non-bridging oxygen atoms in the phosphodiester bond (Figure 1.8). The PS-modification of ASOs still allows the RNase H-mediated degradation of the target mRNA (Chan et al., 2006). This category of modification significantly improved resistance to degradation by nucleases resulting in higher bioavailability of the ASO. In addition, by altering the charge of the ASO, PS-modifications permitted better longevity by elevating the binding to plasma proteins (Rinaldi and Wood, 2018, Chery, 2016, Watts and Corey, 2012). However, PS-modification can slightly decrease the ASO's affinity for the mRNA target, as the ASO-mRNA heteroduplex melting temperature is reduced by about 0.5°C per nucleotide. The PS-modification has also been reported to cause non-specific toxic effects by binding non-specifically to certain serum proteins.

1.3.3.2 Second generation modifications

Even though first generation modifications increased nuclease resistance and bioavailability, PS-modified ASOs normally bind less well to their target mRNA. This drawback was addressed by second generation modifications including OMe and 2'-*O*-methoxy-ethyl (MOE) substitutions of the 2'-position of the ribose sugar in the nucleotide (Figure 1.8) (Chery, 2016). These modifications improved the binding affinity to the target mRNA (Chery, 2016). Furthermore, second generation ASOs, containing a 2'-*O*-substituted nucleotide as well as a PS-backbone, have increased hybridisation affinity to their target mRNA, improved resistance towards degradation by nuclease, and decreased immunostimulatory activity, in comparison with their unmodified counterparts (Rinaldi and Wood, 2018).

1.3.3.3 Third generation modifications

To further augment ASO nuclease resistance, target affinity, and delivery to target sites, third generation ASOs have been designed predominantly by structural modifications of the furanose ring of the nucleotide. Locked nucleic acid (LNA), also known as 2'4'-methylene bridged nucleic acid, peptide nucleic acid (PNA), and phosphoroamidate morpholino oligomer (PMO) are the most common of third generation modifications of ASOs (Figure 1.8) (Chan et al., 2006). Third generation ASOs are not always substrates for RNase H and mainly act by steric blocking (Chan et al., 2006).

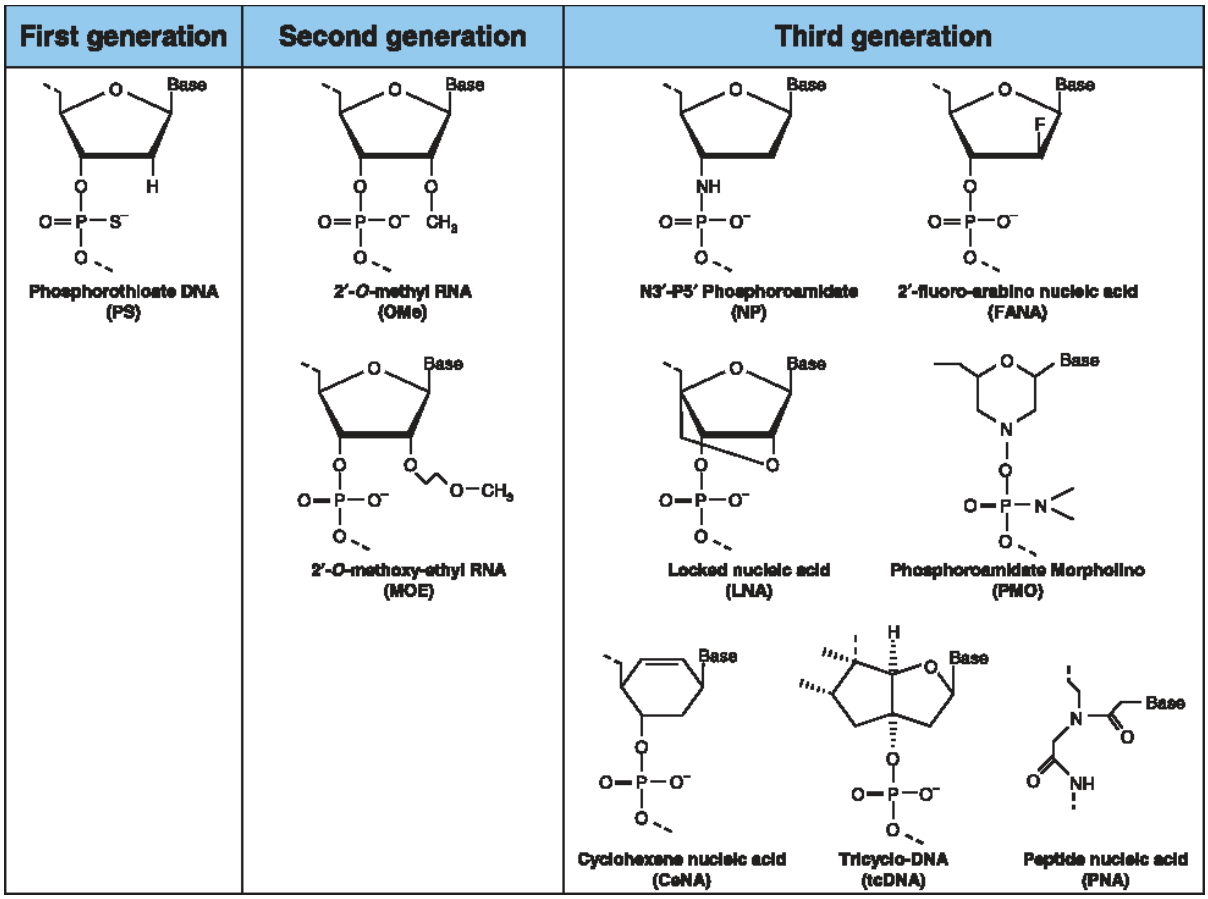


Figure 1.8: Chemical modifications of antisense oligonucleotides.

First generation antisense oligonucleotides (ASOs) are typically designed with phosphorothioate (PS) backbones or unmodified phosphodiester backbones. Second generation modifications are characteristically designed with 2'-*O*-methyl (OMe) and 2'-*O*-methoxy-ethyl (MOE) modifications. Third generation ASOs include variants such as the locked nucleic acid (LNA), the peptide nucleic acid (PNA), and the phosphoroamidate morpholino oligomer (PMO) modifications. The image, from a paper by Chan et al. (2006), was used with kind permission from John Wiley and Sons, Inc. (Hoboken, New Jersey, USA).

1.3.4 Delivery methods for antisense oligonucleotides

In general, there are two strategies for delivering ASOs into the cell. Firstly, structural modification of the ASO molecule itself, and secondly, the use of a carrier such as viral vectors, lipid-based agents, and receptor-targeted ASO-ligand conjugates (Juliano, 2016).

1.3.4.1 Viral vectors

There have been many research studies in the use of viral vectors in ASO delivery, and there are many examples of medication delivered in this manner (Juliano, 2016, Phillips, 1997). Viruses are considered appropriate for delivery owing to their extremely effectual transfection into cells. However, in spite of the increased efficiency, there are safety apprehensions that restrict their application such as inflammatory and immune response induction. Due to this, investigators are presently looking to non-viral approaches to deliver ASOs.

1.3.4.2 Lipid-based reagents

In order to protect ASOs and elevate their bioavailability, lipid-based delivery systems have been developed. Such delivery systems comprise of a lipid bilayer with a hydrophilic aqueous core, which can entrap anionic ASOs to create lipid nanoparticles called liposomes. The structure of liposomes permits them to fuse with cell membranes so as to release their ASO molecules into the cell (Thierry, 1997). Commercial reagents, such as Lipofectamine-2000™ (Invitrogen™), are most frequently used for transfection of cells in culture (Dalby et al., 2004). Even though the phospholipid bilayer of the liposomes has large utility in delivery and facilitates cellular internalisation of ASOs, it can cause non-specific influences on non-targeted cells with potential toxicity (Juliano, 2016). Furthermore, though the cationic nature of liposomes enables lipid nanoparticle formation, this might occur with other anionic molecules as well, for example negatively-charged proteins in the blood (Thierry, 1997).

1.3.4.3 Antibody-based and peptide-based conjugates

Antibody-based and peptide-based ASO conjugates have become an important avenue of research for ASO-based pharmacology. In such delivery systems, the ASO

molecules are delivered selectively into cells by receptor-mediated endocytosis, in most cases via using antibodies or small peptide-ligands.

Covalent conjugation of peptide ligands with ASO molecules can elicit an enhanced uptake by cells (Nakagawa et al., 2010). Nevertheless, ASO-peptide delivery has also met with drawbacks. Predominantly, it is hard to produce an ASO-peptide conjugate without adversely affecting gene-silencing efficiency or receptor interaction. Secondly, such molecules are comparatively small and are cleared quickly by the renal system (Juliano, 2016, Nakagawa et al., 2010).

Nucleic acid-antibody conjugates have been used in other gene-silencing approaches like small-interfering (si) RNAs. In such cases, antibodies are conjugated either to the RNA molecule itself or to its nanoparticle carrier, and thus can induce endocytosis via binding to a particular receptor on the cell surface (Cuellar et al., 2015). The use of ASO-antibody conjugates has been described by researchers from McGill University and the University of Toronto (Arnold et al., 2018). In this study, a conjugate ASO-antibody was developed in order to target and decrease the expression of *DRR/FAM107A* in patient-derived glioblastoma stem cells. The conjugate was successfully internalised, accumulated, and it decreased the expression of the target *DRR/FAM107A* (Arnold et al., 2018). This research also countered that the covalent binding of the delivery apparatus with an ASO can impede RNase H binding to the ASO-mRNA heteroduplex (Arnold et al., 2018, Lima et al., 2004).

1.3.5 Current uses of antisense oligonucleotides as therapeutics

Antisense oligonucleotides are being widely explored as therapeutics with several undergoing clinical trials and many have been approved for usage. Nine ASO drugs representing two mechanisms of action, four chemical classes, and four routes of delivery have been approved for use (Table 1.3) (Crooke et al., 2021a).

The second generation ASO Fomivirsen (Vitravene™) was the first ASO to be approved by the US Food and Drug Administration (FDA) for therapeutic use. In 1998, it was licensed for treating cytomegalovirus (CMV) retinitis and produced its effect by mRNA degradation via RNase H (Stein and Castanotto, 2017).

Nusinersen (Spinraza™) is a more contemporary example of the medical utilisation of antisense technology. This ASO was approved in 2017 for treating spinal muscular atrophy (SMA). It is an 18-nucleotide ASO with PS and MOE modifications and acts via modulating the splicing of survival of motor neuron (SMN) mRNA. Consequently, a functioning SMN protein is produced (Figure 1.9). The ASO has been confirmed as being extremely effective (Stein and Castanotto, 2017, Zanetta et al., 2014).

Even though all the approved ASO drugs are for usage in cases with uncommon diseases, the numerous ASOs are that are currently undergoing clinical development are intended for treating prevalent diseases. These include disorders affecting the nervous and cardiovascular systems as well as cancer and metabolic, muscle, lung, eye, and infectious diseases (Table 1.4) (Crooke et al., 2021a, Dhuri et al., 2020). Furthermore, there is a growing list of ASOs in clinical trials with a large number successfully attaining phase III clinical trials. These should deliver additional results in the future (Crooke et al., 2021a).

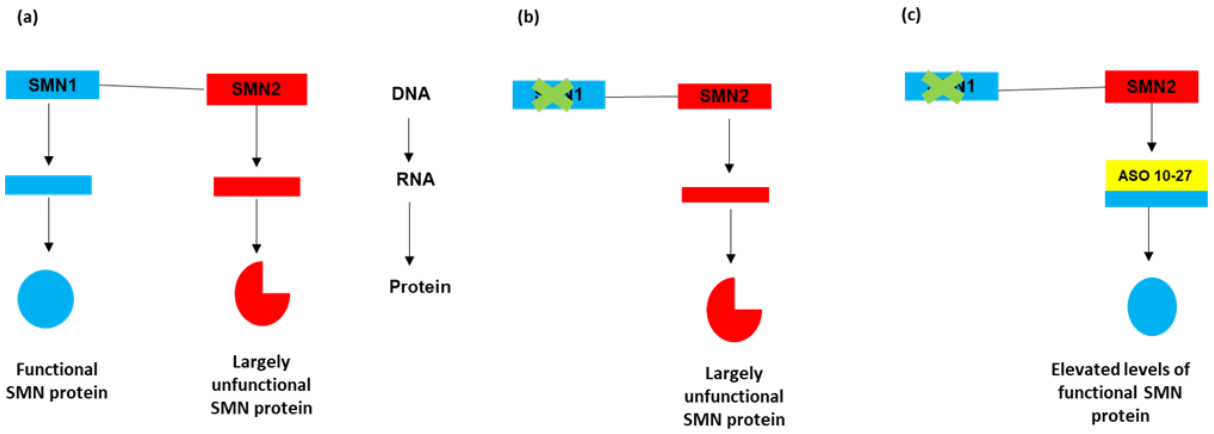


Figure 1.9: Spinraza™ antisense oligonucleotide mechanism for the treatment of spinal muscular atrophy.

There are normally two genes that produce the survival of motor neuron (SMN) protein, namely *SMN1* and *SMN2*. *SMN1* produces the full-length functioning SMN protein. *SMN2* largely produces a shorter unfunctional form of the protein; (b) In patients with spinal muscular atrophy (SMA), *SMN1* is either missing or mutated which leads to a large reduction in the amount of functioning SMN protein; (c) Antisense oligonucleotide 10-27 binds to *SMN2* mRNA and allows the inclusion of important regions needed to make the SMN protein. This leads to the production of more of the full-length SMN functional SMN protein.

Table 1.3: Approved antisense oligonucleotide drugs

ASO drug	Approval	Chemistry/ Action	Delivery route	Target tissue, gene, disease	Dosage	Reference
Casimersen (Amondys 45™)	FDA (2021)	PMO Exon-skipping	Intravenous	Muscle, Dystrophin exon 45, DMD	30 mg/kg once weekly	(Shirley, 2021)
Eteplirsen (Exondys 51™)	FDA (2016), EMA (2018)	PMO Exon-skipping	Intravenous	Muscle, Dystrophin exon 51, DMD	30 mg/kg once weekly	(Cirak et al., 2011)
Fomivirsen (Vitravene™)	FDA (1998), EMA (1999)	PS RNase H	Intravitreal	Eye, *CMVIE2*, CMV retinitis	330 µg per eye once every four weeks	(Vitravene-Study- Group, 2002a, Vitravene-Study- Group, 2002b, Vitravene-Study-Group, 2002c)
Golodirsen (Vyondys 53™)	FDA (2019)	PMO Exon-skipping	Intravenous	Muscle, Dystrophin exon 53, DMD	30 mg/kg once Weekly	(Frank et al., 2020)
Inotersen (Tegsedi™)	FDA (2018), EMA (2018)	PS-MOE RNase H	Subcutaneous	Liver, *TTR*, hATTR	300 mg once weekly	(Benson et al., 2018)
Mipomersen (Kynamro™)	FDA (2013)	PS-MOE RNase H	Subcutaneous	Liver, *APOB100*, HoFH	200 mg once weekly	(Duell and Jialal, 2016, Santos et al., 2015, Raal et al., 2010)
Nusinersen (Spinraza™)	FDA (2016), EMA (2017)	PS-MOE Exon-skipping	Intrathecal	CNS, *SMN2* intron 7, SMA	12 mg once every four months	(Mercuri et al., 2018, Finkel et al., 2017, Chiriboga et al., 2016)
Viltolarsen (Viltepso™)	FDA (2020)	PMO Exon-skipping	Intravenous	Muscle, Dystrophin exon 53, DMD	80 mg/kg once weekly	(Clemens et al., 2020)
Volanesorsen (Waylivra™)	EMA (2019)	PS-MOE RNase H	Subcutaneous	Liver, *APOC3*, familial chylomicronemia syndrome	300 mg once weekly	(Witztum et al., 2019)

APO, apolipoprotein; CMV, cytomegalovirus; CNS, central nervous system; DMD, Duchenne muscular dystrophy; EMA, European Medicines Agency; FDA, US Food and Drug Administration; hATTR, hereditary transthyretin-mediated amyloidosis; HoFH, homozygous familial hypercholesterolemia; IE2, viral transcription factor IE2; MOE, 2'-O-methoxy-ethyl; PMO, phosphoroamidate morpholino oligomer; PS, phosphorothioate; SMA, spinal muscular atrophy; SMN, survival of motor neuron; TTR, transthyretin.

Table 1.4: Antisense oligonucleotide drugs in clinical trials

ASO drug	Target gene, tissue	Chemistry	Delivery route	Indication	Reference
SIS-GCGRRx 02583919	*GCGR*, liver	PS-MOE	Subcutaneous	Type 2 diabetes	(ClinicalTrials.gov, 2018a)
IONIS-PTP1BRx	*PTP1B*, liver	PS-MOE	Subcutaneous	Type 2 diabetes (inactive)	(ClinicalTrials.gov, 2018a)
Apatorsen	*HSP27*, tumour cells	PS-MOE	Intravenous	Cancer (phase II)	(Chi et al., 2016)
Pelacarsen (TQJ230/AKCEA-APO(a)-LRx)	*APO*, liver	MOE-GalNAc	Subcutaneous	Cardiovascular disease (phase III)	(Tsimikas et al., 2020)
ISIS 104838	*TNF*, immune cells	PS-MOE	Subcutaneous	Inflammatory disease (terminated)	(Sewell et al., 2002)
GSK3228836/ IONIS-HBVRx	HBV RNAs, liver	PS-MOE	Subcutaneous	HBV, chronic atypical (phase II)	(Yuen et al., 2019)
ISIS681257 03070782	Lp(a), Liver	PS-MOE	Subcutaneous	Elevated lipoprotein(a), cardiovascular disease	(ClinicalTrials.gov, 2020a)
IONIS DGAT2Rx 03334214	*DGAT2*, liver	PS-MOE	Subcutaneous	Hepatic steatosis	(ClinicalTrials.gov, 2020b, Dhuri et al., 2020)
ISIS-FGFR4RX 02476019	*FGFR4*, fibroblasts	PS-MOE	Subcutaneous	Obesity	(Dhuri et al., 2020, ClinicalTrials.gov, 2018b)
IONIS-TTR RX 02175004	*TTR*, Liver	PS-MOE	Subcutaneous	Familial amyloid polyneuropathy	(ClinicalTrials.gov, 2021, Dhuri et al., 2020)
Tofersen 02623699	*SOD1*, CNS	PS-MOE	Intrathecal	Amyotrophic lateral sclerosis	(Miller et al., 2022, Dhuri et al., 2020)
Volanesorsen 02658175	*APOC3*, Liver	PS-MOE	Subcutaneous	Proteinemia type 1	(Dhuri et al., 2020, ClinicalTrials.gov, 2019)
Tominersen (RG6042/ISIS 443139)	*HTT*, CNS	PS-MOE	Intrathecal	Huntington's disease (phase III)	(Tabrizi et al., 2019)
BIIB080 (IONIS-MAPTRx)	*MAPT*, CNS	PS-MOE	Intrathecal	Alzheimer's disease, FTD (phase II)	(Crooke et al., 2021a)
BIIB094 (ION859)	*LRRK2*, CNS	PS-MOE	Intrathecal	Parkinson's disease (phase I)	(Crooke et al., 2021a)
Drisapersen	Dystrophin exon 51, muscle	PS-MOE	Subcutaneous	DMD (terminated)	(Crooke et al., 2021a, Goemans et al., 2016, Voit et al., 2014)
IONIS-FXIRx/ BAY2306001	*FXI*, liver	PS-MOE	Subcutaneous	Clotting disorders (phase II)	(Crooke et al., 2021a, Büller et al., 2015)

Atesidorsen/ ATL1103	*GHR*, liver	PS-MOE	Subcutaneous	Acromegaly (phase II)	(Trainer et al., 2018)

APO, apolipoprotein; CNS, central nervous system; DGAT, diacylglycerol transferase; DMD, Duchenne muscular dystrophy; FGFR4, fibroblast growth factor receptor 4; FTD, frontotemporal dementia; FXI, factor XI; GalNAc, N-acetylgalactosamine; GCGR, glucagon receptor; GHR, growth hormone receptor; HBV, hepatitis B virus; HSP, heat-shock protein; HTT, Huntingtin; Lp(a), lipoprotein(a); LRRK2, leucine rich repeat kinase 2; MAPT, microtubule associated protein tau; MOE, 2'-O-methoxy-ethyl; PS, phosphorothioate; PTP1B, protein tyrosine phosphatase 1B; SOD1, superoxide dismutase; TNF tumour necrosis factor; TTR, transthyretin.

1.4 The Current Project

1.4.1 Justification for the project

In CD, the high concentration of blood ACTH is caused by the over-expression of POMC in corticotroph adenomas. This excess of ACTH results in the over-production of cortisol by the adrenals with the consequent defining clinical features of weight gain, severe fatigue, muscle weakness, high blood pressure, depression, purplish skin striae, easy bruising, diabetes, and menstrual disorders. Although transsphenoidal surgery is the most effective treatment for CD, the condition can reoccur following removal of the pituitary adenoma. Furthermore, medical treatments are often accompanied by unpleasant side-effects and can leave patients deficient in other anterior pituitary hormones.

Consequently, there is an unmet clinical need for a medical therapy that can drastically decease the high levels of cortisol in patients with CD, and this need was emphasised in the international consensus statement on the treatment of ACTH-dependent Cushing's syndrome (Biller et al., 2008). An ideal therapeutic would have a rapid effect, resolve clinical features, be specific, minimise damage to other pituitary functions, normalise biochemical changes with minimal morbidity, and allow control of the disease in the long-term. Such requirements have led to the idea of selectively reducing circulating ACTH, and therefore cortisol, using a gene-silencing methodology such as small inhibitory (si)RNAs or ASOs.

1.4.2 Justification for the approach

There are now many reports on the successful use of ASOs to treat several different diseases (Section 1.3.5). With respect to endocrine disorders that require the reduction of hormone levels, siRNAs have been used to suppress parathyroid hormone production *in vitro* and *in vivo* in a preliminary study aimed at treating secondary hyperparathyroidism (Kanai et al., 2009). In addition, an antisense oligomer designed to inhibit translation of human growth hormone receptor mRNA has been successful in treating patients with acromegaly (Trainer et al., 2018).

There are also reports of ASOs that can cause a reduction in ACTH production by preventing the translation of *POMC*/*Pomc* mRNA. For example, transfection of AtT-20 cells, a mouse cell line that constitutively expresses *Pomc* and secretes ACTH (Schiller, 2000, Furth et al., 1953a), with ASOs against *Pomc* suppressed successfully the secretion of ACTH (Spampinato et al., 1994). When treated with the same ASOs, a similar effect was observed in a rat model (Spampinato et al., 1994). Furthermore, when human ACTH-secreting adenoma cells were transfected with anti-POMC ASOs (subsequently referred to as POMC ASOs in this study), *POMC* mRNA and ACTH levels were lowered by over 50% (Woloschak et al., 1994). However, so far, such ASOs have not been investigated further with respect to treating CD.

1.4.3 Hypothesis and aims of the project

Hypothesis: Antisense therapy can be used to treat CD by targeting antisense oligonucleotides (ASOs) against *POMC* mRNA and so reduce ACTH production.

Aims: The overall aim of this project was to investigate the applicability of gene-silencing technology as a treatment for CD by analysing the effects of POMC ASOs on the secretion of ACTH from mouse AtT-20 cells, these being used as the model *in vitro* system for CD. The project aimed to:

- Design ASOs against *Pomc* mRNA.
- Assess the effects of different POMC ASOs on the secretion of ACTH from AtT-20 cells.
- Determine the lowest effective POMC ASO concentration and POMC ASO longevity of action.
- Investigate the resistance of POMC ASOs to nuclease degradation.
- Analyse the effects of POMC ASOs on the immune response.
- Analyse effects of POMC ASOs on cell viability.

Chapter 2

Materials and Methods

2 Materials and Methods

Most of the methods used in this work were carried out in accordance with Dr Helen Kemp's protocols (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK).

2.1 Plasticware

Plasticware comprising tissue culture plates and flasks, 25-ml Universal tubes, 0.5-ml and 1.5-ml Eppendorf tubes, 50-ml and 10-ml centrifuge tubes, pipettes, and pipette tips were purchased from Starlab (UK) Ltd. (Milton Keynes, UK), Sarstedt Ltd. (Numbrecht, Germany), Nalgene Nunc International (Rochester, NY, USA), Corning Inc., (Corning, NY, USA), or Bibby Sterilin Ltd. (Bargoed, UK).

2.2 Reagents

Most of the reagents used in this project, comprising chemicals, solvents, buffers, acids, and components of media were bought from Melford Laboratories (Ipswich, UK), Sigma-Aldrich (Poole, UK), or Fisher Scientific UK Ltd. (Loughborough, UK). Usually, they were of molecular biology or analytical grade. The origin of some reagents is given in the text

2.3 Mammalian cell culture

2.3.1 Cell line

Mouse AtT-20/D16v-F2 (AtT-20) adhesive cells were obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA). They secrete ACTH when cultured. With respect to the origin of the AtT-20 cell line, initially, a mouse pituitary tumour was established in LAF1 mice (Furth et al., 1953). The AtT-20 cell line was then cloned from cell cultures that were established following alternate passaging of the mouse pituitary tumour cells via animals and cell culture (Yasumura et al., 1966).

2.3.2 Cell culture medium

The AtT-20 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMAX™ supplement and 4.5 g/L D-glucose (Life Technologies Ltd.,

Paisley, UK). Also added to the medium were 10% foetal bovine serum (Labtech, Heathfield, UK), 50 µg/ml of streptomycin (Life Technologies Ltd.) and 50 units/ml of penicillin (Life Technologies Ltd.).

2.3.3 Cell culturing and passaging

Initially, a cryovial of cells was taken from -80°C storage and thawed in a water bath at 37°C. After adding 1 ml of warm cell culture medium to the thawed cells, they were transferred to 10 ml of culture medium in a Universal tube, and centrifuged at 1000 revolutions per minute (rpm) for 5 min in a MSE Sanyo Harrier 18/80 refrigerated centrifuge (MSE UK Ltd., London, UK). The supernatant was discarded, and the cell pellet was resuspended in 5 ml of cell culture medium. A 10-ml volume of fresh culture medium was added to a T75 culture flask and the resuspended cells were transferred to the flask. The cells were subsequently incubated in a humidified Sanyo MCO-20AIC CO2 Incubator (Sanyo Electric Co. Ltd., Osaka, Japan) in 5% CO_2 at 37°C. The cells underwent at least two passages before being utilised in experiments.

Cells were passaged every 3-4 days to prevent them from becoming fully confluent. To passage cells, the culture medium was removed from the cells grown in a T75 flask. The cells were then washed in phosphate-buffered saline (PBS) (pH 7.4). A 1-ml of aliquot of Trypsin (0.05%)-EDTA (0.02%) Solution (Sigma-Aldrich) was added to lift the adhered cells from the bottom surface of the flask. The flask was then incubated at 37°C for a maximum of 3 min. The dislodged cells were resuspended in 10 ml of culture medium and then transferred to a Universal tube, before being centrifuged at 1000 rpm for 5 min. The supernatant was removed from the cells, and the cell pellet resuspended in 10 ml of culture medium. A 2-ml aliquot of the cell suspension was transferred to a clean T75 flask and a suitable volume of growth medium added to dilute the cells by 1:4 or 1:5. The flask was placed in a humidified incubator with 5% CO_2 at 37°C.

2.3.4 Cell counting

If cell counting was required, cells were harvested and resuspended in culture medium, as detailed above. A 50-µl sample of Trypan Blue Stain (Sigma-Aldrich), a stain that colours dead cells blue, was then mixed with 50 µl of the cell suspension. A

drop of the stained cell suspension was then pipetted under a haemocytometer cover slip. To count unstained viable cells, the haemocytometer was viewed under a light microscope. The cell count from five squares (middle square and the four corner squares) was averaged and then doubled to take into account the 1:1 dilution factor in the Trypan Blue Stain. The number of cells was multiplied by 10^4, to give the number of viable cells per ml.

2.3.5 Cell freezing

To store cells long-term at -80°C, approximately 1 x 10^7 cells were resuspended in 1-2 ml of freezing mixture that consisted of 10% dimethyl sulfoxide in foetal bovine serum. The cells were then transferred to a cryovial ready for storage.

2.3.6 Cell images

Cultured cells were viewed using a Miotic AE2000 inverted microscope (Miotic Incorporation Ltd., Kowloon, Hong Kong). Images were recorded using a Ceti 5 Mpx Digi-Pad microscope tablet camera (Medline Scientific Ltd., Chalgrove, UK).

2.4 Antisense oligonucleotides

The ASO design tool *S*fold-*S*oligo (Wadsworth Center, New York State Department of Health, Albany, NY, USA) (https://sfold.wadsworth.org/cgi-bin/index.pl) (Ding et al., 2004) and OligoAnalyzer (Integrated DNA Technologies, Inc., Coralville, IA, USA) (https://eu.idtdna.com) were used to assist with the design of candidate POMC ASOs. Scrambled POMC ASO sequences were generated using the online tool at GeneScript Biotech Corp (Piscataway, NJ, USA) (https://www.genscript.com).

The ASOs used in this project are listed in Table 2.1. They were obtained from GeneLink Inc. (Hawthorne, NY, USA) or from Qiagen (Hilden, Germany). The ASOs were received as lyophilised samples and were resuspended in sterile water to a concentration of 100 µM (100 pmol/µl) and stored at -20°C until required.

Table 2.1: Antisense oligonucleotides used in the study

ASO name[1]	Sequence[2]
POMC ASO2	5’-GCTCTTCTCGGAGGTCATGA-3’
POMC ASO3	5’-GTTCTTGATGATGGCGTTC-3’
POMC ASO5	5’-GAAGTGACCCATGACGTAC-3’
POMC ASO8	5’-GTAGCAGAATCTCGGCATC-3’
POMC ASO2-PS	5’-G*C*T*C*T*T*C*T*C*G*G*A*G*G*T*C*A*T*G*A-3’
POMC ASO3-PS	5’-G*T*T*C*T*T*G*A*T*G*A*T*G*G*C*G*T*T*C-3’
POMC ASO5-PS	5’-G*A*A*G*T*G*A*C*C*C*A*T*G*A*C*G*T*A*C-3’
POMC ASO8-PS	5’-G*T*A*G*C*A*G*A*A*T*C*T*C*G*G*C*A*T*C-3’
POMC ASO2-OMe	5’-[mG]*[mC]*[mT]*[mC]*[mT]*T*C*T*C*G*G*A*G*G*T*[mC]*[mA]*[mT]*[mG]*[mA]-3’
POMC ASO3-OMe	5’-[mG]*[mT]*[mT]*[mC]*[mT]*T*G*A*T*G*A*T*G*G*[mC]*[mG]*[mT]*[mT]*[mC]-3’
POMC ASO5-OMe	5’-[mG]*[mA]*[mA]*[mG]*[mT]*G*A*C*C*C*A*T*G*A*[mC]*[mG]*[mT]*[mA]*[mC]-3’
POMC ASO8-OMe	5’-[mG]*[mT]*[mA]*[mG]*[mC]*A*G*A*A*T*C*T*C*G*[mG]*[mC]*[mA]*[mT]*[mC]-3’
POMC ASO2-LNA	5’-[G]*[C]*[T]*[C]*[T]*T*C*T*C*G*G*A*G*G*T*[C]*[A]*[T]*[G]*[A]-3’
POMC ASO3-LNA	5’-[G]*[T]*[T]*[C]*[T]*T*G*A*T*G*A*T*G*G*[C]*[G]*[T]*[T]*[C]-3’
POMC ASO5-LNA	5’-[G]*[A]*[A]*[G]*[T]*G*A*C*C*C*A*T*G*A*[C]*[G]*[T]*[A]*[C]-3’
POMC ASO8-LNA	5’-[G]*[T]*[A]*[G]*[C]*A*G*A*A*T*C*T*C*G*[G]*[C]*[A]*[T]*[C]-3’

[1]ASO, antisense oligonucleotide; LNA, locked nucleic acid; OMe, 2’-*O*-methyl; PS, phosphorothioate.

[2][mA] [mC] [mG] or [mT], 2’-*O*-methyl modification; [A] [C] [G] or [T], LNA modification; *, phosphorothioate linkage

2.5 Transfection of AtT-20 cells

Transfections were undertaken using Lipofectamine®-2000 Reagent. This reagent is a cationic lipid that can be used to facilitate nucleic acid delivery into cells (Figure 2.1). Cationic lipids consist of a positively charged head group and one or two hydrocarbon chains and are able to form lipid bilayer structures called liposomes. Liposomes can be used to entrap nucleic acid molecules due to interaction between the positively charged lipid head group and the negatively charged phosphate backbone of the nucleic acid. The positively charged surface of liposomes can interact with negatively charged cell membranes allowing for fusion of the liposome/nucleic acid complex with the cell membrane. The transfection complex then enters the cell through endocytosis forming a membrane surrounded-intracellular vesicle. Inside the cell, the complex escapes the endosomal pathway and nucleic acid is released into the cell cytoplasm.

2.5.1 Transfection protocol

To carry out transfections of AtT-20 cells, the cells were plated in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml of antibiotic-free culture medium. The cells were incubated at 37°C in a 5% CO_2 humidified incubator for 24 h. This gave a cell confluency of approximately 80%. The culture medium in each well was replaced with 500 µl of Opti-MEM® reduced serum medium (Life Technologies Ltd.) and 1 ml of fresh antibiotic-free culture medium per well. The cells were placed back into the incubator until needed for transfection.

To transfect AtT-20 cells with the desired ASO (Tables 2.1 and 2.2), cells were treated in duplicate with 500 µl of the required ASO made in a solution of Lipofectamine®-2000 Reagent (Life Technologies Ltd.) and Opti-MEM®. In the 500-µl sample, the ASO was at a concentration that would give the final required ASO concentration (e.g., 100 nM) once added to the cells in 1.5 ml of culture medium. Equally, Lipofectamine®-2000 Reagent was at a dilution in the 500-µl sample that would give a final dilution on the cells of 1:200, as recommended by the manufacturer.

Cells treated with ASO alone, to assess the transfer efficiency of the ASO in the absence of Lipofectamine®-2000 Reagent, were included in all experiments. Cells treated with Lipofectamine®-2000 Reagent alone, to assess any cytotoxic effects of

the reagent, were also included in all experiments. Untreated cells were also includedin all experiments to measure the baseline secretion of ACTH from the AtT-20 cells. All cells were placed in a 5% CO_2 humidified incubator for 24 h (or to the required time point) at 37°C, prior to various analyses.

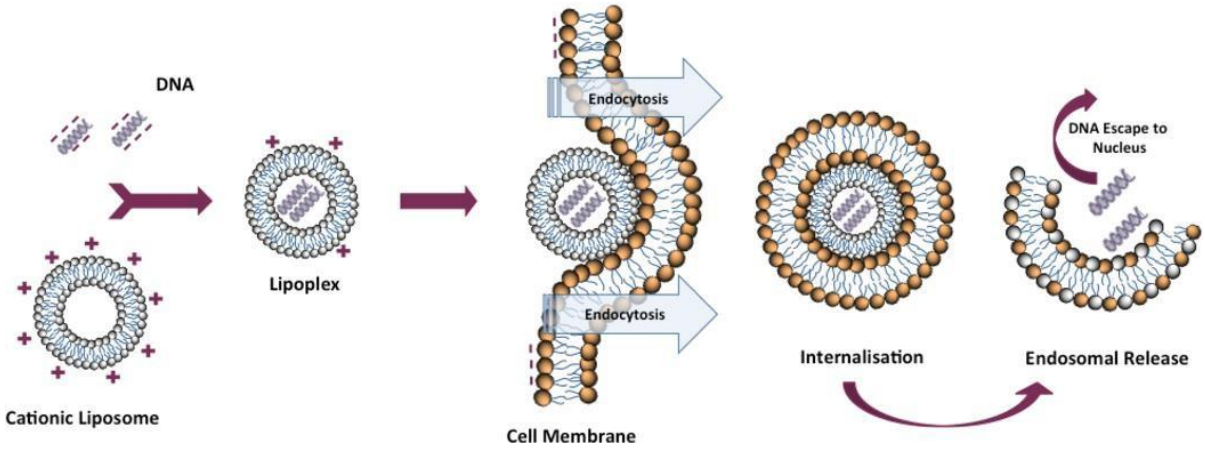


Figure 2.1: Principle of Lipofectamine®-2000-mediated transfection.

Transfections were undertaken using Lipofectamine®-2000 Reagent. This reagent is a cationic lipid that can be used to facilitate nucleic acid delivery into cells. Cationic lipids consist of a positively charged head group and one or two hydrocarbon chains that are able to form lipid bilayer structures called liposomes. Liposomes can be used to entrap nucleic acid molecules due to interaction between the positively charged lipid head group and the negatively charged phosphate backbone of the nucleic acid. The positively charged surface of liposomes can interact with negatively charged cell membranes allowing for fusion of the liposome/nucleic acid complex with the cell membrane. The transfection complex then enters the cell through endocytosis forming a membrane surrounded-intracellular vesicle. Inside the cell, the complex escapes the endosomal pathway and nucleic acid is released into the cell cytoplasm. The image, from a thesis by Moghaddam (2013), is used with kind permission from Aston University (Birmingham, UK).

2.5.2 Transfection efficiency

The efficiency of transfection was determined by transfecting AtT-20 cells with BLOCK-IT™ Fluorescent Oligo (Life Technologies Ltd.), a fluorescein isothiocyanate (FITC)-labelled oligonucleotide, followed by analysis using fluorescence-activated cell sorting (FACS) analysis (Figure 2.2). This technique provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. At the same time, the cells are counted so the proportion of fluorescently-labelled and unlabelled cells can be determined and thus the proportion of cells that have been transfected can be calculated.

As detailed in Section 2.5.1, AtT-20 cells were transfected with BLOCK-IT™ Fluorescent Oligo at a final concentration of 100 nM using Lipofectamine®-2000 Reagent. Controls included cells treated with BLOCK-IT™ Fluorescent Oligo alone or Lipofectamine®-2000 Reagent alone. Untreated cells were also included in the experiments.

After transfection and following 24 h of incubation, the culture medium was removed from the cells. Subsequently, they were washed with 500 µl of PBS, and then treated with Trypsin-EDTA Solution for 3 min at 37°C. The cells were resuspended in 500 µl of antibiotic-free medium before centrifugation in an Eppendorf MiniSpin microcentrifuge at 10,000 rpm for 10 min to pellet the cells. After centrifugation, the supernatant was removed and the cell pellet resuspended in 500 µl of 4% paraformaldehyde fixation buffer (Biolegend®, San Diego, CA, USA) and transferred to a Falcon tube. Tubes were covered with aluminium foil and stored at 4°C until required for FACS analysis using a BD LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

The flow cytometer was calibrated by running a sample of untreated AtT-20 cells to define the cell size as well as to standardise the intensity of background fluorescence. Gating by the FACS analysis software was set to include only viable single cells and to eliminate debris, dead cells, and cell clumps or doublets. From the FACS, the percentage of the total cells that fluoresced was calculated and this defined the transfection efficiency.

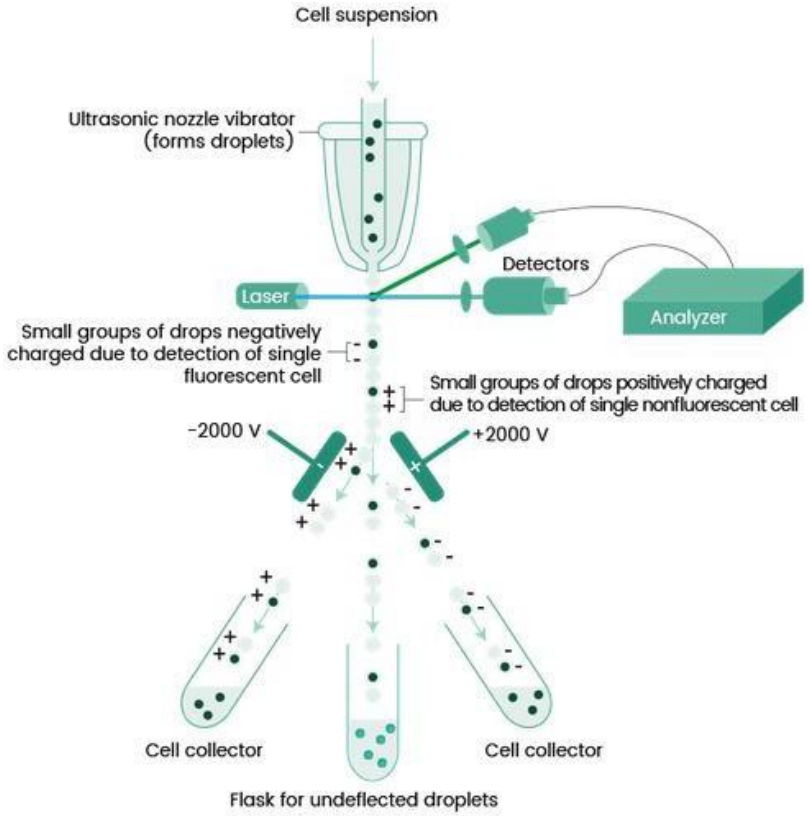


Figure 2.2: Principle of fluorescence-activated cell sorting.

The fluorescence-activated cell sorting technique provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. At the same time, the cells are counted so the proportion of fluorescently-labelled and unlabelled cells can be determined and thus the proportion of cells that have been transfected can be calculated. The image is used with kind permission from Sino Biological (Eschborn, Germany).

2.6 ACTH immunoassays

The concentration of ACTH in AtT-20 cell culture supernatants was detected using an Immulite 2000® ACTH immunoassay (Siemens Healthcare, Camberley, UK). This is a type of sandwich ELISA that uses two different anti-ACTH antibodies (Figure 2.3). Firstly, a monoclonal mouse anti-ACTH (24-39) antibody, which is coated around beads, acts as the capture antibody attaching to ACTH in the sample. Secondly, a polyclonal rabbit anti-ACTH (1-24) antibody attaches to a different part of ACTH to form a sandwich. The polyclonal antibody is attached also to alkaline phosphatase, an enzyme that causes the dephosphorylation of the chemiluminescent substrate, adamantyl dioxetane phosphate. The resultant unstable 1,2-dioxetane decomposes further and emits a glow of light (lambda max 470 nm), which is detected within the Immulite 2000 XPi Immunoassay System (Siemens Healthcare). The light emitted is converted to a pg/ml ACTH value by means of a standard curve.

The specifications of the immunoassay were: intra-assay precision of 6.7-9.5%; inter-assay precision of 6.1-10%; assay range 5-1250 pg/ml; sensitivity, 5 pg/ml; recovery, 89-111%; linearity, 90-109%; and specificity assessed as no reaction with MSH, ACTH (1-18), or ACTH (1-24).

After transfection at 24 h, a 30-µl sample of the culture medium was taken from each plate well. The samples were stored immediately at -80°C. When required for immunoassay, samples were thawed quickly and then diluted in 100 mM phosphate buffer (pH 4.0) to 1:100. In most samples, this dilution factor provided an ACTH concentration that came within the 5-1250 pg/ml range of the assay. Samples (150 µl) were tested at the Clinical Chemistry Laboratory at the Sheffield Teaching Hospitals NHS Foundation Trust (Sheffield, UK). Output concentrations of ACTH were as pg/ml.

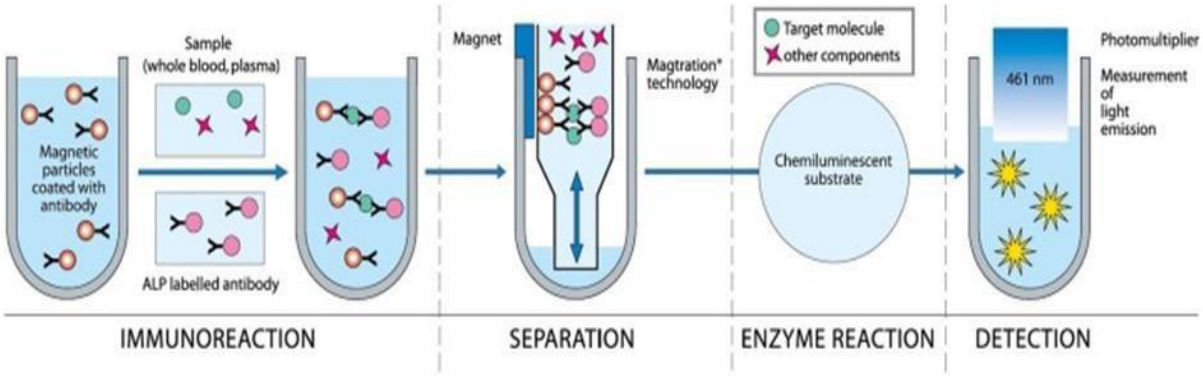


Figure 2.3: Principle of the Immulite 2000® ACTH immunoassay.

The Immulite 2000® ACTH immunoassay is a type of sandwich ELISA that uses two different anti-ACTH antibodies. Firstly, a monoclonal mouse anti-ACTH (24-39) antibody, which is coated around magnetic beads, acts as the capture antibody attaching to ACTH in the sample. Secondly, a polyclonal rabbit anti-ACTH (1-24) antibody attaches to a different part of ACTH to form a sandwich. The polyclonal antibody is attached also to alkaline phosphatase (ALP), an enzyme that causes the dephosphorylation of the chemiluminescent substrate, adamantyl dioxetane phosphate. The resultant unstable 1,2-dioxetane decomposes further and emits a glow of light, which is detected within the Immulite 2000 XPi Immunoassay System. The light emitted is converted to a pg/ml ACTH value by means of a standard curve. The image, from a paper by (Savonnet et al., 2021), was used with kind permission from Elsevier Ltd. (Cambridge, UK).

2.7 Interferon ELISAs

AtT-20 cells were transfected with the required ASOs at 100 nM as per the transfection protocol (Section 2.5.1). In addition, CpG oligonucleotides (Table 2.2) (SYNBIO Technologies, Monmouth Junction, NJ, USA) were employed as positive controls for stimulating the innate immune response, and were also used at 100 nM. Controls included untreated cells, cells transfected with ASOs or CpG oligonucleotides alone, and cells transfected with transfection reagent only. At 24 h and 48 h after transfection, 0.5-ml samples of the cell culture medium were collected and stored at -80°C ready for interferon measurement.

The concentration of interferons in AtT-20 cell culture medium was measured using VeriKine™ Mouse Interferon (IFN)-α and Mouse IFN-β ELISA Kits (PBL Assay Science, Piscataway, NJ, USA). The assays were a sandwich ELISA format and were designed to measure IFN-α or IFN-β in cell culture media. Briefly, IFN-α or IFN-β, contained in samples, was bound to a specific anti-IFN-α or anti-IFN-β antibody, respectively, that was coated onto 96-well plate wells. The binding was detected using a secondary antibody followed by a horse-radish peroxidase (HRP)-streptavidin conjugate. The binding of the conjugate was then detected by adding the HRP chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB). This formed a blue product that became yellow on the addition of acid with an absorbance peak at 450 nm. The parameters of both ELISAs are listed in Table 2.3.

The standards of IFN-α (12.5-400 pg/ml) and IFN–β (15.6-1000 pg/ml) were made up in culture medium. Wash buffer, secondary antibody, and HRP-streptavidin conjugate were all made according to the manufacturer’s protocol. Test samples were diluted in culture medium, if required, to bring the IFN-α and IFN-β concentrations to within the range of the ELISAs (Table 2.3).

To assay IFN-α, 100 µl of standards, samples, and blanks (zero concentration) were added to wells in duplicate. A 50-µl aliquot of secondary antibody was then applied. The ELISA plate was incubated for 1 h at room temperature with shaking. Incubation was then continued at 4°C for 24 h without shaking. Plate wells were washed four times with 300 µl of wash buffer. Then, 100 µl of HRP-streptavidin conjugate were added to each well. The ELISA plate was incubated at room temperature for 2 h.

Afterwards, the plate wells were washed four times with wash buffer and then 100 µl of TMB substrate (TMB with < 1% hydrogen peroxide) were added. The ELISA plate was incubated in the dark at room temperature for 15 min. Subsequently, 100 µl of stop solution were added to each plate well. Within 5 min of stopping the reaction, the absorbance at 450 nm was determined using a Labtech LT-4500 Microplate Reader (Labtech International Ltd., Lewes, UK) and its associated data analysis software (Labtech International Ltd.).

The method was the same for measuring IFN-β, except that the ELISA was carried out within the same day.

The average absorbance of the duplicate wells for each of the standards, samples, and blanks was determined. The average blank absorbance value was then subtracted from the standard and sample absorbance values. Using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA), the log10 values of the IFN-α and IFN-β standard concentrations were plotted against their absorbance values to give a 4-parameter logistic standard curve. The absorbance of each of the test samples was then interpolated to the standard curve to find the log10 values of the IFN-α or IFN-β concentration. The log10 values were then transformed to give an IFN-α or IFN-β concentration in pg/ml.

2.8 Pro-inflammatory cytokine ELISAs

Initially, AtT-20 cells were transfected with the required ASOs and CpG oligonucleotides at 100 nM, exactly as described in Section 2.7. Again, 0.5-ml samples at 24 h and 48 h after transfection were collected and stored at -80°C ready for pro-inflammatory cytokine measurement.

The concentration of pro-inflammatory cytokines in AtT-20 cell culture medium was measured using either Mouse Interleukin (IL)-1β, IL-6, or Tumour Necrosis Factor (TNF)-α Immunoassay Quantikine ELISAs (R&D Systems, Inc., Minneapolis, MN, USA). The assays used a sandwich ELISA format. Cytokines, either IL-1β, IL-6, or TNF-α present in samples, bound to a specific anti-IL-1β, anti-IL-6, or anti-TNF-α antibody present on the wells of a 96-well plate. The binding of IL-1β, IL-6, or TNF-α was then detected using a HRP-conjugated secondary antibody. Conjugate binding

was detected by HRP substrate TMB that becomes yellow on acidification with an absorbance peak at 450 nm. The parameters of each of the cytokine ELISAs are given in Table 2.3.

For the ELISAs, standards of IL-1β (12.5-800 pg/ml), IL-6 (7.8-500 pg/ml), and TNF-α (10.9-700 pg/ml) were prepared in Calibrator Diluent RD5T. Wash buffer and substrate were made as per the manufacturer's protocol. The samples to be tested were diluted in culture medium, if required, to bring the IL-1β, IL-6, or TNF-α concentrations to within the range of the ELISAs (Table 2.3).

To assay IL-1β, IL-6, or TNF-α, 50 µl of Assay Diluent RD1N were added to each well. Then, 50 µl of standards, samples, and blanks were added to wells in duplicate. The plate was incubated for 2 h at room temperature. The wells were washed four times with 400 µl of wash solution before adding 100 µl of HRP-conjugated secondary antibody to each well. The plate was incubated for 2 h at room temperature. Following this, the wells were washed four times with wash solution and then 100 µl of TMB substrate solution were added to each well. The plate was incubated in the dark at room temperature for 30 min, and then 100 µl of stop solution were added to each well. The absorbance at 450 nm was determined using a Labtech LT-4500 Microplate Reader and its associated data analysis software within 30 min of stopping the reaction. The absorbance data were processed as for the interferon ELISAs (Section 2.7) to give an IL-1β, IL-6, or TNF-α concentration in the test samples in pg/ml.

Table 2.2: CpG oligonucleotides and their control oligonucleotides

CpG oligonucleotide[1]	Sequence[2,3]
CpG-1585	5’-G*G*GGTCAA**CG**TTGA*G*G*G*G*G*G-3’
CpG-1585-C	5’-G*G*GGTCAAGCTTGA*G*G*G*G*G*G-3’
CpG-1668	5’-T*C*C*A*T*G*A***C*G***T*T*C*C*T*G*A*T*G*C*T-3’
CpG-1668-C	5’-T*C*C*A*T*G*A*G*C*T*T*C*C*T*G*A*T*G*C*T-3’
CpG-2395	5’-T***C*G***T***C*G***T*T*T*T*T***C*G***G*T***C*G*C*G***T***C*G***C*T***C*G**-3’
CpG-2395-C	5’-T*G*C*T*G*C*T*T*T*T*T*G*G*G*G*G*G*C*C*C*C*C*C*C-3’

[1]C, control oligonucleotides.

[2]*, phosphorothioate linkage.

[3]CG sequences are in bold.

Table 2.3: Interferon and pro-inflammatory cytokine ELISAs

Mouse ELISA[1]	Range (pg/ml)	Sensitivity (pg/ml)	Intra-assay precision (% coefficient of variation)	Inter-assay precision (% coefficient of variation)	Recovery yield (%)
IFN-α	12.5-400	< 12.5	≤ 10.0	≤ 10.0	≥ 94
IFN-β	15.0-1000	< 15.6	≤ 8.0	≤ 8.0	82-135
IL-1β	12.5-800	2.3	3.0-7.5	5.7-8.4	95-119
IL-6	7.8-500	1.6	3.5-6.7	6.2-8.8	86-120
TNF-α	10.9-700	1.9	2.7-3.1	6.2-8.8	94-111

[1]VeriKine™ Mouse Interferon (IFN)-α or Mouse IFN-β ELISA Kit (PBL Assay Science, Piscataway, NJ, USA). Mouse Interleukin (IL)-1β, IL-6, or Tumour Necrosis Factor (TNF)-α Immunoassay Quantikine ELISA (R&D Systems, Inc., Minneapolis, MN, USA).

2.9 RNA purification

2.9.1 RNA preparation method

A RNeasy Mini Kit (Qiagen, Manchester, UK) was used to isolate total RNA from AtT-20 cells, according to the manufacturer's protocol. In brief, 1 x 10^7 of freshly grown AtT-20 cells were harvested. The cell pellet, or cell pellets that had been stored at -80°C, was resuspended in 700 µl of RLT lysis buffer that contained a high concentration of guanidine isothiocycanate, which supports the binding of RNA to silica membranes, and 14 mM dithiothreitol, which inactivates RNAses. The cell lysate was homogenised by passing it five times through a 20-gauge (0.9-mm) needle using a 2-ml syringe. This process was required to decrease the viscosity of the cell lysate. For isolating total RNA from a smaller number (1 x 10^6) of cells, 350 µl of RLT lysis buffer were used in the initial lysis step.

A volume of 700 µl of 70% ethanol was added to the cell lysate, which was then transferred to and split between two RNeasy Mini spin columns that were held in 1.5-ml collection tubes. The columns were centrifuged for 15 sec at 10,000 rpm in an Eppendorf MiniSpin microcentrifuge (Eppendorf AG, Hamburg, Germany). The flow through in the collection tubes discarded. The spin columns were then washed with 700 µl of RW1 buffer, which contained a low concentration of guanidine isothiocyanate, by centrifuging for 15 sec at 10,000 rpm. Again, the flow through was discarded.

Subsequently, each column was washed with 500 µl of RPE buffer that 80% contained ethanol by centrifuging for 15 sec at 10,000 rpm. A second wash with a further 500 µl of RPE buffer and centrifugation of the columns for 2 min at 10,000 rpm was then carried out. Finally, the spin columns were centrifuged at 10,000 rpm for 1 min to dry the silica membranes to which the RNA was bound. The spin columns were transferred into clean 1.5-ml collection tubes. To elute the RNA from the silica membranes, 50 µl of RNase-free water were added to the columns, which were centrifuged for 1 min at 10,000 rpm.

2.9.2 DNase treatment of RNA

To remove any contaminating DNA from RNA samples, the eluted RNA was treated with a TURBO DNA-free Kit (Life Technologies Ltd.), as directed by the manufacturer'smethod. To the RNA sample, a 0.1 volume of 10x TURBO DNase buffer and 1 µl of TURBO DNase enzyme were added and mixed gently. The RNA sample was then incubated at 37°C for 30 min. Subsequently, a 0.1 volume of DNase Inactivation Reagent was added to the RNA. The RNA sample was incubated for 5 min at room temperature with mixing occasionally before centrifugation at 10,000 rpm for 1.5 min in an Eppendorf MiniSpin microcentrifuge. The RNA was transferred to a clean 1.5-ml tube and stored at -80°C.

2.9.3 Analysis of RNA integrity, quantity, and purity

Qualitative analysis of RNA was undertaken using agarose gel electrophoresis (Section 2.12). To quantify the concentration of RNA by way of absorbance spectroscopy at 260 nm, a NanoDrop ND-1000 spectrophotometer (Labtech, Wilmington, DE, USA) was used according to the manufacturer's instructions. The read out was given in ng/µl. The purity of the RNA samples was assessed by the absorbance ratio at 260 nm to 280 nm.

2.10 cDNA preparation

The preparation of cDNA from total RNA was carried out according to a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Reverse transcription reactions were set up on ice in 0.5-ml tubes as shown in Table 2.4. They consisted of up to 2 µg of total RNA and included reverse transcriptase (RT) buffer, random primers, deoxynucleotide triphosphates (dNTPs), MultiScribe™ RT, and RNase Inhibitor in a total reaction volume of 20 µl. A second reaction was set up as a control without any RT added (Table 2.4). After mixing gently, the reactions were incubated in a Techne TC-312 thermal cycler (GeneFlow, Lichfield, UK) for 10 min at 25ºC, followed by 37ºC for 2 h, then 85ºC for 5 min to inactivate the RT, and finally a hold at 4ºC. The RT reactions were stored at -20ºC until needed for polymerase chain reaction (PCR) amplification.

Table 2.4: Reverse transcription reactions

Reaction component[1]	Amount per reaction with RT (µl)	Amount per reaction without RT (µl)	Final concentration
10x RT buffer	2.0	2.0	1x
25x dNTP mix (100 mM)	0.8	0.8	4 mM
10x random primers	2.0	2.0	1x
MultiScribe™ RT (50 units/µl)	1.0	0.0	2.5 units/µl
RNase inhibitor (20 units/µl)	1.0	1.0	1.0 unit/µl
Nuclease-free water	3.2	4.2	-
Total RNA (up to 2 µg)	10.0	10.0	100 ng/µl
Total volume	**20.0**	**20.0**	**-**

[1]All components were from a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) except for RNase inhibitor, which was from Promega (Southampton, UK), and RNA, which was prepared as required. dNTP, deoxynucleotide triphosphate; RT, reverse transcriptase.

2.11 Polymerase chain reaction amplification

The forward and reverse primers for PCR amplification are listed in Table 2.5. They were synthesised to order by Eurofins Genetic Services Ltd. (London, UK). Until required, primers were stored at -40°C in nuclease-free water at a 100 pmol/µl concentration.

2.11.1 Reactions

Polymerase chain reaction (PCR) was used to amplify the required DNA fragments from the RT reactions containing cDNA (Section 2.10). All reactions were set up on ice in 0.2-ml PCR tubes in a total volume of 50 µl (Table 2.6). A reaction without cDNA was included, to check that there was no contaminating DNA in the original RNA preparations, which might act as a template for the PCR process. A control without any template DNA, in which nuclease-free water was used instead of any RT reaction, was used to verify that the PCR components were not DNA-contaminated. The primers used in PCR amplification are given in Table 2.5.

Once the reactions had been set up, PCR was carried out in a Techne TC-312 thermal cycler (GeneFlow). Usually, the cycling conditions were an initial denaturation step at 94°C for 10 min, then denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and an extension at 72°C for 1 min. These steps were repeated for 35 cycles. A final extension at 72°C for 5 min was used to complete the reactions before a hold at 10°C. PCR products were analysed by agarose gel electrophoresis (Section 2.12).

Table 2.5: Primers for polymerase chain reaction amplification and DNA sequencing

Primer	Sequence	Details	Source
POMC-Forward	5'-GAGAGCAACCTGCTGGCTTGC-3'	POMC primers were intron-spanning. They amplified a 514-base pair fragment of *Pomc*.	Eurofins (London, UK)
POMC-Reverse	5'-AGGTCATGAAGCCACCGTAACG-3'		
S15-Forward	5'-TTCCGCAAGTTCACCTACC-3'	Control primers were intron-spanning. They amplified a 361-base pair fragment of the house-keeping gene *Rps15*. This encodes a ribosomal subunit protein. Applicable to both mouse *Rsp15* and human *RPS15*.	
S15-Reverse	5'-CGGGCCGGCCATGCTTTACG-3'		

Table 2.3: Primers for PCR amplification and DNA sequencing

Component[1]	Amount per 50 ul PCR reaction	Final concentration
5x GoTaq Flexi Buffer	10 µl	1x
10 mM dNTP[2] Mix	1 µl	0.2 mM
25 mM $MgCl_2$	3 µl	1.5 mM
Forward primer (5 µM)	2.5 µl	2.5 µM
Reverse primer (5 µM)	2.5 µl	2.5 µM
Go Taq Hot Start Polymerase (5 units/µl)	0.25 µl	1.25 units
cDNA (RT reaction)	1-2 µl	< 500 ng in total
Nuclease-free water	Made to 50 µl	

[1]All components were from Promega (Southampton, UK) except for the primers, which were from Eurofins Genetic Services Ltd. (London, UK), and cDNA (RT reaction), which was prepared as required. cDNA, complementary DNA; dNTP, deoxynucleotide triphosphate; PCR, polymerase chain; reaction RT, reverse transcriptase.

2.12 Agarose gel electrophoresis

Agarose was melted in Tris-acetate-EDTA (TAE) (40 mM Tris-acetate; 1 mM EDTA; pH 8.3) electrophoresis buffer (Promega, Southampton, UK) to give 0.8-1% agarose gels, which were used for analysis of DNA and RNA. For every 50 ml of the gel solution, 1 µl of ethidium bromide solution (10 mg/ml) (Promega) was or 3 µl of Midori Green Advance DNA Stain (GeneFlow) were added. A casting deck of a Sub-Cell® Horizontal Electrophoresis System (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) was set up with a suitable gel comb ready for the molten agarose to be poured into after cooling.

Once the gel had solidified, the gel comb was taken out and the gel put into the electrophoresis tank, which was filled with TAE buffer to cover the gel. Samples of DNA or RNA were mixed with 6x Blue/Orange Loading Dye (0.4% orange G; 0.03% bromphenol blue; 0.03% xylene cyanol FF; 15% Ficoll® 400; 10 mM Tris-hydrochloride, pH 7.5; 50 mM EDTA, pH 8.0) (Promega) at 1/6th of the DNA or RNA volume. The samples were immediately loaded into the gel slots. On each gel, a DNA marker lane was included. It comprised a 0.5-1.0-µg sample of either a 100-bp DNA Ladder (100-1,500-base pair DNA fragments) (Promega), a 1-kb DNA Ladder (250-10,000-base pair DNA fragments) (Promega), or a 1-kb DNA Ladder (500-10,000-bp DNA fragments) (New England Biolabs, Ipswich, MA, USA).

After that, gels were run at 50-70 volts utilising a PowerPac Basic Power Supply (Bio-Rad Laboratories Ltd.). The gels were examined and recorded using a G:BOX gel documentation system (Syngene, Cambridge, UK) and GeneSnap image acquisition software (Syngene).

2.13 Purification of DNA fragments

To prepare DNA fragments for sequencing, they were separated initially by agarose gel electrophoresis employing Midori Green Advance DNA Stain for visualisation (Section 2.12). Afterwards, DNA fragments were recovered from the agarose gel using a Wizard® PCR Preps DNA Purification Kit (Promega). Briefly, the relevant band of DNA was visualised using a FastGene® Blue/Green LED Transilluminator-XL (Nippon Genetics Europe, Dueren, Germany). Using a clean scalpel, the DNA band was

excised from the gel and placed in a 1.5-ml tube. A 1-ml sample of DNA PurificationResin Wizard® PCR Preps was used to dissolve the gel slice and the resulting mixture was applied to a Wizard® Minicolumn, using a 2-ml syringe. Subsequently, 2 ml of 80% isopropanol were used to wash the column. To remove excess isopropanol, the column was then centrifuged in an Eppendorf MiniSpin microcentrifuge at 10,000 rpm for 2 min. Subsequently, 50 µl of nuclease-free water were added to the column, which was then incubated at room temperature for 1 min. The purified DNA fragment was eluted from the column by centrifugation at 10,000 rpm for 20 sec. DNA fragments were stored at -20˚C until required. They were also analysed by agarose gel electrophoresis (Section 2.12) and DNA sequencing (Section 2.14), to ensure that the correct DNA fragment had been purified.

2.14 Sequencing of DNA

Sequencing primers were synthesised to order by Eurofins Genetic Services Ltd. and they were kept at -40°C in sterile nuclease-free water at a 100 pmol/µl concentration. Sanger sequencing of DNA was carried out by Genewiz at Azenta Life Sciences (South Plainfield, NJ, USA). DNA samples and DNA sequencing primers (Table 2.5) were provided to the service at concentrations of 5-10 ng/µl and 5 pmol/µl, respectively.

2.15 Analysis of DNA sequences

Analysis of DNA sequences was performed using a combination of the Lasergene® Core Suite version 11.0 (DNASTAR, Inc., Madison, WI, USA), and the online facilities of the ExPASy Bioinformatics Resources Portal (SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland) (http://web.expasy.org), the European Bioinformatics Institute-European Molecular Biology Laboratory (EBI-EMBL) (Cambridge, UK) (http://www.ebi.ac.uk), and the National Center for Biotechnology Information (NCBI) (Bethesda, MD, USA) (www.ncbi.nlm.nih.gov).

2.16 Nuclease resistance analysis

The methods used for assessing the degradation of ASOs by exonucleases and in complex biological media were adapted from previous protocols (Meng et al., 2018).

After processing, all samples were stored at -80°C until they were required for analysis by electrophoresis in 1.5% agarose gels.

2.16.1 ASO degradation in cell culture medium

The degradation of ASOs in cell culture medium was measured by adding ASOs, at a final concentration of 20 pmol/µl (20 µM), to pre-warmed cell culture medium containing 10% foetal bovine serum (Section 2.3.2). The ASO-cell culture medium samples were incubated at 37°C, and 2-µl samples taken at specific time points.

2.16.2 ASO degradation in human plasma

The resistance of ASOs to degradation in plasma was analysed using pooled human plasma (Biotrend, Cologne, Germany). The required ASO was mixed, at a final concentration of 20 pmol/µl, with human plasma. The mixture was incubated at 37°C, and 2-µl samples were taken at specific time points.

2.16.3 ASO degradation in AtT-20 cell lysate

The resistance of ASOs to degradation in cell lysates was analysed using lysate prepared from the AtT-20 cell line. AtT-20 cells were cultured, according to the protocol in Section 2.3.3, transferred to PBS containing Protease Inhibitor Cocktail (Sigma-Aldrich), and then washed three times. The cell pellet was resuspended in lysate buffer (150 mM sodium chloride; 25 mM sodium phosphate (pH 6.9); 1% Triton X-100; 0.5% Nonidet P40; Protease Inhibitor Cocktail), and sonicated using an Ultrasonic Processor (Jencons Scientific Ltd., Leighton Buzzard, UK) four times for 20 sec with the samples on ice. The cell lysate was then centrifuged in an Eppendorf MiniSpin microcentrifuge at 10,000 rpm for 30 min at 4°C. The supernatant was collected and stored at -80°C. This part of the protocol was undertaken by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK).

The required ASO was mixed, at a final concentration of 20 pmol/µl, with AtT-20 cell lysate. The mixture was incubated at 37°C, and 2-µl samples were taken at the specific time points.

2.16.4 ASO 3’-exonuclease degradation

The resistance of ASOs to 3’-exonuclease-mediated degradation was analysed using phosphodiesterase I purified from *Crotalus adamanteus* venom (Sigma-Aldrich). Immediately before use, the enzyme was dissolved in cold purified water to 100 units/ml, according to the manufacturer’s protocol. The required ASO was mixed, at a final concentration of 20 pmol/µl, with glycine buffer (200 mM glycine; 15 mM magnesium chloride; pH 9.0) at 0°C. The enzyme solution was added to a final concentration of 5 mUnits/µl. The reaction was then incubated at 37°C, and 2-µl samples were taken at specific time points.

2.16.5 ASO 5’-exonuclease degradation

The resistance of ASOs to 5’-exonuclease-mediated degradation was analysed using phosphodiesterase II purified from bovine spleen (Sigma-Aldrich). The enzyme was dissolved in cold purified water to 5 units/ml. The required ASO was mixed, at a final concentration of 20 pmol/µl, with acetate buffer (100 mM ammonium acetate; 1 mM EDTA; 1 mM Tween 80; pH 7.0) at 0°C. The enzyme solution was added to a final concentration of 1 mUnit/µl. The reaction was then incubated at 37°C, and 2-µl samples were taken at specific time points.

2.17 Statistical analysis

Data were analysed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to determine whether there were any statistically significant differences between the means of two or more independent sets of continuous data. Unpaired t tests were used to compare two sets of continuous data. In all statistical analyses, P values < 0.05 were considered significant.

Chapter 3

Design of POMC antisense oligonucleotides

3 Design of POMC antisense oligonucleotides

3.1 Introduction

Antisense oligonucleotides recognise and bind to mRNA molecules via the Watson and Crick base-pairing rules, so it might be expected that simply selecting a sequence within a target mRNA would result in effective gene-silencing. However, several considerations need to be taken into account when designing ASOs to ensure their success and, although there are still no exact design criteria, there are guidelines that can aid the process (Chan et al., 2006, Stein, 2001). Overall, good design can optimise factors such as cellular uptake, tissue distribution, hybridisation affinity, nuclease resistance, immune response induction, and protein binding activities that can otherwise adversely affect ASO potency and specificity (Section1.3.2). Several considerations are discussed below.

3.1.1 ASO design considerations

3.1.1.1 The ASO molecule

Nucleotide length: The optimum length of an ASO is generally 12-28 nucleotides (Harding et al., 2007, Errington et al., 2003). Longer ASOs usually have a lower uptake into cells, and those that are shorter have a higher chance of hybridising to unintended targets (Harding et al., 2007).

Overall G-C content: The G-C content of an ASO correlates strongly with the thermodynamic stability of an ASO-mRNA heteroduplex and with RNase H activity (Ho et al., 1996). Antisense oligonucleotides with a G-C content of at least 55% have stronger gene-silencing effects than those with less than 45% (Ho et al., 1996). Overall, a balanced base composition of 40-60% G-C residues is more favourable for antisense activity (Phillips and Zhang, 2000).

G-quartets: If included in an ASO, a stretch of four or more G residues can lead to the formation of G-quartets (Sen and Gilbert, 1992). Such secondary structures can reduce the amount of available single-stranded ASO and also allow binding to proteins such as transcription factors and heparin-binding proteins, thus interfering with gene-

silencing efficacy (Stein, 2001, Stein, 1999). Substitution of a single G residue with 7-deazaguanosine can overcome this issue.

Functional motifs: Several functional nucleotide motifs, such as CCAC, TCCC, ACTC, GCCA, and CTCT, enhance the efficiency of ASOs, whereas others, including ACTG, AAA, and TAA have the deleterious effects (Matveeva et al., 2000). Enhancing motifs are thought to increase ASO-mRNA thermal stability (Matveeva et al., 2000).

Immunostimulatory motifs: If possible, ASOs should lack CpG and CpsG motifs, which can stimulate the immune response (Ballas et al., 1996, Klinman et al., 1996, Krieg et al., 1995). To reduce immunostimulation by a significant degree, the C residue within these motifs can be replaced by 5-methylcytosine (Krieg et al., 1995).

Self-complementarity: Antisense oligonucleotide self-complementarity can lead to the formation of internal secondary structures such as hairpins or of dimers between ASO molecules (Agrawal and Kandimalla, 2000). These have the potential to have deleterious effects upon ASO gene-silencing activity as there is less single-stranded ASO available and secondary structures can also bind proteins such that other cellular functions may be inadvertently affected (Agrawal and Kandimalla, 2000). However, there is conflicting evidence with regard to the contribution of self-complementarity to ASO efficacy. For example, one report suggested that ASOs with weaker secondary structure and a lesser ability to dimerise had better antisense activity (Matveeva et al., 2003). In contrast, other studies have indicated that ASOs forming more stable dimers were more effective (Aartsma-Rus et al., 2009), and that ASO secondary structure stability had no impact upon gene-silencing efficacy (Aartsma-Rus et al., 2009, Shao et al., 2006).

Chemical modifications: Chemical modifications have been used to better ASO binding affinity and nuclease resistance, and to reduce their potential for inducing adverse side-effects. The lower binding affinities and sequence-independent consequences associated with first-generation PS-modified ASOs (Shaw et al., 1997, Galbraith et al., 1994) led researchers to examine chemical alterations that would improve these parameters. The polyanion-related effects of the ASO PS-backbone, such as the activation of complement and prolonging of coagulation, were largely negated by the incorporation of OMe-modifications (Kandimalla et al., 1998), which

also improved binding affinities and imparted greater nuclease resistance. Third-generation ASO modifications such as LNA are extremely useful for increasing binding affinity and resistance to nucleases (Vester and Wengel, 2004, Kurreck et al., 2002). As previously reviewed, several other modifications to improve the gene-silencing activity of the selected ASO sequence can be considered (Chan et al., 2006).

Structure: A final ASO design feature, and indeed one that has been widely used, is that of the 'gapmer' (Shimojo et al., 2019, Marrosu et al., 2017). Such ASOs consist of an internal 'gap' of DNA nucleotides and flanking regions that usually consist of OMe-modified, MOE-modified or LNA-modified nucleotides (Figure 3.1). Their binding affinity to their target mRNA is very strong and the central ASO-mRNA heteroduplex acts as a RNase H substrate such that degradation of the target transcript can occur.

3.1.1.2 mRNA target site

Nucleotide homology: The ASO target sequence should be checked for homology to other sequences so that off-target hybridisation that might result in toxic effects can be avoided.

Secondary structures: An early study indicated that targeting ASOs against a single-stranded region of RNA was three orders of magnitude stronger, in terms of binding affinity, than if targeting a RNA hairpin structure (Lima et al., 1992). Since then, several studies using computational methods (Lu and Mathews, 2008, Shao et al., 2006, Andronescu et al., 2005, Ding et al., 2004, Zuker, 2003, Mathews et al., 1999, Patzel et al., 1999, Walton et al., 1999), as well as experimental approaches (Vickers et al., 2000, Milner et al., 1997), have reported that the lower the energy cost for disrupting secondary structures in the target mRNA to allow ASO access, the better the antisense activity.

Features: It is also apt to consider if the target region is a site for interaction with cellular components such as ribosomes, spliceosomes, and RNA-binding proteins, as they can affect ASO binding activity (Lima et al., 2014). Furthermore, ASOs can act as gene-silencing agents at locations such as the untranslated 5'-end and 3'-end of mRNA, where they can block the translational machinery (Kretschmer-Kazemi Far et al., 2001). Indeed, three regions that are considered to be good targets on mRNA for

designing effective ASOs are the 5'-cap region, the ATG translation start codon, and the 3'-untranslated region (Phillips and Zhang, 2000).

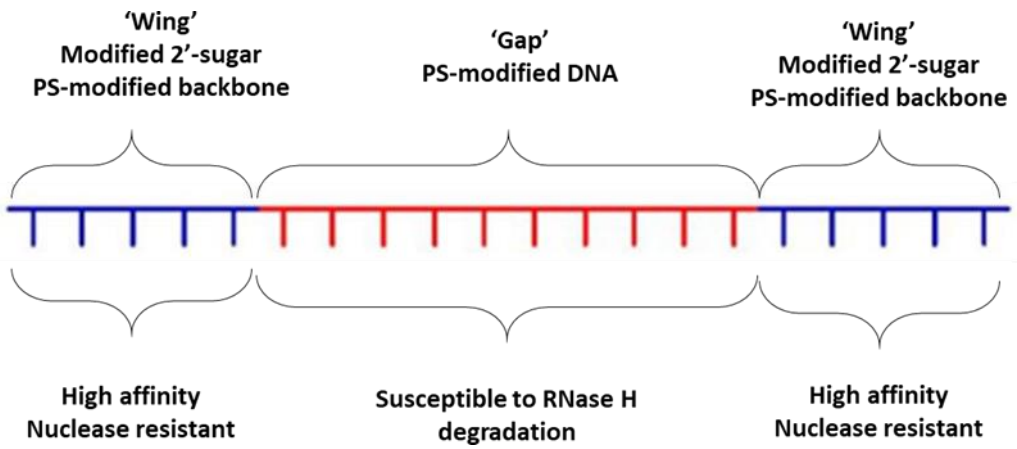


Figure 3.1: The structure of an antisense oligonucleotide gapmer.

An ASO gapmer consists of an internal 'gap' of DNA nucleotides with flanking 'wing' regions that usually consist of 2'-*O*-methyl (OMe)-modified, 2-*O*-methyl-ethyl (MOE)-modified or locked nucleic acid (LNA)-modified nucleotides. The backbone of the ASO has phosphorothioate (PS) linkages. The binding affinity of ASO gapmers to their target mRNA is very strong and the central ASO-mRNA heteroduplex acts as a RNase H substrate such that degradation of the target transcript can occur.

3.1.1.3 ASO-mRNA interaction

An early study suggested a positive correlation between the hybridisation affinity of an ASO and its activity (Monia et al., 1992). Usually, hybridisation affinities are measured by determining the melting temperature of an ASO-mRNA hybrid (Freier and Altmann, 1997). However, this method does not take into account mRNA secondary structures or RNA-binding proteins that might affect ASO-mRNA affinity within cells. Now, several computer programs are available to predict the thermodynamic properties of ASO-mRNA target interaction (Lu and Mathews, 2008, Mathews et al., 1999). Using these facilities, reports have suggested that ASOs forming a more stable duplex with mRNA have better antisense activity (Fei and Zhang, 2005, Matveeva et al., 2003), although, in later studies, the correlation of ASO-mRNA stability with ASO effectiveness was not confirmed (Aartsma-Rus et al., 2009, Shao et al., 2006).

Although there are reported differences in the relative importance of different thermodynamic interactions on ASO activity (Lu and Mathews, 2008), it is prudent to consider them in the context of ASO-mRNA stability, as well as ASO self-complementarity and the secondary structures that may be present in the target mRNA, during the ASO design process.

3.1.2 ASO design approaches

There are several strategies available to design ASOs that have potent activity including experimental and computer-aided methods. For example, oligonucleotide arrays (Cho et al., 2001), mRNA walking (Sohail and Southern, 2000), and RNase H mapping (Ho et al., 1998) have all been used to screen ASOs in the laboratory. However, these techniques are high in terms of cost and labour, and also need specialised equipment. In contrast, to aid ASO design, several software packages and online facilities (Table 3.1) are available at no cost and these are often successful in generating effective ASOs (Chan et al., 2006, Chalk and Sonnhammer, 2002). Overall no available computer program will design ASOs that are assured to be 100% effective, so in general, there is still some trial and error involved in identifying potent ASOs (Aartsma-Rus et al., 2009).

Table 3.1: Software for aiding antisense oligonucleotide design

Software	Website	Reference/Supplier
PFizer RNAi Enumeration and Design (PFRED)	https://github.com/pfred/	(Sciabola et al., 2021)
Antisense Architect	https://www.dnasoftware.com	DNA Software (Plymouth, MI, USA)
RNAstructure: Oligoscreen tool	https://rna.urmc.rochester.edu/RNAstructureWeb/	(Reuter and Mathews, 2010, Mathews et al., 2004) University of Rochester Medical Center, Rochester, NY, USA
LNASO	https://iomics.ugent.be	University of Ghent, Ghent, Belgium
*S*fold: *S*oligo tool	https://sfold.wadsworth.org/cgi-bin/index.pl	(Ding et al., 2004, Ding and Lawrence, 2003, Ding and Lawrence, 2001) Wadsworth Center New York State Department of Health (Albany, NY, USA)
OligoAnalyzer	https://eu.idtdna.com	Integrated DNA Technologies, Inc. (Coralville, IA, USA)
TargetFinder[1]	http://www.bioit.org.cn/ao/targetfinder.htm	(Bo and Wang, 2005)
AOpredict[1]	http://www.cgb.ki.se/AOpredict	(Chalk and Sonnhammer, 2002)

[1]Not accessible during this project.

3.2 Aims and Objectives

The aim of this part of the study was to design ASOs targeting *Pomc* mRNA with the expectation that they would have the potential to reduce the production of ACTH.

The specific objectives were to:

- Obtain the sequence for the mouse *Pomc* gene and mRNA transcript from the GenBank sequence database using the online facility at the National Center for Biotechnology Information (NCBI) (Bethesda, MD, USA) (www.ncbi.nlm.nih.gov).

- Use selected computer-aided design programs (Table 3.1) to design candidate POMC ASOs that could potentially be used to reduce *Pomc* expression, as measured by ACTH secretion from AtT-20 cells.

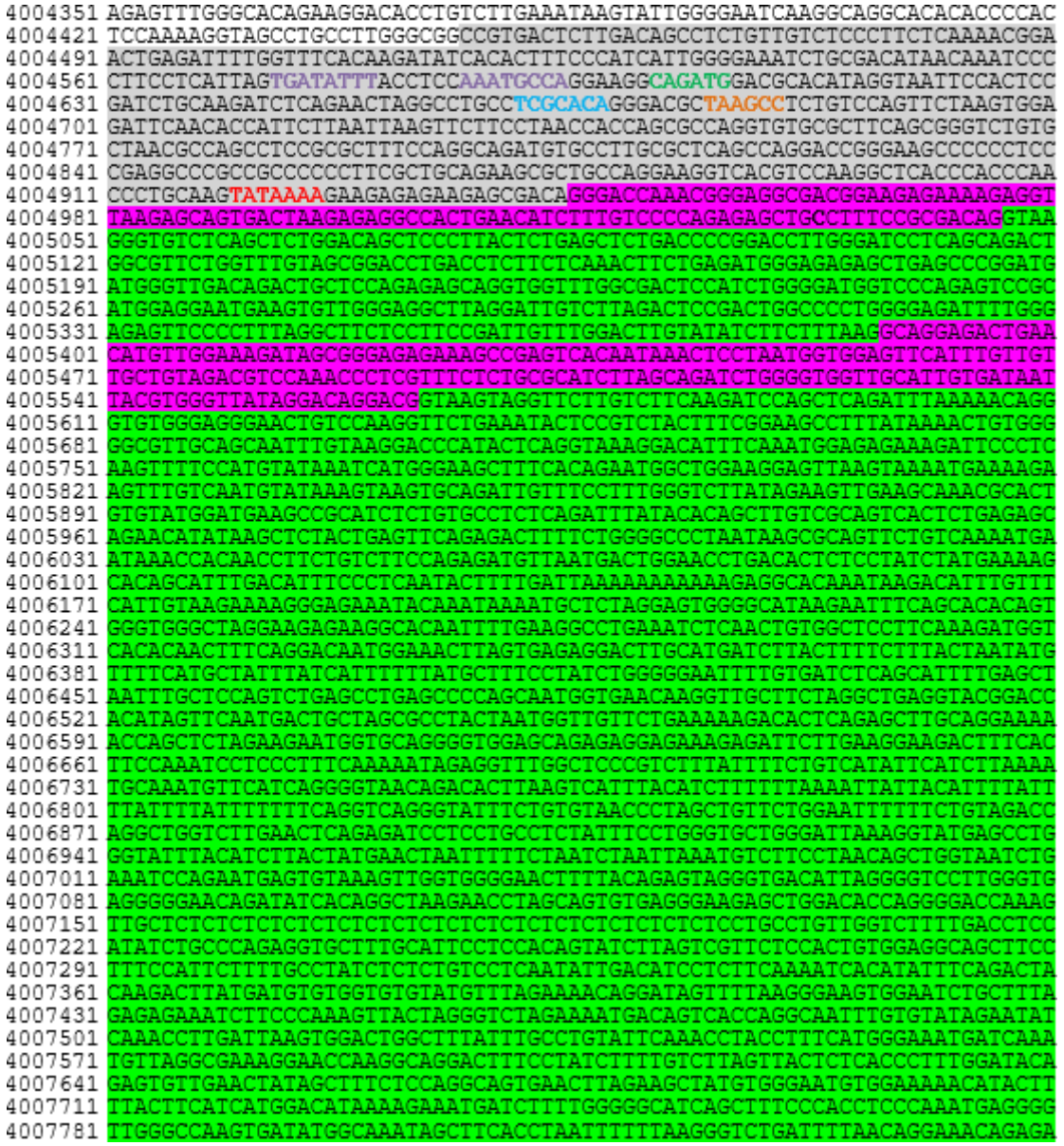
3.3 Results

3.3.1 Sequence analysis of the mouse *Pomc* gene

Since the initial *in vitro* experiments involved the use of mouse AtT-20 cells to test the effects of POMC ASOs on ACTH secretion, the sequence of the mouse *Pomc* gene (*Mus musculus* strain C57BL/6J chromosome 12, GRCm39) was downloaded from GenBank sequence database at the online facility at the National Center for Biotechnology Information (NCBI) (Bethesda, MD, USA) (www.ncbi.nlm.nih.gov), in order to aid in the design of appropriate ASOs. The sequence of the gene and the 5699-base pair primary transcript with three introns and four exons (RefSeq accession number: NC_000078.7) is shown in Figure 3.2.

The *Pomc* gene promoter region and transcription factor binding sites were located and these are annotated in Figure 3.2. The important features identified included the TATA box for RNA polymerase binding, the Pitx-response element for PITX1 transcription factor binding, the Tpit-response element for TPIT transcription factor binding, the Nur-response element for NUR77 transcription factor binding, and the E-box$_{neuro}$ for NEUROD1 transcription factor binding (Murakami et al., 2007). TPIT and NEUROD1 act with PITX1 to drive corticotroph-specific *Pomc* gene expression, and NUR77 antagonises the negative feedback of glucocorticoids on *Pomc* expression (Murakami et al., 2007).

The mouse mature *Pomc* transcript spliced to exclude the three introns was also downloaded from the NCIB online facility. The sequence (RefSeq accession number: NM_001278581.1) was 1214 base pairs, and is shown in Figure 3.3.


4004351 AGAGTTTGGGCACAGAAGGACACCTGTCTTGAAATAAGTATTGGGGAATCAAGGCAGGCACACACCCCAC
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4004771 CTAACGCCAGCCTCCGCGCTTTCCAGGCAGATGTGCCTTGCGCTCAGCCAGGACCGGGAAGCCCCCCTCC
4004841 CGAGGCCCGCCGCCCCCCTTCGCTGCAGAAGCGCTGCCAGGAAGGTCACGTCCAAGGCTCACCCACCCAA
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4005121 GGCGTTCTGGTTTGTAGCGGACCTGACCTCTTCTCAAACTTCTGAGATGGGAGAGAGCTGAGCCCGGATG
4005191 ATGGGTTGACAGACTGCTCCAGAGAGCAGGTGGTTTGGCGACTCCATCTGGGGATGGTCCCAGAGTCCGC
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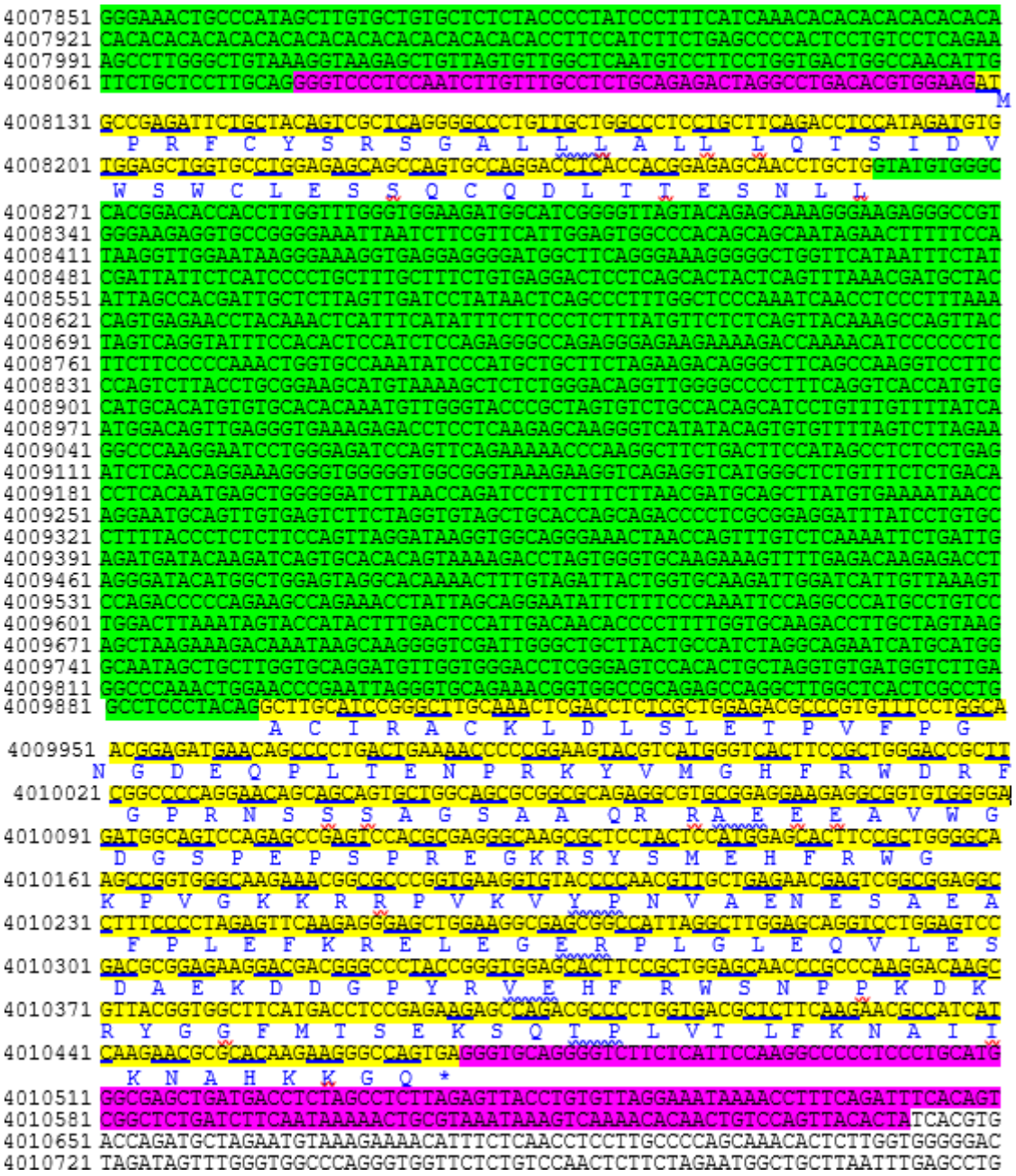


Figure 3.2: Sequence of the mouse *Pomc* gene.

Features on the gene that are indicated include the promoter region (grey highlight), introns (green highlight), the translated regions of the mature transcript (yellow highlights), the untranslated regions of the mature transcript (pink highlights), the TATA box (red font), the Pitx-response element (orange font), the Tpit-response element (blue font), the Nur-response element (purple font), and the E-boxneuro (green font). The primary transcript is 5699 base pairs inclusive of the intronic and exonic regions (RefSeq accession number: NC_000078.7).

```
1    GGGACCAAAC GGGAGGCGAC GGAAGAGAAA AGAGGTTAAG AGCAGTGACT AAGAGAGGCC
61   ACTGAACATC TTTGTCCCCA GAGAGCTGCC TTTCCGCGAC AGGCAGGAGA CTGAACATGT
121  TGGAAAGATA GCGGGAGAGA AAGCCGAGTC ACAATAAACT CCTAATGGTG GAGTTCATTT
181  GTTGTTGCTG TAGACGTCCA AACCCTCGTT TCTCTGCGCA TCTTAGCAGA TCTGGGGTGG
241  TTGCATTGTG ATAATTACGT GGGTTATAGG ACAGGACGGG GTCCCTCCAA TCTTGTTTGC
301  CTCTGCAGAG ACTAGGCCTG ACACGTGGAA GATGCCGAGA TTCTGCTACA GTCGCTCAGG
361  GGCCCTGTTG CTGGCCCTCC TGCTTCAGAC CTCCATAGAT GTGTGGAGCT GGTGCCTGGA
421  GAGCAGCCAG TGCCAGGACC TCACCACGGA GAGCAACCTG CTGGCTTGCA TCCGGGCTTG
481  CAAACTCGAC CTCTCGCTGG AGACGCCCGT GTTTCCTGGC AACGGAGATG AACAGCCCCT
541  GACTGAAAAC CCCCGGAAGT ACGTCATGGG TCACTTCCGC TGGGACCGCT TCGGCCCCAG
601  GAACAGCAGC AGTGCTGGCA GCGCGGCGCA GAGGCGTGCG GAGGAAGAGG CGGTGTGGGG
661  AGATGGCAGT CCAGAGCCGA GTCCACGCGA GGGCAAGCGC TCCTACTCCA TGGAGCACTT
721  CCGCTGGGGC AAGCCGGTGG GCAAGAAACG GCGCCCGGTG AAGGTGTACC CCAACGTTGC
781  TGAGAACGAG TCGGCGGAGG CCTTTCCCCT AGAGTTCAAG AGGGAGCTGG AAGGCGAGCG
841  GCCATTAGGC TTGGAGCAGG TCCTGGAGTC CGACGCGGAG AAGGACGACG GGCCCTACCG
901  GGTGGAGCAC TTCCGCTGGA GCAACCCGCC CAAGGACAAG CGTTACGGTG GCTTCATGAC
961  CTCCGAGAAG AGCCAGACGC CCCTGGTGAC GCTCTTCAAG AACGCCATCA TCAAGAACGC
1021 GCACAAGAAG GGCCAGTGAG GGTGCAGGGG TCTTCTCATT CCAAGGCCCC CTCCCTGCAT
1081 GGGCGAGCTG ATGACCTCTA GCCTCTTAGA GTTACCTGTG TTAGGAAATA AAACCTTTCA
1141 GATTTCACAG TCGGCTCTGA TCTTCAATAA AAACTGCGTA AATAAAGTCA AAACACAACT
1201 GTCCAGTTAC ACTA
```

Figure 3.3: Sequence of the mouse mature *Pomc* transcript.

The mouse mature *Pomc* transcript excluding introns (RefSeq accession number: NM_001278581.1) was 1214 nucleotides. The sequence for translation is highlighted in yellow. The translational start ATG codon and the translational stop TGA codon are underlined. The coding sequence of exon 3 is from position 332 to 463, and the coding sequence of exon 4 is from position 464 to 1039.

3.3.2 Design of POMC ASOs

Initially, the *S*fold program (Table 3.1) was used to predict potential target sites for POMC ASO binding. The program is based on algorithms that predict secondary structures, and therefore accessible regions within RNA molecules, and generates a probability profile of potential single-stranded regions on the target mRNA. The regions that have high probabilities of being single-stranded are predicted to be accessible sites where ASO binding could be facilitated. A probability profile was generated for the mouse mature *Pomc* transcript (Figure 3.3) and this is shown in Figure 3.4. This indicated several mRNA sites had a high probability of being single-stranded and so were predicted to be accessible for ASO binding.

Within the *S*fold program, the *S*oligo module can aid in the design of ASOs by combining the prediction of secondary structures and accessibility on the target mRNA with empirical ASO design rules. This allows output data to indicate the percentage base composition of and the presence of G-quartets in potential target sequences. A binding energy value (kcal/mol) for the interaction of each ASO with its target sequence can also be calculated. The more negative the value, the stronger the predicted binding of the ASO to its target.

The *S*oligo module was used, therefore, to predict ASO targets on the mouse mature *Pomc* transcript for stretches of 19 nucleotides, a length within the optimum for ASO molecules. The results are shown in Appendix I, where the full output data for the percentage G-C content, the presence of GGGG residues in target sequences, and the binding energy values are indicated.

Applying empirical guidelines for ASO design within the *Pomc* coding sequences, target sites with a G-C content of below 40% or above 60% and those having four or more consecutive G residues were avoided. In addition, sites having a positive binding energy value (≥ 0 kcal/mol) were excluded. The potential POMC ASO sequences themselves were then examined for the presence of G-quartets and deleterious motifs such as TAA, AAA, and ACTG sequences, so that these could be avoided. The remaining candidate POMC ASOs and their target sites are shown in Figure 3.5.

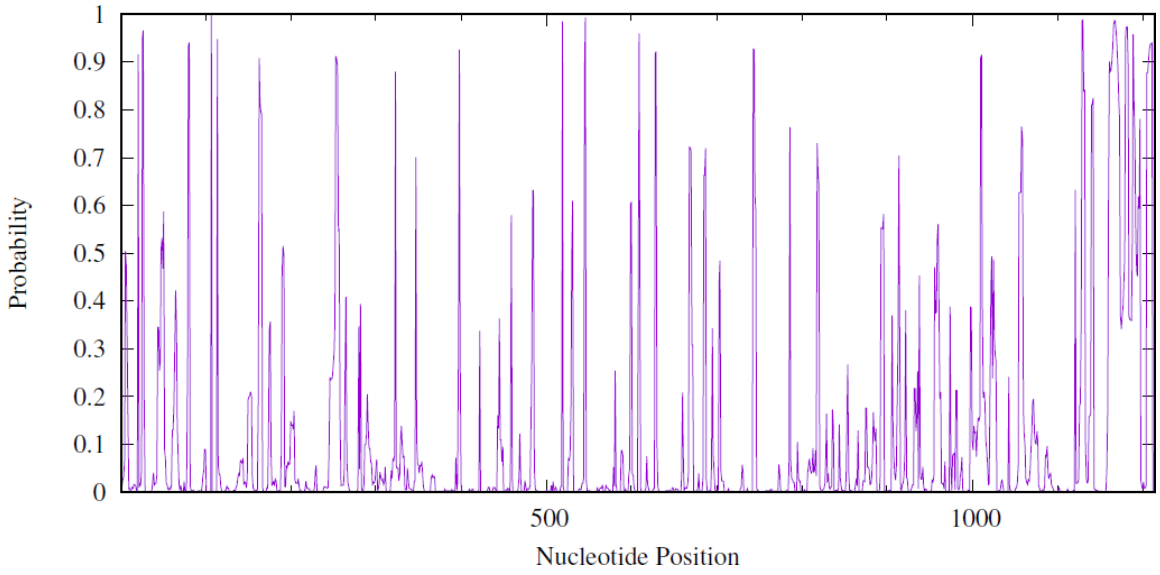


Figure 3.4: Single-stranded RNA probability profile of mouse mature *Pomc* mRNA.

The *S*fold program was used to generate a probability profile of potential single-stranded regions on the mouse *Pomc* mRNA from nucleotide positions 1 to 1214 (Figure 3.3). The sites with higher probabilities of being single-stranded are also predicted to be accessible sites.

316-	334	GCCUGACACGUGGAAGAUG	CATCTTCCACGTGTCAGGC	57.9%	-6.1	0
317-	335	CCUGACACGUGGAAGAUGC	GCATCTTCCACGTGTCAGG	57.9%	-6.3	0
318-	336	CUGACACGUGGAAGAUGCC	GGCATCTTCCACGTGTCAG	57.9%	-6.4	0
319-	337	UGACACGUGGAAGAUGCCG	CGGCATCTTCCACGTGTCA	57.9%	-6.3	0
320-	338	GACACGUGGAAGAUGCCGA	TCGGCATCTTCCACGTGTC	57.9%	-6.3	0
321-	339	ACACGUGGAAGAUGCCGAG	CTCGGCATCTTCCACGTGT	57.9%	-5.3	0
322-	340	CACGUGGAAGAUGCCGAGA	TCTCGGCATCTTCCACGTG	57.9%	-5.2	0
323-	341	ACGUGGAAGAUGCCGAGAU	ATCTCGGCATCTTCCACGT	52.6%	-5.3	0
324-	342	CGUGGAAGAUGCCGAGAUU	AATCTCGGCATCTTCCACG	52.6%	-3.4	0
325-	343	GUGGAAGAUGCCGAGAUUC	GAATCTCGGCATCTTCCAC	52.6%	-1.8	0
326-	344	UGGAAGAUGCCGAGAUUCU	AGAATCTCGGCATCTTCCA	47.4%	-0.9	0
327-	345	GGAAGAUGCCGAGAUUCUG	CAGAATCTCGGCATCTTCC	52.6%	-0.8	0
328-	346	GAAGAUGCCGAGAUUCUGC	GCAGAATCTCGGCATCTTC	52.6%	-0.7	0
330-	348	AGAUGCCGAGAUUCUGCUA	TAGCAGAATCTCGGCATCT	47.4%	-0.1	0
331-	349	GAUGCCGAGAUUCUGCUAC	GTAGCAGAATCTCGGCATC	52.6%	-1.6	0
332-	350	AUGCCGAGAUUCUGCUACA	TGTAGCAGAATCTCGGCAT	47.4%	-2.1	0
333-	351	UGCCGAGAUUCUGCUACAG	CTGTAGCAGAATCTCGGCA	52.6%	-1.4	0
381-	399	UGCUUCAGACCUCCAUAGA	TCTATGGAGGTCTGAAGCA	47.4%	-1.0	0
382-	400	GCUUCAGACCUCCAUAGAU	ATCTATGGAGGTCTGAAGC	47.4%	-1.6	0
383-	401	CUUCAGACCUCCAUAGAUG	CATCTATGGAGGTCTGAAG	47.4%	-3.1	0
384-	402	UUCAGACCUCCAUAGAUGU	ACATCTATGGAGGTCTGAA	42.1%	-3.4	0
385-	403	UCAGACCUCCAUAGAUGUG	CACATCTATGGAGGTCTGA	47.4%	-3.4	0
386-	404	CAGACCUCCAUAGAUGUGU	ACACATCTATGGAGGTCTG	47.4%	-3.4	0
387-	405	AGACCUCCAUAGAUGUGUG	CACACATCTATGGAGGTCT	47.4%	-3.4	0
388-	406	GACCUCCAUAGAUGUGUGG	CCACACATCTATGGAGGTC	52.6%	-3.4	0
389-	407	ACCUCCAUAGAUGUGUGGA	TCCACACATCTATGGAGGT	47.4%	-3.3	0
390-	408	CCUCCAUAGAUGUGUGGAG	CTCCACACATCTATGGAGG	52.6%	-1.6	0
391-	409	CUCCAUAGAUGUGUGGAGC	GCTCCACACATCTATGGAG	52.6%	-1.5	0
392-	410	UCCAUAGAUGUGUGGAGCU	AGCTCCACACATCTATGGA	47.4%	-1.5	0
393-	411	CCAUAGAUGUGUGGAGCUG	CAGCTCCACACATCTATGG	52.6%	-1.5	0
394-	412	CAUAGAUGUGUGGAGCUGG	CCAGCTCCACACATCTATG	52.6%	-1.6	0
395-	413	AUAGAUGUGUGGAGCUGGU	ACCAGCTCCACACATCTAT	47.4%	-1.8	0
396-	414	UAGAUGUGUGGAGCUGGUG	CACCAGCTCCACACATCTA	52.6%	-1.8	0
397-	415	AGAUGUGUGGAGCUGGUGC	GCACCAGCTCCACACATCT	57.9%	-1.8	0
399-	417	AUGUGUGGAGCUGGUGCCU	AGGCACCAGCTCCACACAT	57.9%	-0.7	0
438-	456	ACCUCACCACGGAGAGCAA	TTGCTCTCCGTGGTGAGGT	57.9%	-2.3	0
441-	459	UCACCACGGAGAGCAACCU	AGGTTGCTCTCCGTGGTGA	57.9%	-1.7	0
449-	467	GAGAGCAACCUGCUGGCUU	AAGCCAGCAGGTTGCTCTC	57.9%	-2.0	0
450-	468	AGAGCAACCUGCUGGCUUG	CAAGCCAGCAGGTTGCTCT	57.9%	-1.8	0
452-	470	AGCAACCUGCUGGCUUGCA	TGCAAGCCAGCAGGTTGCT	57.9%	-0.9	0
453-	471	GCAACCUGCUGGCUUGCAU	ATGCAAGCCAGCAGGTTGC	57.9%	-1.0	0
454-	472	CAACCUGCUGGCUUGCAUC	GATGCAAGCCAGCAGGTTG	57.9%	-1.2	0
455-	473	AACCUGCUGGCUUGCAUCC	GGATGCAAGCCAGCAGGTT	57.9%	-1.0	0
467-	485	UGCAUCCGGGCUUGCAAAC	GTTTGCAAGCCCGGATGCA	57.9%	-0.2	0
468-	486	GCAUCCGGGCUUGCAAACU	AGTTTGCAAGCCCGGATGC	57.9%	-0.9	0
469-	487	CAUCCGGGCUUGCAAACUC	GAGTTTGCAAGCCCGGATG	57.9%	-2.0	0
470-	488	AUCCGGGCUUGCAAACUCG	CGAGTTTGCAAGCCCGGAT	57.9%	-3.3	0
471-	489	UCCGGGCUUGCAAACUCGA	TCGAGTTTGCAAGCCCGGA	57.9%	-3.2	0
474-	492	GGGCUUGCAAACUCGACCU	AGGTCGAGTTTGCAAGCCC	57.9%	-3.0	0
475-	493	GGCUUGCAAACUCGACCUC	GAGGTCGAGTTTGCAAGCC	57.9%	-3.0	0
476-	494	GCUUGCAAACUCGACCUCU	AGAGGTCGAGTTTGCAAGC	52.6%	-2.9	0
477-	495	CUUGCAAACUCGACCUCUC	GAGAGGTCGAGTTTGCAAG	52.6%	-2.9	0
478-	496	UUGCAAACUCGACCUCUCG	CGAGAGGTCGAGTTTGCAA	52.6%	-2.4	0
479-	497	UGCAAACUCGACCUCUCGC	GCGAGAGGTCGAGTTTGCA	57.9%	-2.3	0
480-	498	GCAAACUCGACCUCUCGCU	AGCGAGAGGTCGAGTTTGC	57.9%	-2.3	0
481-	499	CAAACUCGACCUCUCGCUG	CAGCGAGAGGTCGAGTTTG	57.9%	-2.3	0
482-	500	AAACUCGACCUCUCGCUGG	CCAGCGAGAGGTCGAGTTT	57.9%	-2.3	0
483-	501	AACUCGACCUCUCGCUGGA	TCCAGCGAGAGGTCGAGTT	57.9%	-2.1	0
513-	531	UUCCUGGCAACGGAGAUGA	TCATCTCCGTTGCCAGGAA	52.6%	-3.6	0
514-	532	UCCUGGCAACGGAGAUGAA	TTCATCTCCGTTGCCAGGA	52.6%	-4.2	0

515-	533	CCUGGCAACGGAGAUGAAC	GTTCATCTCCGTTGCCAGG	57.9%	-5.5	0
516-	534	CUGGCAACGGAGAUGAACA	TGTTCATCTCCGTTGCCAG	52.6%	-6.0	0
517-	535	UGGCAACGGAGAUGAACAG	CTGTTCATCTCCGTTGCCA	52.6%	-6.0	0
518-	536	GGCAACGGAGAUGAACAGC	GCTGTTCATCTCCGTTGCC	57.9%	-6.0	0
519-	537	GCAACGGAGAUGAACAGCC	GGCTGTTCATCTCCGTTGC	57.9%	-6.0	0
520-	538	CAACGGAGAUGAACAGCCC	GGGCTGTTCATCTCCGTTG	57.9%	-3.3	0
553-	571	CCGGAAGUACGUCAUGGGU	ACCCATGACGTACTTCCGG	57.9%	-1.6	0
554-	572	CGGAAGUACGUCAUGGGUC	GACCCATGACGTACTTCCG	57.9%	-1.6	0
555-	573	GGAAGUACGUCAUGGGUCA	TGACCCATGACGTACTTCC	52.6%	-1.6	0
556-	574	GAAGUACGUCAUGGGUCAC	GTGACCCATGACGTACTTC	52.6%	-1.5	0
557-	575	AAGUACGUCAUGGGUCACU	AGTGACCCATGACGTACTT	47.4%	-1.3	0
558-	576	AGUACGUCAUGGGUCACUU	AAGTGACCCATGACGTACT	47.4%	-1.3	0
559-	577	GUACGUCAUGGGUCACUUC	GAAGTGACCCATGACGTAC	52.6%	-1.3	0
560-	578	UACGUCAUGGGUCACUUCC	GGAAGTGACCCATGACGTA	52.6%	-1.3	0
561-	579	ACGUCAUGGGUCACUUCCG	CGGAAGTGACCCATGACGT	57.9%	-1.3	0
563-	581	GUCAUGGGUCACUUCCGCU	AGCGGAAGTGACCCATGAC	57.9%	-1.6	0
564-	582	UCAUGGGUCACUUCCGCUG	CAGCGGAAGTGACCCATGA	57.9%	-1.6	0
598-	616	CAGGAACAGCAGCAGUGCU	AGCACTGCTGCTGTTCCTG	57.9%	-8.6	0
599-	617	AGGAACAGCAGCAGUGCUG	CAGCACTGCTGCTGTTCCT	57.9%	-8.6	0
602-	620	AACAGCAGCAGUGCUGGCA	TGCCAGCACTGCTGCTGTT	57.9%	-7.5	0
693-	711	GCAAGCGCUCCUACUCCAU	ATGGAGTAGGAGCGCTTGC	57.9%	-3.0	0
694-	712	CAAGCGCUCCUACUCCAUG	CATGGAGTAGGAGCGCTTG	57.9%	-3.0	0
695-	713	AAGCGCUCCUACUCCAUGG	CCATGGAGTAGGAGCGCTT	57.9%	-2.9	0
696-	714	AGCGCUCCUACUCCAUGGA	TCCATGGAGTAGGAGCGCT	57.9%	-2.8	0
699-	717	GCUCCUACUCCAUGGAGCA	TGCTCCATGGAGTAGGAGC	57.9%	-1.7	0
700-	718	CUCCUACUCCAUGGAGCAC	GTGCTCCATGGAGTAGGAG	57.9%	-1.6	0
701-	719	UCCUACUCCAUGGAGCACU	AGTGCTCCATGGAGTAGGA	52.6%	-1.6	0
702-	720	CCUACUCCAUGGAGCACUU	AAGTGCTCCATGGAGTAGG	52.6%	-1.6	0
703-	721	CUACUCCAUGGAGCACUUC	GAAGTGCTCCATGGAGTAG	52.6%	-1.6	0
704-	722	UACUCCAUGGAGCACUUCC	GGAAGTGCTCCATGGAGTA	52.6%	-1.0	0
705-	723	ACUCCAUGGAGCACUUCCG	CGGAAGTGCTCCATGGAGT	57.9%	-0.7	0
730-	748	CAAGCCGGUGGGCAAGAAA	TTTCTTGCCCACCGGCTTG	57.9%	-3.6	0
731-	749	AAGCCGGUGGGCAAGAAAC	GTTTCTTGCCCACCGGCTT	57.9%	-4.0	0
770-	788	CCCAACGUUGCUGAGAACG	CGTTCTCAGCAACGTTGGG	57.9%	-4.3	0
771-	789	CCAACGUUGCUGAGAACGA	TCGTTCTCAGCAACGTTGG	52.6%	-5.2	0
772-	790	CAACGUUGCUGAGAACGAG	CTCGTTCTCAGCAACGTTG	52.6%	-5.4	0
773-	791	AACGUUGCUGAGAACGAGU	ACTCGTTCTCAGCAACGTT	47.4%	-5.4	0
774-	792	ACGUUGCUGAGAACGAGUC	GACTCGTTCTCAGCAACGT	52.6%	-5.4	0
775-	793	CGUUGCUGAGAACGAGUCG	CGACTCGTTCTCAGCAACG	57.9%	-3.4	0
776-	794	GUUGCUGAGAACGAGUCGG	CCGACTCGTTCTCAGCAAC	57.9%	-1.8	0
777-	795	UUGCUGAGAACGAGUCGGC	GCCGACTCGTTCTCAGCAA	57.9%	-1.7	0
807-	825	CCCUAGAGUUCAAGAGGGA	TCCCTCTTGAACTCTAGGG	52.6%	-6.0	0
808-	826	CCUAGAGUUCAAGAGGGAG	CTCCCTCTTGAACTCTAGG	52.6%	-5.9	0
809-	827	CUAGAGUUCAAGAGGGAGC	GCTCCCTCTTGAACTCTAG	52.6%	-5.7	0
810-	828	UAGAGUUCAAGAGGGAGCU	AGCTCCCTCTTGAACTCTA	47.4%	-5.7	0
811-	829	AGAGUUCAAGAGGGAGCUG	CAGCTCCCTCTTGAACTCT	52.6%	-5.8	0
812-	830	GAGUUCAAGAGGGAGCUGG	CCAGCTCCCTCTTGAACTC	57.9%	-6.5	0
813-	831	AGUUCAAGAGGGAGCUGGA	TCCAGCTCCCTCTTGAACT	52.6%	-7.0	0
814-	832	GUUCAAGAGGGAGCUGGAA	TTCCAGCTCCCTCTTGAAC	52.6%	-7.0	0
815-	833	UUCAAGAGGGAGCUGGAAG	CTTCCAGCTCCCTCTTGAA	52.6%	-7.0	0
816-	834	UCAAGAGGGAGCUGGAAGG	CCTTCCAGCTCCCTCTTGA	57.9%	-7.1	0
846-	864	UAGGCUUGGAGCAGGUCCU	AGGACCTGCTCCAAGCCTA	57.9%	-1.2	0
851-	869	UUGGAGCAGGUCCUGGAGU	ACTCCAGGACCTGCTCCAA	57.9%	-2.5	0
906-	924	AGCACUUCCGCUGGAGCAA	TTGCTCCAGCGGAAGTGCT	57.9%	-8.2	0
944-	962	UACGGUGGCUUCAUGACCU	AGGTCATGAAGCCACCGTA	52.6%	-3.2	0
945-	963	ACGGUGGCUUCAUGACCUC	GAGGTCATGAAGCCACCGT	57.9%	-4.1	0
948-	966	GUGGCUUCAUGACCUCCGA	TCGGAGGTCATGAAGCCAC	57.9%	-5.6	0
949-	967	UGGCUUCAUGACCUCCGAG	CTCGGAGGTCATGAAGCCA	57.9%	-5.7	0
950-	968	GGCUUCAUGACCUCCGAGA	TCTCGGAGGTCATGAAGCC	57.9%	-5.6	0
951-	969	GCUUCAUGACCUCCGAGAA	TTCTCGGAGGTCATGAAGC	52.6%	-5.7	0

952- 970	CUUCAUGACCUCCGAGAAG	CTTCTCGGAGGTCATGAAG	52.6%	-5.8	0
953- 971	UUCAUGACCUCCGAGAAGA	TCTTCTCGGAGGTCATGAA	47.4%	-5.9	0
954- 972	UCAUGACCUCCGAGAAGAG	CTCTTCTCGGAGGTCATGA	52.6%	-5.9	0
955- 973	CAUGACCUCCGAGAAGAGC	GCTCTTCTCGGAGGTCATG	57.9%	-5.8	0
956- 974	AUGACCUCCGAGAAGAGCC	GGCTCTTCTCGGAGGTCAT	57.9%	-5.7	0
957- 975	UGACCUCCGAGAAGAGCCA	TGGCTCTTCTCGGAGGTCA	57.9%	-5.6	0
959- 977	ACCUCCGAGAAGAGCCAGA	TCTGGCTCTTCTCGGAGGT	57.9%	-6.4	0
981- 999	CCCUGGUGACGCUCUUCAA	TTGAAGAGCGTCACCAGGG	57.9%	-3.0	0
982-1000	CCUGGUGACGCUCUUCAAG	CTTGAAGAGCGTCACCAGG	57.9%	-3.1	0
983-1001	CUGGUGACGCUCUUCAAGA	TCTTGAAGAGCGTCACCAG	52.6%	-2.4	0
984-1002	UGGUGACGCUCUUCAAGAA	TTCTTGAAGAGCGTCACCA	47.4%	-2.4	0
985-1003	GGUGACGCUCUUCAAGAAC	GTTCTTGAAGAGCGTCACC	52.6%	-2.5	0
986-1004	GUGACGCUCUUCAAGAACG	CGTTCTTGAAGAGCGTCAC	52.6%	-2.7	0
987-1005	UGACGCUCUUCAAGAACGC	GCGTTCTTGAAGAGCGTCA	52.6%	-3.0	0
988-1006	GACGCUCUUCAAGAACGCC	GGCGTTCTTGAAGAGCGTC	57.9%	-3.2	0
989-1007	ACGCUCUUCAAGAACGCCA	TGGCGTTCTTGAAGAGCGT	52.6%	-3.7	0
990-1008	CGCUCUUCAAGAACGCCAU	ATGGCGTTCTTGAAGAGCG	52.6%	-2.1	0
991-1009	GCUCUUCAAGAACGCCAUC	GATGGCGTTCTTGAAGAGC	52.6%	-2.2	0
992-1010	CUCUUCAAGAACGCCAUCA	TGATGGCGTTCTTGAAGAG	47.4%	-2.4	0
993-1011	UCUUCAAGAACGCCAUCAU	ATGATGGCGTTCTTGAAGA	42.1%	-3.2	0
994-1012	CUUCAAGAACGCCAUCAUC	GATGATGGCGTTCTTGAAG	47.4%	-4.6	0
995-1013	UUCAAGAACGCCAUCAUCA	TGATGATGGCGTTCTTGAA	42.1%	-5.4	0
996-1014	UCAAGAACGCCAUCAUCAA	TTGATGATGGCGTTCTTGA	42.1%	-6.4	0
997-1015	CAAGAACGCCAUCAUCAAG	CTTGATGATGGCGTTCTTG	47.4%	-7.2	0
998-1016	AAGAACGCCAUCAUCAAGA	TCTTGATGATGGCGTTCTT	42.1%	-7.4	0
999-1017	AGAACGCCAUCAUCAAGAA	TTCTTGATGATGGCGTTCT	42.1%	-6.8	0
1000-1018	GAACGCCAUCAUCAAGAAC	GTTCTTGATGATGGCGTTC	47.4%	-7.6	0
1001-1019	AACGCCAUCAUCAAGAACG	CGTTCTTGATGATGGCGTT	47.4%	-7.3	0
1002-1020	ACGCCAUCAUCAAGAACGC	GCGTTCTTGATGATGGCGT	52.6%	-6.9	0
1003-1021	CGCCAUCAUCAAGAACGCG	CGCGTTCTTGATGATGGCG	57.9%	-6.5	0
1004-1022	GCCAUCAUCAAGAACGCGC	GCGCGTTCTTGATGATGGC	57.9%	-7.0	0
1005-1023	CCAUCAUCAAGAACGCGCA	TGCGCGTTCTTGATGATGG	52.6%	-7.3	0
1006-1024	CAUCAUCAAGAACGCGCAC	GTGCGCGTTCTTGATGATG	52.6%	-8.0	0
1007-1025	AUCAUCAAGAACGCGCACA	TGTGCGCGTTCTTGATGAT	47.4%	-7.8	0
1008-1026	UCAUCAAGAACGCGCACAA	TTGTGCGCGTTCTTGATGA	47.4%	-8.6	0
1009-1027	CAUCAAGAACGCGCACAAG	CTTGTGCGCGTTCTTGATG	52.6%	-9.3	0
1010-1028	AUCAAGAACGCGCACAAGA	TCTTGTGCGCGTTCTTGAT	47.4%	-9.7	0
1011-1029	UCAAGAACGCGCACAAGAA	TTCTTGTGCGCGTTCTTGA	47.4%	-9.2	0
1012-1030	CAAGAACGCGCACAAGAAG	CTTCTTGTGCGCGTTCTTG	52.6%	-8.4	0
1013-1031	AAGAACGCGCACAAGAAGG	CCTTCTTGTGCGCGTTCTT	52.6%	-8.3	0
1014-1032	AGAACGCGCACAAGAAGGG	CCCTTCTTGTGCGCGTTCT	57.9%	-7.4	0

Figure 3.5: Candidate antisense oligonucleotide targets on *Pomc* transcripts.

The *S*oligo module in the *S*fold program was used to predict ASO targets on the mouse mature 1214-base pair *Pomc* transcript for stretches of 19 nucleotides. Empirical guidelines for ASO design were then applied to the full output data for the predicted ASO target sites (Appendix 1) on the *Pomc* coding sequences. Target sites with a G-C content of below 40% or above 60%, those having four or more consecutive G residues, and those with a positive binding energy value (≥ 0 kcal/mol) were excluded. Potential POMC ASO sequences containing G-quartets and deleterious motifs such as TAA, AAA, and ACTG sequences were also excluded. The remaining candidate POMC ASOs and their target sites are shown as: the target position on the mRNA sequence (column 1); the target sequence (5'-end to 3'-end) (column 2); the ASO sequence (5'-end to 3'-end) (column 3); the percentage G-C content (column 4); the ASO binding energy (kcal/mol) (column 5); and the GGGG indicator, where 1 indicates at least one GGGG in the target sequence and 0 indicates otherwise (column 6).

3.3.3 Selection of POMC antisense oligonucleotides

As ASO self-complementarity can lead to the formation of internal secondary structures such as hairpins or of dimers between ASO molecules, the OligoAnalyzer program (Integrated DNA Technologies, Inc., Coralville, IA, USA) (https://eu.idtdna.com) was used to analyse the possibility of such structures forming in the potential POMC ASO molecules (Figure 3.5). The program calculates binding energies (ΔG°37) for all possible ASO-ASO interactions with values set at ≥ −1.1 kcal/mol and ≥ −8.0 kcal/mol to represent weaker secondary structures and a lesser ability to dimerise, respectively (Matveeva et al., 2003). Analysis of the potential POMC ASOs indicated that the majority had favourable binding energies with respect ASO-ASO interactions.

Guidelines for the design of ASO experimental studies strongly suggest that they include at least two different on-target ASOs, so that the outcomes are robust and interpretable (Gagnon and Corey, 2019). For this study, four different POMC ASOs were selected for experimentation. These are shown in Figure 3.6, where their nucleotide sequences and their target locations on the mature mouse *Pomc* 1214-base pair transcript are illustrated. POMC ASO2, POMC ASO3, and POMC ASO5 were targeted against exon 4, and ASO8 against exon 3, including the translation start point.

The features of the selected POMC ASOs are summarised in Table 3.2. All POMC ASOs were within the optimum length of 12-28 nucleotides, and had a G-C content ranging from 47-55%. None of the ASOs contained G-quartets or other potentially detrimental motifs. Only POMC ASO2 contained a motif that could enhance ASO function. Self-complementarity, in terms of strong hairpin secondary structures, was not evident in any of the POMC ASOs. One stronger self-dimer formation was evident in POMC ASO2.

The Basic Local Alignment Search Tool (BLAST) hosted at the online facility of the National Center for Biotechnology Information (NCBI) (Bethesda, MD, USA) (www.ncbi.nlm.nih.gov) was used to search and align any DNA sequences present in the National Institutes of Health (NIH) GenBank genetic sequence database (www.ncbi.nlm.nih.gov) with each of the POMC ASO sequences. None of the four

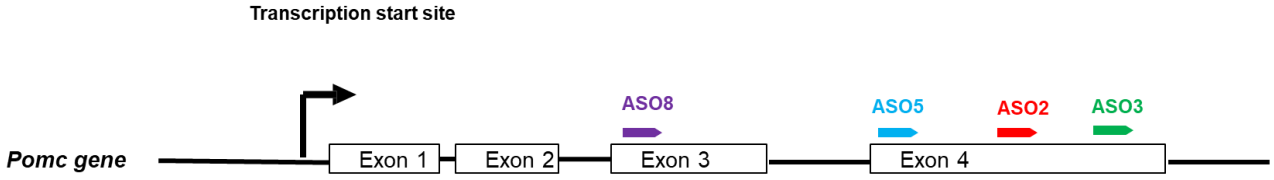
selected POMC ASO targets had any significant sequence homology to genes other than *Pomc* and, hypothetically, this would restrict any off-target impacts.

3.3.4 Homology between the selected POMC antisense oligonucleotide targets and human *POMC*

With a view that any selected POMC ASO could eventually be used in humans, it was essential to check their target sequence homology with the human *POMC* sequence. The human mature *POMC* transcript spliced to exclude introns was downloaded from the NCIB online facility. The sequence (RefSeq accession number: NM_000939.4) was 1128 base pairs (Appendix II). The EMBOSS Needle Pairwise Sequence Alignment online tool of the European Bioinformatics Institute-European Molecular Biology Laboratory (EBI-EMBL) (Cambridge, UK) (http://www.ebi.ac.uk) was used to align both mouse *Pomc* and human *POMC* transcripts (Appendix III).

The selected POMC ASO target sequences were then compared to the human *POMC* sequence, and this comparison is shown in Table 3.3. The target sequence of POMC ASO2 had 100% homology between mouse and human, but there were differences of at least one nucleotide for the remaining three POMC ASOs.

(a)



(b)

```
1     GGGACCAAAC GGGAGGCGAC GGAAGAGAAA AGAGGTTAAG AGCAGTGACT AAGAGAGGCC
61    ACTGAACATC TTTGTCCCCA GAGAGCTGCC TTTCCGCGAC AGGCAGGAGA CTGAACATGT
121   TGGAAAGATA GCGGGAGAGA AAGCCGAGTC ACAATAAACT CCTAATGGTG GAGTTCATTT
181   GTTGTTGCTG TAGACGTCCA AACCCTCGTT TCTCTGCGCA TCTTAGCAGA TCTGGGGTGG
241   TTGCATTGTG ATAATTACGT GGGTTATAGG ACAGGACGGG GTCCCTCCAA TCTTGTTTGC
301   CTCTGCAGAG ACTAGGCCTG ACACGTGGAA GATGCCGAGA TTCTGCTACA GTCGCTCAGG
361   GGCCCTGTTG CTGGCCCTCC TGCTTCAGAC CTCCATAGAT GTGTGGAGCT GGTGCCTGGA
421   GAGCAGCCAG TGCCAGGACC TCACCACGGA GAGCAACCTG CTGGCTTGCA TCCGGGCTTG
481   CAAACTCGAC CTCTCGCTGG AGACGCCCGT GTTTCCTGGC AACGGAGATG AACAGCCCCT
541   GACTGAAAAC CCCCGGAAGT ACGTCATGGG TCACTTCCGC TGGGACCGCT TCGGCCCCAG
601   GAACAGCAGC AGTGCTGGCA GCGCGGCGCA GAGGCGTGCG GAGGAAGAGG CGGTGTGGGG
661   AGATGGCAGT CCAGAGCCGA GTCCACGCGA GGGCAAGCGC TCCTACTCCA TGGAGCACTT
721   CCGCTGGGGC AAGCCGGTGG GCAAGAAACG GCGCCCGGTG AAGGTGTACC CCAACGTTGC
781   TGAGAACGAG TCGGCGGAGG CCTTTCCCCT AGAGTTCAAG AGGGAGCTGG AAGGCGAGCG
841   GCCATTAGGC TTGGAGCAGG TCCTGGAGTC CGACGCGGAG AAGGACGACG GGCCCTACCG
901   GGTGGAGCAC TTCCGCTGGA GCAACCCGCC CAAGGACAAG CGTTACGGTG GCTTCATGAC
961   CTCCGAGAAG AGCCAGACGC CCCTGGTGAC GCTCTTCAAG AACGCCATCA TCAAGAACGC
1021  GCACAAGAAG GGCCAGTGAG GGTGCAGGGG TCTTCTCATT CCAAGGCCCC CTCCCTGCAT
1081  GGGCGAGCTG ATGACCTCTA GCCTCTTAGA GTTACCTGTG TTAGGAAATA AAACCTTTCA
1141  GATTTCACAG TCGGCTCTGA TCTTCAATAA AAACTGCGTA AATAAAGTCA AAACACAACT
```

(c)

POMC ASO2	5'-GCTCTTCTCGGAGGTCATGA-3'
POMC ASO3	5'-GTTCTTGATGATGGCGTTC-3'
POMC ASO5	5'-GAAGTGACCCATGACGTAC-3'
POMC ASO8	5'-GTAGCAGAATCTCGGCATC-3'

Figure 3.6: Selected POMC antisense oligonucleotides.

A schematic representation of the POMC ASO target positions on the mouse *Pomc* gene. The sequence of the mouse mature *Pomc* 1214-base pair transcript showing the target positions of the four selected POMC ASOs at nucleotide positions 331-349 for POMC ASO8 (purple font), 559-577 for POMC ASO5 (blue font), 954-973 for POMC ASO2 (red font), and 1000-1018 for POMC ASO3 (green font). The translational start ATG codon and the translational stop TGA codon are underlined. (c) The sequences of the four selected POMC ASOs.

Table 3.2: Summary of the selected POMC antisense oligonucleotides

Feature	POMC ASO2	POMC ASO3	POMC ASO5	POMC ASO8
Position of target sequence on mRNA transcript[1]	954-973 (exon 4)	1000-1018 (exon 4)	559-577 (exon 4)	331-349 (exon 3)
Length in nucleotides	20	19	19	19
G-C content (%)	55.0	47.4	52.6	52.6
G-quartets	None	None	None	None
Enhancing motifs[2]	1 (CTCT)	None	None	None
Detrimental motifs[3]	None	None	None	None
CpG	1	1	1	1
Strong hairpin secondary structures with ΔG°37 ≤ −1.1 kcal/mol[4,5]	None	None	None	None
Strong self-dimer formation with ΔG°37 ≤ −8.0 kcal/mol[4,5]	1 (ΔG°37 = -8.53 kcal/mol)	None	None	None

[1]Figure 3.6.

[2]Enhancing motifis CCAC, TCCC, ACTC, GCCA, and CTCT.

[3]Detrimental motifs ACTG, AAA, and TAA.

[4]Values determined using OligoAnalyzer (Integrated DNA Technologies, Inc., Coralville, IA, USA) (https://eu.idtdna.com).

[5]Values as in Matveeva et al. (2003).

Table 3.3: Comparison of POMC ASO target sequences in mouse and human

ASO	Target sequence on mouse *Pomc*[1]	Target sequence on human *POMC*[2]	Sequence homology (%)
POMC ASO2	5'-TCATGACCTCCGAGAAGAGC-3'	5'-TCATGACCTCCGAGAAGAGC-3'	100
POMC ASO3	5'-GAACGCCATCATCAAGAAC-3'	5'-**A**AACGCCATCATCAAGAAC-3'	95
POMC ASO5	5'-GTACGTCATGGGTCACTTC-3'	5'-GTACGTCATGGG**C**CACTTC-3'	95
POMC ASO8	5'-GATGCCGAGATTCTGCTAC-3'	5'-GATGCCGAGAT**CG**TGCT**G**C-3'	85

[1]Mouse mature *Pomc* transcript RefSeq accession number: NM_001278581.1.

[2]Human mature *POMC* transcript RefSeq accession number: NM_000939.4.

3.3.5 Chemical modifications and gapmer design

Three groups of modified POMC ASOs were designed to allow an improvement in their pharmacological properties. They had identical sequences to the unmodified ASOs shown in Figure 3.6, but with specific modifications to the nucleic acid backbone and/or to some of the constituent nucleotides.

The first group of modified ASOs were designed to incorporate PS bonds to give a PS-modified backbone (Figure 3.7). The PS modification protects ASOs from degradation by nuclease enzymes and enhances ASO stability and target mRNA affinity.

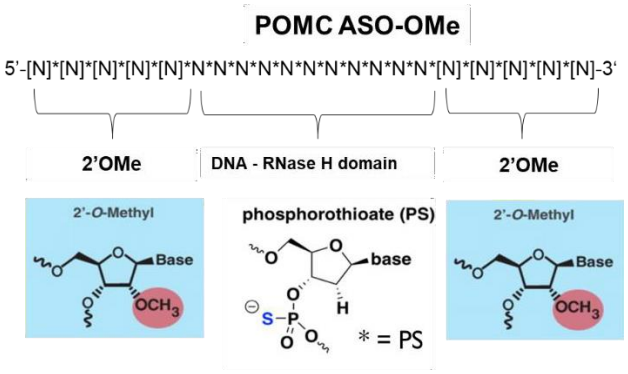
The second group had a PS-modified backbone, internal DNA nucleotides, and five OMe nucleotides at each end of the molecule (Figure 3.7). The OMe modification augments ASO affinity to their target mRNA which theoretically reduces non-specific impacts as well as improves stability. The gapmer design should allow the binding of RNase H and the subsequent degradation of the targeted mRNA.

The third group also had a PS-modified backbone, internal DNA nucleotides, and five LNA nucleotides at each end of the molecule (Figure 3.7). The LNA modification increases ASO-binding affinity and resistance to nucleases. Again, the gapmer design should allow the binding of RNase H with subsequent degradation of the mRNA target.

(a)

POMC ASO2-PS	5’-G*C*T*C*T*T*C*T*C*G*G*A*G*G*T*C*A*T*G*A-3’
POMC ASO3-PS	5’-G*T*T*C*T*T*G*A*T*G*A*T*G*G*C*G*T*T*C-3’
POMC ASO5-PS	5’-G*A*A*G*T*G*A*C*C*C*A*T*G*A*C*G*T*A*C-3’
POMC ASO8-PS	5’-G*T*A*G*C*A*G*A*A*T*C*T*C*G*G*C*A*T*C-3’
POMC ASO2-OMe	5’-[mG]*[mC]*[mT]*[mC]*[mT]*T*C*T*C*G*G*A*G*G*T*[mC]*[mA]*[mT]*[mG]*[mA]-3’
POMC ASO3-OMe	5’-[mG]*[mT]*[mT]*[mC]*[mT]*T*G*A*T*G*A*T*G*G*[mC]*[mG]*[mT]*[mT]*[mC]-3’
POMC ASO5-OMe	5’-[mG]*[mA]*[mA]*[mG]*[mT]*G*A*C*C*C*A*T*G*A*[mC]*[mG]*[mT]*[mA]*[mC]-3’
POMC ASO8-OMe	5’-[mG]*[mT]*[mA]*[mG]*[mC]*A*G*A*A*T*C*T*C*G*[mG]*[mC]*[mA]*[mT]*[mC]-3’
POMC ASO2-LNA	5’-[G]*[C]*[T]*[C]*[T]*T*C*T*C*G*G*A*G*G*T*[C]*[A]*[T]*[G]*[A]-3’
POMC ASO3-LNA	5’-[G]*[T]*[T]*[C]*[T]*T*G*A*T*G*A*T*G*G*[C]*[G]*[T]*[T]*[C]-3’
POMC ASO5-LNA	5’-[G]*[A]*[A]*[G]*[T]*G*A*C*C*C*A*T*G*A*[C]*[G]*[T]*[A]*[C]-3’
POMC ASO8-LNA	5’-[G]*[T]*[A]*[G]*[C]*A*G*A*A*T*C*T*C*G*[G]*[C]*[A]*[T]*[C]-3’

(b)



(c)

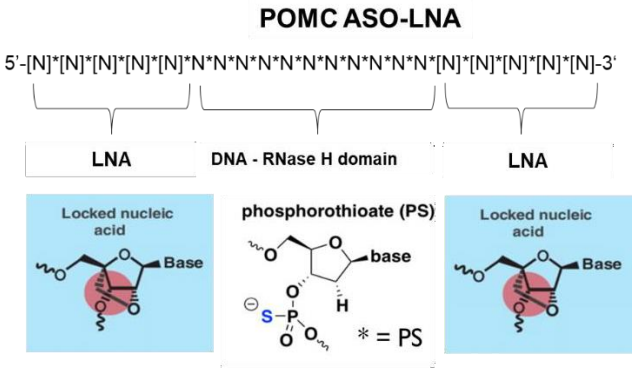


Figure 3.7: Chemical modifications and gapmer design of POMC antisense oligonucleotides.

(a)The sequence and modifications of the four selected POMC ASOs are listed. The 2’-*O*-methyl modifications are shown as [mA] [mC] [mG] or [mT], the LNA modifications are shown as [A] [C] [G] or [T], and the phosphorothioate (PS) linkages are represented by an asterisk (*). (b) Representation of the POMC ASO-OMe gapmer design with PS-modified backbone. N, nucleotide; *, PS linkage. (c) Representation of the POMC ASO-LNA gapmer design with PS-modified backbone. N, nucleotide; *, PS linkage.

3.3.6 Manufacture of POMC antisense oligonucleotides

The unmodified, PS-modified, and OMe-modified POMC ASOs were ordered and purchased from GeneLink Inc. (Hawthorne, NY, USA). The LNA-modified POMC ASOs were from Qiagen (Hilden, Germany). All ASOs arrived as lyophilised samples. They were resuspended in sterile water to a final concentration of 100 µM and stored at -20°C, prior to use in experiments.

3.4 Discussion

This part of the study aimed to design ASOs targeting mouse *Pomc* mRNA with the expectation that they would decrease ACTH secretion from AtT-20 cells after being transfected with them. Several considerations were taken into account when designing the ASOs in order to maximise the chances of successful gene-silencing. Some of the considered parameters related to the ASO molecule, some to the mRNA target site, and others to the interaction of ASOs with their target on the mRNA.

The POMC ASOs were designed using computer-aided methods, specifically the *S*fold program (Ding et al., 2004), which predicted the potential accessible regions on the pomc mRNA where ASO binding could be possible. The *S*oligo tool within the *S*fold program indicated the percentage G-C content, the presence of GGGG residues, and the binding energy values for the interaction of the mRNA with potential ASOs. These parameters were used to eliminate unfavorable POMC ASO sequences. Subsequently, the OligoAnalyzer program was used to investigate self-complementary structures forming in the remaining potential POMC ASOs. The results indicated that the majority had favorable binding energies with respect to ASO-ASO interactions from both inter- and intra-molecular base pairing (Matveeva et al., 2003).

Following the guidelines for the design of experimental studies using ASOs (Gagnon and Corey, 2019), four POMC ASOs were selected for further study. Three of the ASOs, POMC ASO2, POMC ASO3, and POMC ASO5, were targeted against exon 4 of the mouse pomc gene. The fourth, POMC ASO8, was targeted against exon 3. The selected OMe and LNA modifications, as well as the gapmer design of the POMC ASOs, were chosen to improve, theoretically, their gene-silencing effects (Chery, 2016). Using the Basic Local Alignment Search Tool (BLAST), it was found that none of the four selected POMC ASO targets had any significant sequence homology to genes other than *Pomc*. Hypothetically, this would restrict any potential off-target impacts. Although it was of great importance for any future *ex vivo* work using human pituitary tumour cells or any clinical trials using human patients, only the target of POMC ASO2 had complete homology to target in the human *POMC* gene. The other three POMC ASOs would need at least one nucleotide change to allow their use in human systems.

Chapter 4

Effect of POMC antisense oligonucleotides on ACTH production by AtT-20 cells

4 Analysis of the expression of corticotrophin-releasing hormone receptor 1 on AtT20 cells

4.1 Introduction

Antisense oligonucleotides are single-stranded, short-length oligonucleotides that have been designed to target a specific mRNA and, via sequence complementarity, produce DNA-RNA heteroduplexes. As discussed previously (Section 1.3.1), heteroduplex formation can allow the target mRNA to be degraded by RNase H, stop the translation of the mRNA by blocking the progression of ribosomes, obstruct the binding of RNA-binding proteins, or modify the mature mRNA by modulating splicing factor action.

The effects of ASOs are determined by measuring their impact upon the production of the protein encoded by their target mRNA. In some cases, ASO effects can be investigated easily, especially if a hormone or enzyme is encoded by the gene of interest as such products can easily be detected in convenient assays. In the case of CD, the effectiveness of ASOs designed to target and prevent the translation of *Pomc* mRNA can be assessed by measuring the effects on ACTH production by cultured AtT-20 cells.

The AtT-20 cells are mouse corticotrophs that can be used as a model *in vitro* system for CD. When grown in culture, they constitutively produce ACTH, so it is easy to determine the effects of ASO treatment by measuring the secretion of the hormone. Indeed, a previous study investigated an unmodified ASO against *Pomc* (Spampinato et al., 1994), and showed that after transfection of AtT-20 cells, the ASO reduced the secretion of ACTH by 48-50% at 24 h when used at a 100 nM concentration. This indicated that the cells were a valid model for testing the effects of POMC ASO molecules.

4.2 Aims and Objectives

The overall aim of this part of the project was to investigate the effects of the POMC ASOs designed in Chapter 3 on ACTH secretion from AtT-20 cells.

The specific objectives were to:

- Confirm the phenotype of AtT-20 cells, with respect to the expression of the *Pomc* gene, by detecting *Pomc* mRNA using RT-PCR and the production of ACTH using ELISA.
- Standardise a system for transfecting AtT-20 cells by using a fluorescent oligonucleotide and FACS analysis.
- Analyse the effects of POMC ASOs on ACTH secretion by transfecting AtT-20 cells and measuring the subsequent production of ACTH by ELISA.
- Analyse the effects of control ASOs, as described above.
- Determine the lowest effective concentration of POMC ASOs, as described above.
- Investigate the longevity of action of POMC ASOs, as described above.
- Investigate the effect of different POMC ASO combinations, as described above.
- Analyse the effects of POMC ASOs on cell viability by transfecting AtT-20 cells and subsequently counting viable cells.

4.3 Results

4.3.1 Analysis of the AtT-20 cell phenotype

Initially, the expression of the *Pomc* gene in AtT-20 cells needed to be confirmed by RT-PCR and ACTH immunoassay, before other experiments were undertaken.

4.3.1.1 Image of AtT-20 cells in culture

AtT-20 cells were grown in a T75 culture flask. Once confluent, which was usually after a period of five days, the cells were viewed using a Miotic AE2000 inverted microscope and images were recorded using a Ceti 5 Mpx Digi-Pad microscope tablet camera. An image of the cells at a magnification of 400x is shown in Figure 4.1. In morphology, the cells presented as elongated and fusiform, and they resembled AtT-20 cells shown in several previous studies (Ikeda et al., 2004, Mains et al., 1999, Aguado et al., 1997, Vedder, 1990).

4.3.1.2 Analysis of ACTH secretion from AtT-20 cells

To confirm that the AtT-20 cells secreted measurable levels of ACTH and to determine the baseline levels of ACTH overtime, cells were plated at a density of 2 x 10^5 cells/well in 6-well plates with 2 ml of culture medium. They were incubated in 5% CO_2 in a humidified incubator at 37°C for 24 h. At this time point, a 30-µl sample of the culture medium was taken from two wells. The samples were analysed using an Immulite 2000® ACTH immunoassay and an average ACTH concentration in pg/ml was calculated. In addition, a viable cell count in cells/ml using Trypan Blue staining (Section 2.3.4) was undertaken for the four remaining wells and an average value recorded. Other plates were incubated for periods of 48, 72, 96 or 120 h. At each time point, the culture medium was sampled and viable cell counting was performed, as described for the 24 h time point.

The results of the ACTH determinations and of the viable cell counting over the five-day period are illustrated in Figure 4.2 and represent the mean of three separate experiments. The number of viable cells increased from 1 x 10^5 cells/ml (initial number of cells plated) to 9.6 x 10^5 cells/ml (mean) at 120 h. The levels of ACTH were seen to rise from 5.2 x 10^4 pg/ml (mean) at 24 h to 1.61 x 10^5 pg/ml (mean) at 120 h. On Figure

4.2, the viable cell counts are represented as cells x 10^{-1}/ml for ease of plotting the values alongside the ACTH levels. Viable cell counts compared with ACTH concentrations showed that the two variables increased at the same rate over the five-day time period.

In conclusion, expression of *Pomc* in AtT-20 cells, in terms ACTH production, was confirmed in the experiments.

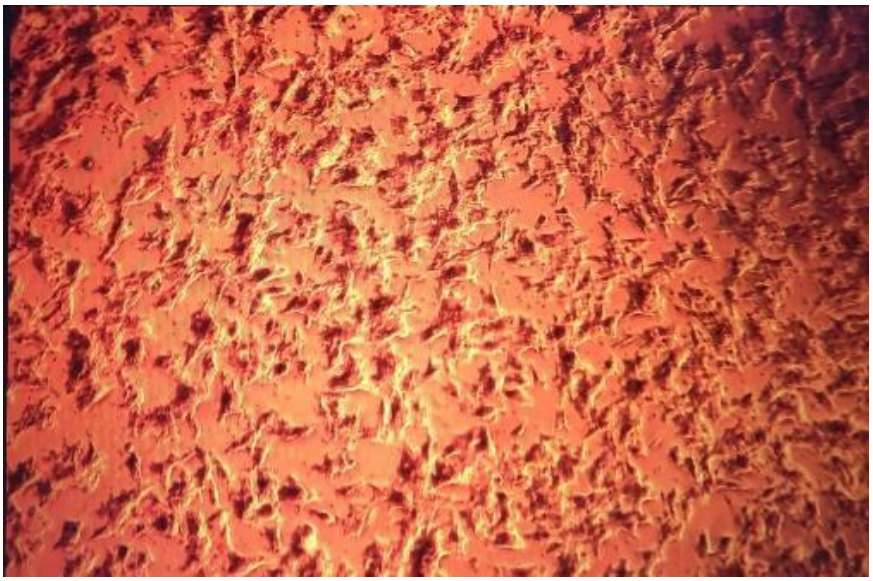


Figure 4.1: Image of AtT-20 cells in culture.

AtT-20 cells were grown in a T75 culture flask. After 120 h, they were viewed using a Miotic AE2000 inverted microscope and images were recorded using a Ceti 5 Mpx Digi-Pad microscope tablet camera. The cells are at a magnification of 400x.

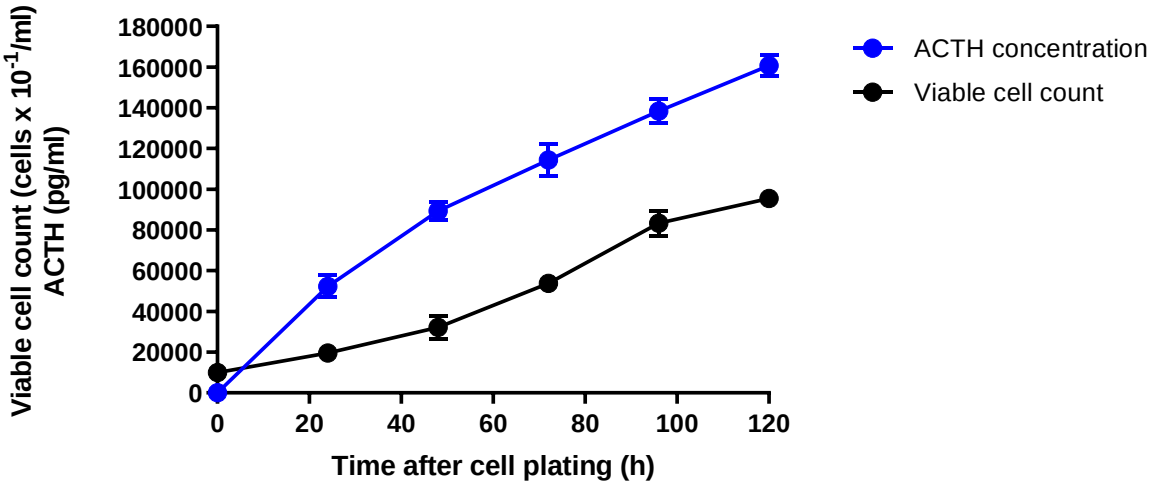


Figure 4.2: Levels of ACTH secreted over time by AtT-20 cells.

The ACTH levels (mean pg/ml ± SD of three experiments) secreted from AtT-20 cells over five days are shown. The viable cell count (mean cells x 10^{-1}/ml ± SD of three experiments) over the same period are also illustrated.

4.3.1.3 Preparation of RNA and cDNA from AtT-20 cells

AtT-20 cells were grown in a T75 flask and then collected for RNA preparation. Total RNA was extracted from pelleted cells according to a RNAeasy Mini Kit (Section 2.9.1) and treated with a TURBO DNA-free Kit (Section 2.9.2) to remove any contaminating genomic DNA. RNA concentrations were estimated by a NanoDrop 2000 spectrophotometer.

The total RNA prepared from samples of 1 x 10^7 AtT-20 cells was 3.7 ± 1.2 µg (mean ± SD; *n* = 12). To analyse the quality of the RNA, samples were electrophoresed in 1% agarose gels. In most samples, bands representing 28S and 18S ribosomal RNAs were observed as sharp bands and at a ratio of approximately 2:1, respectively (Figure 4.3) indicating that the RNA samples were of good quality and without degradation. Ribosomal RNA bands that appeared as a smear were judged to have undergone degradation either before or during purification of the RNA and were discarded. Pure RNA has an absorbance ratio at 260 nm to 280 nm of 2.0. Usually, the absorbance ratio of RNA samples were close to this value, so they were considered of sufficient purity for use.

To prepare AtT-20 cell cDNA, total RNA was subjected to reversed transcription using a High-Capacity cDNA Reverse Transcription Kit with MultiScribe™ RT, as detailed in Section 2.10. As a control for the presence of contaminating genomic DNA in the RNA preparations, a reaction without adding RT was also set up.

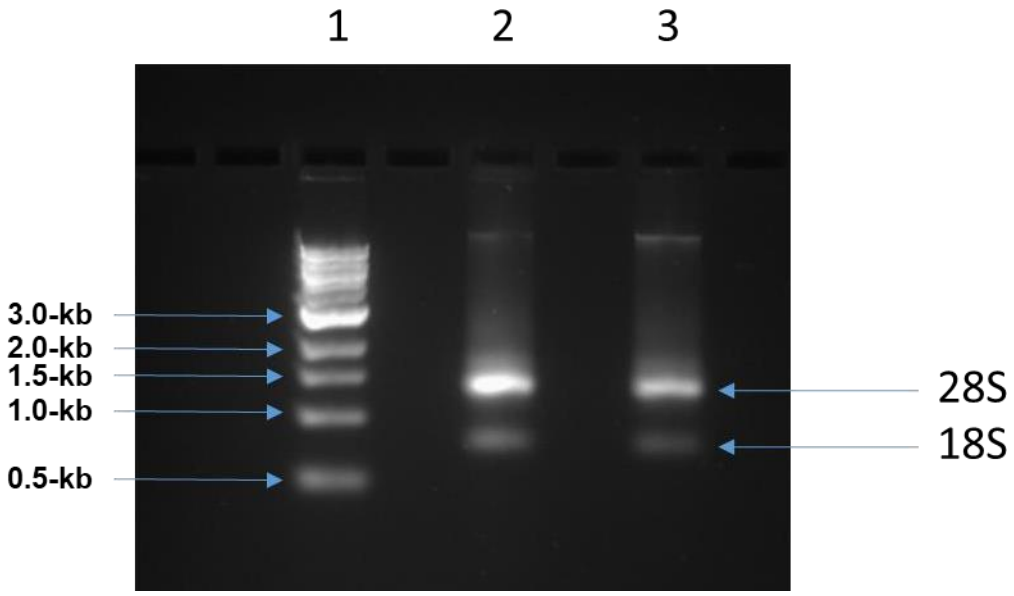


Figure 4.3: Agarose gel of AtT-20 cell total RNA preparations.

Following total RNA extraction from AtT-20 cells, 5-µl samples were elecrophoresed on a 1% agarose gel. Lane 1, 1-kb (kilobase) DNA markers; lane 2, total AtT-20 RNA; lane 3, total AtT-20 RNA. The ribosomal 28S and 18S RNA bands are indicated.

4.3.1.4 Polymerase chain reaction amplification of *Pomc*

The RT reactions (with and without RT) were used as templates for PCR amplification using primers that were specific for *Pomc*, POMC-Forward and POMC-Reverse (Table 2.5). The primers were designed to be intron-skipping (Figure 4.4). This avoids PCR amplification from any contaminating genomic DNA that might be in the RNA samples. The PCR amplification product was expected to be 514 base pairs (Figure 4.4). In addition, intron-skipping S15 control primers (Table 2.5) were used to compare the quality of cDNA samples. These primers amplified the *Rps15* house-keeping gene that encodes a ribosomal subunit protein and were expected to yield a 361-base pair PCR amplification product.

Firstly, PCR amplification reactions of the RT samples (with and without RT) were set up as described in Section 2.11.2 using POMC-specific or S15-specific primers. PCR reactions without any RT sample at all were also included to check that the PCR reagents were uncontaminated with DNA. After 35 cycles of PCR amplification, the PCR products were run on an agarose gel. The results are illustrated in Figure 4.5.

The results showed that PCR products of the anticipated size, 514 base pairs for *Pomc* and 361 base pairs for *Rps15*, had been amplified in the PCR reactions where RT samples (with RT) had been added. PCR products were not observed from PCR reactions containing RT samples without RT confirming that there was no contaminating genomic DNA in the initial RNA preparations. In addition, PCR products were not seen on the gel in lanes containing PCR reactions that were set up without any RT reaction being added, indicating that none of the PCR reagents was contaminated with DNA.

Figure 4.4: Target sites of POMC primers on the mouse *Pomc* gene.

Part of the mouse *Pomc* gene sequence showing translated regions in yellow, untranslated regions in pink, and introns in green. The target sites of the intron-skipping POMC-Forward and POMC-Reverse PCR primers are in bold type and are underlined.

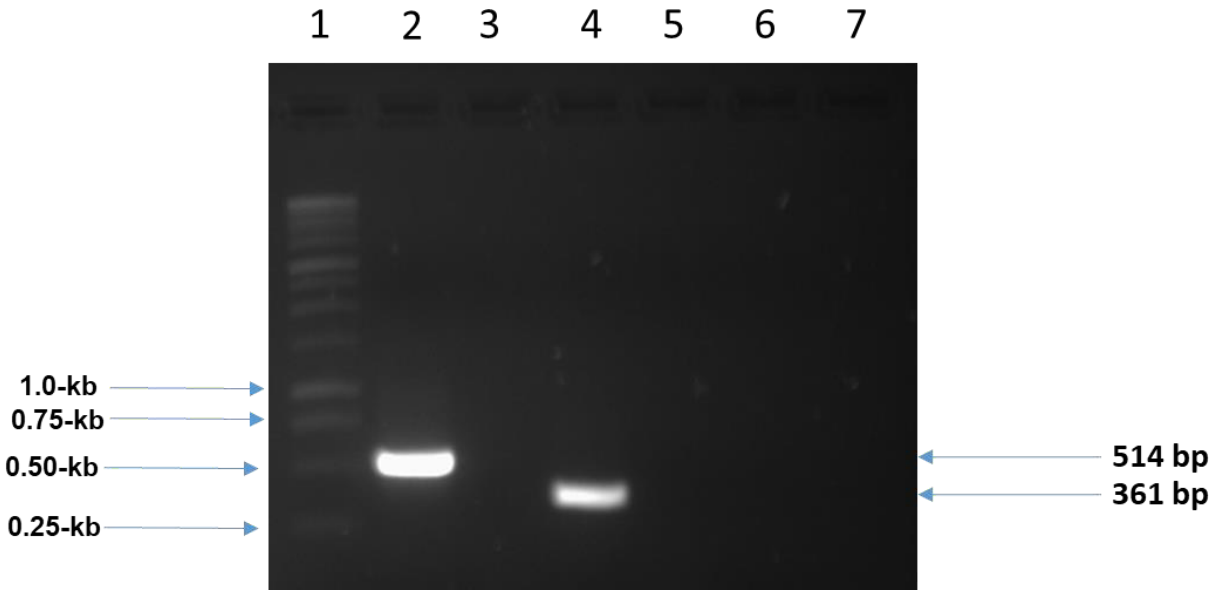


Figure 4.5: Agarose gel of AtT-20 *Pomc* and *Rps15* PCR products.

Following PCR amplification, 5-µl samples of the PCR reactions were run on a 1% agarose gel. Lane 1, 1-kb (kilobase) DNA markers; lane 2, RT sample with RT and POMC primers; lane 3, RT sample without RT and POMC primers; lane 4, RT sample with RT and S15 primers; lane 5, RT sample without RT and S15 primers; lane 6, PCR reaction without any RT reaction added and POMC primers; lane 7, PCR reaction without any RT reaction added and S15 primers. The *Pomc* PCR product at 514 base pairs (bp) and the *Rps15* PCR product at 361 bp are indicated.

4.3.1.5 Sequencing of the PCR amplification product

To check that the PCR product amplified by the POMC primers was as expected with respect to its sequence, it was excised from a 1% agarose gel and then purified using a Wizard PCR Preps DNA Purification System (Section 2.13). Sequencing of the PCR product, using POMC-Forward and POMC-Reverse primers (Table 2.5), was carried out by the Genewiz at Azenta Life Sciences service (Section 2.14).

The result of the sequencing is shown in Figure 4.6. Using the EMBOSS Needle Pairwise Sequence Alignment online tool at the European Bioinformatics Institute-European Molecular Biology Laboratory (Cambridge, UK) (http://www.ebi.ac.uk), the sequenced PCR product was compared with the mouse mature *Pomc* transcript (Figure 3.3). This showed 100% homology between the two sequences (Figure 4.6) and confirmed that the PCR product obtained was the one expected.

Overall, expression of *Pomc* by AtT-20 cells, in terms of mRNA synthesis, was confirmed by the RT-PCR analysis.

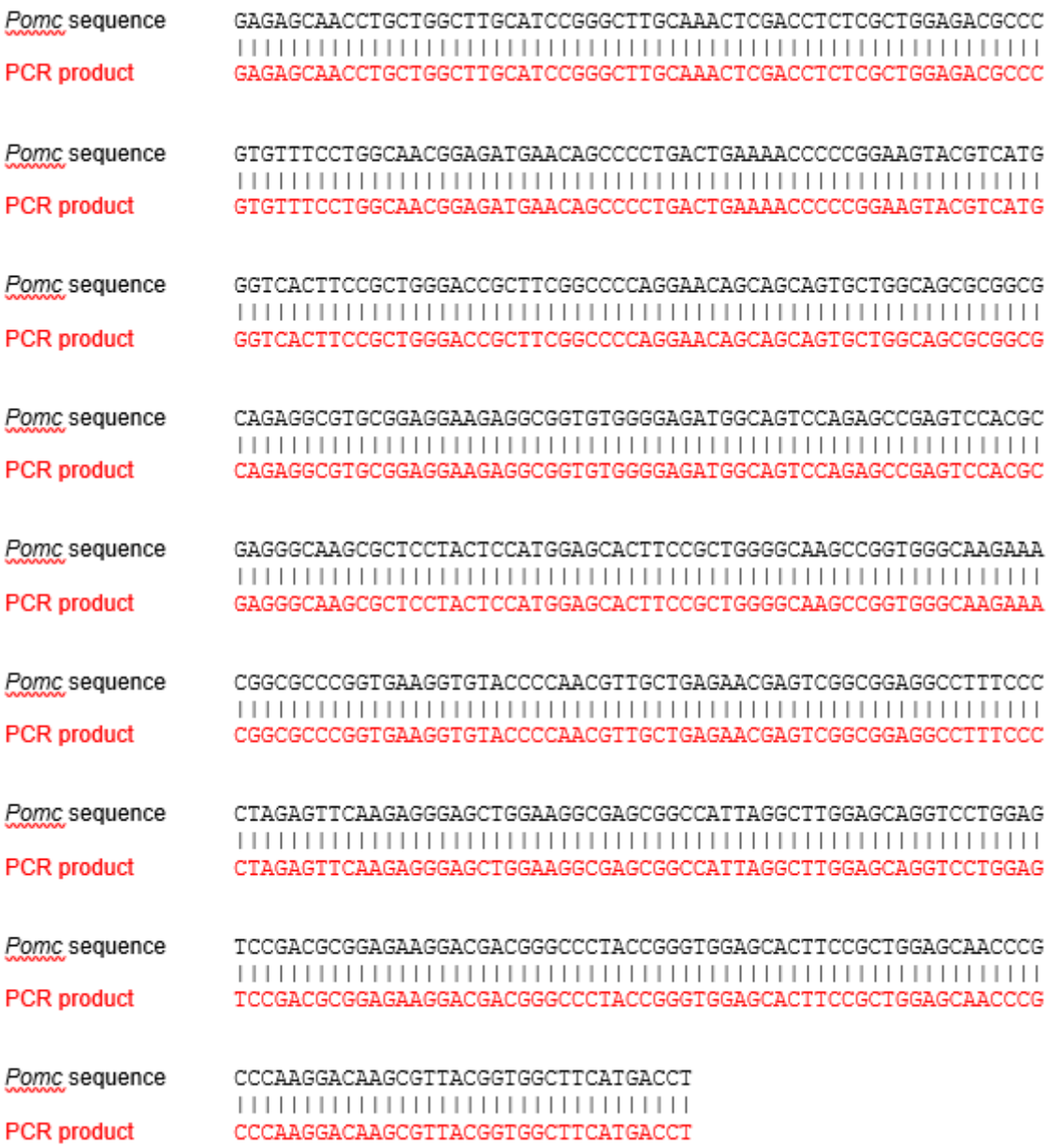


Figure 4.6: Alignment of the mouse mature *Pomc* transcript and PCR product.

The sequence of the 514-base pair PCR product amplified with POMC-specific primers was aligned with the mouse mature *Pomc* transcript using the EMBOSS Needle Pairwise Sequence Alignment online tool at the European Bioinformatics Institute-European Molecular Biology Laboratory (EBI-EMBL) (Cambridge, UK) (http://www.ebi.ac.uk).

4.3.2 Transfection of AtT-20 cells

Transfection of AtT-20 cells was essential to deliver POMC ASOs in order that their effects on *Pomc* expression could be analysed. Initially, therefore, the transfection efficiency of AtT-20 cells needed to be determined, and any deleterious effects of the transfection reagent on cell viability needed to be assessed, as this could affect the output results.

4.3.2.1 Determination of AtT-20 cell transfection efficiency

To estimate the transfection efficiency of AtT-20 cells, cells were plated into 6-well plates at 2 x 10^5 cells/well in 2 ml of culture medium. After 24 h, the cells were transfected in duplicate with BLOCK-IT™ Fluorescent Oligo at a final concentration of 100 nM and Lipofectamine®-2000 Reagent at a final dilution of 1:200 (recommended). Control treatments of AtT-20 cells were BLOCK-IT™ Fluorescent Oligo alone and Lipofectamine®-2000 Reagent alone. Untreated AtT-20 cells were also included to set the FACS analyser gate. Following 24 h of incubation, the cells were prepared for FACS analysis as in Section 2.5.2.

The FACS analyser was calibrated using untreated cells to standardise the background fluorescence and to determine the modal cell size. A doublet-discrimination gate was also set so to exclude non-viable cells and aggregates of cells. The calibration meant that a fluorescence cut-off threshold of 10^3 was set on the x-axis of the FACS histogram and that only single cells were counted. Cells to the right of the fluorescence cut-off threshold were counted as being transfected and the percentage of the total cells that were fluorescent was the transfection efficiency.

In Figure 4.7, the results of one FACS analysis experiment, following transfection of AtT-20 cells, are illustrated. For treatment with BLOCK-IT™ Fluorescent Oligo alone, 0.5% and 0.7% (duplicate samples) of the AtT-20 cells were fluorescently-labelled. This indicated that to enter the cells, the BLOCK-IT™ Fluorescent Oligo required the presence of Lipofectamine®-2000 Reagent (Figure 4.7a). For treatment with Lipofectamine®-2000 Reagent alone, 0.4% and 0.2% (duplicate samples) of the AtT-20 cells showed fluorescence. This indicated that the cells did not have background auto-fluorescence to any significant level (Figure 4.7b). In contrast, for AtT-20 cells

treated with BLOCK-IT™ Fluorescent Oligo and Lipofectamine®-2000 Reagent, 80.0% and 84.1% (duplicate samples) of cells fluoresced and caused a right-shift on the FACS analysis histogram (Figure 4.7c). This indicated that the AtT-20 cells had been transfected successfully.

Overall, the AtT-20 cell transfection efficiency ranged from 67.9-87.9% (mean ± SD = 81.0% ± 5.95%; *n* = 9) (Table 4.1; Figure 4.8). Although transfection efficiency was variable, AtT-20 cells were transfected to an acceptable level using Lipofectamine®-2000 Reagent at a 1:200 final dilution. On treatment with Lipofectamine®-2000 Reagent alone or BLOCK-IT™ Fluorescent Oligo alone, AtT-20 cells showed only 0.11-0.45% fluorescence (mean ± SD = 0.32% ± 0.11%; *n* = 9) and 0.60-2.65% fluorescence (mean ± SD = 1.44% ± 0.71%; *n* = 9), respectively, (Table 4.1 and Figure 4.8).

The same protocol was used to test the efficiency of transfection of AtT-20 cells when using the Lipofectamine®-2000 Reagent at a final dilution of 1:50, 1:100, 1:500, and 1:1000. The results are summarised in Table 4.2. Final dilutions of 1:500 and 1:1000 gave lower transfection efficiencies of 39.8-49.3% and 15.6-23.5%, respectively, compared with that obtained at the recommended 1:200 dilution. In contrast, at final dilutions of 1:100 and 1:50, the transfection efficiencies were slightly higher at 79.6-90.3% and 80.1-91.4%, respectively.

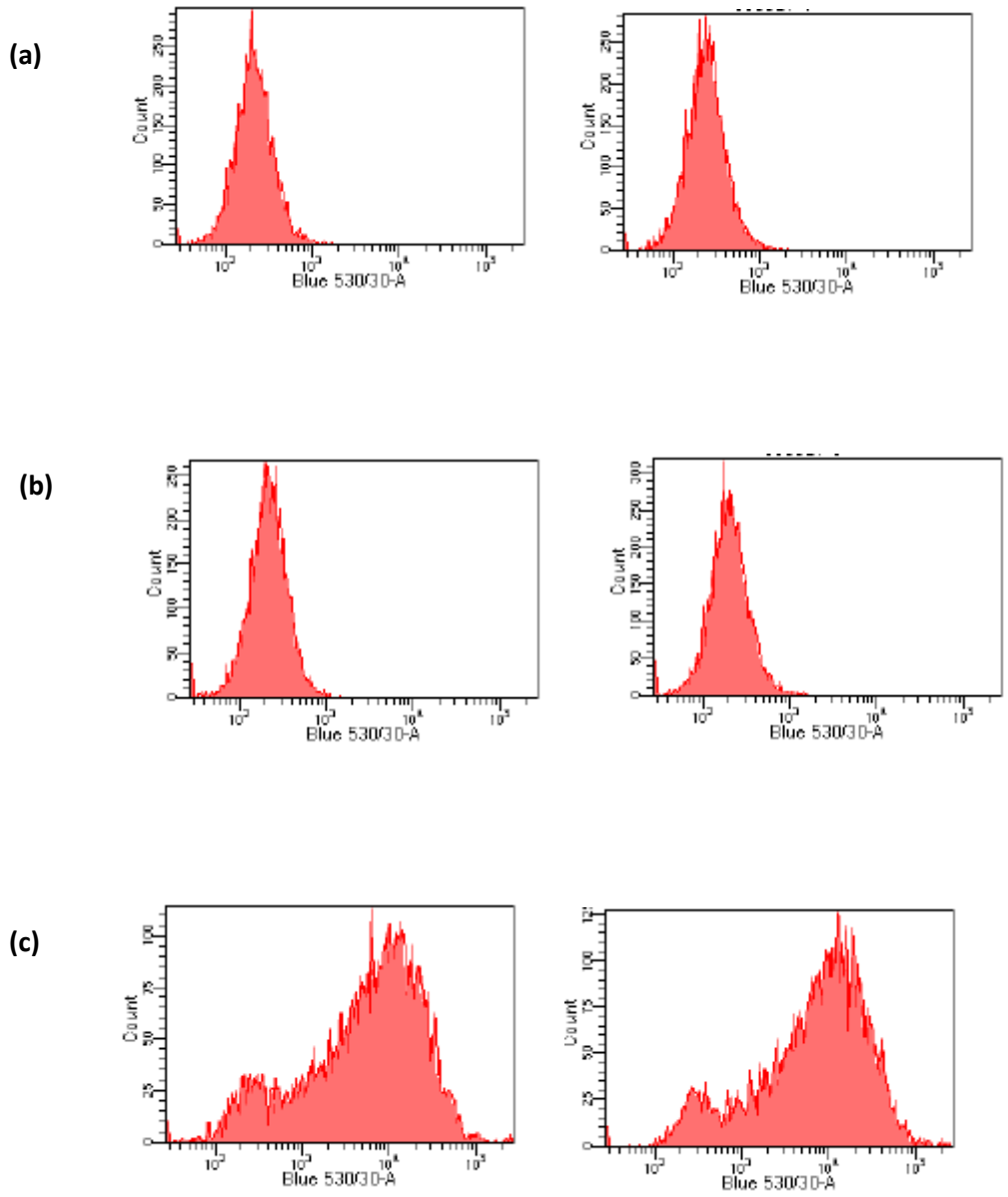


Figure 4.7: AtT-20 cell transfection efficiency.

(a) In AtT-20 cells treated with BLOCK-IT™ Fluorescent Oligonucleotide alone, 0.5% and 0.7% (duplicate samples) of cells were fluorescent; (b) In AtT-20 cells treated with Lipofectamine®-2000 Reagent alone, 0.4% and 0.2% (duplicate samples) were fluorescent; (c) In AtT-20 cells treated with BLOCK-IT™ Fluorescent Oligo and Lipofectamine®-2000 Reagent, 80.0% and 84.1% (duplicate samples) of cells were fluorescent. The fluorescence cut-off threshold of 10^3 is shown on the x-axis of each histogram.

Table 4.1: Efficiency of transfection for AtT-20 cells

Experiment number	Transfection efficiency (% fluorescently-labelled cells after treatment with BLOCK-IT™ Fluorescent Oligo alone)[1]	Transfection efficiency (% fluorescently-labelled cells after treatment with Lipofectamine®-2000 Reagent alone)[1]	Transfection efficiency (% fluorescently-labelled cells after treatment with BLOCK-IT™ Fluorescent Oligo and Lipofectamine®-2000 Reagent)[1]
1	2.65	0.40	77.1
2	0.75	0.15	67.9
3	1.15	0.35	78.1
4	0.60	0.30	82.1
5	1.15	0.30	84.6
6	1.30	0.15	84.7
7	1.60	0.40	82.0
8	1.30	0.40	84.3
9	2.50	0.45	87.9
Mean	**1.44**	**0.32**	**81.0**
SD	**0.71**	**0.11**	**5.95**
Range	**0.60-2.65**	**0.11-0.45**	**67.9-87.9**

[1]The transfection efficiency is the mean of two samples carried out within the same experiment.

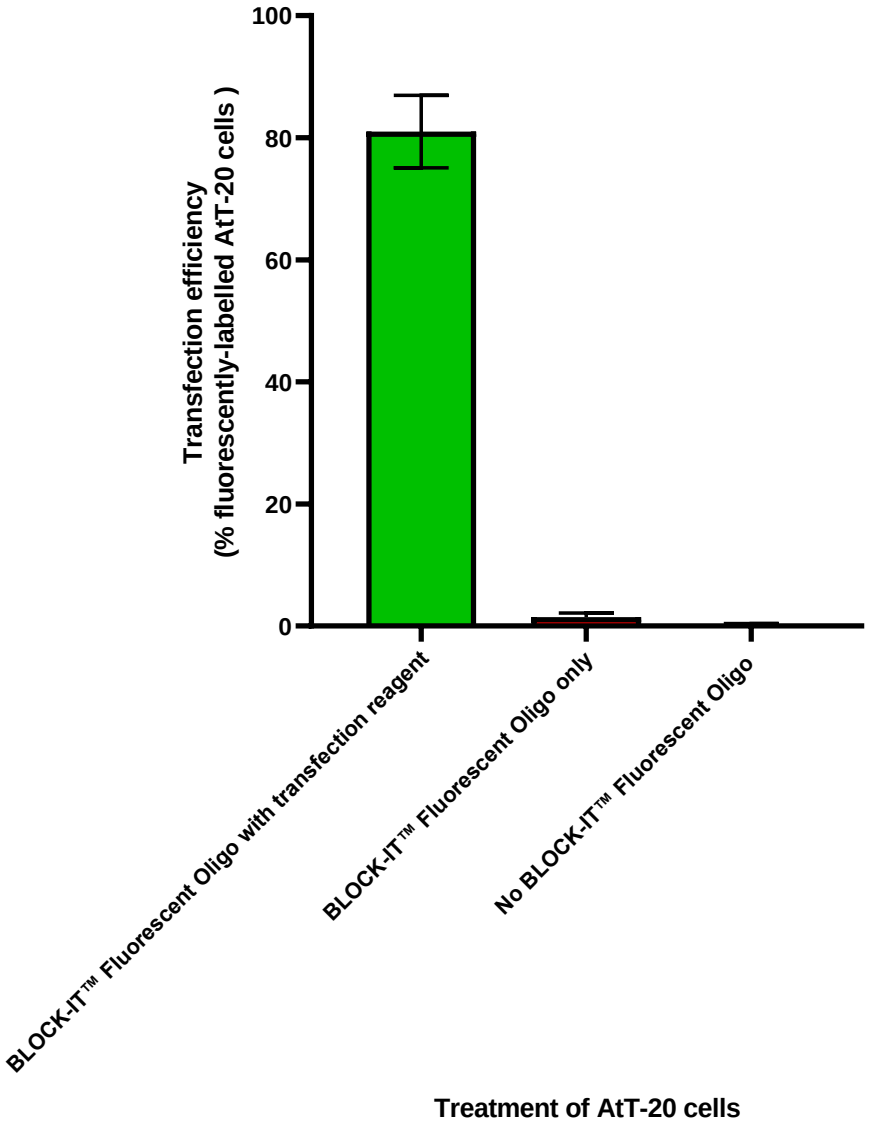


Figure 4.8: Graph showing the transfection efficiency of AtT-20 cells using Lipofectamine®-2000 Reagent and BLOCK-IT™ Fluorescent Oligo.

AtT-20 cells were transfected with BLOCK-IT™ Fluorescent Oligo at 100 nM using Lipofectamine®-2000 Reagent. Control transfections were AtT-20 cells treated with the oligonucleotide alone or treated with Lipofectamine®-2000 Reagent alone. The results shown for transfection efficiency are the mean (± SD) of nine separate experiments. Compared with the two control transfections, there was a significant increase in the percentage of fluorescently-labelled cells when they were treated with both the BLOCK-IT™ Fluorescent Oligo and Lipofectamine®-2000 Reagent (Unpaired t tests, $P < 0.05$).

Table 4.2: AtT-20 cell transfection efficiency at different dilutions of Lipofectamine®-2000 Reagent

Lipofectamine®-2000 Reagent final dilution	Transfection efficiency of AtT-20 cells (%)[1]
1:50	80.1-91.4[2]
1:100	79.6-90.3[2]
1:200	67.9-87.9
1:500	39.8-49.3[2]
1:1000	15.6-23.5[2]

[1]Transfection efficiency is the percentage of fluorescently-labelled AtT-20 cells after treatment with BLOCK-IT™ Fluorescent Oligo and Lipofectamine®-2000 Reagent.

[2]These data were provided by Dr Helen Kemp (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK).

4.3.2.2 Effect of transfection reagent on AtT-20 cell viability

Although AtT-20 cells were transfected to a reasonable efficiency 67.9-87.9% using Lipofectamine®-2000 Reagent at the recommended 1:200 dilution, such liposomal reagents can be cytotoxic and thus adversely affect experimental results. Therefore, the toxicity profile of the Lipofectamine®-2000 Reagent was investigated, with respect to AtT-20 cells, to ensure a high transfection rate could be balanced against any potential cellular toxicity.

AtT-20 cells were plated in 6-well plates at a density of 2 x 10^5 cells per well with 2 ml of culture medium. They were incubated in 5% CO_2 in a humidified incubator at 37°C for 24 h. The cells were then treated with Lipofectamine®-2000 Reagent at a final dilution of 1:200 (recommended), 1:50, 1:100, 1:500, and 1:1000. Cells without any treatment were also included in the experiments. After 24 h incubation, viable cells were counted using Trypan Blue staining (Section 2.3.4).

The results of four separate experiments are shown in Figure 4.9. The mean viable cell count at 1:200, 1:500, and 1:1000 dilutions of Lipofectamine®-2000 Reagent was 3.07 x 10^5, 3.09 x 10^5, and 3.08 x 10^5 cells/ml, respectively. Compared with untreated cells at 3.39 x 10^5 cells/ml, there was a slight but not significant reduction in the viable cell count (Unpaired t test, $P > 0.05$). At a 1:50 or a 1:100 dilution of Lipofectamine®-2000 Reagent, there was a significant decrease in the viable cell count with 2.76 x 10^5 cells/ml and 1.39 x 10^5 cells/ml, respectively (Unpaired t test, $P < 0.0001$ and $P = 0.0094$, respectively).

The results indicated that use of the recommended final dilution of 1:200 of Lipofectamine®-2000 Reagent in transfection experiments, which also gave a transfection efficiency of 67.9-87.9% (Table 4.2), did not have a significant effect upon cell viability at 24 h post-transfection. Lower dilutions of 1:50 and 1: 100, although providing slightly higher transfection efficiencies (Table 4.2), they had a negative impact on cell viability. Higher dilutions of 1:500 and 1:1000 did not affect the viability of AtT-20 cells significantly, but transfections efficiencies were reduced (Table 4.2). Therefore, a 1:200 final dilution of Lipofectamine®-2000 Reagent was used in further transfection experiments.

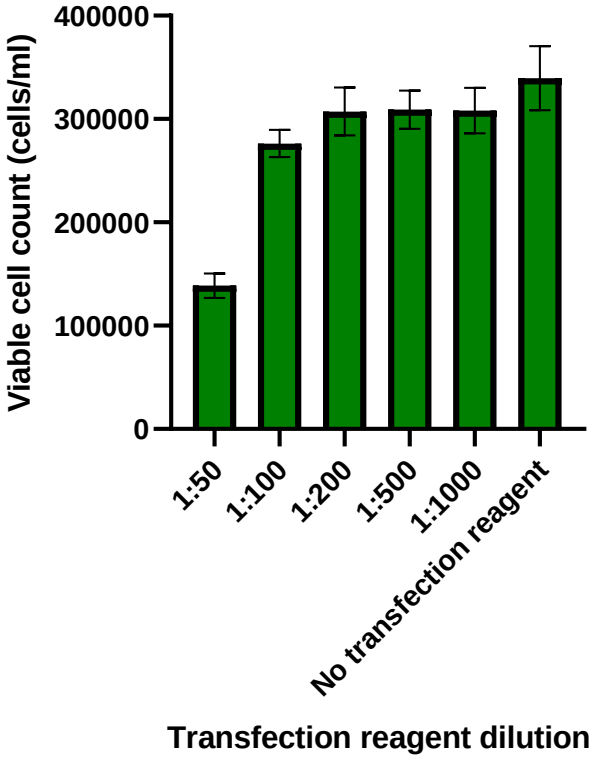


Figure 4.9: Cell viability in relation to treatment with Lipofectamine®-2000 Reagent.

AtT-20 cells were treated with Lipofectamine®-2000 Reagent at dilutions of 1:200 (recommended), 1:50, 1:100, 1:500, and 1:1000. Untreated cells were also included. After 24 h, the cells were counted. The mean (± SD) viable cell counts of four separate experiments are shown. Treatment with Lipofectamine®-2000 Reagent at dilution of 1:200, 1:500, and 1:1000 compared with untreated cells had no significant effect on the viable cell count (Unpaired t test, P = > 0.05). At a 1:50 or a 1:100 dilution of Lipofectamine®-2000 Reagent, there was a decrease in the viable cell count (Unpaired t test, $P < 0.0001$ and $P = 0.0094$, respectively).

4.3.3 Investigation of the effects of different POMC ASOs on ACTH secretion from cultured AtT-20 cells

4.3.3.1 Effect of unmodified POMC ASOs on ACTH secretion

The effect that POMC-targeted ASOs might have on ACTH secretion from AtT-20 cells was investigated initially by transfecting them with unmodified POMC ASO2, 3, 5, and 8 (Table 2.1).

As detailed in Section 2.5, AtT-20 cells were plated in 6-well plates at 2 x 10^5 cells per well. At 24 h, the cells were transfected in duplicate with ASO at a final concentration of 100 nM. Control treatments included Lipofectamine®-2000 Reagent only and ASO only. Untreated AtT-20 cells were also included in all experiments so that baseline ACTH secretion could be determined. At 24 h, a 30-µl aliquot of the culture medium was collected and the ACTH concentration determined using an Immulite 2000® ACTH immunoassay (Section 2.6).

The results of four individual experiments are in Figure 4.10. They indicated that treating AtT-20 cells with unmodified POMC ASO2, 3, 5, or 8 reduced the concentration of ACTH in the culture medium significantly when compared with untreated cells (Unpaired t test, $P < 0.0001$). In contrast, there was no significant effect upon ACTH secretion when cells were treated with either POMC ASOs alone or with Lipofectamine®-2000 Reagent alone (Unpaired t test, $P > 0.05$).

The effects of the unmodified POMC ASOs on ACTH secretion from AtT-20 cells are compared in Table 4.3. Unmodified POMC ASO5 was the most effective, reducing secreted ACTH to 28% of that normally secreted from untreated AtT-20 cells. The levels of ACTH reduction achieved with ASO5 and ASO8 were significantly more than ASO2. ASO5 was also significantly more effective than ASO3.

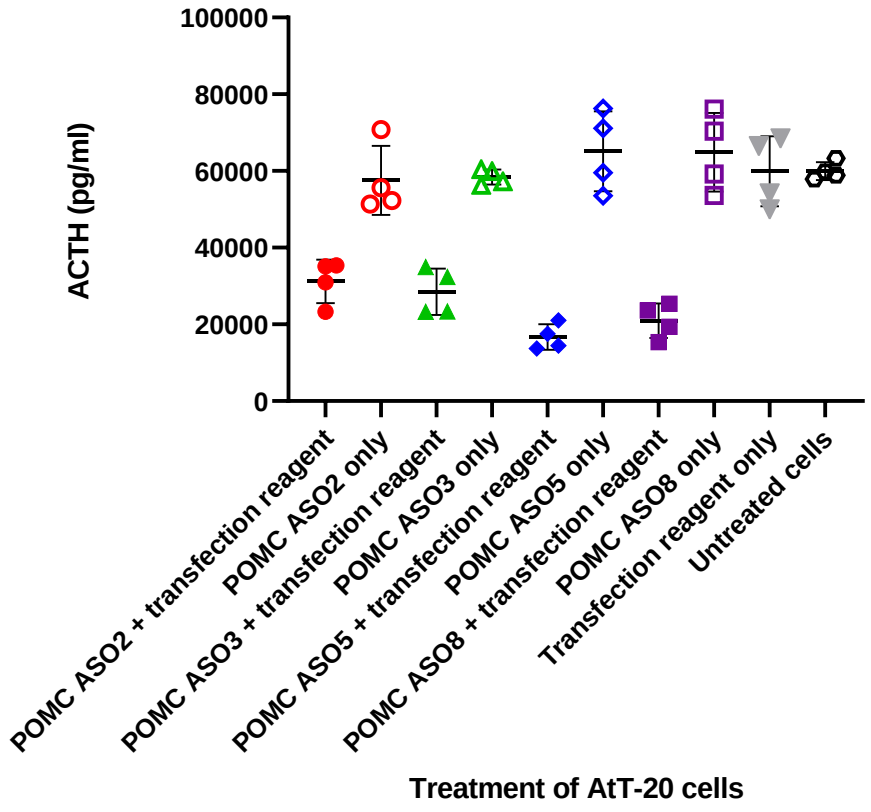


Figure 4.10: ACTH levels secreted by AtT-20 cells following transfection with unmodified POMC ASOs.

AtT-20 cells were transfected with unmodified POMC ASO2, 3, 5, or 8 at 100 nM. The levels of ACTH in the culture medium were measured in samples taken 24 h post-transfection. Control treatments used were transfection reagent alone and ASO alone. To give a baseline level of ACTH secretion, untreated cells were also included in the experiments. The results show the mean (± SD) ACTH concentrations measured in four individual experiments. All four unmodified POMC ASOs reduced ACTH levels significantly when compared to untreated cells (Unpaired t test, $P < 0.0001$). There was no significant effect on ACTH secretion when cells were treated with ASO only or with Lipofectamine®-2000 Reagent only (Unpaired t test, $P > 0.05$).

Table 4.3: Comparison of the effectiveness of unmodified POMC ASOs in reducing ACTH secretion from AtT-20 cells

Unmodified POMC ASO used to treat AtT-20 cells	**ACTH secreted compared with untreated AtT-20 cells compared with levels secreted by untreated cells**
ASO2	52%
ASO3	48%
ASO5	28%
ASO8	35%
Unmodified POMC ASOs compared	***P* value (Unpaired t test)[1]**
ASO2 vs ASO3	0.54
ASO2 vs ASO5	**0.0045**
ASO2 vs ASO8	**0.029**
ASO3 vs ASO5	**0.014**
ASO3 vs ASO8	0.091
ASO5 vs ASO8	0.18

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified unmodified ASOs. Significant *P* values are shown in bold type.

4.3.3.2 Effect of PS-modified POMC ASOs on ACTH secretion

Subsequently, the effect that PS-modified POMC ASOs had upon the secretion of ACTH from AtT-20 cells was investigated. The ASOs tested were POMC ASO2-PS, ASO3-PS, ASO5-PS, and ASO8-PS (Table 2.1).

The experiments were undertaken exactly as described in Section 4.3.3.1, with 30-µl samples of cell culture medium being collected at 24 h post-transfection, and the ACTH concentration determined using an Immulite 2000® ACTH immunoassay (Section 2.6).

The results of four separate experiments are shown in Figure 4.11. They indicated that treating AtT-20 cells with PS-modified POMC ASOs decreased the levels of ACTH in the culture medium significantly when compared with untreated cells (Unpaired t test, $P < 0.0001$). In contrast, there was no significant effect upon ACTH secretion when cells were treated with either POMC ASOs alone or with Lipofectamine®-2000 Reagent alone (Unpaired t test, $P > 0.05$).

The effects of PS-modified ASOs on the secretion of ACTH from AtT-20 cells are compared in Table 4.4. POMC ASO5-PS was the most effective, reducing secreted ACTH to 23% of that normally secreted from untreated AtT-20 cells. The levels of ACTH reduction achieved with ASO3-PS, ASO5-PS, and ASO8-PS were significantly higher than ASO2-PS. ASO5-PS and ASO8-PS were also significantly more effective than ASO3-PS, and ASO5-PS was more effective than ASO8-PS.

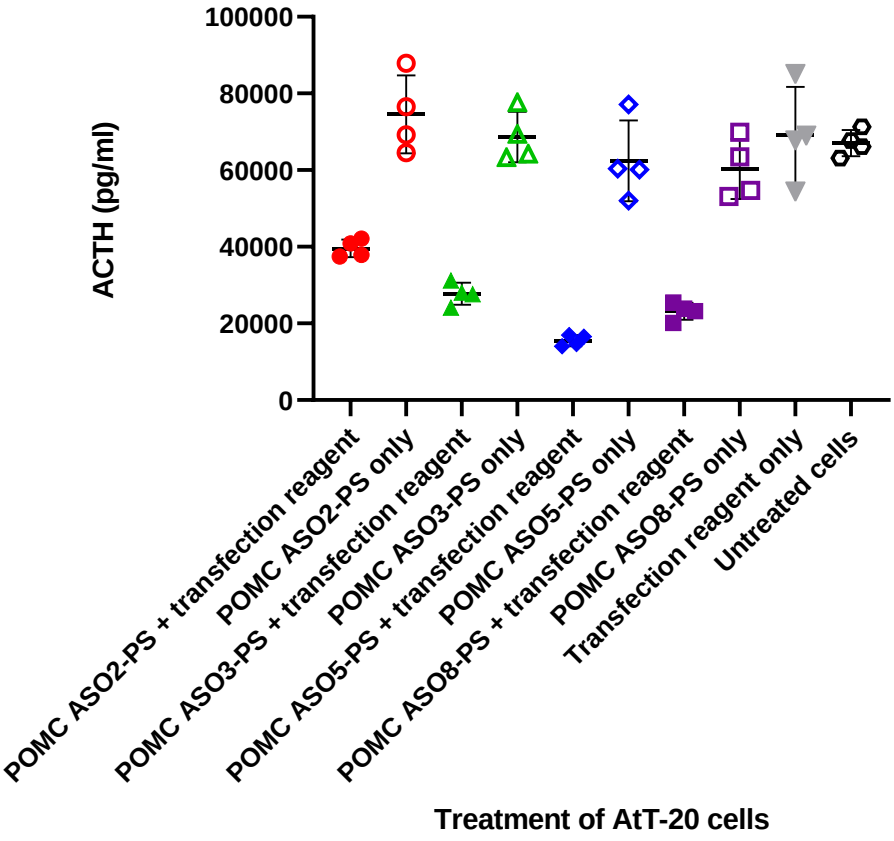


Figure 4.11: ACTH levels secreted by AtT-20 cells following transfection with PS-modified POMC ASOs.

AtT-20 cells were transfected with PS-modified POMC ASO2, 3, 5, or 8 at 100 nM. The levels of ACTH in the culture medium were measured in samples taken 24 h post-transfection. Control treatments used were transfection reagent alone and ASO alone. To give a baseline level of ACTH secretion, untreated cells were also included in the experiments. The results show the mean (± SD) ACTH concentrations measured in four individual experiments. All four PS-modified POMC ASOs reduced ACTH levels significantly when compared to untreated cells (Unpaired t test, $P < 0.0001$). There was no significant effect on ACTH secretion when cells were treated with ASO only or with Lipofectamine®-2000 Reagent only (Unpaired t test, $P > 0.05$).

Table 4.4: Comparison of the effectiveness of PS-modified POMC ASOs in reducing ACTH secretion from AtT-20 cells

PS-modified POMC ASO used to treat AtT-20 cells	**ACTH secreted compared with levels secreted by untreated AtT-20 cells**
ASO2-PS	59%
ASO3-PS	41%
ASO5-PS	23%
ASO8-PS	35%
PS-modified POMC ASOs compared	***P* value (Unpaired t test)[1]**
ASO2-PS vs ASO3-PS	**0.0007**
ASO2-PS vs ASO5-PS	**< 0.0001**
ASO2-PS vs ASO8-PS	**< 0.0001**
ASO3-PS vs ASO5-PS	**0.0003**
ASO3-PS vs ASO8-PS	**0.046**
ASO5-PS vs ASO8-PS	**0.0012**

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified PS-modified ASOs. Significant *P* values are shown in bold type.

4.3.3.3 Effect of OMe-modified POMC ASOs on ACTH secretion

Next, the effect that OMe-modified POMC ASOs might have on the secretion of ACTH was investigated by transfecting AtT-20 cells with POMC ASO2-OMe, ASO3-OMe, ASO5-OMe, and ASO8-OMe (Table 2.1).

The experiments were carried out as described in Section 4.3.3.1, with the collection of 30-µl samples of cell culture medium at 24 h post-transfection. The concentration of ACTH was then determined using an Immulite 2000® ACTH immunoassay (Section 2.6).

The results of four individual experiments are in Figure 4.12. They indicated that treating AtT-20 cells with OMe-modified POMC ASOs reduced ACTH concentration in the culture medium significantly when compared with untreated cells (Unpaired t test, $P < 0.0001$). In contrast, there was no significant effect upon secretion of ACTH when cells were treated with either OMe-modified POMC ASOs alone or with Lipofectamine®-2000 Reagent alone (Unpaired t test, $P > 0.05$).

The effects of the OMe-modified POMC ASOs on the secretion of ACTH from AtT-20 cells are compared in Table 4.5. POMC ASO5-OMe was the most effective, decreasing secreted ACTH to 16% of that normally secreted from untreated AtT-20 cells. The levels of ACTH reduction achieved with ASO3-OMe, ASO5-OMe and ASO8-OMe were significantly more than ASO2-OMe. ASO5-OMe and ASO8-OMe were also significantly more effective than ASO3-OMe.

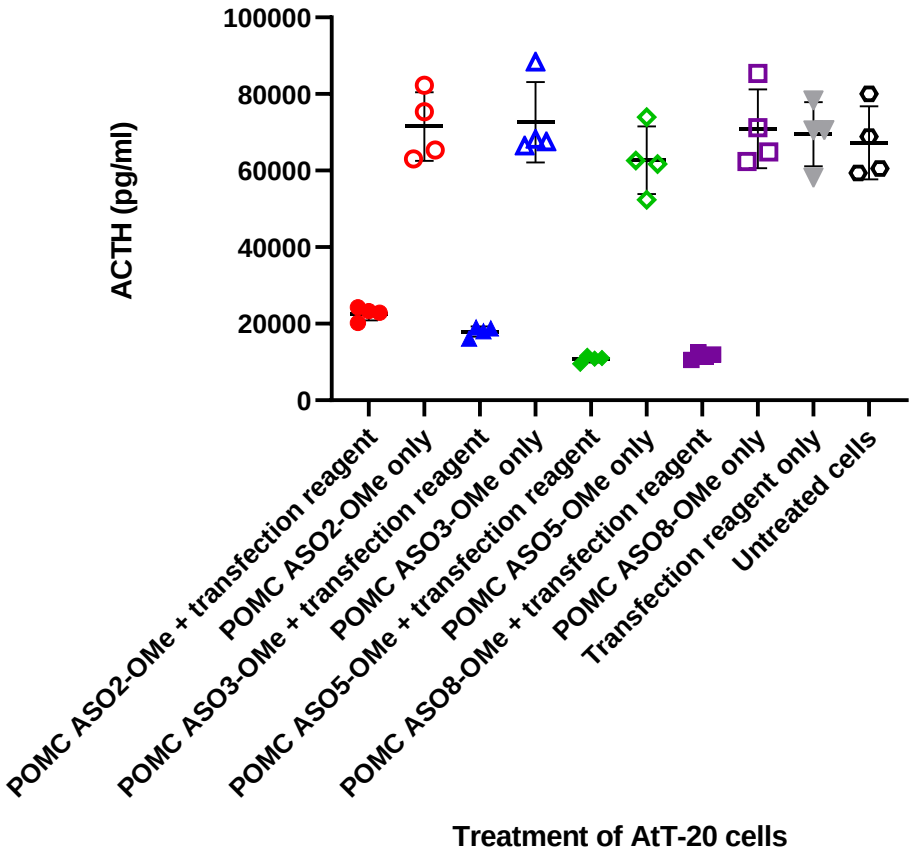


Figure 4.12: ACTH levels secreted by AtT-20 cells following transfection with OMe-modified POMC ASOs.

AtT-20 cells were transfected with OMe-modified POMC ASO2, 3, 5, or 8 at 100 nM. The levels of ACTH in the culture medium were measured in samples taken 24 h post-transfection. Control treatments used were transfection reagent alone and ASO alone. To give a baseline level of ACTH secretion, untreated cells were also included in the experiments. The results show the mean (± SD) ACTH concentrations measured in four individual experiments. All four OMe-modified POMC ASOs reduced ACTH levels significantly when compared to untreated cells (Unpaired t test, $P < 0.0001$). There was no significant effect on ACTH secretion when cells were treated with ASO only or with Lipofectamine®-2000 Reagent only (Unpaired t test, $P > 0.05$).

Table 4.5: Comparison of the effectiveness of OMe-modified POMC ASOs in reducing ACTH secretion from AtT-20 cells

OMe-modified POMC ASO used to treat AtT-20 cells	**ACTH secreted compared with levels secreted by untreated AtT-20 cells**
ASO2-OMe	34%
ASO3-OMe	27%
ASO5-OMe	16%
ASO8-OMe	17%
OMe-modified POMC ASOs compared	***P* value (Unpaired t test)[1]**
ASO2-OMe vs ASO3-OMe	**0.0055**
ASO2-OMe vs ASO5-OMe	**< 0.0001**
ASO2-OMe vs ASO8-OMe	**< 0.0001**
ASO3-OMe vs ASO5-OMe	**< 0.0001**
ASO3-OMe vs ASO8-OMe	**0.0002**
ASO5-OMe vs ASO8-OMe	0.22

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified OMe-modified ASOs. Significant *P* values are shown in bold type.

4.3.3.4 Effect of LNA-modified POMC ASOs on ACTH secretion

Finally, the effect that LNA-modified POMC ASOs had upon ACTH secretion was examined by transfecting AtT-20 cells with POMC ASO2-LNA, ASO3-LNA, ASO5-LNA, and ASO8-LNA (Table 2.1).

The exact steps of the transfection experiment described in Section 4.3.3.1 were also carried out for LNA-modified POMC ASOs. A 30-µl sample of cell culture medium was collected at 24 h after transfection, and the concentration of ACTH was determined using an Immulite 2000® ACTH immunoassay (Section 2.6).

The results of four separate experiments are shown in Figure 4.13. They indicated that treating AtT-20 cells with LNA-modified POMC ASOs decreased ACTH levels in the culture medium significantly when compared with untreated cells (Unpaired t test, $P < 0.0001$). In contrast, there was no significant effect upon ACTH secretion when cells were treated with either LNA-modified POMC ASOs alone or with Lipofectamine®-2000 Reagent alone (Unpaired t test, $P > 0.05$).

The effects of the LNA-modified ASOs on the ACTH secretion from AtT-20 cells are compared in Table 4.6. POMC ASO3-LNA was the most effective, decreasing secreted ACTH to 14% of that normally secreted from untreated AtT-20 cells. The levels of ACTH reduction achieved with ASO3-LNA, ASO5-LNA, and ASO8-LNA were significantly higher than ASO2-LNA. ASO8-LNA was also significantly more effective than ASO3-LNA.

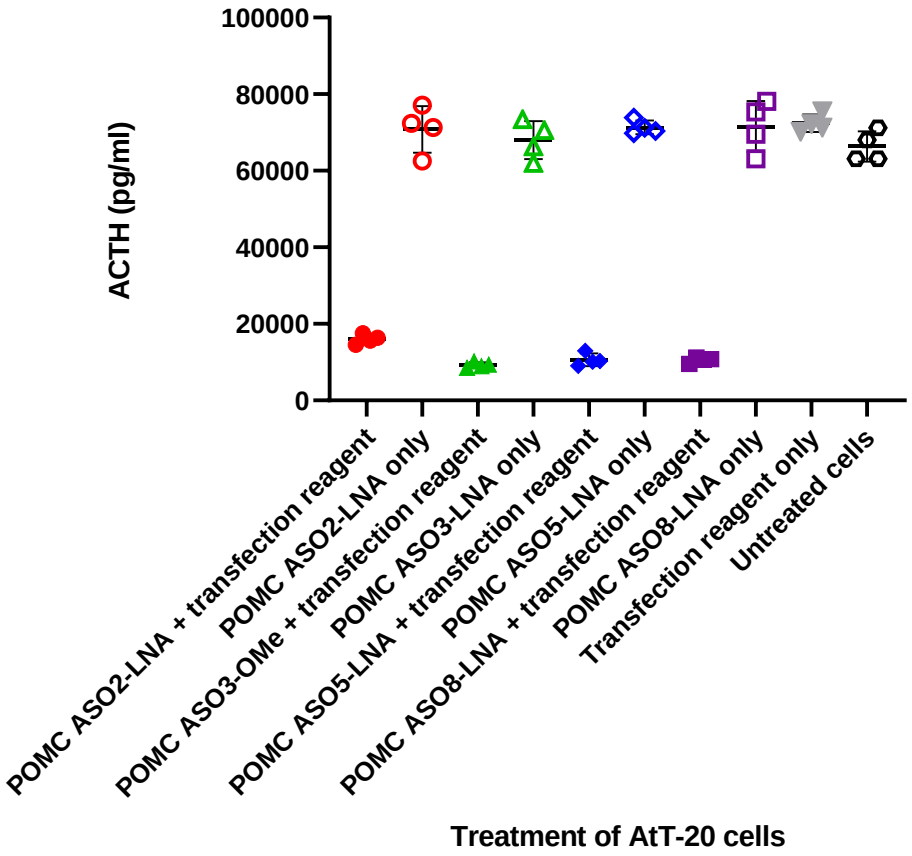


Figure 4.13: ACTH levels secreted by AtT-20 cells following transfection with LNA-modified POMC ASOs.

AtT-20 cells were transfected with LNA-modified POMC ASO2, 3, 5, or 8 at 100 nM. The levels of ACTH in the culture medium were measured in samples taken 24 h post-transfection. Control treatments used were transfection reagent alone and ASO alone. To give a baseline level of ACTH secretion, untreated cells were also included in the experiments. The results show the mean (± SD) ACTH concentrations measured in four individual experiments. All four LNA-modified POMC ASOs reduced ACTH levels significantly when compared to untreated cells (Unpaired t test, $P < 0.0001$). There was no significant effect on ACTH secretion when cells were treated with ASO only or with Lipofectamine®-2000 Reagent only (Unpaired t test, $P > 0.05$).

Table 4.6: Comparison of the effectiveness of LNA-modified POMC ASOs in reducing ACTH secretion from AtT-20 cells

LNA-modified POMC ASO used to treat AtT-20 cells	**ACTH secreted compared with levels secreted by untreated AtT-20 cells**
ASO2-LNA	24%
ASO3-LNA	14%
ASO5-LNA	16%
ASO8-LNA	16%
LNA-modified POMC ASOs compared	***P* value (Unpaired t test)[1]**
ASO2-LNA vs ASO3-LNA	**< 0.0001**
ASO2-LNA vs ASO5-LNA	**0.0021**
ASO2-LNA vs ASO8-LNA	**0.0003**
ASO3-LNA vs ASO5-LNA	0.18
ASO3-LNA vs ASO8-LNA	**0.044**
ASO5-LNA vs ASO8-LNA	0.94

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified LNA-modified ASOs. Significant *P* values are shown in bold type.

4.3.3.5 Summary of effectiveness of different POMC ASO sequences in reducing ACTH secretion from AtT-20 cells

The results detailed in Section 4.3.3.4 indicated that, with the exception of the LNA-modified version, POMC ASO5 was the most effective ASO sequence at reducing secreted ACTH from AtT-20 cells. Unmodified POMC ASO5, POMC ASO5-PS, and POMC ASO5-OMe reduced ACTH secretion to 28%, 23%, and 16%, respectively, of the levels normally secreted from untreated AtT-20 cells (Tables 4.3, 4.4, and 4.5, respectively). POMC ASO3-LNA was the most effective ASO sequence in relation to the LNA-modification, decreasing secreted ACTH to 14% of normal (Table 4.6).

4.3.3.6 Comparison of the effectiveness of different POMC ASO modifications in reducing ACTH secretion from AtT-20 cells

The results in Section 4.3.3.4 indicated that all of the unmodified and modified POMC ASOs showed significant effectiveness in reducing ACTH secretion from AtT-20 cells. Next, the effects of the different modifications of the same POMC ASO on ACTH secretion were compared.

Comparing the reduced levels of ACTH secreted from AtT-20 cells between the different POMC ASO2 modifications revealed that POMC ASO2-LNA was the most effective, and decreased secreted ACTH to 24% of that normally secreted from untreated AtT-20 cells (Table 4.7 and Figure 4.14a). Similar results were found for POMC ASO3 modifications in that POMC ASO3-LNA was the most effective, decreasing secreted ACTH to 14% of normal (Table 4.7 and Figure 4.14b). For POMC ASO5 modifications, POMC ASO5-OMe and ASO5-LNA were the most and equally effective, decreasing secreted ACTH to 16% and 17% of normal, respectively (Table 4.7 and Figure 4.14c). Finally, for POMC ASO8, POMC ASO8-OMe and ASO8-LNA were the most and equally effective, decreasing secreted ACTH to 17% and 16% of the usual level (Table 4.7 and Figure 4.14d).

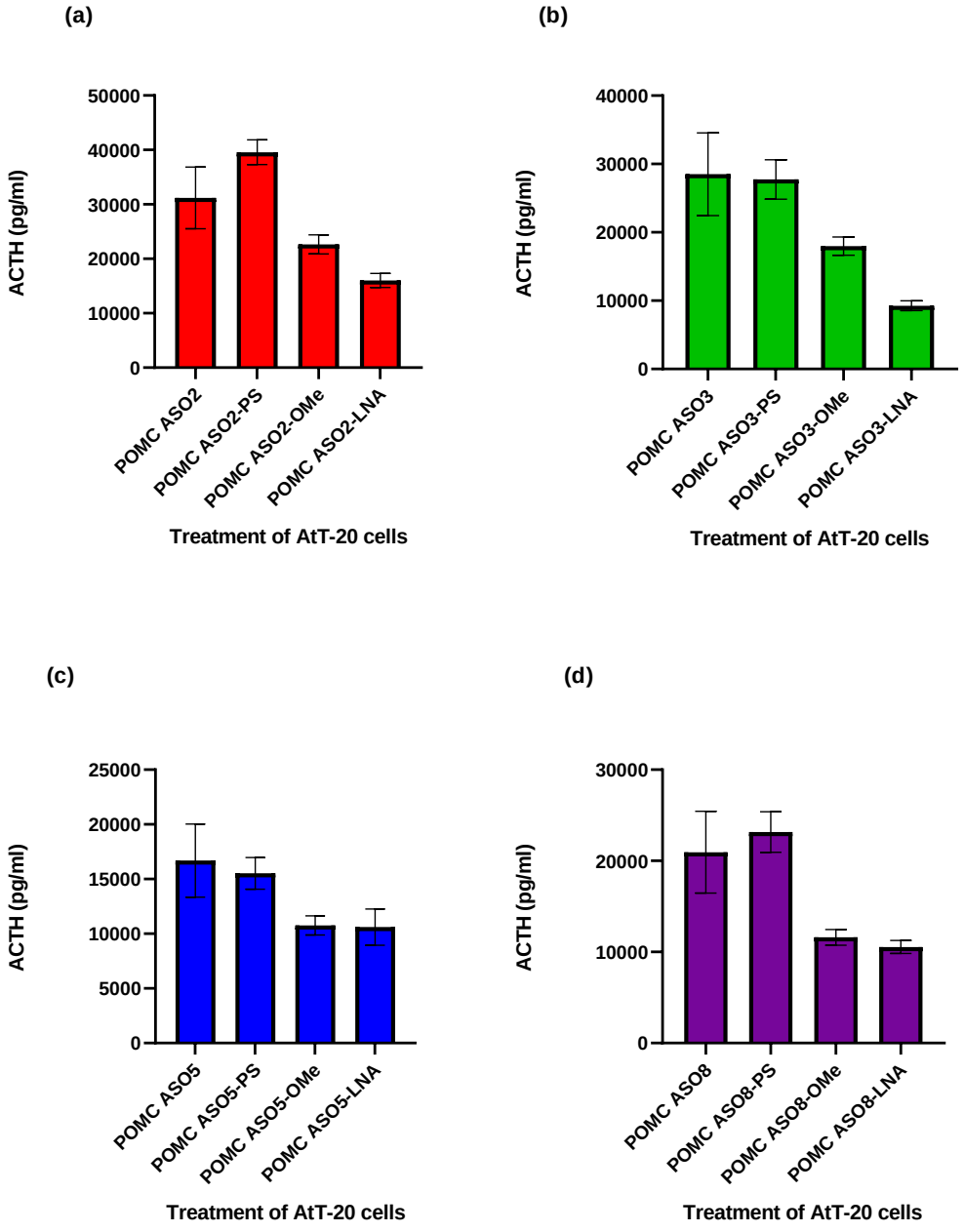


Figure 4.14: Comparison of secreted ACTH levels after transfection of AtT-20 cells with POMC ASO unmodified and modified versions.

The results show the mean (± SD) ACTH concentrations measured in four individual experiments for unmodified and modified versions of (a) POMC ASO2, (b) POMC ASO3, (c) POMC ASO5, and (d) POMC ASO8.

Table 4.7: Comparison of levels of secreted ACTH after transfection of AtT-20 cells with unmodified and modified versions of POMC ASOs

POMC ASO version used to treat AtT-20 cells	ACTH secreted compared with untreated AtT-20 cells
Unmodified ASO2	52%
ASO2-PS	59%
ASO2-OMe	34%
ASO2-LNA	24%
Unmodified ASO3	48%
ASO3-PS	41%
ASO3-OMe	27%
ASO3-LNA	14%
Unmodified ASO5	34%
ASO5-PS	27%
ASO5-OMe	16%
ASO5-LNA	17%
Unmodified ASO8	35%
ASO8-PS	35%
ASO8-OMe	17%
ASO8-LNA	16%
POMC ASO versions compared	***P* value (Unpaired t test)[1]**
ASO2 vs ASO2-PS	**0.033**
ASO2 vs ASO2-OMe	**0.028**
ASO2 vs ASO2-LNA	**0.002**
ASO2-PS vs ASO2-OMe	**< 0.0001**
ASO2-PS vs ASO2-LNA	**< 0.0001**
ASO2-OMe vs ASO2-LNA	**0.0009**
ASO3 vs ASO3-PS	**0.082**
ASO3 vs ASO3-OMe	**0.015**
ASO3 vs ASO3-LNA	**0.0007**
ASO3-PS vs ASO3-OMe	**0.0009**
ASO3-PS vs ASO3-LNA	**< 0.0001**
ASO3-OMe vs ASO3-LNA	**< 0.0001**
ASO5 vs ASO5-PS	0.55
ASO5 vs ASO5-OMe	**0.014**
ASO5 vs ASO5-LNA	**0.018**
ASO5-PS vs ASO5-OMe	**0.0013**
ASO5-PS vs ASO5-LNA	**0.0042**
ASO5-OMe vs ASO5-LNA	0.88
ASO8 vs ASO8-PS	0.41
ASO8 vs ASO8-OMe	**0.0064**
ASO8 vs ASO8-LNA	**0.0038**
ASO8-PS vs ASO8-OMe	**< 0.0001**
ASO8-PS vs ASO8-LNA	**< 0.0001**
ASO8-OMe vs ASO8-LNA	0.11

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified POMC ASO. Significant *P* values are shown in bold type.

4.3.4 Examination of the effects of transfection with POMC ASOs on the viability of cultured AtT-20 cells

The initial results indicated that transfecting AtT-20 cells with POMC ASOs decreased the level of ACTH secreted (Section 4.3.3). However, it was essential to examine if treating AtT-20 cells with POMC ASOs actually adversely affected cell viability and that this in turn was responsible for the reduced levels of ACTH secretion that had been detected.

For the experiments, AtT-20 cells were plated in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml of culture medium. At 24 h, the cells were treated in duplicate with POMC ASOs at a final concentration of 100 nM. Treatment with Lipofectamine®-2000 Reagent alone and POMC ASOs alone were included as controls. All experiments included untreated cells as a baseline for ACTH levels. After 24 h, a sample of 30 µl of the culture medium was taken for quantification of ACTH using an Immulite 2000 ACTH immunoassay (Section 2.6). In addition, AtT-20 cells were harvested and the viable cell counts determined (Section 2.3.4).

The results of three experiments for each POMC ASO are shown in Figure 4.15a-d. They indicated that treatment of AtT-20 cells with POMC ASOs, of any sequence or with any modification, had no significant effect on the number of viable cells that were counted at 24 h post-transfection (One-way ANOVA, $P > 0.05$). This suggested that the significant decrease in the level of ACTH in the culture medium following transfection with POMC ASOs was not due to adverse effects upon cell viability.

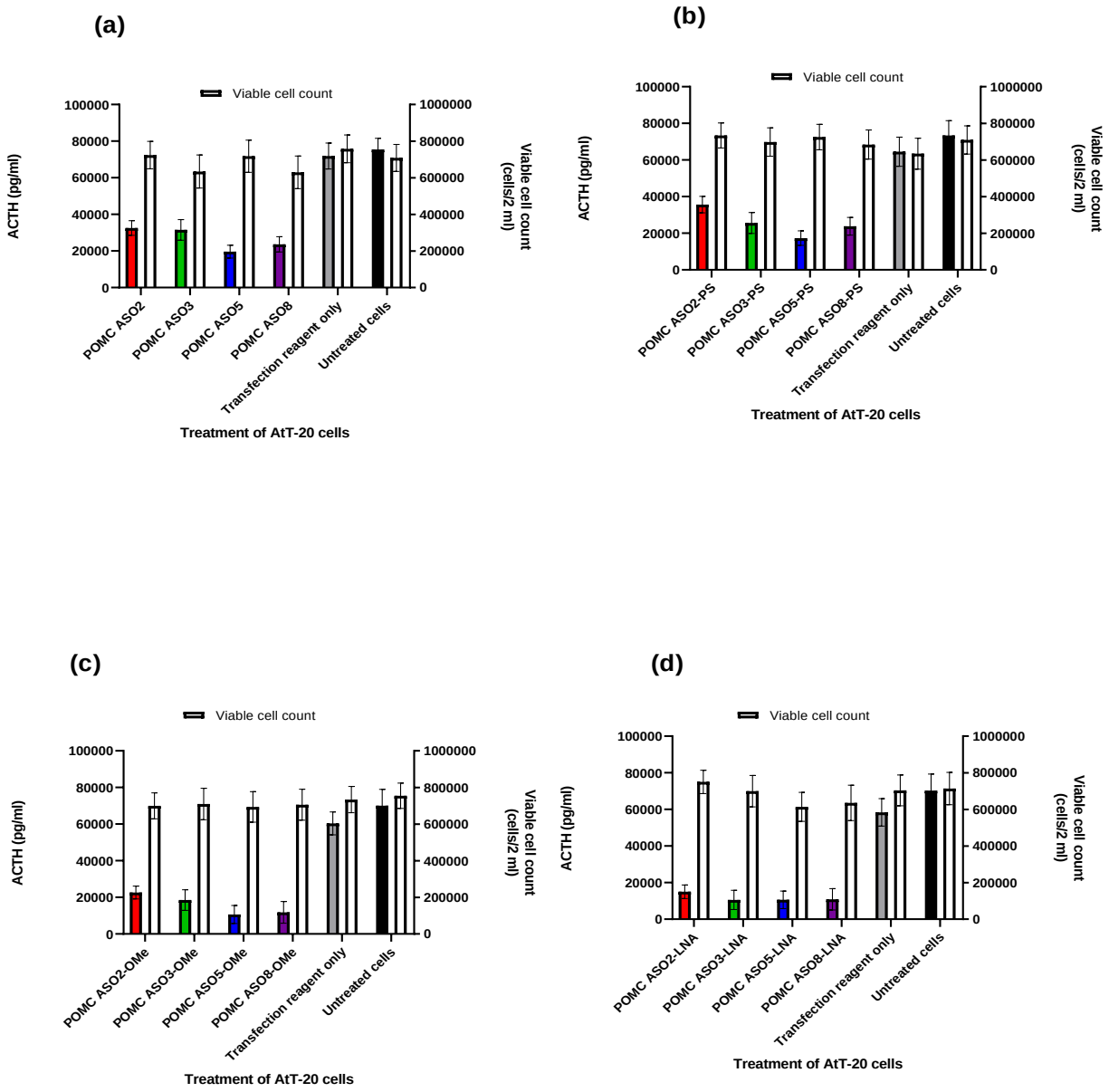


Figure 4.15: Secreted ACTH levels and viable cell counts after transfecting AtT-20 cells with unmodified and modified POMC ASOs.

After transfection of AtT-20 cells with POMC ASOs at 100 nM, the viable cells were counted and the ACTH levels measured, 24 h later. POMC ASOs alone and alone Lipofectamine®-2000 Reagent were used as controls. Cells without treatment were also included. The results show the mean (± SD) ACTH concentrations and viable cell counts from three separate experiments for unmodified and modified versions of (a) POMC ASO2, (b) POMC ASO3, (c) POMC ASO5, and (d) POMC ASO8. No significant effect on the number of viable cells was noted when treatments were compared (One-way ANOVA, $P > 0.05$).

4.3.5 Examining the effects of scrambled and mismatched POMC ASOs on the secretion of ACTH from AtT-20 cells

In the study so far, the effects of four on-target POMC ASOs have been investigated and all have been shown to reduce ACTH production in AtT-20 cells (Section 4.3.3). The use of multiple POMC ASOs fitted with the premise that an experimental plan should include at least two different on-target ASOs, so as to produce robust interpretable outcomes (Gagnon and Corey, 2019). However, to reasonably conclude that the observable effects of ASOs are due to their action upon their target mRNA, the experimental design also needs to include control ASOs (Gagnon and Corey, 2019). If different on-target ASOs have the same effect upon expression of the targeted mRNA, and the control ASOs do not, then it is a reasonable assumption that the observable results are not due to some confounding off-target effects of the gene-silencing ASOs.

Antisense oligonucleotide controls need to be of the same length and with the same chemical modifications as the on-target molecules. So, for example, OMe-modified ASOs would not serve as good controls for LNA-modified ASOs, and so forth. The inclusion of at least two control ASOs is recommended, these being categorised as either mismatched (MM) or scrambled (Gagnon and Corey, 2019). Scrambled ASO controls have the same nucleotide composition as the on-target version, but the sequence of the nucleotides is altered. Mismatched ASOs have one or more nucleotides that are mismatched in relation to the targeted sequence, and they have been shown to negate the effects of their cognate antisense molecule (Swayze et al., 2007, Sharma et al., 2005, Flanagan et al., 1996). However, the extent of the negative effect can depend upon the exact base pairs involved in the mismatch and the adjacent nucleotides (Hagedorn et al., 2017).

The next step, therefore, was to look at the effects on ACTH secretion of scrambled and MM POMC ASO variants, as listed in Tables 4.8 and 4.9, respectively. For this analysis, the OMe-modified and LNA-modified POMC ASOs were chosen for creating the control versions because they proved to be the most effective in silencing POMC, in the earlier investigations (Section 4.3.3). The scrambled POMC ASOs (Table 4.8) were generated using the online tool at GeneScript Biotech Corp (Piscataway, NJ,

USA) (https://www.genscript.com). Each scrambled ASO had the same nucleotide composition as the input ASO sequence (Table 2.1) and had no match to any mRNA of the selected mouse database. In addition, the software algorithm excluded nucleotide sequences in the final scrambled ASO that are known to exert an immune response or other toxic off-target effects (Table 1.2) (Burdick et al., 2014, Đapić et al., 2003, Krieg, 1999). The mismatched POMC ASOs contained one, two or three nucleotide changes within the internal RNase H domain (Table 4.9).

For the experiments, AtT-20 cells were plated in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml of culture medium. At 24 h, the cells were transfected in duplicate with OMe-modified or LNA-modified POMC ASOs or their MM or scrambled versions at a final concentration of 100 nM. Lipofectamine®-2000 Reagent alone and ASOs alone were included as control treatments. Untreated cells were also included in all experiments. After 24 h, a sample of 30 µl of the culture medium was taken for ACTH quantification using an Immulite 2000 ACTH immunoassay (Section 2.6).

The results of four experiments for OMe-modified and LNA-modified POMC ASOs are shown in Figure 4.16a-d and 4.17a-d, respectively. They suggested that the mismatches in all four OMe-modified and LNA-modified POMC ASOs negated the original gene-silencing effects in that the secretion of ACTH from AtT-20 cells was no longer reduced in comparison to untreated cells (One-way ANOVA, $P > 0.05$). Furthermore, scrambled versions of all four OMe-modified and LNA-modified POMC ASOs did not affect the ACTH levels secreted from AtT-20 cells (Figures 4.16a-d and 4.17a-d, respectively).

Table 4.8: Scrambled antisense oligonucleotides

Scrambled POMC ASO[1,2]	Sequence[3]
POMC ASO2-OMe or LNA Scrambled	5'-[A]*[T]*[T]*[A]*[G]*G*T*C*T*C*C*G*A*T*G*[G]*[C]*[G]*[C]*[T]-3'
POMC ASO3-OMe or LNA Scrambled	5'-[A]*[G]*[C]*[G]*[T]*G*T*T*G*A*T*C*T*C*[G]*[T]*[T]*[G]*[T]-3'
POMC ASO5-OMe or LNA Scrambled	5'-[A]*[C]*[C]*[T]*[C]*A*C*T*A*G*C*G*A*G*[T]*[A]*[G]*[G]*[A]-3'
POMC ASO8-OMe or LNA Scrambled	5'-[A]*[C]*[C]*[A]*[G]*G*A*T*C*A*T*C*G*T*[A]*[T]*[G]*[C]*[G]-3'

[1]The scrambled POMC ASOs were generated using the online tool at GeneScript Biotech Corp (Piscataway, NJ, USA) (https://www.genscript.com).

[2]ASO, antisense oligonucleotide; LNA, locked nucleic acid; OMe, 2'-*O*-methyl.

[3][A] [C] [G] [T], 2'-*O*-methyl or LNA modification of nucleotide; *, phosphorothioate linkage.

Table 4.9: Mismatched antisense oligonucleotides

ASO[1]	Sequence[2]
POMC ASO2-OMe/LNA	5’-[G]*[C]*[T]*[C]*[T]*T*C*T*C*G*G*A*G*G*T*[C]*[A]*[T]*[G]*[A]-3’
POMC ASO2-OMe/LNA MM1	5’-[G]*[C]*[T]*[C]*[T]*T*C*T*A*G*G*A*G*G*T*[C]*[A]*[T]*[G]*[A]-3’
POMC ASO2-OMe/LNA MM2	5’-[G]*[C]*[T]*[C]*[T]*T*C*G*T*G*G*A*G*G*T*[C]*[A]*[T]*[G]*[A]-3’
POMC ASO2-OMe/LNA MM3	5’-[G]*[C]*[T]*[C]*[T]*T*C*C*G*A*G*A*G*G*T*[C]*[A]*[T]*[G]*[A]-3’
POMC ASO3-OMe/LNA	5’-[G]*[T]*[T]*[C]*[T]*T*G*A*T*G*A*T*G*G*[C]*[G]*[T]*[T]*[C]-3’
POMC ASO3-OMe/LNA MM1	5’-[G]*[T]*[T]*[C]*[T]*T*G*A*T*C*A*T*G*G*[C]*[G]*[T]*[T]*[C]-3’
POMC ASO3-OMe/LNA MM2	5’-[G]*[T]*[T]*[C]*[T]*T*G*A*G*A*A*T*G*G*[C]*[G]*[T]*[T]*[C]-3’
POMC ASO3-OMe/LNA MM3	5’-[G]*[T]*[T]*[C]*[T]*T*G*A*C*T*G*T*G*G*[C]*[G]*[T]*[T]*[C]-3’
POMC ASO5-OMe/LNA	5’-[G]*[A]*[A]*[G]*[T]*G*A*C*C*C*A*T*G*A*[C]*[G]*[T]*[A]*[C]-3’
POMC ASO5-OMe/LNA MM1	5’-[G]*[A]*[A]*[G]*[T]*G*A*C*C*G*A*T*G*A*[C]*[G]*[T]*[A]*[C]-3’
POMC ASO5-OMe/LNA MM2	5’-[G]*[A]*[A]*[G]*[T]*G*A*C*T*A*A*T*G*A*[C]*[G]*[T]*[A]*[C]-3’
POMC ASO5-OMe/LNA MM3	5’-[G]*[A]*[A]*[G]*[T]*G*A*C*T*C*G*T*G*A*[C]*[G]*[T]*[A]*[C]-3’
POMC ASO8-OMe/LNA	5’-[G]*[T]*[A]*[G]*[C]*A*G*A*A*T*C*T*C*G*[G]*[C]*[A]*[T]*[C]-3’
POMC ASO8-OMe/LNA MM1	5’-[G]*[T]*[A]*[G]*[C]*A*G*A*A*A*C*T*C*G*[G]*[C]*[A]*[T]*[C]-3’
POMC ASO8-OMe/LNA MM2	5’-[G]*[T]*[A]*[G]*[C]*A*G*A*G*C*C*T*C*G*[G]*[C]*[A]*[T]*[C]-3’
POMC ASO8-OMe/LNA MM3	5’-[G]*[T]*[A]*[G]*[C]*A*G*A*C*G*A*T*C*G*[G]*[C]*[A]*[T]*[C]-3’

[1]ASO, antisense oligonucleotide; LNA, locked nucleic acid; OMe, 2’-*O*-methyl.

[2][A] [C] [G] [T], 2’-*O*-methyl or LNA modification of nucleotide; *, phosphorothioate linkage. Mismatched nucleotides, in comparison to the relevant POMC ASO, are in red.

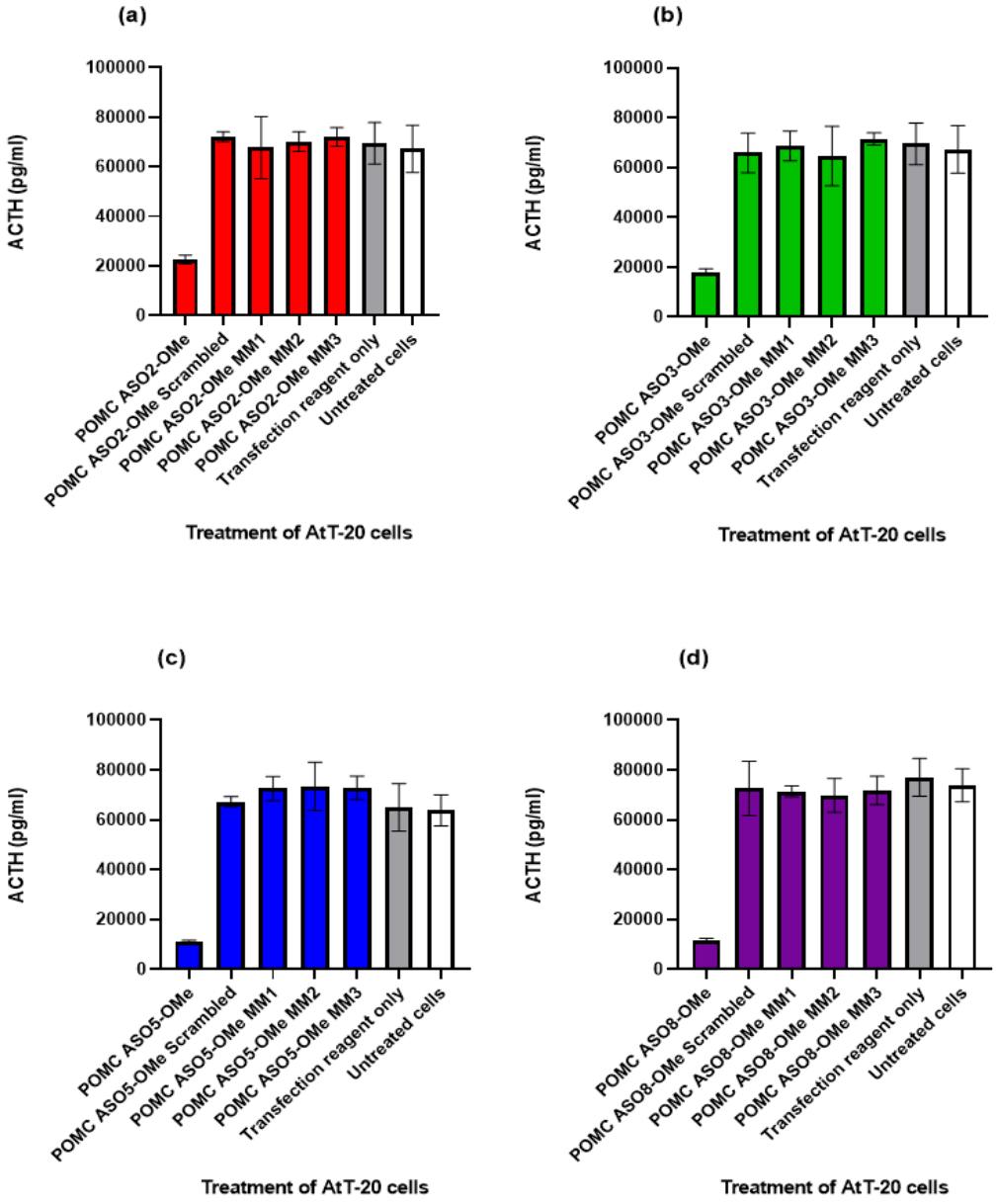


Figure 4.16: Secreted ACTH levels following transfection of AtT-20 cells with scrambled or mismatched OMe-modified POMC ASOs.

Cells were transfected with OMe-modified ASOs and their scrambled and mismatched (MM) versions at 100 nM. OMe-modified ASOs alone and Lipofectamine®-2000 Reagent alone were used as control treatments. Cells without treatment were also included in all experiments. After 24 h, the ACTH levels in the culture medium were measured. The mean (± SD) ACTH concentration measured in four experiments is shown for (a) ASO2-OMe, (b) ASO3-OMe, (c) ASO5-OMe, and (d) ASO8-OMe. Neither the scrambled nor the MM ASO-OMe molecules had a significant effect on ACTH secretion in comparison with untreated cells (One-way ANOVA, $P > 0.05$).

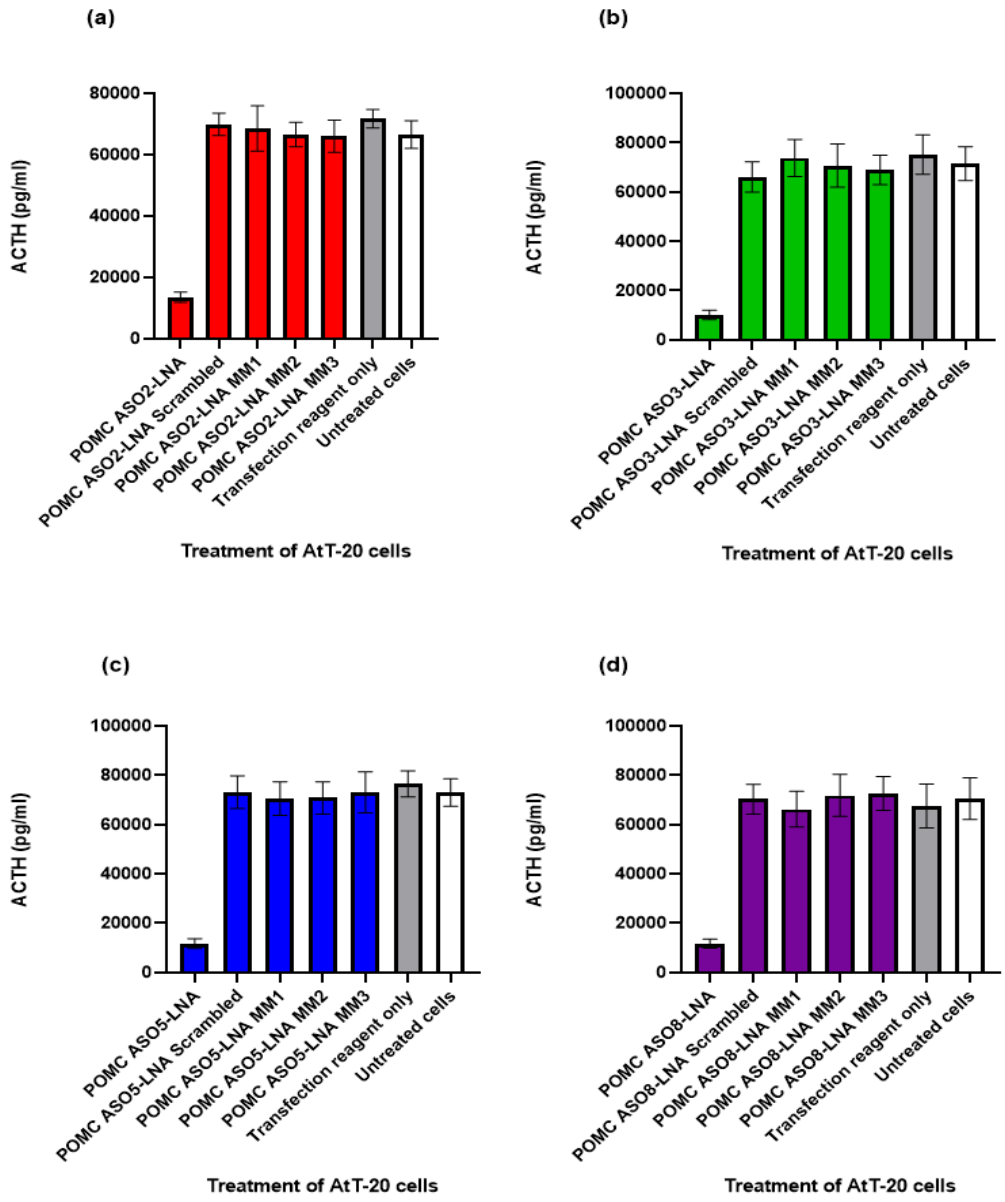


Figure 4.17: Secreted ACTH levels following transfection of AtT-20 cells with scrambled or mismatched LNA-modified POMC ASOs.

Cells were transfected with LNA-modified ASOs and their scrambled and mismatched (MM) versions at 100 nM. LNA-modified ASOs alone and Lipofectamine®-2000 Reagent alone were used as control treatments. Cells without treatment were also included in all experiments. After 24 h, the ACTH levels in the culture medium were measured. The mean (± SD) ACTH concentration measured in four experiments is shown for (a) ASO2-LNA, (b) ASO3-LNA, (c) ASO5-LNA, and (d) ASO8-LNA. Neither the scrambled nor the MM ASO-LNA molecules had a significant effect on ACTH secretion in comparison with untreated cells (One-way ANOVA, $P > 0.05$).

4.3.6 Effect of different concentrations of OMe-modified and LNA-modified POMC ASOs on ACTH secretion from AtT-20 cells

As higher doses of ASOs are more likely to produce off-target effects and induce the immune response (Ottesen et al., 2021, Chi et al., 2017). The effect of different ASO concentrations on the secretion of ACTH from AtT-20 cells was investigated. Usually, in cell culture experiments, the final concentration of ASOs used is less than 1 μM (Ducoli et al., 2021, Ämmälä et al., 2018, Swayze et al., 2007). For this study, POMC ASO concentrations of 1 nM up to 100 nM were chosen, as such levels had been used successfully to suppress ACTH secretion from AtT-20 cells, in a previous study (Spampinato et al., 1994).

For the experiments, AtT-20 cells were plated in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml of culture medium. After 24 h, the cells were transfected in duplicate with different concentrations (100 nM, 30 nM, 10 nM, and 1 nM) of OMe-modified or LNA-modified POMC ASOs. As control treatments, cells were treated with ASO alone or Lipofectamine®-2000 Reagent alone. Untreated cells were also included in all experiments. At 24 h post-transfection, a 30-μl sample of the culture medium was taken for ACTH measurement using an Immulite 2000 ACTH immunoassay (Section 2.6).

The results of four experiments using OMe-modified and LNA-modified POMC ASOs are illustrated in Figures 4.18 and 4.19, respectively. For the different concentrations of both OMe-modified and LNA-modified POMC ASOs, all of them were shown to decrease the level of ACTH statistically significantly when compared to cells without treatment (One-way ANOVA, $P < 0.0001$).

The effectiveness of different concentrations of OMe-modified and LNA-modified POMC ASOs in suppressing ACTH secretion from AtT-20 cells is summarised in Table 4.10. In general, for the ASO-OMe versions, higher concentrations gave better gene-silencing effects. For ASO2-LNA, ASO5-LNA, and ASO8-LNA, their effects were comparable at the different concentrations used. For ASO3-LNA, a 100 nM concentration was slightly more effective at reducing ACTH secretion than the lower concentrations.

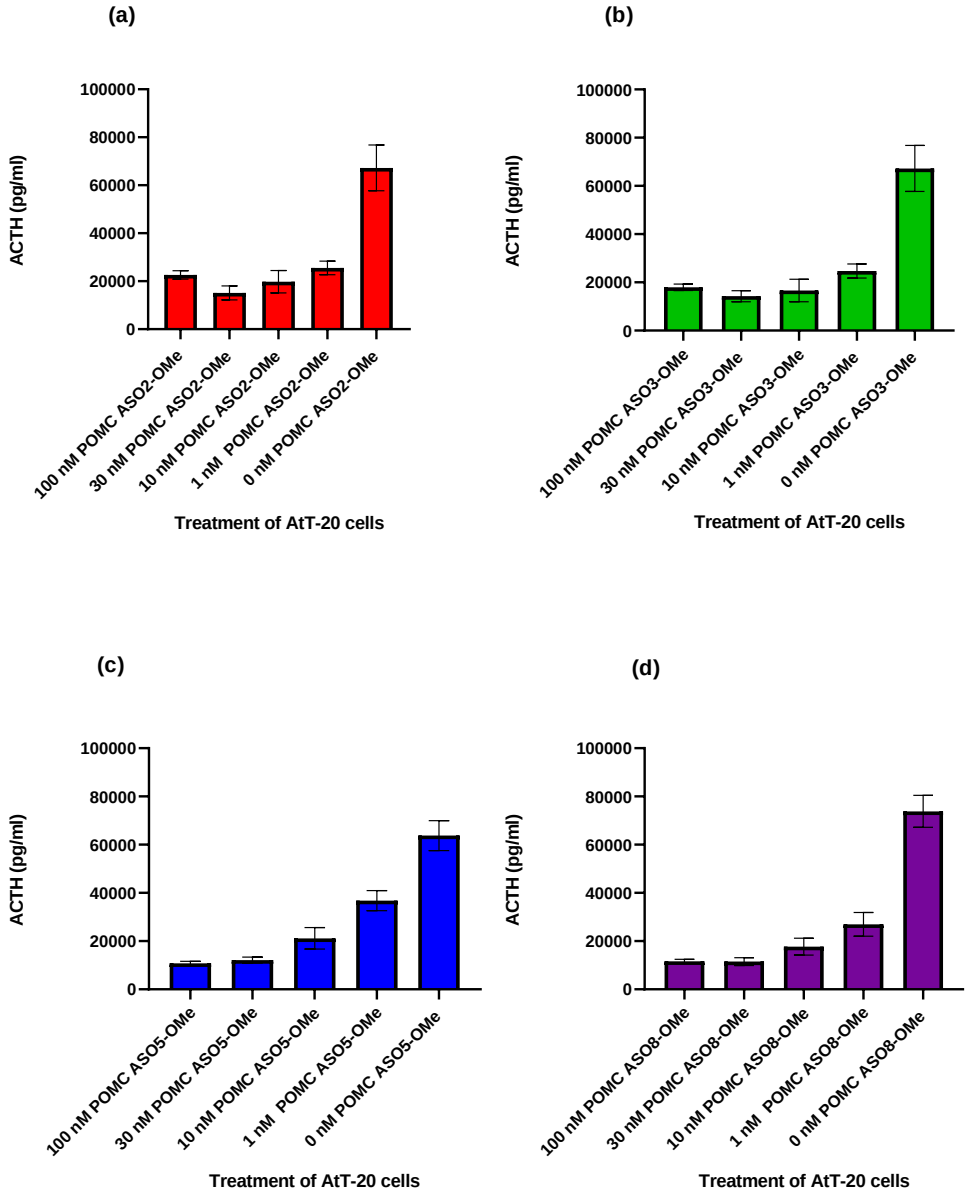


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Figure 4.18: ACTH levels secreted from AtT-20 cells after transfection of different concentrations of OMe-modified POMC ASOs.

Transfection of AtT-20 cells with OMe-modified ASOs were carried out with concentrations of 100 nM, 30 nM, 10 nM, and 1 nM. OMe-modified ASOs alone and Lipofectamine®-2000 Reagent alone were used as control treatments. Cells without treatment were included. After 24h, the ACTH levels in the culture medium were measured. The results illustrate the mean (± SD) of ACTH concentrations measured in four experiments for (a) ASO2-OMe, (b) ASO3-OMe, (c) ASO5-OMe, and (d) ASO8-OMe. The level of secreted ACTH was significantly decreased by all ASO-OMe concentrations in comparison with untreated cells (One-way ANOVA, $P < 0.0001$).

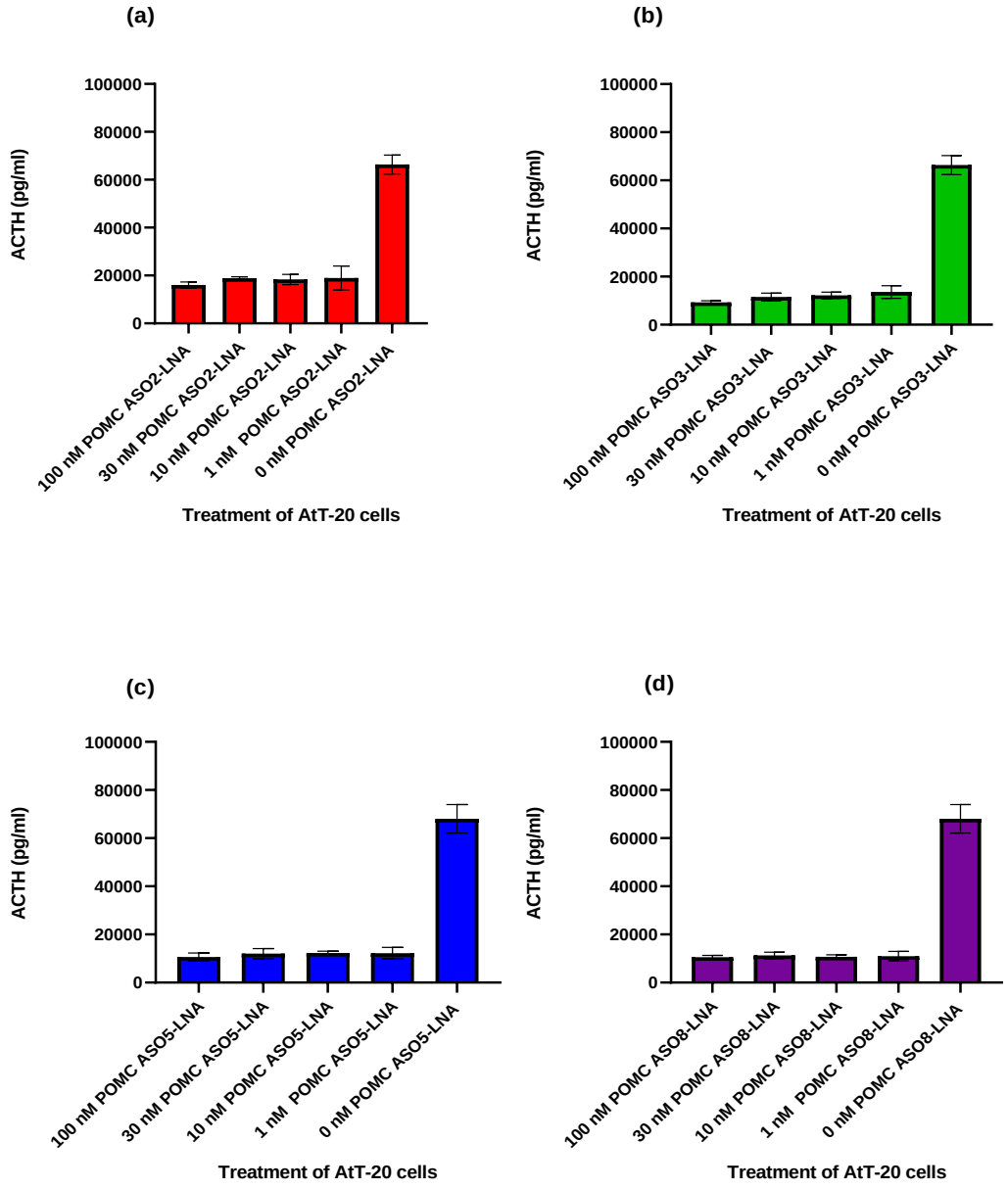


Figure 4.19: ACTH levels secreted from AtT-20 cells after transfection of different concentrations of LNA-modified POMC ASOs.

Transfection of AtT-20 cells with LNA-modified ASOs were carried out with concentrations of 100 nM, 30 nM, 10 nM, and 1 nM. LNA-modified ASOs alone and Lipofectamine®-2000 Reagent alone were used as control treatments. Cells without treatment were included. After 24h, the ACTH levels in the culture medium were measured. The results illustrate the mean (± SD) of ACTH concentrations measured in four experiments for (a) ASO2-LNA, (b) ASO3-LNA, (c) ASO5-LNA, and (d) ASO8-LNA. The level of secreted ACTH was significantly decreased by all ASO-LNA concentrations in comparison with untreated cells (One-way ANOVA, $P < 0.0001$).

Table 4.10: Comparison of the effectiveness of different concentrations of OMe-modified or LNA-modified POMC ASOs

POMC ASO	ASO-OMe or ASO-LNA concentration (nM)	ACTH concentration as a % of that secreted from untreated AtT-20 cells	*P* value (Unpaired t test)[1]		
			30 nM	10 nM	1 nM
ASO2-OMe	100	34	**0.0043**	0.29	0.13
	30	23	-	0.14	**0.0021**
	10	29	-	-	0.08
	1	38	-	-	-
ASO3-OMe	100	27	**0.029**	0.61	**0.0057**
	30	21	-	0.39	**0.0013**
	10	25	-	-	**0.027**
	1	37	-	-	-
ASO5-OMe	100	17	0.13	**0.0037**	**< 0.0001**
	30	19	-	**0.0077**	**< 0.0001**
	10	33	-	-	**0.0021**
	1	58	-	-	-
ASO8-OMe	100	16	0.92	**0.014**	**0.0008**
	30	16	-	**0.018**	**0.001**
	10	24	-	-	**0.022**
	1	37	-	-	-
ASO2-LNA	100	24	**0.0076**	0.11	0.30
	30	28	-	0.66	0.97
	10	28	-	-	0.83
	1	29	-	-	-
ASO3-LNA	100	14	**0.042**	**0.0076**	**0.019**
	30	17	-	0.52	0.22
	10	18	-	-	0.38
	1	21	-	-	-
ASO5-LNA	100	16	0.34	0.11	0.31
	30	18	-	0.79	0.89
	10	18	-	-	0.94
	1	18	-	-	-
ASO8-LNA	100	15	0.38	0.83	0.69
	30	17	-	0.48	0.82
	10	16	-	-	0.78
	1	16	-	-	-

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified POMC ASO. Significant *P* values are shown in bold type.

The reduced secretion of ACTH following AtT-20 cell transfection with the same concentrations of OMe-modified and LNA-modified POMC ASOs is compared in Figures 4.20 and 4.21, respectively. The comparisons showed that with OMe-modified ASOs at 30 nM and 10 nM, the reduction of ACTH secretion did not differ significantly between them (One-way ANOVA, $P > 0.05$). At 1 nM, ASO5-OMe appeared to be the least effective in reducing ACTH production (One-way ANOVA, $P = 0.0025$). For LNA-modified ASOs, ASO2-LNA was the least effective at all the concentrations used (One-way ANOVA, $P < 0.05$).

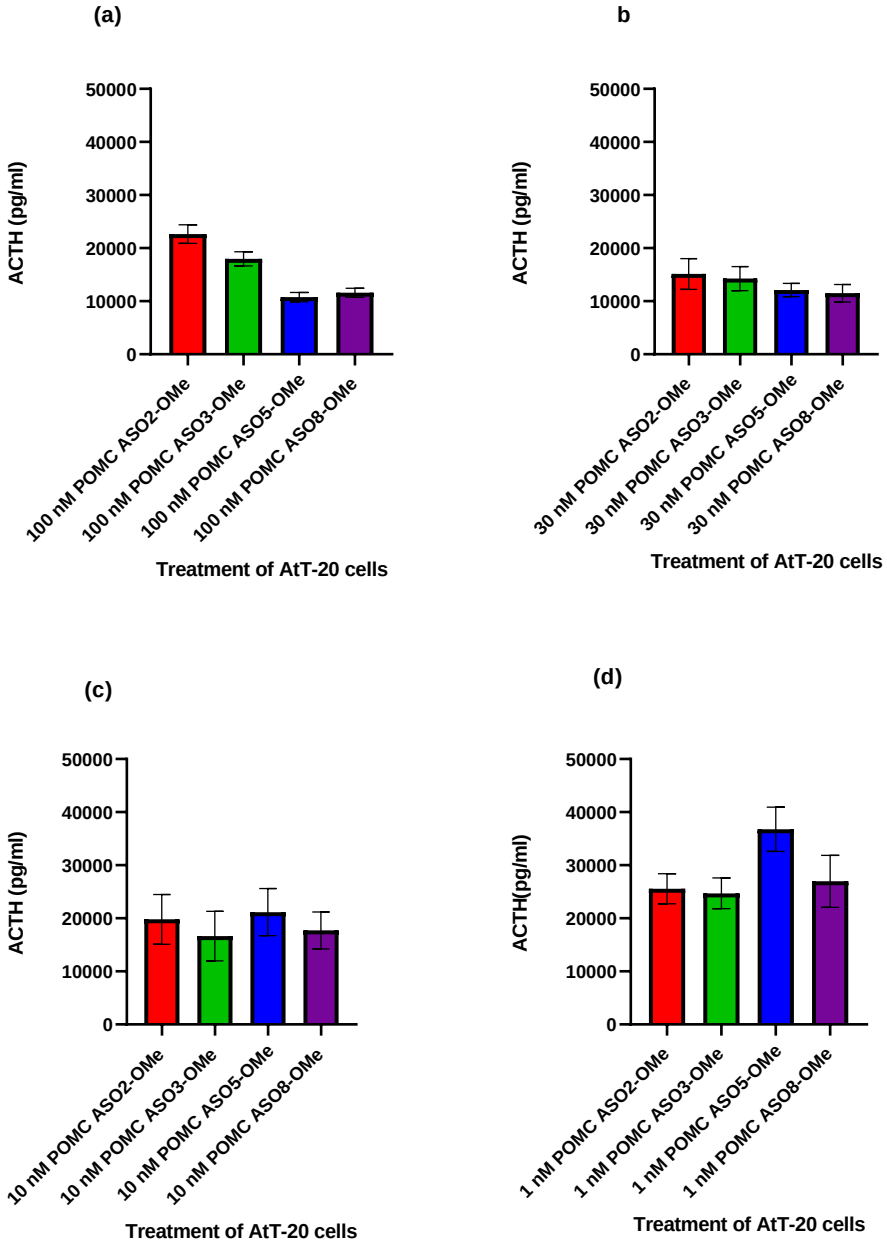


Figure 4.20: Comparison of ACTH levels secreted from AtT-20 cells following transfection with different concentrations of OMe-modified POMC ASOs.

The reduction in ACTH levels secreted from AtT-20 cells following transfection with different concentrations of each of the OMe-modified POMC ASOs is shown for (a) 100 nM (One-way ANOVA, $P < 0.0001$), (b) 30 nM (One-way ANOVA, $P > 0.05$), (c) 10 nM (One-way ANOVA, $P > 0.05$), and (d) 1 nM (One-way ANOVA, $P = 0.0025$).

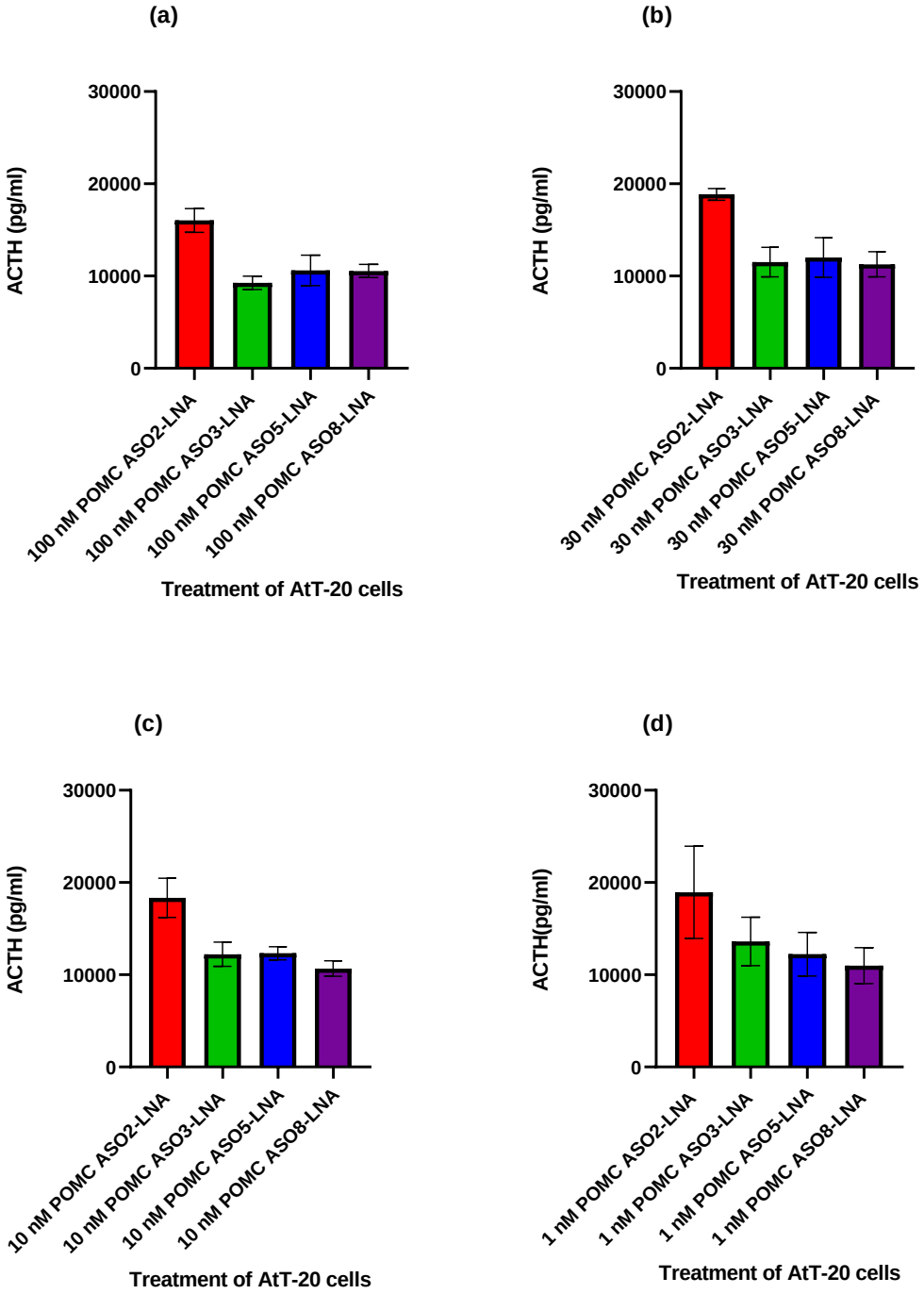


Figure 4.21: Comparison of ACTH levels secreted from AtT-20 cells following transfection with different concentrations of LNA-modified POMC ASOs.

The reduction in ACTH levels secreted from AtT-20 cells following transfection with different concentrations of each of the LNA-modified POMC ASOs is shown for (a) 100 nM (One-way ANOVA, $P < 0.0001$), (b) 30 nM (One-way ANOVA, $P < 0.0001$), (c) 10 nM (One-way ANOVA, $P < 0.0001$), and (d) 1 nM (One-way ANOVA, $P = 0.021$).

4.3.7 Longevity of action of OMe-modified and LNA-modified POMC ASOs on ACTH secretion from AtT-20 cells

The longevity of ASO action is an important aspect of any antisense therapeutic that needs to be investigated. Longer acting ASOs will negate repeated application of the drug to the patient in the course of their treatment. Well-designed ASOs have been shown to reduce specific protein expression by up to three weeks in animal models and longer in human patients (Anderson et al., 2021, Burghes and McGovern, 2010).

For the experiments, At-T20 cells were plated in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml of culture medium. After 24 h, the cells were transfected with OMe-modified or LNA-modified POMC ASOs at final concentrations of 100 nM, 30 nM, and 10 nM, and 1 nM. As controls, cells were treated with POMC ASO or Lipofectamine®-2000 Reagent alone. Untreated cells were also included. Samples of 30 µl of the culture medium were taken at 24, 48, 72, 96, and 120 h post-transfection for measuring ACTH using an Immulite 2000 ACTH immunoassay (Section 2.6).

The results of four experiments of OMe-modified ASOs are shown in Figure 4.22. In comparison with untreated cells, 100 nM, 30 nM, and 10 nM concentrations of all the OMe-modified ASOs gave prolonged and significant suppression of the secretion of ACTH up to and including 120 h (Unpaired t tests, all *P* values < 0.05). The use of OMe-modified ASOs at 1 nM significantly suppressed ACTH secretion from AtT-20 cells for up to 72 h for ASO5-OMe and ASO8-OMe, and for up to 96 h for ASO2-OMe and ASO3-OMe (Unpaired t tests, all *P* values < 0.05).

For LNA-modified ASOs, the results are shown in Figure 4.23. In comparison with untreated cells, all concentrations of all the LNA-modified ASOs significantly reduced ACTH secretion from AtT-20 cells up to and including the 120 h time point (Unpaired t tests, all *P* values < 0.05).

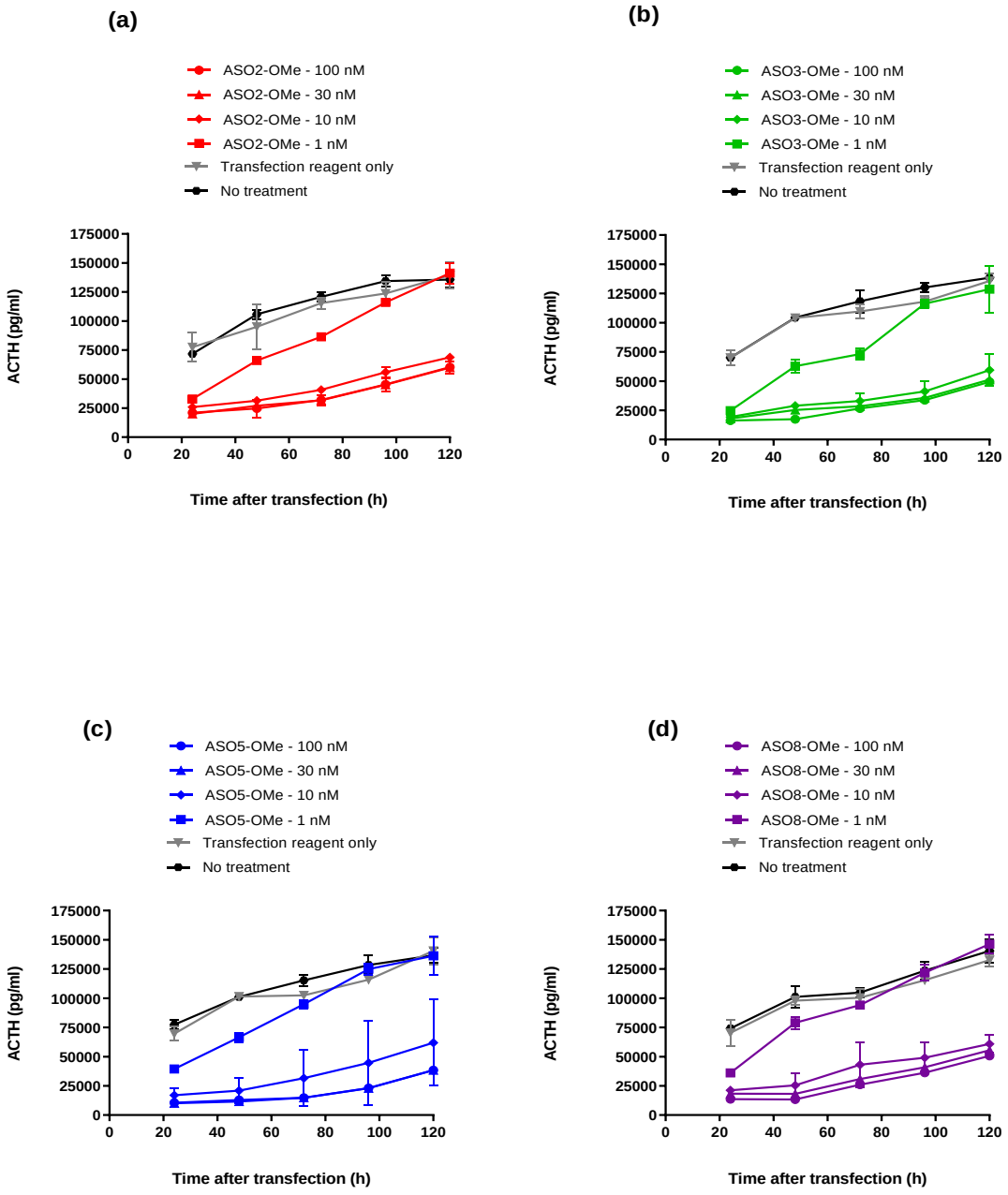


Figure 4.22: Longevity of action of OMe-modified POMC ASOs on ACTH secretion from AtT-20 cells.

Transfection of AtT-20 cells with OMe-modified POMC ASOs were carried out with concentrations of 100 nM, 30 nM, 10 nM, and 1 nM. OMe-modified ASOs alone and Lipofectamine®-2000 Reagent alone were used as control treatments. Cells without treatment were included. The ACTH levels in the cell culture medium were measured after 24, 48, 72, 96, and 120 h. The results are the mean (± SD) ACTH concentrations measured in four experiments and are shown for (a) ASO2-OMe, (b) ASO3-OMe, (c) ASO5-OMe, and (d) ASO8-OMe at the ASO concentrations used.

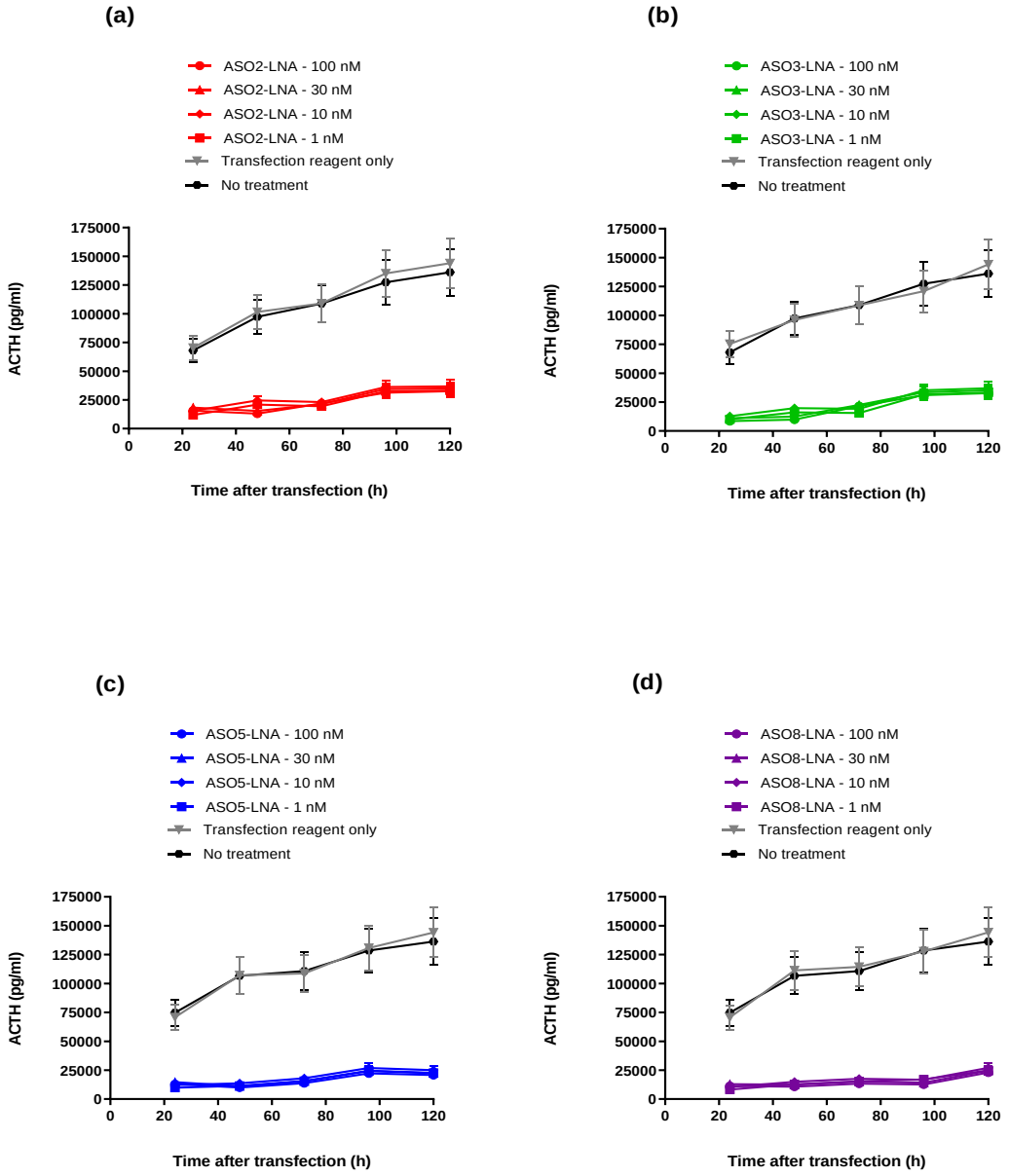


Figure 4.23: Longevity of action of LNA-modified POMC ASOs on ACTH secretion from AtT-20 cells.

Transfection of AtT-20 cells with LNA-modified POMC ASOs were carried out with concentrations of 100 nM, 30 nM, 10 nM, and 1 nM. OMe-modified ASOs alone and Lipofectamine®-2000 Reagent alone were used as control treatments. Cells without treatment were included. The ACTH levels in the cell culture medium were measured after 24, 48, 72, 96, and 120 h. The results are the mean (± SD) ACTH concentrations measured in four experiments and are shown for (a) ASO2-LNA, (b) ASO3-LNA, (c) ASO5-LNA, and (d) ASO8-LNA at the ASO concentrations used.

4.3.8 The effect of combinations of OMe-modified and LNA-modified POMC ASOs on ACTH secretion from AtT-20 cells

POMC ASOs were tested together at low concentrations to investigate if different combinations were more potent in reducing ACTH secretion from AtT-20 cells.

For the experiments, At-T20 cells were plated in 6-well plates at a density of 2 x 10^5 cells per well in 2 ml of culture medium. After 24 h, the cells were transfected with OMe-modified or LNA-modified POMC ASO combinations with each ASO at a final concentration of 1 nM. As controls, cells were treated with POMC ASO or Lipofectamine®-2000 Reagent alone. Untreated cells were also included. At 24 h post-transfection, a 30-µl sample of the culture medium was taken for ACTH measurement using an Immulite 2000 ACTH immunoassay (Section 2.6).

The results of three experiments for OMe-modified ASOs are shown in Figure 4.23. Comparison of the results when using ASO combinations as opposed to a single ASO indicated that only in three cases was there no significant decrease in ACTH secretion when two ASOs were used together (Unpaired t test, $P > 0.05$) (Table 4.11). These were ASO2-OMe and ASO3-OMe *vs* ASO3-OMe; ASO2-OMe and ASO8-OMe *vs* ASO8-OMe; and ASO3-OMe and ASO8-OMe *vs* ASO8-OMe (Table 4.11).

For LNA-modified ASOs, the results are shown in Figure 4.24. Comparison of the results when using ASO combinations as opposed to a single ASO indicated that in the majority of cases there was no significant decrease in ACTH secretion when two ASOs were used together (Unpaired t test, $P > 0.05$) (Table 4.11). In one case, ASO2-LNA and ASO5-LNA *vs* ASO5-LNA (Table 4.11), the single ASO5-LNA was more effective at reducing ACTH than the two ASOs together (Unpaired t test, $P < 0.05$).

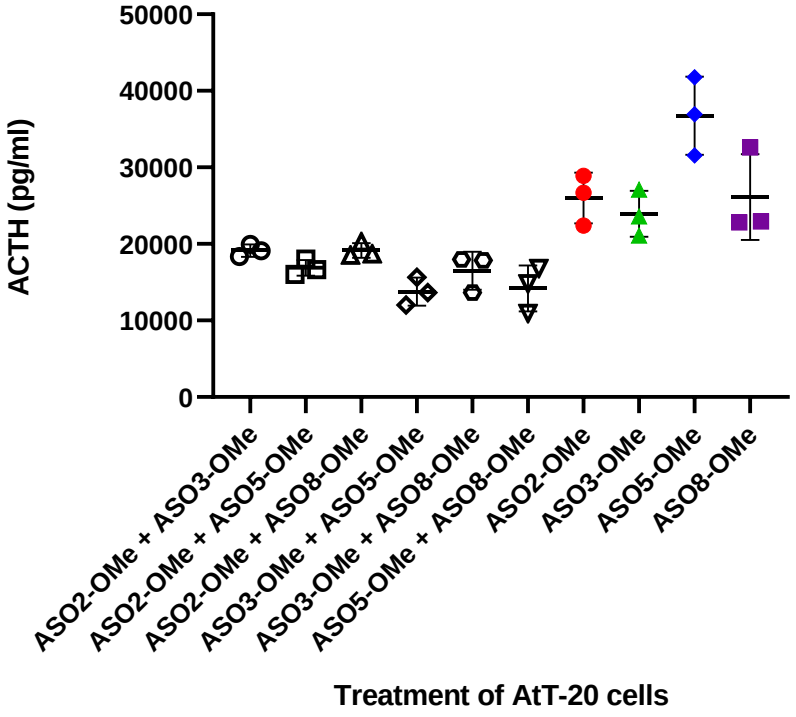


Figure 4.24: Comparison of ACTH levels secreted from AtT-20 cells following transfection with different combinations of OMe-modified POMC ASOs.

The reduction in ACTH levels secreted from AtT-20 cells following transfection with different combinations of OMe-modified POMC ASOs is shown as the mean (± SD) ACTH concentration from duplicate measurements in three experiments.

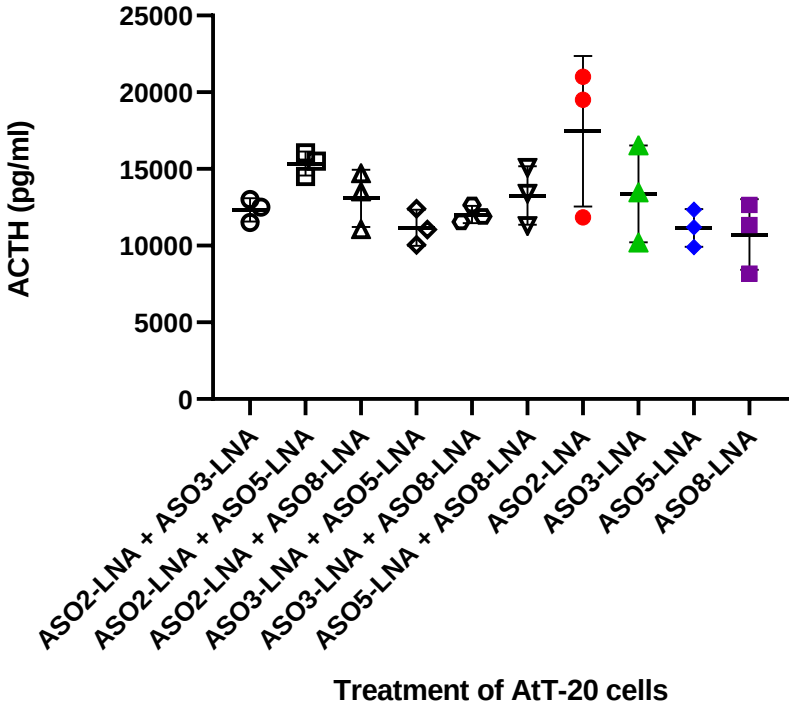


Figure 4.25: Comparison of ACTH levels secreted from AtT-20 cells following transfection with different combinations of LNA-modified POMC ASOs.

The reduction in ACTH levels secreted from AtT-20 cells following transfection with different combinations of LNA-modified POMC ASOs is shown as the mean (± SD) ACTH concentration from duplicate measurements in three experiments.

Table 4.11: Comparison of ACTH levels secreted from AtT-20 cells following transfection with different combinations of OMe-modified and LNA-modified POMC ASOs

Combined POMC ASO	Single POMC ASO	*P* value (Unpaired t test)[1]
ASO2-OMe + ASO3-OMe	ASO2-OMe	**0.025**
	ASO3-OMe	0.056
ASO2-OMe + ASO5-OMe	ASO2-OMe	**0.010**
	ASO5-OMe	**0.0027**
ASO2-OMe + ASO8-OMe	ASO2-OMe	**0.026**
	ASO8-OMe	0.10
ASO3-OMe + ASO5-OMe	ASO3-OMe	**0.0075**
	ASO5-OMe	**0.0018**
ASO3-OMe + ASO8-OMe	ASO3-OMe	**0.029**
	ASO8-OMe	0.053
ASO5-OMe + ASO8-OMe	ASO5-OMe	**0.0027**
	ASO8-OMe	**0.032**
ASO2-LNA + ASO3-LNA	ASO2-LNA	0.15
	ASO3-LNA	0.61
ASO2-LNA + ASO5-LNA	ASO2-LNA	0.50
	ASO5-LNA	**0.0075**
ASO2-LNA + ASO8-LNA	ASO2-LNA	0.22
	ASO8-LNA	0.24
ASO3-LNA + ASO5-LNA	ASO3-LNA	0.32
	ASO5-LNA	0.99
ASO3-LNA + ASO8-LNA	ASO3-LNA	0.51
	ASO8-LNA	0.39
ASO5-LNA + ASO8-LNA	ASO5-LNA	0.18
	ASO8-LNA	0.21

[1]Comparison of ACTH levels (pg/ml) secreted from AtT-20 cells after treatment with specified POMC ASO. Significant *P* values are shown in bold type.

4.4 Discussion

This part of the project aimed to examine how effective four POMC ASOs were at suppressing ACTH secretion from AtT-20 cells, which is an *in vitro* model of CD. The cells secrete ACTH when growing in culture medium, so the effect of new treatments that may modulate ACTH secretion can be evaluated. Two earlier studies examined the effects of ASOs on ACTH secretion from cells in culture. One study utilised an unmodified ASO against *Pomc* exon 3 (Spampinato et al., 1994). After transfection of AtT-20 cells with the ASO at 100 nM, ACTH secretion was reduced by 48-50% at 24 h. In a second study, an unmodified ASO against exon 1 of *POMC* reduced ACTH secretion from human corticotroph adenoma cells by 48-58% after 18 h when used at a concentration of 50 μM (Woloschak et al., 1994).

The findings in the current study confirmed the previously reported results; when used in transfections, POMC ASOs lowered ACTH secretion from cultured AtT-20 cells to 14-59% of normal levels. In terms of nucleotide sequence, POMC ASO5 was the most effective ASO, apart from its LNA-modified version, where POMC ASO3-LNA had the strongest gene-silencing effect.

In relation to different ASO modifications, the LNA-modified versions were the most effective for POMC ASO2 and POMC ASO3. The OMe-modified and LNA-modified versions of POMC ASO5 and POMC ASO8 were equally effective.

In order to minimise adverse outcomes such as immune reactions and off-target effects, AtT-20 cells were transfected with increasingly lower concentrations of each of the OMe-modified and LNA-modified POMC ASOs. Generally, the results showed that higher concentrations of OMe-modified versions of the ASOs were more effective at reducing ACTH secretion, and that for LNA-modified ASOs, concentrations as low as 1 nM were as effective as the higher concentrations tested.

When considering the longevity of POMC ASO action, LNA-modified ASOs were the most effective over time at reducing ACTH secretion from AtT-20 cells, even at the 1 nM lower concentration. In contrast, OMe-modified ASOs were less effective at gene silencing over time when used at 1 nM, although they were efficient at reducing ACTH secretion over time at higher concentrations.

Experiments using different combinations of OMe-modified POMC ASOs indicated that, except for three cases, they were more effective at reducing ACTH secretion from AtT-20 cells than their cognate single ASO. In contrast, using LNA-modified ASOs together had no significant effect compared with single ASOs.

The importance of controls cannot be overstated in relation to measuring the downstream effects of ASOs, as there is always the possibility that off-target mRNA binding will have an adverse influence on the measured outcomes (Gagnon and Corey, 2019). In this study, viable cell counting data discounted the chance that Lipofectamine®-2000 Reagent was killing AtT-20 cells due to cytotoxicity and thereby lowering ACTH secretion; experiments indicated that there was no statistically significant difference between the number of viable cells in Lipofectamine®-2000 Reagent-treated and untreated groups. This result was due to the use of a Lipofectamine®-2000 Reagent dilution (1:200) that allowed a high transfection efficiency of 81% ± 5.9% (mean ± SD; n = 9) without unwanted effects upon AtT-20 cell viability.

Further to optimising the concentration of transfection agent to avoid deleterious effects upon the AtT-20 cells, the experimental design also needed to include control ASOs (Gagnon and Corey, 2019). If different on-target ASOs have the same effect upon expression of the targeted mRNA, and control ASOs do not, then it is reasonable to assume that the observable results are not likely due to confounding off-target effects of the gene-silencing ASOs. In the present study, all four on-target POMC ASOs tested reduced the secretion of ACTH from AtT-20 cells. The chosen controls were MM and scrambled ASOs (Gagnon and Corey, 2019). Scrambled ASOs have the same nucleotide composition as the on-target version, but with a changed nucleotide sequence, and MM ASOs have one or more nucleotides that have been changed within the internal site of the molecule (Swayze et al., 2007, Sharma et al., 2005, Flanagan et al., 1996). The experiments carried out with control ASOs indicated that scrambled and MM versions of the on-target POMC ASOs did not cause a reduction in ACTH secretion from AtT-20 cells. This indicated, not only the importance of including controls, but also that the reduction of ACTH secretion was due to the targeting of *Pomc* mRNA rather than unintended side-effects on the cells or cell processes.

In conclusion, the four POMC ASOs tested could be used to effectively silence *Pomc* expression in AtT-20 cells and reduce the levels of ACTH secreted by them.

Chapter 5

Susceptibility of POMC antisense oligonucleotides to nuclease degradation

5 Susceptibility of POMC antisense oligonucleotides to nuclease degradation

5.1 Introduction

The POMC ASOs investigated in this study have been shown to mediate gene silencing in the *in vitro* model of AtT-20 cells, and so could possibly be a potential treatment for CD. However, clinical usage has many more barriers to the successful use of a new therapeutic ASO. Issues include nuclease degradation, poor target binding, toxicity, cellular delivery, rapid renal clearance, and inadequate tissue distribution (Section 1.3.2). Such problems have been addressed by introducing various chemical modifications into ASOs (Section 1.3.3) and by the use of carrier systems (Section 1.3.4), all of which can improve the bioavailability of ASO molecules.

With respect to nuclease degradation, ASOs are susceptible to endonuclease and exonuclease activity that hydrolyses phosphodiester bonds (Geary et al., 2015b, McGinnis et al., 2012, Geary, 2009). Such enzymes are found in many types of cells and so pose a threat to ASO bioavailability (Schildkraut, 2001). Many chemical modifications to the basic nucleic acid backbone have been investigated with the aim of improving ASO resistance to enzyme digestion (Deleavey and Damha, 2012, Cobb, 2007). For example, PS-modified backbones can prevent cleavage by nucleases whilst maintaining the native structure of the ASO (Eckstein, 1985). In addition, modified ASOs containing 2'-*O*-substituted nucleotides have improved resistance towards degradation by nucleases (Rinaldi and Wood, 2018). Furthermore, better nuclease resistance has been achieved by structural modifications of the nucleotide furanose ring such as those found in LNA, PNA, and PMO ASOs (Chan et al., 2006).

Investigations of clinically approved ASOs such as Spinraza™ and Kynamro™ have indeed shown that they are broken down by intracellular endonucleases and exonucleases (Crooke and Geary, 2013). Furthermore, enzymatic activity has been shown to be dependent upon the modifications used in the ASO. In the case of Spinraza™, incorporation of MOE-modified nucleobases and a PS-modified backbone resulted in slower degradation by 3'- and 5'- exonucleases (Crooke and Geary, 2013). In contrast, Kynamro™, which is a gapmer with MOE-modified nucleotides at either end, unmodified internal nucleotides, and a PS-modified backbone, was initially subjected to endonuclease digestion of the gapmer molecules before further

exonuclease degradation occurred (Geary et al., 2015a, Crooke and Geary, 2013, Yu et al., 2007).

5.2 Aims and Objectives

Previously in this study (Chapter 4), unmodified, PS-modified, OMe-modified, and LNA-modified POMC ASOs were shown to reduce ACTH secretion from AtT-20 cells, albeit to differing degrees in terms of concentration and effectiveness over time. The aim of this part of the project was to investigate the resistance of POMC ASOs to nuclease degradation in complex biological media and in exonuclease-containing buffered solutions, since any differences in effectiveness might be due to differing susceptibility to enzymatic hydrolysis.

The specific objectives were to:

- Analyse the susceptibility to nuclease degradation of POMC ASOs in cell culture medium, human plasma, and AtT-20 cell lysate using agarose gel electrophoresis and DNA concentration measurements over time.
- Analyse the susceptibility of POMC ASOs to 3'- and 5'-exonuclease digestion, as above.

5.3 Results

5.3.1 Degradation of POMC ASOs in cell culture medium

The susceptibility of POMC ASOs to enzymatic degradation in cell culture medium was investigated by a 120-h incubation at 37°C, as detailed in Section 2.16.1. Samples (2 µl) of the ASO-cell culture medium were removed after 0, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h of incubation and were analysed by electrophoresis in 1.5% agarose gels (Section 2.12). The gels were examined and recorded using a G:BOX gel documentation system and GeneSnap image acquisition software. The degradation of each ASO across the time points was examined from the images.

The results of the agarose gel analysis, which was undertaken by Dr Jacob Whatmore (Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK), are shown in Figure 5.1a-d. Unmodified POMC ASO2, ASO3, ASO5, and ASO8 were not detectable at the 72-h, 96-h, 96-h, and 24-h time-points, respectively (Figure 5.1a). All PS-modified, OMe-modified, and LNA-modified POMC ASOs were still visible on the gels at the 120-h time point (Figure 5.1b, c, and d, respectively). The degradation patterns were consistent in repeated experiments.

The agarose gel analysis was a qualitative method for monitoring any degradation of POMC ASO molecules in cell culture medium. For a quantitative approach, the experiments were repeated with the ASO concentration in the collected samples being measured using a NanoDrop ND-1000 spectrophotometer (Section 2.9.3).

The results are illustrated in Figure 5.2a-d, and show that POMC ASO degradation followed a similar time course to that using the qualitative analysis in agarose gels. Unmodified ASOs showed significant degradation over the 120-h incubation (Figure 5.2a), whilst the modified ASOs appeared to be more stable to degradation over the same time-period (Figure 5.2b-d).

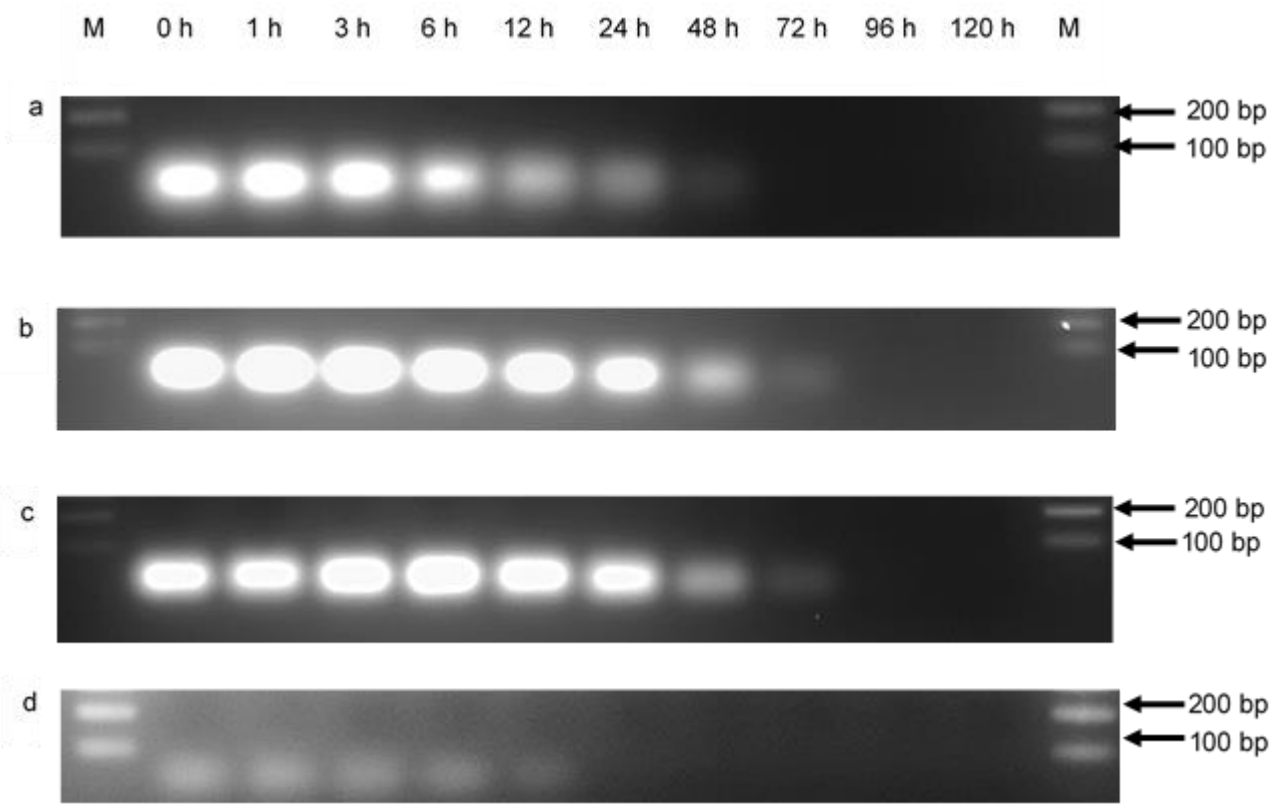


Figure 5.1a: Agarose gel electrophoresis of unmodified POMC ASOs after incubation in cell culture medium.

Unmodified POMC ASOs were incubated in cell culture medium. Samples (2 µl) were removed at 0, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) ASO2, (b) ASO3, (c) ASO5, and (d) ASO8. M, 100-bp DNA markers. This is a representative result of three separate experiments.

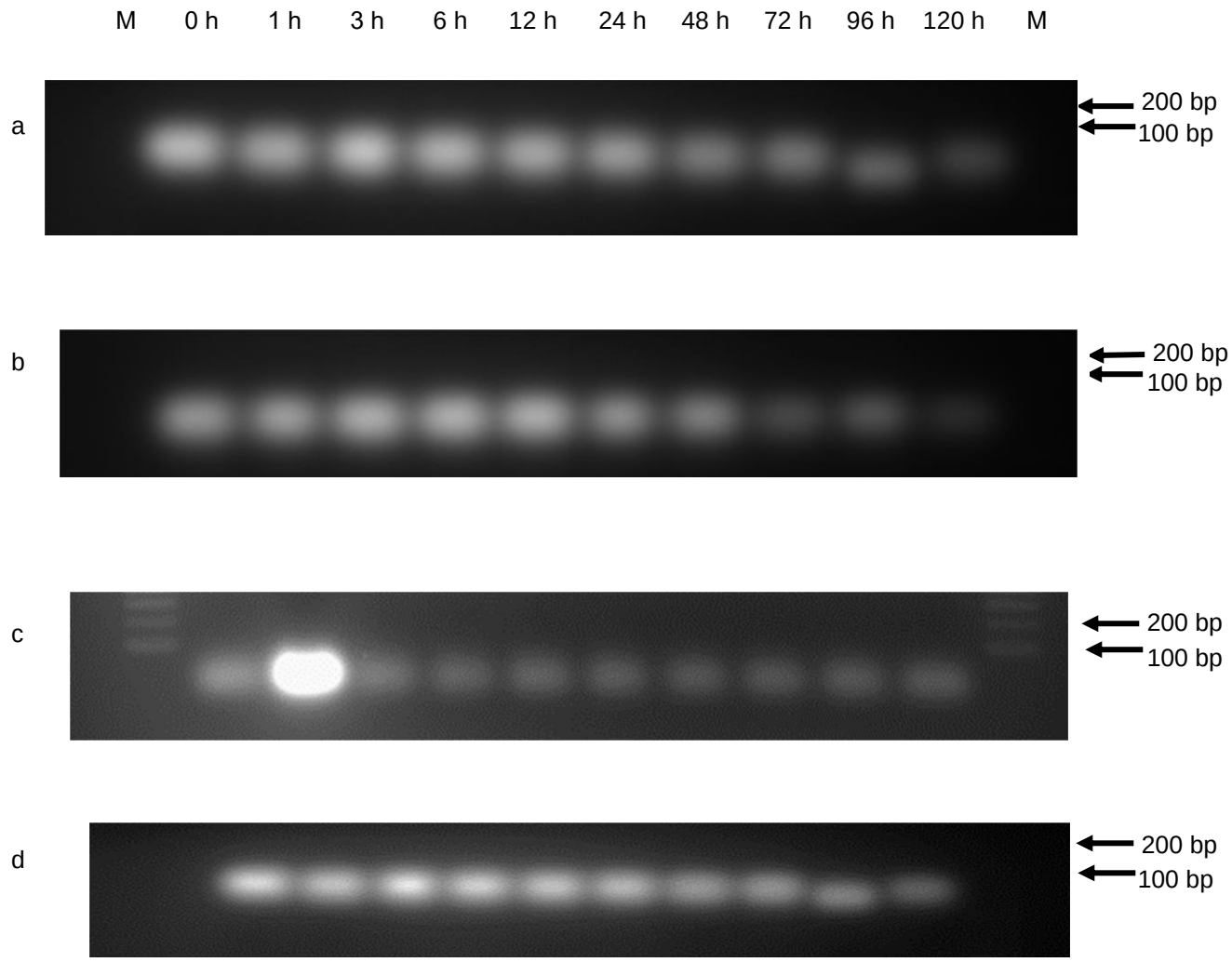


Figure 5.1b: Agarose gel electrophoresis of PS-modified POMC ASOs after incubation in cell culture medium.

PS-modified POMC ASOs were incubated in cell culture medium. Samples (2 µl) were removed at 0, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) ASO2-PS, (b) ASO3-PS, (c) ASO5-PS, and (d) ASO8-PS. M, 100-bp DNA markers. This is a representative result of three separate experiments.

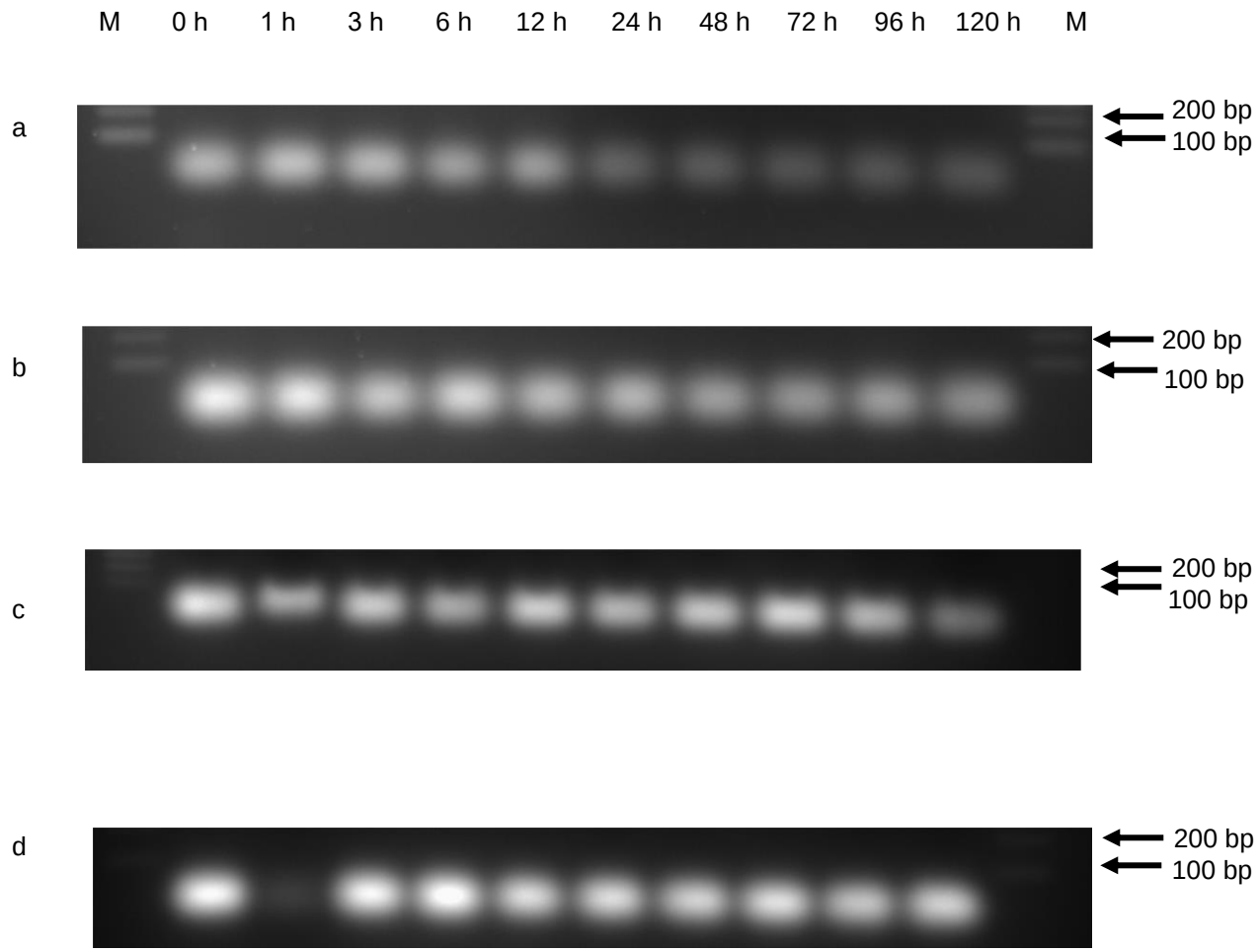


Figure 5.1c: Agarose gel electrophoresis of POMC OMe-modified ASOs after incubation in cell culture medium.

OMe-modified POMC ASOs were incubated in cell culture medium. Samples (2 µl) were removed at 0, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) ASO2-OMe, (b) ASO3-OMe, (c) ASO5-OMe, and (d) ASO8-OMe. M, 100-bp DNA markers. This is a representative result of three separate experiments.

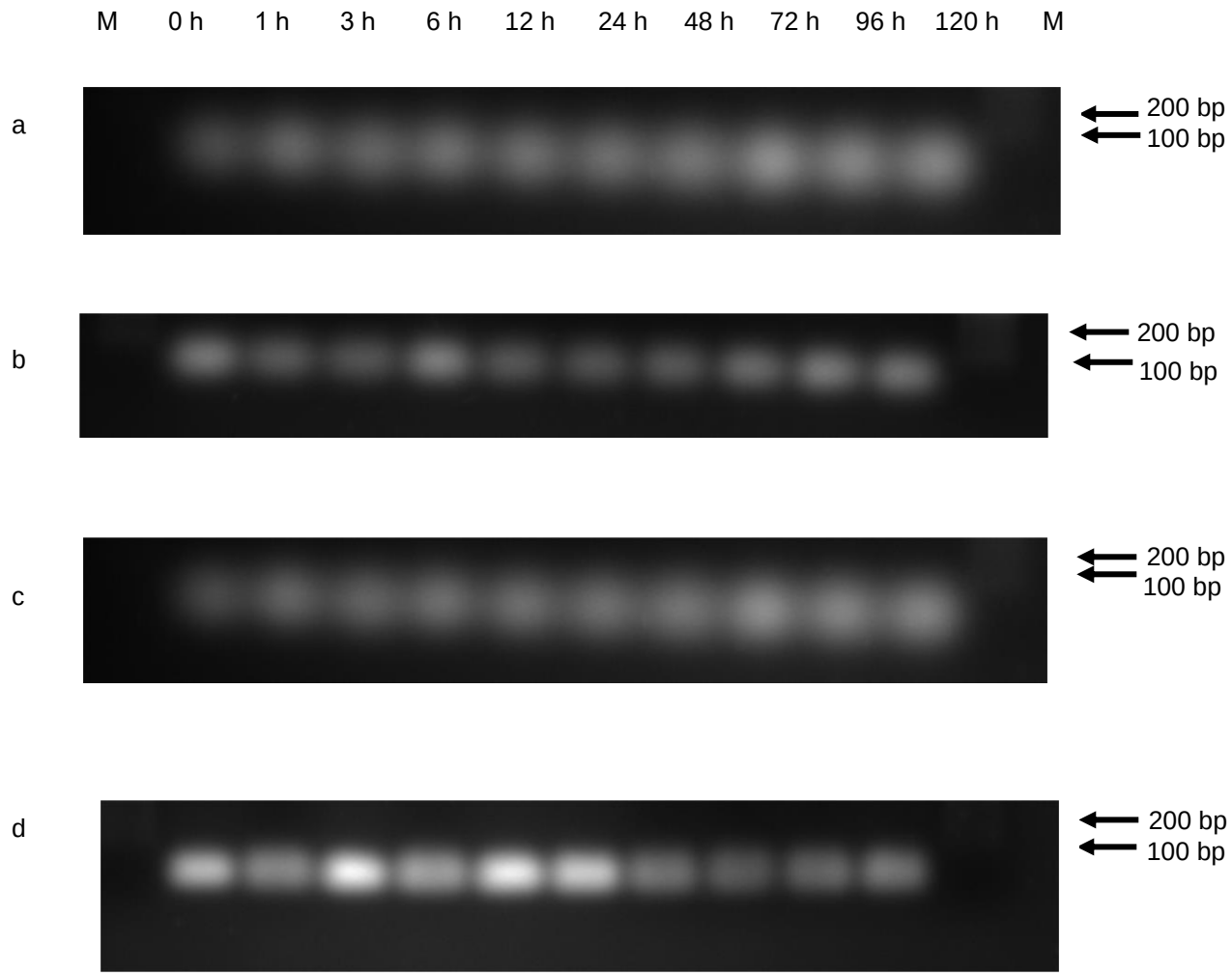


Figure 5.1d: Agarose gel electrophoresis of POMC LNA-modified ASOs after incubation in cell culture medium.

LNA-modified POMC ASOs were incubated in cell culture medium. Samples (2 µl) were removed at 0, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) ASO2-LNA, (b) ASO3-LNA, (c) ASO5-LNA, and (d) ASO8-LNA. M, 100-bp DNA markers. This is a representative result of three separate experiments.

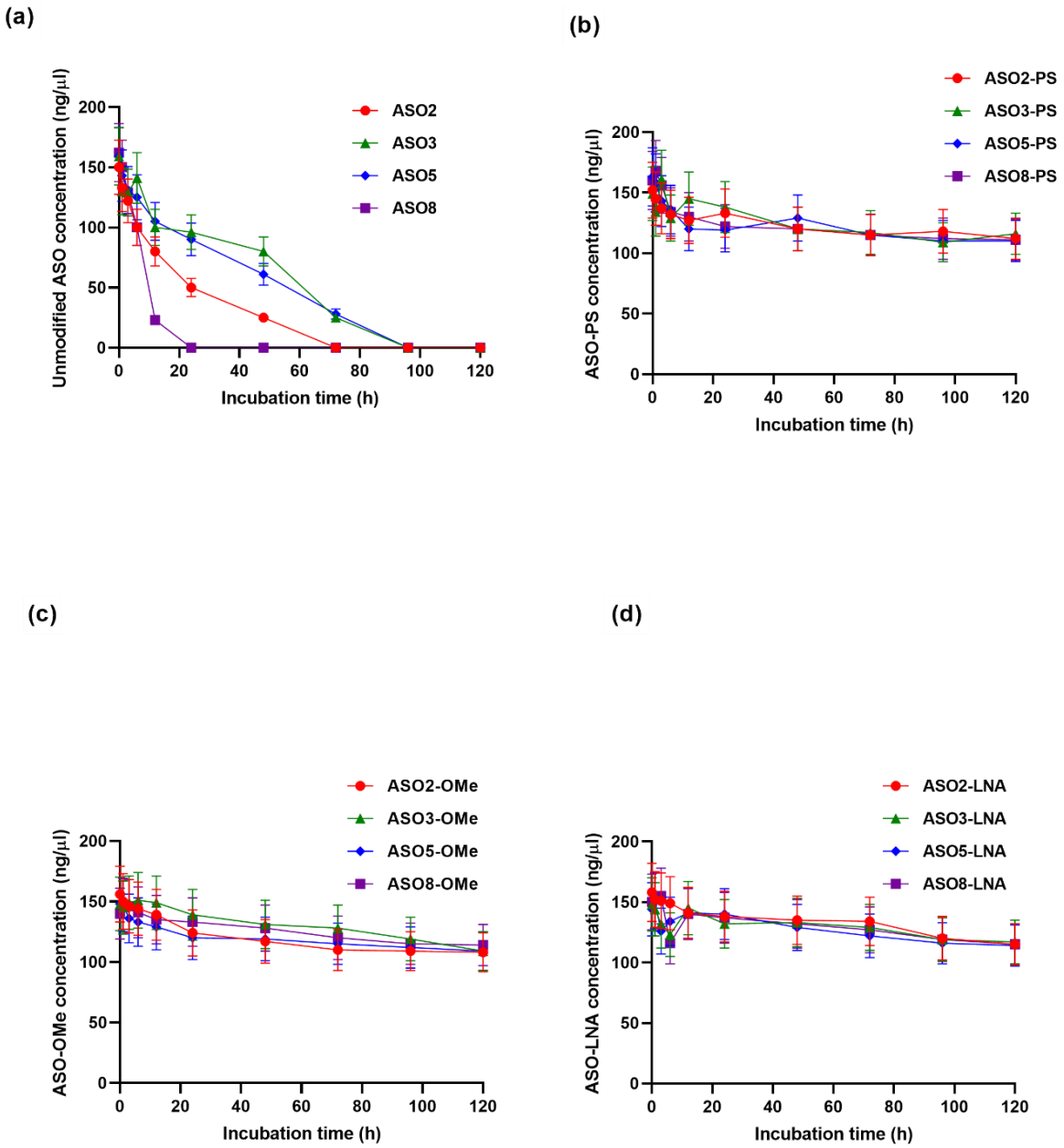


Figure 5.2: Degradation of POMC ASOs in cell culture medium.

POMC ASOs were incubated in cell culture medium. Samples (2 µl) were removed at 0, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h, and were analysed for ASO concentration (ng/µl) in a NanoDrop ND-1000 spectrophotometer. The results shown are the mean ASO concentration (ng/µl) and SD of three experiments for: (a) Unmodified POMC ASOs, (b) PS-modified POMC ASOs, (c) OMe-modified POMC ASOs, and (d) LNA-modified POMC ASOs.

5.3.2 Degradation of POMC ASOs in human plasma

The susceptibility of POMC ASOs to enzymatic degradation in human plasma was investigated in an 8-h incubation at 37°C, as described in Section 2.16.2. Samples (2 µl) of the human plasma containing the ASO were removed after 0, 2, 4, 6, and 8 h of incubation, and were analysed by agarose gel electrophoresis and nanodrop measurements, as detailed in Section 5.3.1.

The results of the agarose gel analysis indicated that the unmodified POMC ASOs were undetectable at the 4-h time-point. All PS-modified, OMe-modified, and LNA-modified POMC ASOs were still visible on the gels at the 8-h time-point. As examples, Figure 5.3a, b, c, and d, respectively, show the results for unmodified ASO2, ASO3-PS, ASO5-OMe, and ASO8-LNA.

The experiments were repeated with the ASO concentration in the collected samples being measured using a NanoDrop ND-1000 spectrophotometer. The results indicated that unmodified ASOs showed degradation over the 8-h incubation period (Figure 5.4a). In contrast, the modified ASOs appeared to be relatively stable to degradation over the same 8-h time-period (Figure 5.4b-d).

To investigate if ASO degradation was due to nuclease activity, the human plasma was heated to 85°C to denature any enzymes present before an 8-h incubation with unmodified POMC ASOs. The results are shown in Figure 5.4e. Less degradation of ASOs was evident over the same incubation period, compared with ASOs in non-heat treated plasma.

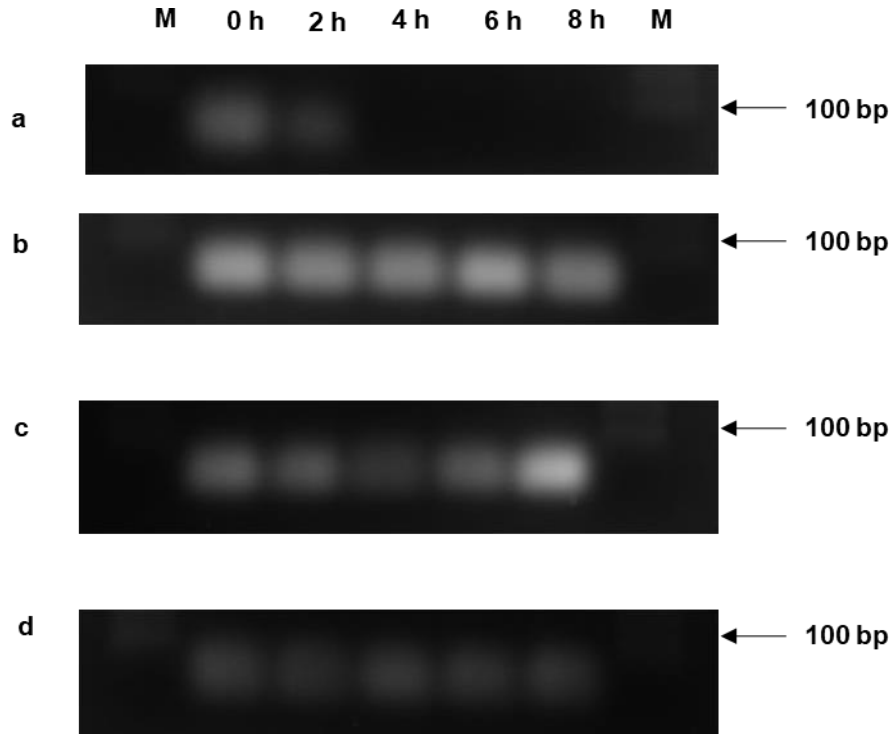


Figure 5.3: Agarose gel electrophoresis of POMC ASOs after incubation in human plasma.

POMC ASOs were incubated in human plasma. Samples (2 µl) were removed at 0, 2, 4, 6, and 8 h, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) Unmodified ASO2, (b) ASO3-PS, (c) ASO5-OMe, and (d) ASO8-LNA. M, 100-bp DNA markers. This is a representative result of three separate experiments.

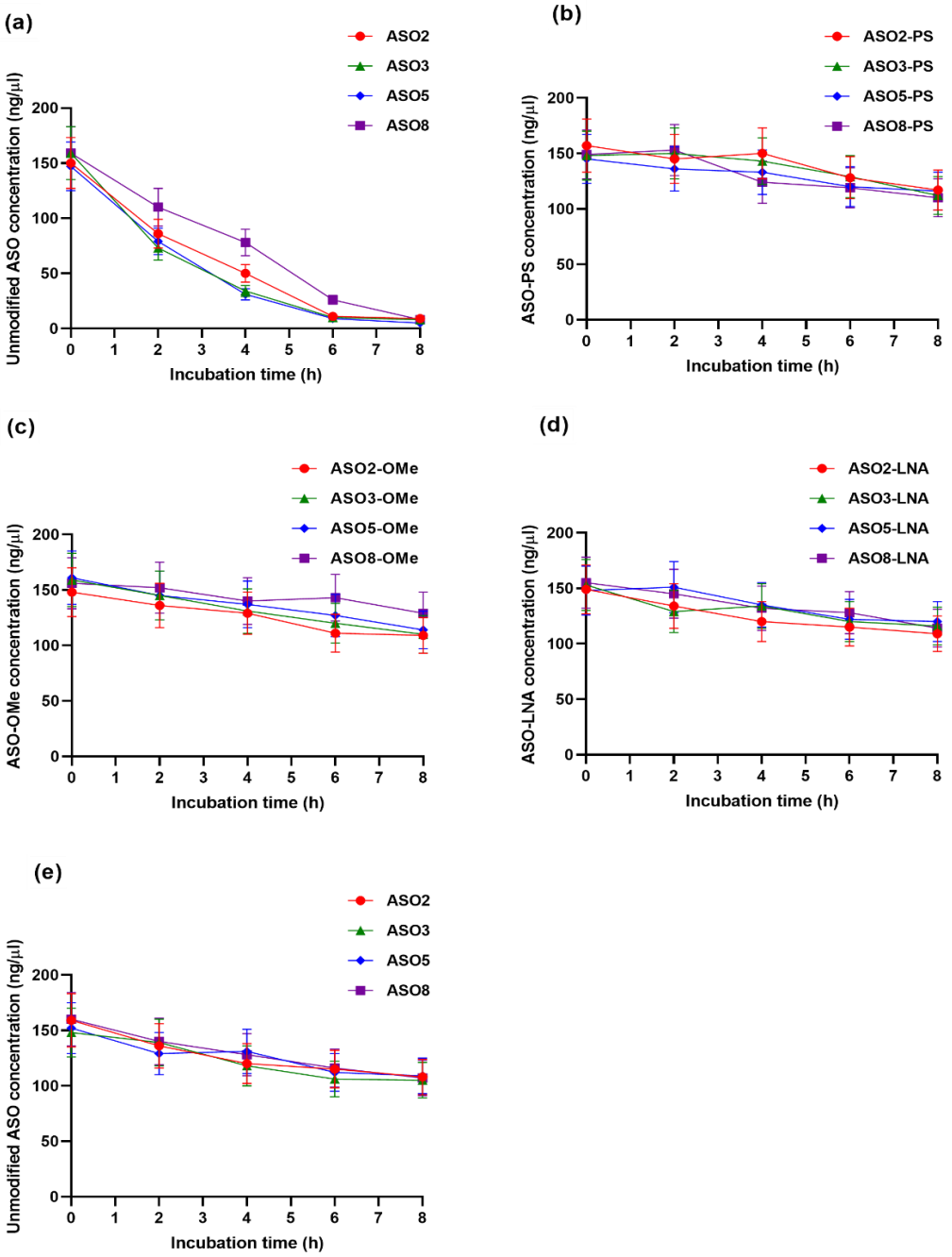


Figure 5.4: Degradation of POMC ASOs in human plasma.

POMC ASOs were incubated in human plasma. Samples (2 µl) were removed at 0, 2, 4, 6, and 8 h, and were analysed for ASO concentration (ng/µl) in a NanoDrop ND-1000 spectrophotometer. The results shown are the mean ASO concentration (ng/µl) and SD of three experiments for: (a) Unmodified POMC ASOs, (b) PS-modified POMC ASOs, (c) OMe-modified POMC ASOs, (d) LNA-modified POMC ASOs, and (e) Unmodified POMC ASOs in heat-treated plasma.

5.3.3 Degradation of POMC ASOs in AtT-20 cell lysate

The susceptibility of POMC ASOs to enzymatic degradation in AtT-20 cell lysate was investigated in an 8-h incubation at 37°C, as described in Section 2.16.3. Samples (2 µl) of the AtT-20 cell lysate containing the ASO were removed after 0, 2, 4, 6, and 8 h of incubation, and were analysed by agarose gel electrophoresis and nanodrop measurements, as detailed in Section 5.3.1.

The results of the agarose gel analysis indicated that the unmodified POMC ASOs were not detectable at the 4-h time-point. All PS-modified, OMe-modified, and LNA-modified POMC ASOs were still visible on the gels at the 8-h time-point. As examples, Figure 5.5a, b, c, and d, respectively, show the results for unmodified ASO2, ASO3-PS, ASO5-OMe, and ASO8-LNA.

The experiments were repeated with the ASO concentration in the collected samples being measured using a NanoDrop ND-1000 spectrophotometer. The results indicated that unmodified ASOs showed degradation over the 8-h incubation period (Figure 5.6a). In contrast, the modified ASOs appeared to be relatively stable to degradation over the same 8-h time-period (Figure 5.6b-d).

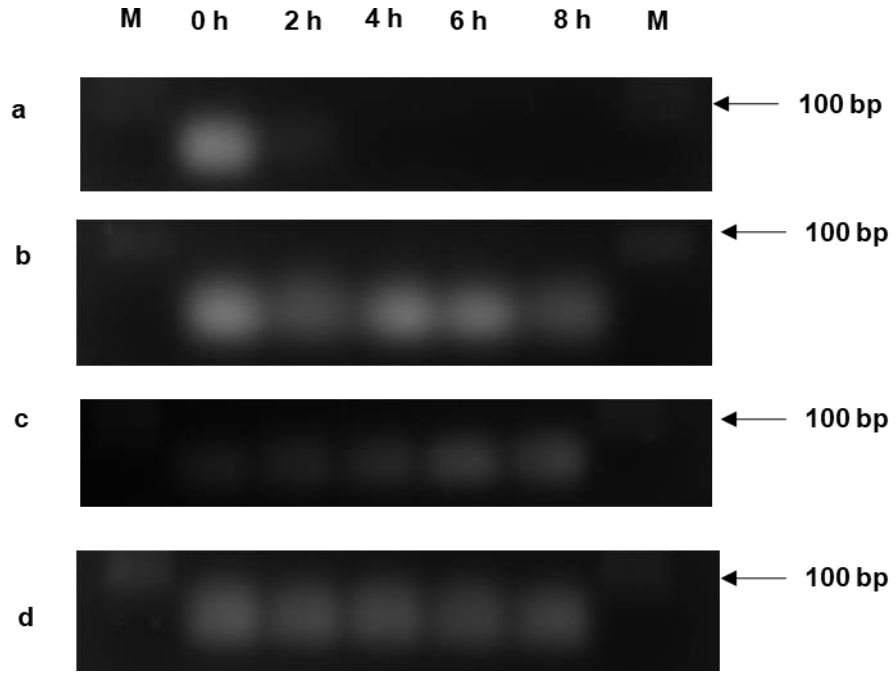


Figure 5.5: Agarose gel electrophoresis of POMC ASOs after incubation in AtT-20 cell lysate.

POMC ASOs were incubated in AtT-20 cell lysate. Samples (2 µl) were removed at 0, 2, 4, 6, and 8 h, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) Unmodified ASO2, (b) ASO3-PS, (c) ASO5-OMe, and (d) ASO8-LNA. M, 100-bp DNA markers. This is a representative result of three separate experiments.

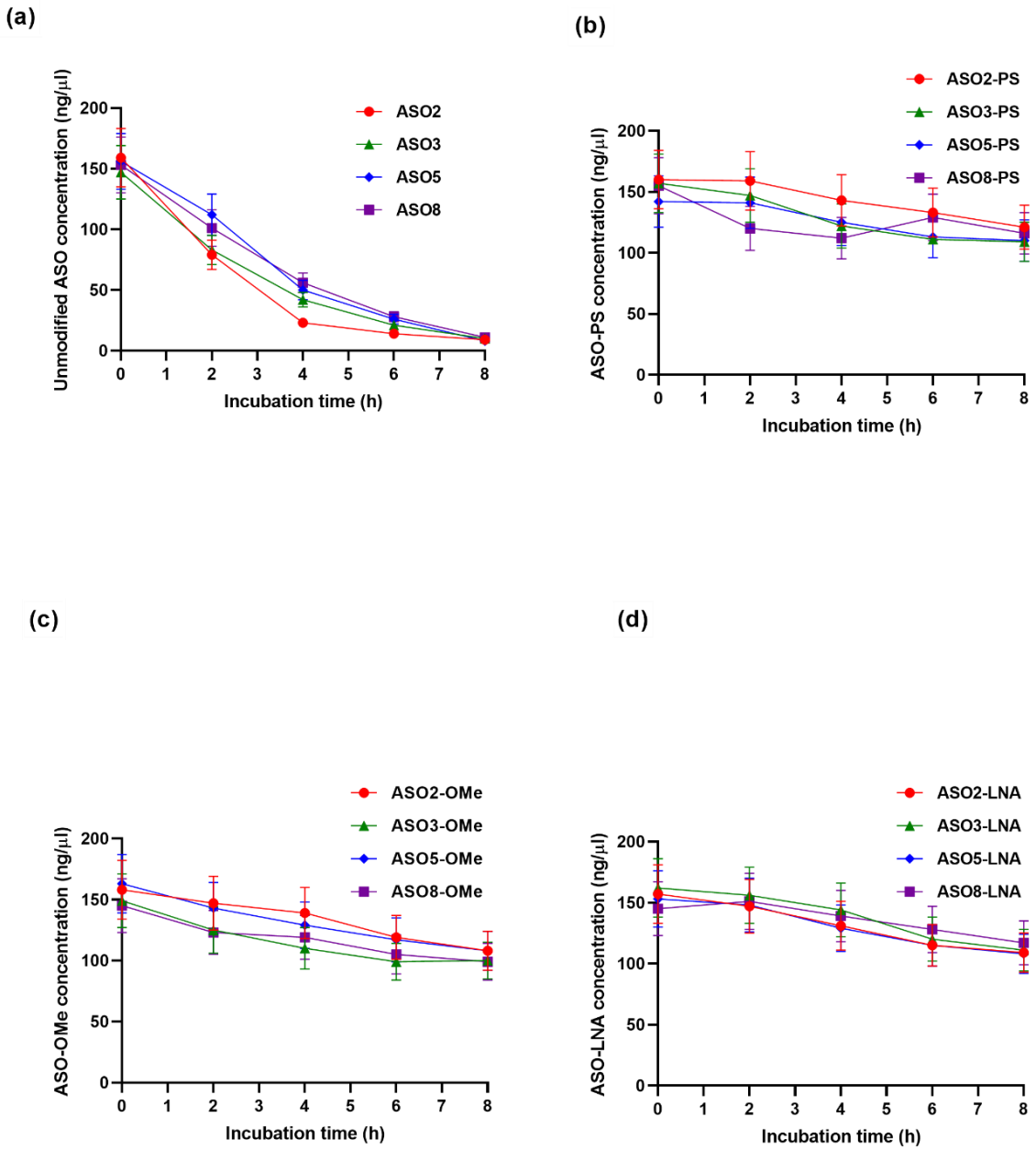


Figure 5.6: Degradation of POMC ASOs in AtT-20 cell lysate.

POMC ASOs were incubated in AtT-20 cell lysate. Samples (2 µl) were removed at 0, 2, 4, 6, and 8 h, and were analysed for ASO concentration (ng/µl) in a NanoDrop ND-1000 spectrophotometer. The results shown are the mean ASO concentration (ng/µl) and SD of three experiments for: (a) Unmodified POMC ASOs, (b) PS-modified POMC ASOs, (c) OMe-modified POMC ASOs, and (d) LNA-modified POMC ASOs.

5.3.4 Degradation of POMC ASOs by 3'-exonuclease

The susceptibility of POMC ASOs to degradation by the 3'-exonuclease was investigated in a 120-min incubation at 37°C, as described in Section 2.16.4. Samples (2 µl) of the buffer containing the 3'-exonuclease and the ASO were removed after 0, 10, 30, 60, and 120 min of incubation, and were analysed by agarose gel electrophoresis and nanodrop measurements, as detailed in Section 5.3.1.

The results of the agarose gel analysis indicated that the unmodified POMC ASOs were not detectable at the 30-min time-point. All PS-modified, OMe-modified, and LNA-modified POMC ASOs were still visible on the gels at the 120-min time-point. As examples, Figure 5.7a, b, c, and d, respectively, show the results for ASO2, ASO3-PS, ASO5-OMe, and ASO8-LNA.

The experiments were repeated with the ASO concentration in the collected samples being measured using a NanoDrop ND-1000 spectrophotometer. The results indicated that unmodified ASOs showed degradation over the 120-min incubation period (Figure 5.8a). In contrast, the modified ASOs appeared to be relatively stable to degradation over the same 120-min time-period (Figure 5.8b-d).

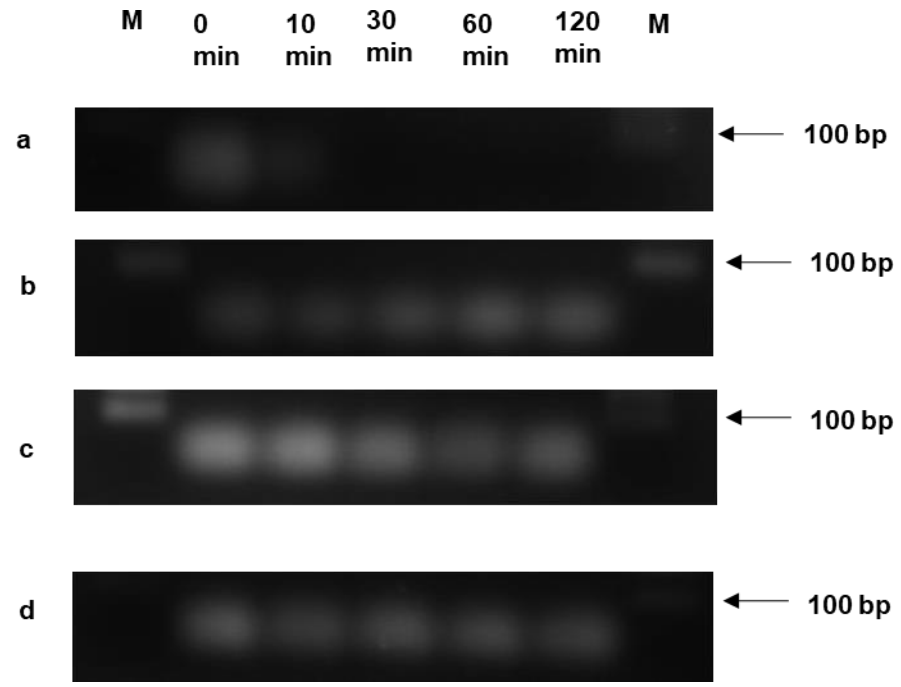


Figure 5.7: Agarose gel electrophoresis of POMC ASOs after incubation with 3'-exonuclease.

POMC ASOs were incubated in buffer containing the 3'-exonuclease. Samples (2 µl) were removed at 0, 10, 30, 60, and 120 min, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) Unmodified ASO2, (b) ASO3-PS, (c) ASO5- OMe, and (d) ASO8-LNA. M, 100-bp DNA markers. This is a representative result of three separate experiments.

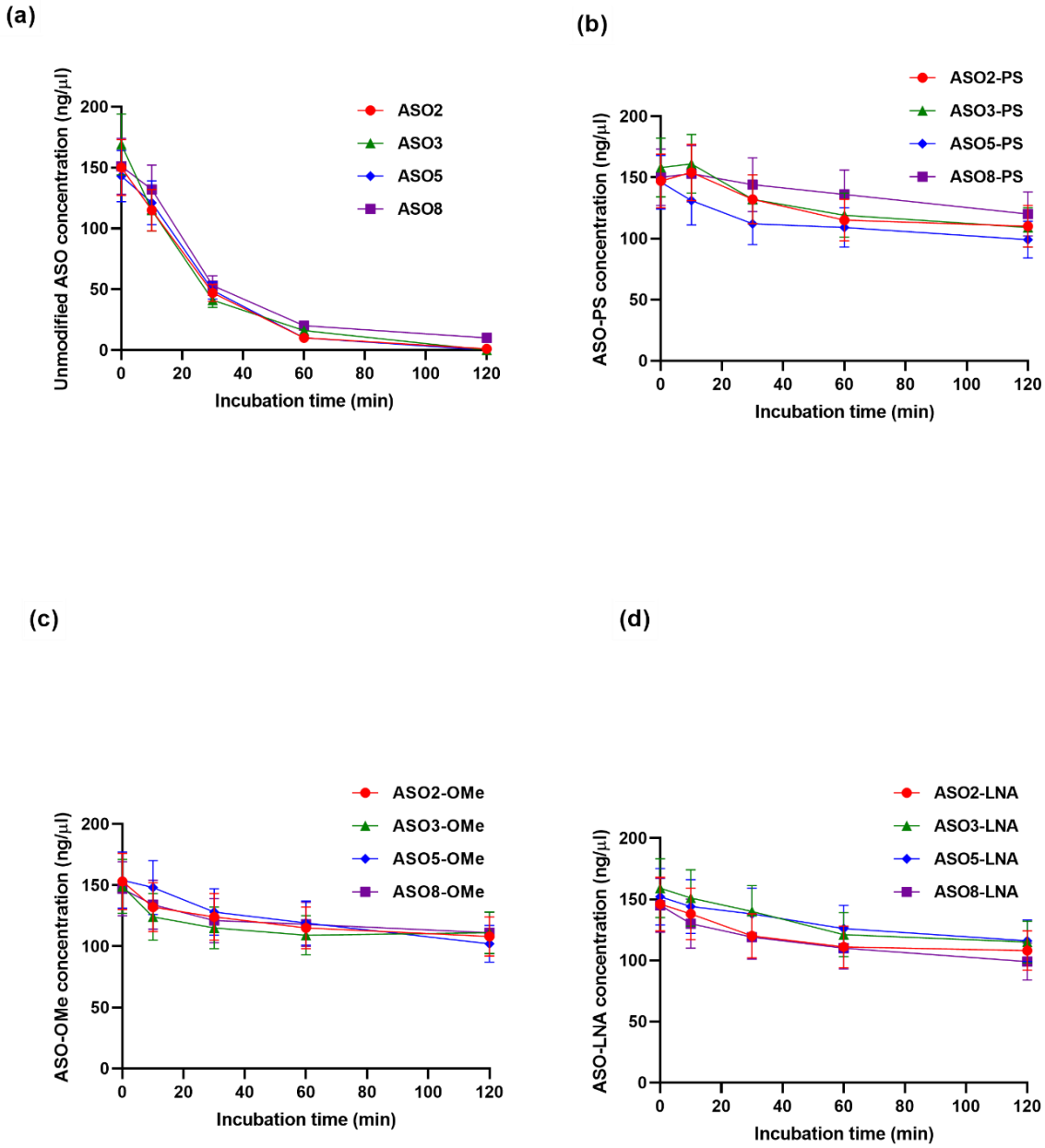


Figure 5.8: Degradation of POMC ASOs by 3'-exonuclease.

POMC ASOs were incubated in buffer containing 3'-exonuclease. Samples (2 µl) were removed at 0, 10, 30, 60, and 120 min, and were analysed for ASO concentration (ng/µl) in a NanoDrop ND-1000 spectrophotometer. The results shown are the mean ASO concentration (ng/µl) and SD of three experiments for: (a) Unmodified POMC ASOs, (b) PS-modified POMC ASOs, (c) OMe-modified POMC ASOs, and (d) LNA-modified POMC ASOs.

5.3.5 Degradation of POMC ASOs by 5’-exonuclease

The susceptibility of POMC ASOs to degradation by the 5’-exonuclease was investigated in a 120-min incubation at 37°C, as described in Section 2.16.5. Samples (2 µl) of the buffer containing the 5’-exonuclease and the ASO were removed after 0, 10, 30, 60, and 120 min of incubation, and were analysed by agarose gel electrophoresis and nanodrop measurements, as detailed in Section 5.3.1.

The results of the agarose gel analysis indicated that the unmodified POMC ASOs could not be detected by the 30-min time-point. All PS-modified, OMe-modified, and LNA-modified POMC ASOs were still visible on the gels at the 120-min time-point. As examples, Figure 5.9a, b, c, and d, respectively, show the results for ASO2, ASO3-PS, ASO5-OMe, and ASO8-LNA.

The experiments were repeated with the ASO concentration in the collected samples being measured using a NanoDrop ND-1000 spectrophotometer. The results indicated that unmodified ASOs showed degradation over the 120-min incubation period (Figure 5.10a). In contrast, the modified ASOs appeared to be relatively stable to degradation over the same 120-min time-period (Figure 5.10b-d).

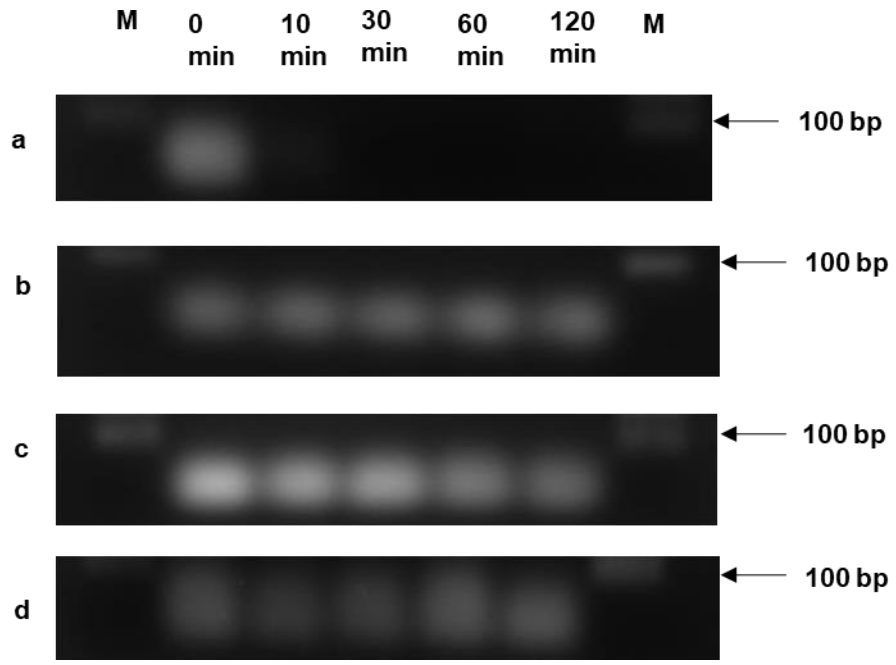


Figure 5.9: Agarose gel electrophoresis of POMC ASOs after incubation with 5'-exonuclease.

POMC ASOs were incubated in buffer containing the 5'-exonuclease. Samples (2 µl) were removed at 0, 10, 30, 60, and 120 min, and were analysed by electrophoresis in 1.5% agarose gels. The results are shown for: (a) Unmodified ASO2, (b) ASO3-PS, (c) ASO5-OMe, and (d) ASO8-LNA. M, 100-bp DNA markers. This is a representative result of three separate experiments.

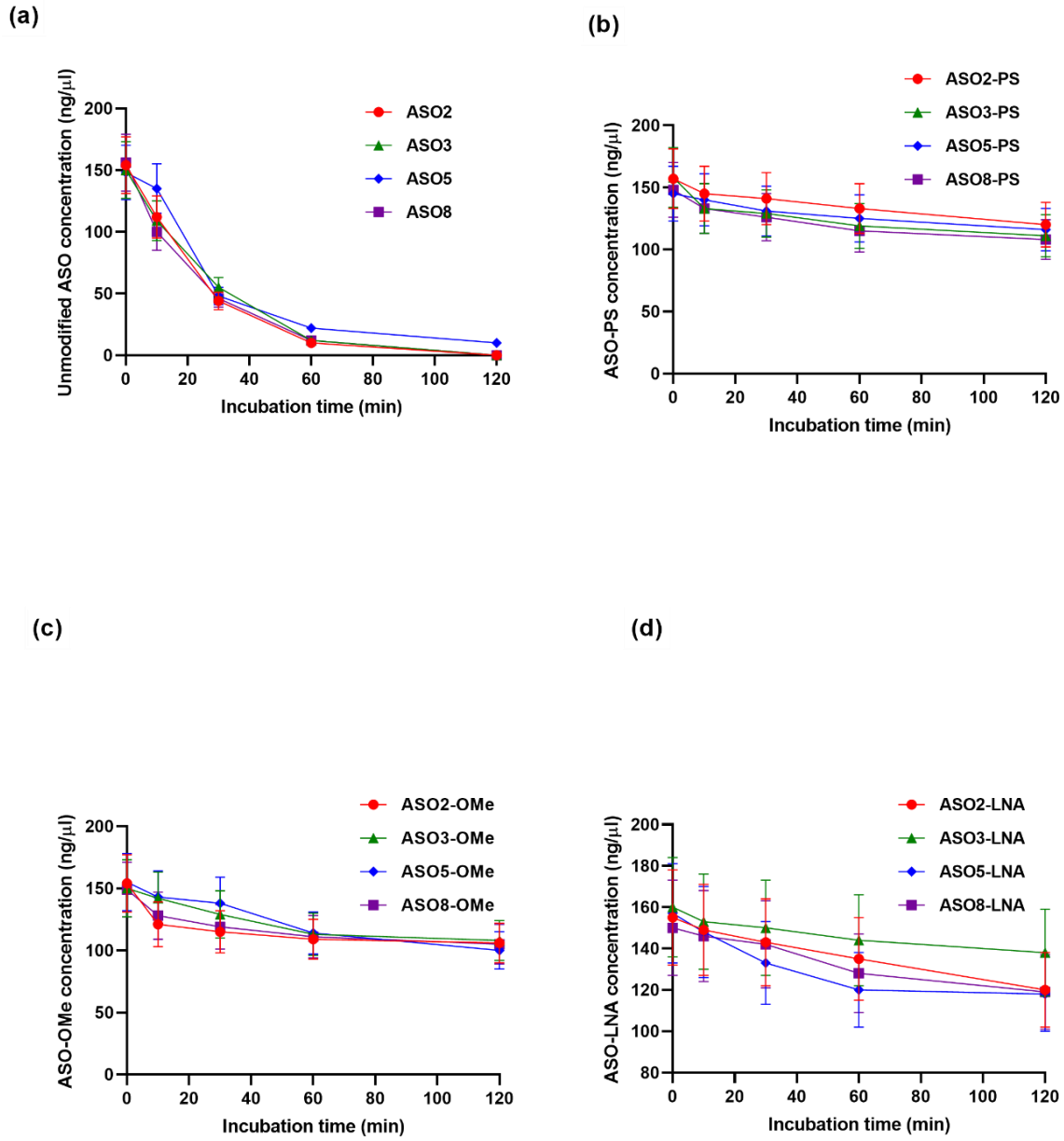


Figure 5.10: Degradation of POMC ASOs by 5'-exonuclease.

POMC ASOs were incubated in buffer containing 5'-exonuclease. Samples (2 µl) were removed at 0, 10, 30, 60, and 120 min, and were analysed for ASO concentration (ng/µl) in a NanoDrop ND-1000 spectrophotometer. The results shown are the mean ASO concentration (ng/µl) and SD of three experiments for: (a) Unmodified POMC ASOs, (b) PS-modified POMC ASOs, (c) OMe-modified POMC ASOs, and (d) LNA-modified POMC ASOs.

5.3.6 Comparison of nuclease degradation of POMC ASOs

The mean percentage of each ASO remaining at the incubation end-point in each of the media used is listed in Table 5.1. The results indicated that in all complex biological media and in buffer containing exonucleases, the unmodified POMC ASOs were degraded to leave 0-7% of the starting ASO concentration. In comparison, the modified POMC ASOs were degraded to leave 65-86% of their original concentration across the different incubation media investigated. The type of modification (PS, OMe or LNA) or the ASO sequence did not appear to have a significant effect on the level of nuclease degradation.

Table 5.1: Comparison of nuclease degradation of POMC ASOs

POMC ASO[1]	Percentage ASO remaining at the end time-point of incubation[2]				
	Cell culture medium (120 h)	Human plasma (8 h)	AtT-20 cell lysate (8 h)	3'-exonuclease (2 h)	5'-exonuclease (2 h)
ASO2-unmodified	0	6	6	1	0
ASO3-unmodified	0	5	7	0	0
ASO5-unmodified	0	3	5	0	7
ASO8-unmodified	0	5	7	7	0
ASO2-PS	74	75	76	75	76
ASO3-PS	78	76	69	69	70
ASO5-PS	67	80	77	68	80
ASO8-PS	69	74	75	80	73
ASO2-OMe	69	74	68	71	69
ASO3-OMe	74	69	67	74	72
ASO5-OMe	74	71	66	66	65
ASO8-OMe	81	83	68	76	70
ASO2-LNA	73	73	69	74	77
ASO3-LNA	79	76	69	72	86
ASO5-LNA	79	81	71	76	75
ASO8-LNA	77	74	81	68	79

[1]LNA, locked nucleic acid; OMe, 2'-*O* methyl; PS, phosphorothioate.

[2]The percentage ASO remaining is the mean of three experiments.

5.4 Discussion

Investigations into the ACTH-suppressing effects of POMC ASOs showed that they suppressed expression of ACTH in an *in vitro* model of CD (Chapter 4). In addition, it was found that the POMC ASOs incorporating LNA or OMe nucleotide modifications had greater potency in suppressing ACTH secretion. This is likely due to an increased binding affinity and better stability in that they are more resistant to degradation by exonucleases. The aim of the work in the current Chapter was to explore the idea that ASO modifications increased their resistance to nuclease activity. The degradation of ASOs in different complex biological media and by exonucleases in buffered solutions was investigated over time via qualitative agarose gel electrophoresis and quantitative nanodrop measurements.

The results showed that unmodified ASOs degraded over time following incubation in all of the complex biological media and in the solutions containing exonucleases. In contrast, PS-modified, OMe-modified, and LNA-modified POMC ASOs showed much more stability over the same period of incubation. Interestingly, the relative stability of unmodified ASOs during at least the first 24 h of incubation in cell culture medium could account for the fact that they were able to reduce ACTH secretion from AtT-20 cells (Chapter 4). It may be that the foetal bovine serum included in the cell culture medium contained a low concentration and/or activity of nucleases, since when exposed to exonucleases in buffered solutions, the unmodified POMC ASOs were rapidly degraded. The degradation of unmodified ASOs could be mitigated by prior heat treatment of the incubation medium (human plasma). Such treatment was expected to denature any enzymes such as the exonucleases, so the results indicated that an enzymatic process was the likely causative factor in unmodified ASO degradation.

The results also supported the idea that the increased stability of the OMe-modified and LNA-modified ASO molecules was, at least in part, due to their resistance to degradation by exonucleases. As such, the stability of the modified POMC ASOs correlated with the increased and extended suppression of ACTH production by AtT-20 cells (Chapter 4). Although the results are from *in vitro* experiments and studies will

need to be done in animals and humans, the data gives an initial impression of the stability of POMC ASOs.

Chapter 6

Effect of POMC antisense oligonucleotides on the immune response

6 Effect of POMC antisense oligonucleotides on the immune response

6.1 Introduction

The POMC ASOs examined in this study have been shown to effectively silence *Pomc* expression in the *in vitro* AtT-20 cell model, and so could potentially be a novel treatment for CD. However, there are several obstacles to the successful clinical usage of a new therapeutic ASO, and one of the challenges is the potential stimulatory effects of ASOs on the immune system (Frazier, 2015). A major cause of immune reactivity is the presence of unmethylated cytosine-phosphorus-guanine (CpG) motifs in an ASO molecule (Krieg, 2002, Agrawal, 1999, Krieg et al., 1995).

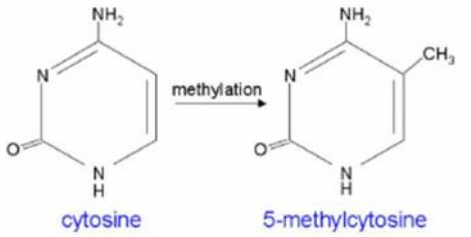
It is well-known that the DNA of humans and other vertebrates contain not only very low numbers of CpG sequences, but that the majority of them are methylated at the 5' position of the cytosine molecule (Figure 6.1a) (Bird, 1987). By contrast, CpG dinucleotides are prevalent in viral and bacterial DNA where they remain unmethylated (Krieg et al., 1995). Furthermore, research has established that in vertebrates, the non-methylated CpG motif acts as a signal of infection, activating the host's immune system to initiate the necessary innate and acquired immune responses (Krieg, 2002, Krieg et al., 1995). Specifically, unmethylated CpG is recognised by the toll-like receptor 9 (TLR9), a pattern recognition receptor that is found on innate immune cells including macrophages and dendritic cells (Vollmer, 2006, Krieg, 2002, Bauer et al., 2001). Once activated by its ligand, the TLR9, via various intracellular pathways, induces B cell activation and maturation of dendritic cells, as well as the expression of type 1 interferons (e.g., IFN-α and IFN-β) and type 1 T helper (Th1) cell pro-inflammatory cytokines (e.g., IL-1β, IL-2, IL-12, TNF-α) (Figure 6.1b) (Krug et al., 2001). Subsequently, the production of such interferons and cytokines enhances the immune response by activating effector cells including natural killer (NK) cells and cytotoxic T cells (Krieg, 2002, Ballas et al., 1996).

Previously, the type Th1 immune response initiated by CpG motifs in oligonucleotides has been applied therapeutically, including for infectious diseases and cancer, as well as vaccine adjuvants (Vollmer, 2006, Ballas et al., 2001, Klinman, 2006, Elkins et al., 1999). However, in the case of oligonucleotides being used as gene-silencing treatments, the immune response against CpG dinucleotides is an unwanted side

effect (Agrawal, 1999). Therefore, although not always possible, it is better to avoid the use of unmethylated CpGs in ASO design (Krieg et al., 1995).

Where an ASO has to include a CpG dinucleotide, the immune response can be reduced or even prevented by nucleobase modifications (Vollmer, 2006). For example, 5-methylcytosine has been used to replace the cytosine molecule (Figure 6.1a) in CpG motifs resulting in a dampening of immune reactions (Krieg et al., 1995). In addition, ASOs with MOE or OMe modifications at the CpG dinucleotides have been shown to have reduced immune stimulatory effects (Vollmer, 2006), as has replacing the PS-linkage of the CpG motif by a methylphosphonate linkage (Agrawal, 1999).

a)



(b)

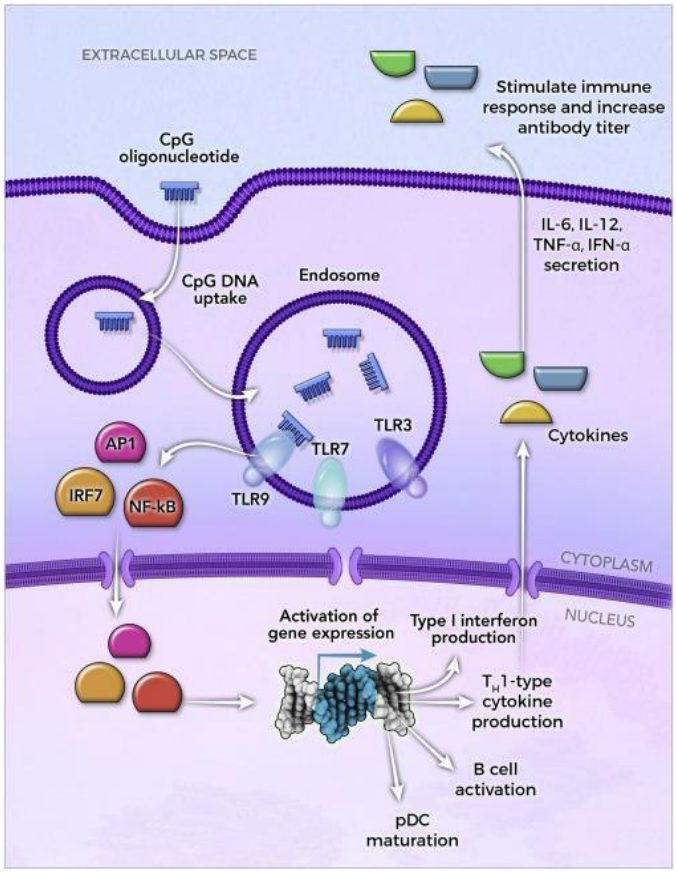


Figure 6.1: Cytosine and 5-methylcytosine structure and CpG induction of the immune response.

Cytosine and 5-methylcytosine molecules; (b) Pathway of the induction of the immune response by CpG motifs via toll-like receptor 9 (TLR9). AP1, activator protein 1; IFN, interferon; IRF7, interferon regulatory factor 7; IL, interleukin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; pDC, plasmocytoid dendritic cells; Th1, type 1 T helper; TNF-α, tumour-necrosis factor-α. The image, from a paper by (Scharner and Aznarez, 2021) (https://doi.org/10.1016/j.ymthe.2020.12.022), was used with kind permission from Elsevier Ltd. (Cambridge, UK), under the Creative Commons Attribution-Non Commercial-No Derivatives License (CC BY NC ND) (https://creativecommons.org/licenses/by-nc-nd/4.0/).

6.2 Aims and Objectives

The overall aim of this part of the project was to investigate the ability of the POMC ASOs to induce the immune response, since all four ASOs contained one CpG sequence (Table3.2). Previously, AtT-20 cells were shown to express important components of the immune response pathways such as toll-like receptors (Iwasaki et al., 2008) and to respond to the immune-stimulant polyinosinic:polycytidylic acid (Dauletbaev et al., 2015) by producing several interferons and cytokines (Alzahrani, 2021). Initially, AtT-20 cells were tested to see if they could be stimulated by commercially available CpG-containing oligonucleotides (Table 2.2) to produce interferons and pro-inflammatory cytokines. The cells were then used to look at the effects of POMC ASOs on the immune response.

The specific objectives were to:

- Transfect AtT-20 cells with commercially available CpG oligonucleotides, which are positive controls for eliciting the immune response, and analyse the cell culture medium for the secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL1-β, and of interferons IFN-α and IFN-β using interferon-specific ELISAs.

- Transfect AtT-20 cells with POMC ASOs and analyse the cell culture medium for the secretion of IFN-α, IFN-β, TNF-α, IL-6, and IL-1β using cytokine-specific ELISAs.

Results

Stimulation of the immune response in At-T20 cells by CpG oligonucleotides

Initial experiments were undertaken to investigate if the immune response could be induced in AtT-20 cells and detected by ELISAs. For this, the cells were transfected with CpG oligonucleotides (Table 2.2) that can act as immune-stimulants for the induction of interferons and pro-inflammatory cytokines (Figure 6.1).

The AtT-20 cells were plated in 6-well plates at 2 x 10^5 cells per well in 2 ml of cell culture medium. At 24 h, the cells were transfected in duplicate with CpG oligonucleotides or control oligonucleotides (Table 2.2) at a 100 nM final concentration. Control transfections of the AtT-20 cells included treatment with CpG oligonucleotides or control oligonucleotides only and with transfection reagent only. Cells without any treatment were included in all experiments, providing a further control. Following 24-h or 48-h periods of incubation, 0.5-ml samples of the cell culture medium were collected and tested for the presence of IFN-α, IFN-β, TNF-α, IL-6, and IL-1β using interferon or cytokine-specific ELISAs, which were carried out as detailed in Sections 2.7 and 2.8. As well as testing samples in ELISAs without dilution, cell culture medium samples were also diluted 1:10, so that the concentration of any the interferons or pro-inflammatory cytokines would come within the measurable range of the ELISAs (Table 2.3).

The results in Figures 6.2a, 6.3a, and 6.4a represent three separate experiments for the concentration (pg/ml) of either interferons or pro-inflammatory cytokines in the cell culture medium for AtT-20 cells treated with transfection reagent and CpG oligonucleotides together. IFN-α, IFN-β, TNF-α, IL-6, and IL-1β were detected in the cell culture medium at 24 h and at 48 h, although the concentrations were dependent upon the exact CpG oligonucleotide used. In contrast, transfection of AtT-20 cells with CpG oligonucleotide controls did not elicit an equivalent expression of either interferons or pro-inflammatory cytokines (Figure 6.2b, 6.3b, and 6.4b). Furthermore, after treatment of AtT-20 cells with either CpG oligonucleotides alone or transfection reagent alone, and for untreated cells, interferons and pro-inflammatory cytokines were not detected in the culture medium.

The results indicated that AtT-20 cells could express interferons and pro-inflammatory cytokines when transfected with CpG oligonucleotides, and so may prove useful in investigating if POMC ASOs could stimulate the immune response.

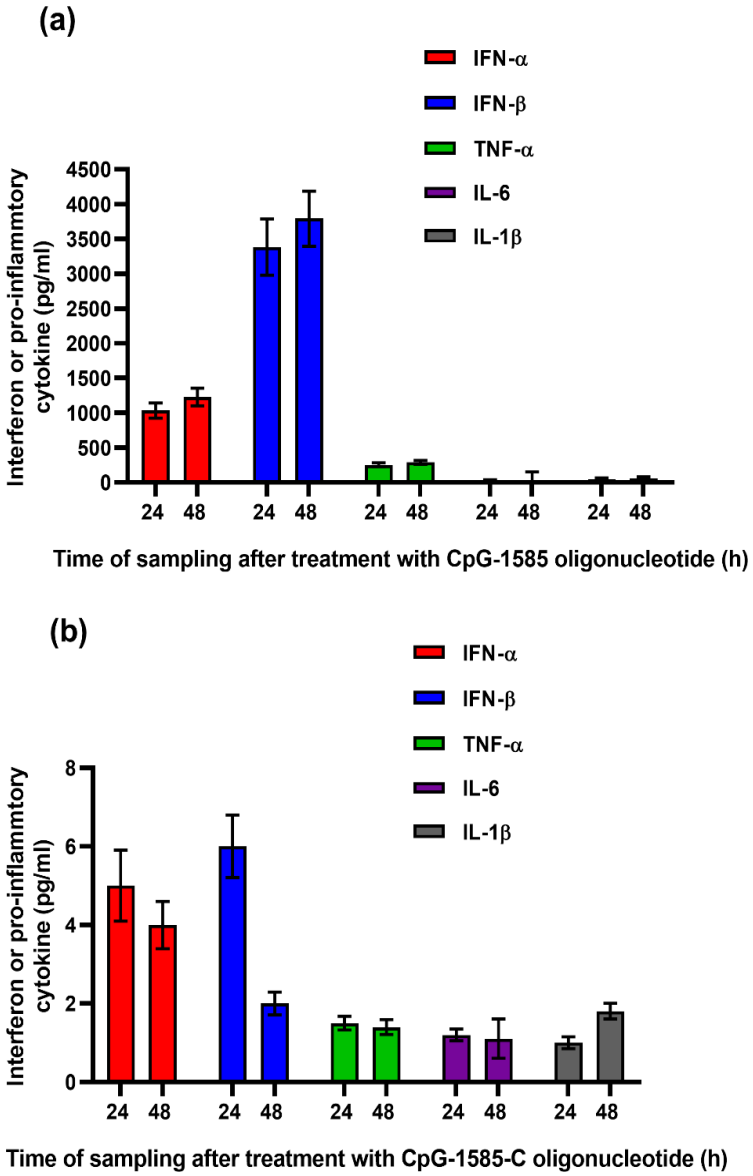


Figure 6.2: Effect of CpG-1585 oligonucleotide and control treatment on the immune response in AtT-20 cells.

AtT-20 cells were transfected with CpG-1585 oligonucleotides or control at a final concentration of 100 nM. Subsequently, the levels of IFN-α, IFN-β, TNF-α, IL-6, and IL-1β in the cell culture medium, at 24 h and 48 h following treatment, were measured using cytokine-specific ELISAs. The results show the mean (± SD) concentrations (pg/ml) of interferons and pro-inflammatory cytokines measured from three individual experiments following transfection of AtT-20 cells with (a) CpG-1585 (5'-G*G*GGTCAACGTTGA*G*G*G*G*G*G-3') plus transfection reagent, and (b) CpG-1585-C (5'-G*G*GGTCAAGCTTGA*G*G*G*G*G*G-3') plus transfection reagent.

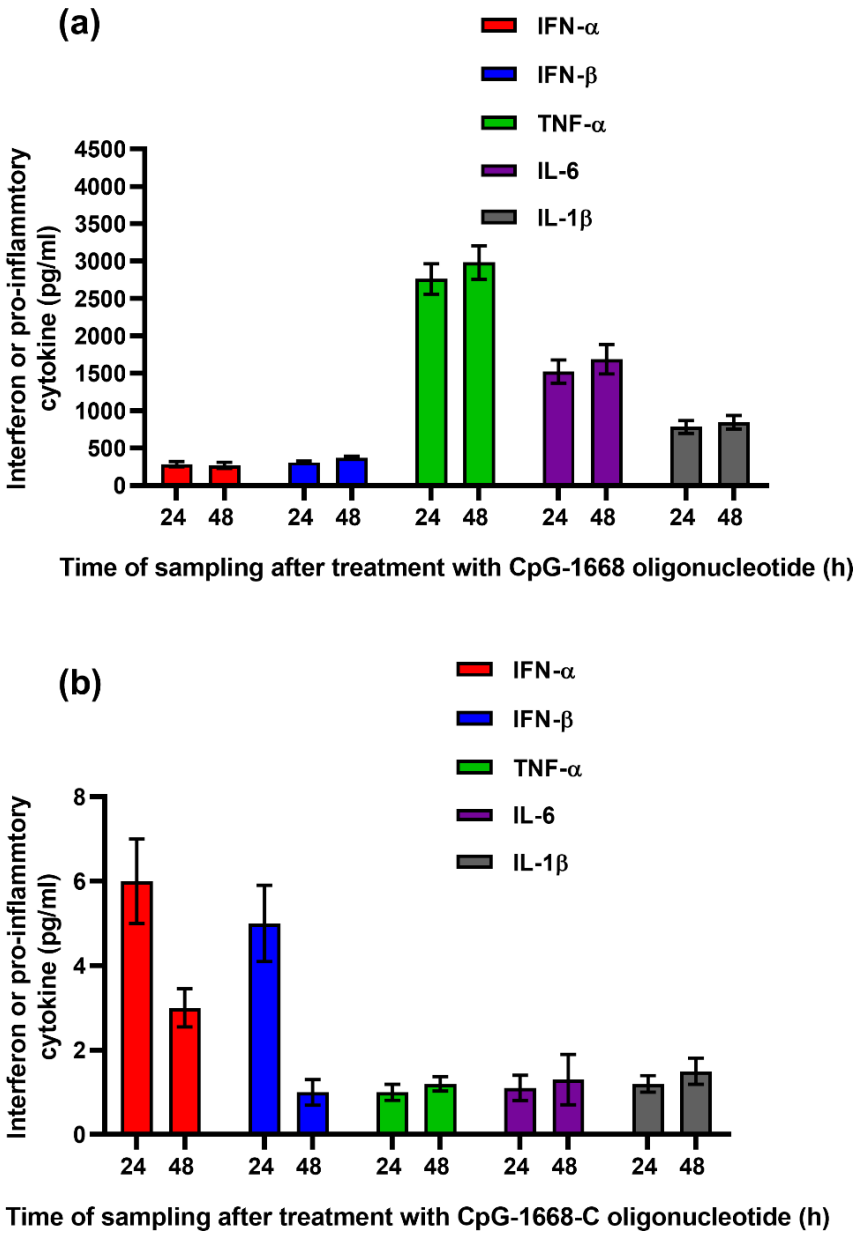


Figure 6.3: Effect of CpG-1666 oligonucleotide and control treatment on the immune response in AtT-20 cells.

AtT-20 cells were transfected with CpG-1668 oligonucleotides or control at a final concentration of 100 nM. Subsequently, the levels of IFN-α, IFN-β, TNF-α, IL-6, and IL-1β in the cell culture medium, at 24 h and 48 h following treatment, were measured using cytokine-specific ELISAs. The results show the mean (± SD) concentrations (pg/ml) of interferons and pro-inflammatory cytokines measured from three individual experiments following transfection of AtT-20 cells with (a) CpG-1668 (5'-T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*T*G*C*T-3') plus transfection reagent, and (b) CpG-1668-C (5'-T*C*C*A*T*G*A*G*C*T*T*C*C*T*G*A*T*G*C*T-3') plus transfection reagent.

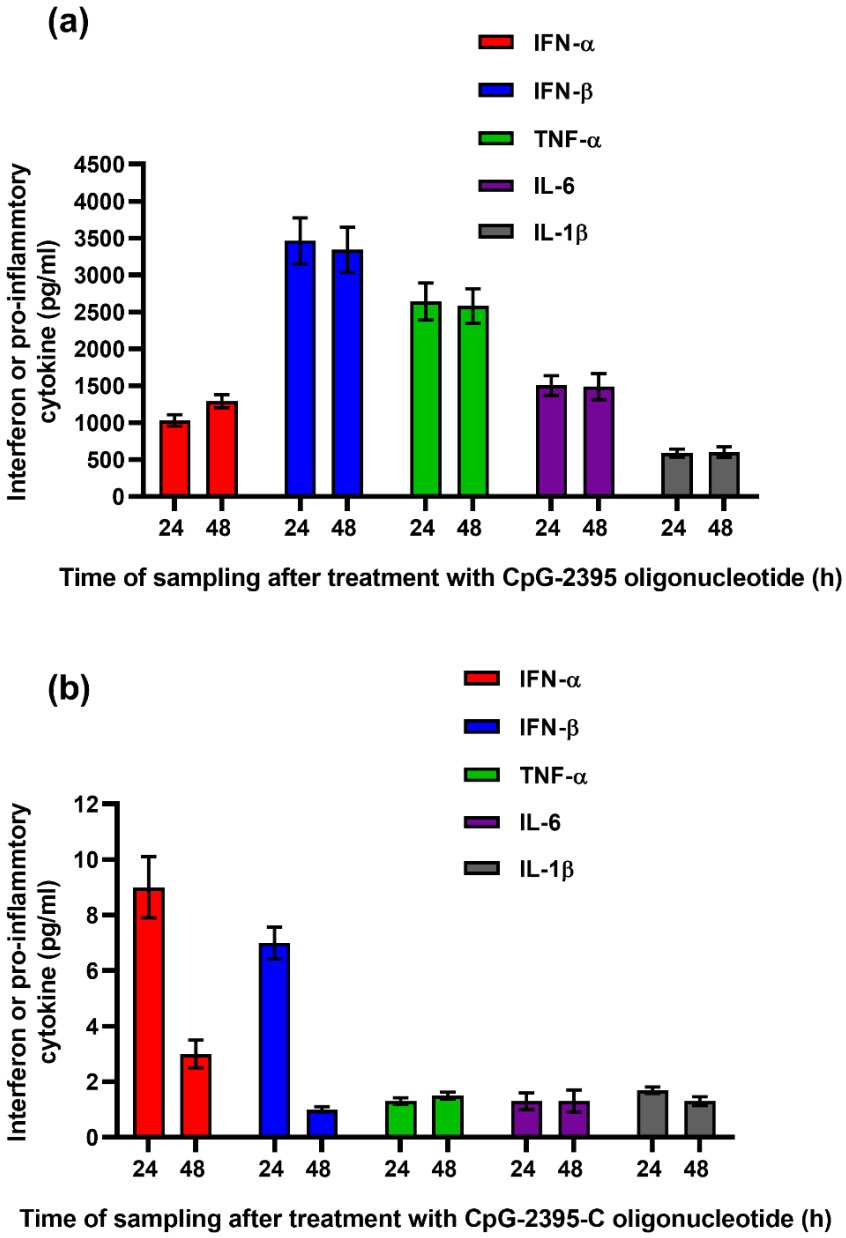


Figure 6.4: Effect of CpG-2395 oligonucleotide and control treatment on the immune response in AtT-20 cells.

AtT-20 cells were transfected with CpG-2395 oligonucleotides or control at a final concentration of 100 nM. Subsequently, the levels of IFN-α, IFN-β, TNF-α, IL-6, and IL-1β in the cell culture medium, at 24 h and 48 h following treatment, were measured using cytokine-specific ELISAs. The results show the mean (± SD) concentrations (pg/ml) of interferons and pro-inflammatory cytokines measured from three individual experiments following transfection of AtT-20 cells with (a) CpG-2395 (5'-T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G-3') plus transfection reagent, and (b) CpG-2395-C (5'-T*G*C*T*G*C*T*T*T*T*G*G*G*G*G*C*C*C*C*C*C-3') plus transfection reagent.

6.3 Effect of POMC ASOs on the immune response in At-T20 cell

To investigate if POMC ASOs could stimulate the immune response, AtT-20 cells were plated in 6-well plates at 2 x 105 cells per well in 2 ml of cell culture medium. After 24 h, the cells were transfected in duplicate with POMC ASOs at a final concentration of 100 nM. Control transfections were POMC ASO alone and transfection reagent alone. In addition, untreated cells were included in the experiments. Following 24 h and 48 h of incubation, 0.5-ml samples of the cell culture medium were removed for analysis in ELISAs for measuring IFN-α, IFN-β, TNF-α, IL-6, or IL-1β (Sections 2.7 and 2.8).

The results (Table 6.1) indicated that, in the majority of samples, IFN-α, IFN-β, TNF-α, IL-6, and IL-1β could not be detected in the cell culture medium at 24 h or at 48 h after AtT-20 cells were transfected with POMC ASOs. Very low concentrations of IL-1β, which were only just above the ELISA sensitivity value, were detected in six samples (Table 6.1). In addition, after treatment of AtT-20 cells with POMC ASO alone or transfection reagent alone, and for untreated cells, interferons and pro-inflammatory cytokines were not detected in the culture medium.

Overall, the results suggested that none of the POMC ASOs could significantly induce the expression of interferons or pro-inflammatory cytokines in AtT-20 cells.

Table 6.1: Results of interferon and pro-inflammatory cytokine ELISAs

POMC ASO	IFN-α[1] (pg/ml) at 24 h/48 h	IFN-β[1] (pg/ml) at 24 h/48 h	IL-1β[1,2] (pg/ml) at 24 h/48 h	IL-6[1] (pg/ml) at 24 h/48 h	TNF-α[1] (pg/ml) at 24 h/48 h
ASO2	1.39/1.13	0.49/0.49	**3.18/**0.72	0.0	0.0
ASO3	1.45/1.48	0.0	1.06/**2.49**	0.66/0.0	0.0
ASO5	0.89/1.06	0.0	**2.89/2.57**	0.0	0.0
ASO8	1.14/1.42	0.0	**2.42/2.89**	0.0	0.0
ASO2-PS	4.08/1.10	0.0	2.22/0.0	0.0	0.0
ASO3-PS	1.36/1.33	0.0	0.0/1.44	0.0	0.0
ASO5-PS	1.53/1.53	0.0	2.16/1.85	0.0	0.0
ASO8-PS	1.54/1.48	0.0	1.65/2.16	0.0	0.0
ASO2-OMe	1.17/0.95	0.0	**2.35/**0.0	0.0	0.0
ASO3-OMe	0.94/1.74	0.0	0.0/1.74	0.0	0.0
ASO5-OMe	1.07/1.20	0.0	2.25/1.94	0.0	0.0
ASO8-OMe	1.64/1.07	0.0	1.73/2.25	0.0	0.0
ASO2-LNA	0.0	0.0	0.0	0.0	0.0
ASO3-LNA	0.0	0.0	0.0	0.0	0.0
ASO5-LNA	0.0	0.0	0.0	0.0	0.0
ASO8-LNA	0.0	0.0	0.0	0.0	0.0

[1]ELISA sensitivity values: IFN-α (< 12.5 pg/ml); IFN-β (< 15.6 pg/ml); IL-1β (2.3 pg/ml); IL-6 (1.6 pg/ml); TNF-α (1.9 pg/ml).

[2]Concentrations of IL-1β above the sensitivity value of the ELISA are shown in bold type.

6.4 Discussion

This part of the project aimed to examine if any of the four POMC ASOs, which reduced ACTH secretion from AtT-20 cells, were also able to cause an induction of the immune response since each one contained a CpG dinucleotide. Previously, AtT-20 cells were shown to express toll-like receptors, important components of immune response pathways (Iwasaki et al., 2008), and to respond to the polyinosinic:polycytidylic acid (Dauletbaev et al., 2015) by producing several interferons and cytokines (Alzahrani, 2021). For these reasons, AtT-20 cells were used to investigate any immune stimulatory effects of both commercially available oligonucleotides containing CpG sequences, which acted as positive controls, and the POMC ASOs.

Initially, AtT-20 cells were transfected with CpG oligonucleotides and, subsequently, the cell culture medium analysed for the presence of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, and of interferons IFN-α and IFN-β using specific ELISAs. Interestingly, albeit to differing levels, all three positive control CpG-containing oligonucleotides stimulated the production of IL-1β, IL-6, TNF-α, IFN-α, and IFN-β in AtT-20 cells, indicating that the cells expressed the necessary immune response pathways.

The three CpG-containing oligonucleotides were of different classifications, as, in addition to the CpG motif, their immune modulatory effects also depends upon the sequence composition, any sugar, base, or backbone modifications, as well as secondary and tertiary structures that they may adopt (Vollmer, 2006). For example, the CpG-1585 oligonucleotide (5'-G*G*GGTCAA**CG**TTGA*G*G*G*G*G*G-3') was of class A due to its palindromic sequence containing a CpG motif at its centre, poly-G sequences at both ends, and partial PS-modifications of the molecule's backbone. It can activate plasmocytoid dendritic cells and induce the expression of several type 1 interferons, but has low activity against B cells. The CpG-1668 oligonucleotide (5'-T*C*C*A*T*G*A***C*G***T*T*T*C*C*T*G*A*T*G*C*T-3') was designated as class B with full PS-modifications of the backbone. Although this type of oligonucleotide cannot activate plasmocytoid dendritic cells, it strongly stimulates B cells. Finally, the CpG-2395 oligonucleotide (5'-T***C*G***T***C*G***T*T*T*T*T***C*G***G***C*G*C*G*C*G***C*C***C*G**-3') was of class C. Via the palindromic sequence, it can form a dimer, and has the activity

of both class A and class B CpG oligonucleotides. It stimulates both B cells and plasmocytoid dendritic cells. Following stimulation of AtT-20 cells with CpG oligonucleotides, the pattern of interferon and pro-inflammatory cytokine expression was broadly consistent with that expected from the different classes of CpG-containing molecules.

In order to examine if any of the POMC ASOs were able to induce immune reactivity, they were used to transfect AtT-20 cells after which samples of the cell culture medium were assayed for pro-inflammatory cytokines and for interferons. The results indicated that treating AtT-20 cells with the different POMC ASOs did not elicit any measurable immune response, even though all four contained one CpG motif (Table 3.2).

Of interest, the unmodified POMC ASOs did not appear to stimulate AtT-20 cells to produce either interferons or cytokines. This would suggest that the different ASO modifications tested (PS-, OMe- and LNA-modified) did not have a significant impact upon reducing immune reactivities. It may be that the context of the CpG dinucleotide in the different POMC ASO sequences was not conducive to stimulating an immune response (Vollmer, 2006). Alternatively, the POMC ASOs may not adopt secondary or tertiary structures that can aid in eliciting the production of interferons or cytokines (Vollmer, 2006). Indeed, not one of the POMC ASOs could form strong hairpin secondary structures (Table 3.2), and only POMC ASO2 was capable of forming a strong self-dimer (Table 3.2).

There are several limitations to these preliminary immune activation studies. For example, repeating the experiments using human pituitary corticotrophs would be of interest, as they may not react in an identical manner to murine AtT-20 cells. More importantly, the effects of POMC ASOs on cells such as dendritic cells and macrophages need to be studied, particularly in an *in vivo* setting where such immune cells would be encountered. If POMC ASOs do stimulate the immune system, they may increase blood pressure, induce chills and fever, and, more seriously, elicit a cytokine storm causing respiratory distress syndrome, cardiovascular shock, and even death (Suntharalingam et al., 2006). Side-effects as dangerous as these, mean it is crucial to investigate and to control any immuno-stimulatory responses when using ASOs in both animal models and in clinical trials with human participants.

Chapter 7

General Discussion

7 General Discussion

7.1 Project Justifications and Aims

Cushing's disease is caused by prolonged exposure to increased levels of cortisol which are caused by a pituitary tumour that hyper-secretes ACTH. It is a devastating disease if untreated with an increased mortality of five-fold owing to cardiovascular comorbidities, stroke, and raised vulnerability to infection (Lacroix et al., 2015, Castinetti et al., 2012). Although surgery is the only method that can provide relief, its use is linked to a high relapse rate and remission is achieved in only 65% of cases (Buliman et al., 2016, Nieman et al., 2015). Various medical treatments are currently used, but they have an adjunctive role and are often accompanied by unpleasant effects (Colao et al., 2012, Fleseriu et al., 2012, Pivonello et al., 2009). So, there is a need for a medical treatment for CD that acts by decreasing levels of ACTH (Biller et al., 2008). Such a therapeutic agent needs to be specific and selective, rapidly act to reduce ACTH, and should not adversely affect the other functions of the anterior pituitary (Biller et al., 2008).

Antisense oligonucleotides are short, single-stranded DNA molecules that act by binding to their target mRNA through complementary base-pairing. As a result, translation of the encoded protein or peptide can be inhibited by one of several different mechanisms (Scherer and Rossi, 2003). There are now many reports on the successful use of ASOs to treat several diseases including cancer and those caused by defective genes (Section 1.3.5). With respect to endocrine disorders that require the reduction of hormone levels, gene-silencing siRNAs have been used to suppress parathyroid hormone production aimed at treating secondary hyperparathyroidism (Kanai et al., 2009), and an ASO designed to inhibit translation of human growth hormone receptor mRNA has been successful in treating acromegaly (Trainer et al., 2018). Furthermore, two ASOs against *Pomc* mRNA have been shown to suppress the secretion of ACTH in *in vitro*, *ex vivo*, and *in vivo* models (Spampinato et al., 1994, Woloschak et al., 1994). However, as a treatment for CD, such POMC ASOs have not been investigated in any more detail. The current project hypothesised that ASOs could be designed and utilised to specifically and effectively inhibit the translation of

Pomc mRNA in ACTH-hypersecreting AtT-20 cells. Ultimately, such POMC ASOs could be the basis of a novel therapy for CD.

The aims of the project were, firstly, to design ASOs targeting mouse *Pomc* mRNA using computer-aided methods (Chapter 3). Secondly, to investigate the effectiveness of POMC ASOs at suppressing ACTH secretion from AtT-20 cells, which are an *in vitro* model of CD (Chapter 4). Thirdly, to examine the resistance of POMC ASOs to nuclease degradation (Chapter 5), and finally, to look at the effects of POMC ASOs on the immune response (Chapter 6). The results of these investigations are summarised in the next sections, along with the limitations of the experiments undertaken.

7.2 Results Summary and Experimental Limitations

7.2.1 POMC ASO design

Several considerations were taken into account when designing the ASOs in order to maximise the chances of successful gene-silencing. Some of the considered parameters related to the ASO molecule, some to the mRNA target site, and others to the interaction of ASOs with their target on the mRNA.

The POMC ASOs were designed using the *S*fold program (Ding et al., 2004). Initially, this allowed the prediction of accessible regions on the *Pomc* mRNA for ASO binding. Subsequent use of the *S*oligo tool allowed the identification of optimum G-C content (40-60%), sequences without GGGG residues, and favourable binding energy values (≥ 0 kcal/mol) for the interaction of the mRNA with potential ASOs. Once unfavorable POMC ASO sequences had been eliminated, OligoAnalyzer online tool was used to investigate those with favourable interactions with respect to both inter- and intra-molecular base-pairing (Matveeva et al., 2003). Following such criteria and using computer-aided methods for ASO design has previously proven very successful in selecting effective antisense molecules (Shao et al., 2006, Matveeva et al., 2003).

The guidelines for the design of ASO experimental studies (Gagnon and Corey, 2019) indicated that using two or more ASOs would allow more robust conclusions to be made about the results of antisense experiments. Therefore, four POMC ASOs were selected for further study; three against exon 4 and one against exon 3 of the mouse *Pomc* gene. In addition, in an effort to improve their gene-silencing effects (Chery, 2016), OMe- and LNA-modifications were chosen for the ASOs, as well as a gapmer design.

One limitation of the design process was the sole use of computer programs. Despite their reported success, it may be that there are experimental methods such as oligonucleotide arrays (Cho et al., 2001), mRNA walking (Sohail and Southern, 2000), and RNase H mapping (Ho et al., 1998) that could be employed to select effective ASOs. A second limitation was that only POMC ASO2 had complete homology to the human *POMC* gene. The remaining three POMC ASOs would need at least one

nucleotide change to allow their use in *ex vivo* work with human pituitary tumour cells or any clinical trials with human participants.

7.2.2 Effectiveness of POMC ASOs in reducing ACTH production

The effectiveness of the four POMC ASOs at suppressing ACTH secretion was investigated in AtT-20 cells, an *in vitro* model of CD.

The results showed that the POMC ASOs lowered ACTH secretion from cultured AtT-20 cells to 14-59% of normal levels. In terms of nucleotide sequence, POMC ASO5 was the most effective ASO, apart from its LNA-modified version, where POMC ASO3-LNA had the strongest gene-silencing effect.

For the different ASO modifications, LNA-modified versions were the most effective for POMC ASO2 and POMC ASO3, with OMe-modified and LNA-modified versions of POMC ASO5 and POMC ASO8 being equally effective. The improved effectiveness of the sugar-modified ASOs was in keeping with previous reports that suggested OMe- and LNA-modifications conferred better gene-silencing activity upon ASOs compared with their unmodified counterparts (Rinaldi and Wood, 2018, Chery, 2016).

One important consideration when using ASOs therapeutically would be to use the lowest effective dose. This minimises potential adverse outcomes caused by immune reactions and off-target effects (Rinaldi and Wood, 2018). In general, higher concentrations (10 nM and above) of OMe-modified versions of the POMC ASOs were more effective at reducing ACTH secretion from AtT-20 cells. Using LNA-modified POMC ASOs, it was found that concentrations as low as 1 nM were as effective as the higher concentrations (10 nM and above) tested. This might be due to a better binding affinity of the LNA-modified ASOs to their target mRNA or greater resistance to nuclease digestion (Rinaldi and Wood, 2018). The LNA-modified POMC ASOs also had the greatest longevity being the most effective over five days at reducing ACTH secretion from AtT-20 cells, even at a concentration of 1 nM.

The importance of controls cannot be overstated in relation to measuring the downstream effects of ASOs, as there is always the possibility that off-target mRNA binding will have an adverse influence on the measured outcomes (Gagnon and

Corey, 2019). In this study, it was shown that the reduction in ACTH secretion from AtT-20 cells was not due to detrimental effects upon AtT-20 cell viability. In addition, the experimental design included control ASOs (Gagnon and Corey, 2019); scrambled POMC ASOs with the same nucleotide composition as the on-target version, but with a changed nucleotide sequence, and MM POMC ASOs, with one or more changed nucleotides within the internal site of the molecule (Swayze et al., 2007, Sharma et al., 2005, Flanagan et al., 1996). Neither type of POMC ASO control caused a reduction in ACTH secretion from AtT-20 cells. These results, along with the fact that different on-target POMC ASOs had the same effect upon ACTH secretion, suggested it was reasonable to assume that the observable outcomes were not due to confounding off-target gene-silencing effects of the POMC ASOs.

Overall, all four POMC ASOs tested, effectively reduced the levels of ACTH secreted by AtT-20 cells. Further work needs to be undertaken to firmly establish that the reduction in ACTH is due to the action of the POMC ASOs on *Pomc* mRNA, so this remains a limitation of the study.

7.2.3 Nuclease resistance of POMC ASOs

Since the POMC ASOs may eventually be used to treat humans with CD, they need to reach the appropriate cells or tissue without undergoing significant nuclease degradation. Therefore, the susceptibility of POMC ASOs to nuclease attack was investigated.

The results of experiments to analyse nuclease degradation of POMC ASOs showed that unmodified ASOs degraded overtime in complex biological media and in buffered exonuclease solutions. In contrast, PS-modified, OMe-modified, and LNA-modified POMC ASOs were more stable over the same period of incubation. The results supported the idea that the improved effectiveness of ASOs containing OMe- and LNA-modifications may be, in part, due to their increased resistance to degradation by exonuclease activity (Rinaldi and Wood, 2018, Chery, 2016).

Overall, the results are limited by the *in vitro* nature of the experiments. Studies will need to be undertaken in animals and in humans to properly assess the stability of POMC ASOs when they are used *in vivo.*

7.2.4 Stimulation of the immune response by POMC ASOs

For POMC ASOs to be used successfully as therapeutics, there is a need to avoid stimulatory effects on the immune system. The POMC ASOs were, therefore, tested for any potential to induce the expression of interferons or pro-inflammatory cytokines.

The results indicated that, although all four of the POMC ASOs used in this study contained one CpG motif, they did not appear to induce any immune responses in AtT-20 cells, even in an unmodified form. This would suggest that the different ASO modifications tested (PS-, OMe- and LNA-modified) did not have a significant impact upon reducing immune reactivities. It may be that the context of the CpG dinucleotide in the different POMC ASO sequences was not conducive to stimulating an immune response (Vollmer, 2006). Alternatively, the POMC ASOs may not adopt secondary or tertiary structures that can aid in eliciting the production of interferons or cytokines (Vollmer, 2006). Indeed, not one of the POMC ASOs could form strong hairpin secondary structures, and only POMC ASO2 was capable of forming a strong self-dimer.

There are several limitations to these preliminary immune activation studies. For example, repeating the experiments using human pituitary corticotrophs and immune cells such as dendritic cells and macrophages would be of interest, as they may not react in an identical manner to murine AtT-20 cells. More importantly, the effect of POMC ASOs needs to be studied in an *in vivo* setting where immune cells would be naturally encountered.

7.3 Future Work

The future directions for the research are outlined below.

7.3.1 Mechanism of POMC ASO action

All four POMC ASOs effectively reduced the levels of ACTH secreted by AtT-20 cells. However, further work needs to be undertaken to firmly establish that the reduction in ACTH was due to the direct action of the POMC ASOs on the levels of *Pomc* mRNA. The POMC ASOs were designed as gapmers, which would be expected to act via the mechanism of RNase H degradation (Crooke, 2017). This would be investigated using quantitative real-time PCR to compare the quantity of *Pomc* mRNA in untreated and POMC ASO-treated AtT-20 cells. If *Pomc* mRNA levels in treated cells were significantly reduced, it would suggest that *Pomc* mRNA was degraded by RNase H. Alternatively, RNase H inhibitors (Kirby et al., 2012) could be added to AtT-20 cells prior to transfection with POMC ASOs. A reduction in the gene-silencing effects of the ASOs would suggest that the mechanism of action was indeed RNase H-dependent. However, initial investigations were not successful in identifying a commercial supplier for RNase H-specific inhibitors that could be used in such experiments.

7.3.2 Investigation of POMC ASO off-target effects

A major concern regarding the use of ASOs is that of unpredicted off-target effects, which occur due to inappropriate base-pairing of the ASO with unrelated mRNAs (Frazier, 2015). In this project, a cautious approach to the design of the POMC ASOs and the use of rigorous BLAST searches aimed to mitigate such adverse potential. In addition, higher doses of ASOs are more likely to produce off-target effects and to induce the immune response (Ottesen et al., 2021, Chi et al., 2017). The data from the current project indicated that low doses (1 nM) of certain POMC ASOs were potent suppressors of *Pomc* expression and that, in some instances, this gene-silencing effect could be maintained over a five-day time-period. However, even with careful design and low dosage, there still remains a possibility that serious side-effects could arise from the therapeutic use of POMC ASOs. These may only be realised in an in vivo biological system. Preliminary investigations using microarray analysis (Bilanges and Stokoe, 2005) of *in vitro* gene-silencing experiments could provide information

about POMC ASO specificity and any potential effects they may have upon the expression of unrelated genes.

In addition to expression in the corticotrophs of the anterior pituitary, *Pomc* is also expressed in the hypothalamus (in neurones of the arcuate nucleus, the dorsomedial hypothalamus, and the brainstem) and in skin melanocytes. Gene-silencing of *Pomc* in the hypothalamus could have effects on energy balance that would result in changes in fat mass. However, the aim of POMC ASO treatment would be to reduce *Pomc* expression, not to abolish it completely, so such unwanted side-effects may be minimal. This would need to be tested in an *in vivo* model of CD.

7.3.3 Animal and *ex vivo* studies

Further work needs to be done to examine the effectiveness of POMC ASOs in both *ex vivo* and *in vivo* models of CD.

Two previous *ex vivo* studies used human corticotrophs isolated from CD patient pituitary adenomas to test the effects of POMC ASOs on ACTH secretion (Woloschak et al., 1994, Frankel et al., 1999). In one study, an ASO targeted at exon 1 of *POMC* was used to treat cultured human corticotroph adenoma cells at a concentration of 50 μM. After 18 h, the treatment was seen to reduce both *POMC* mRNA and ACTH levels by over 50% (Woloschak et al., 1994). The second study tested an ASO against exon 3 of *POMC*. Cultured human corticotrophs were transfected with the ASO at 5 μM. At 24 h post-transfection, ACTH secretion from the cells was decreased by 72% (Frankel et al., 1999). The four POMC ASOs used in the current study targeted different sites on *POMC* mRNA to the ones in the earlier investigations, so it would be of interest to determine the effects of the new POMC ASOs in *ex vivo* experiments. Any gene-silencing effects on other hormones secreted by the corticotrophs could also be examined.

An animal model will be essential to further the research into the potential of POMC ASOs as a therapeutic for CD. One early study used adult male Sprague-Dawley rats to test the effects of an ASO against *Pomc* exon 3 on ACTH production (Spampinato et al., 1994). Following a 60-h infusion of the ASO at 0.625 μM into the hypothalamic

arcuate nucleus of the animals, the number of ACTH-immuno-positive neurons was seen to be significantly reduced, compared with rats infused with control ASOs.

In the literature, several other animal models have been reported for studying ACTH-dependent Cushing's syndrome (Nishiyama et al., 2022). One model comprises a subcutaneous implantation of AtT-20 cells to BALB/c AnN Crl-nu BR nude mice (Leung et al., 1982), and this has been used previously to test the effects of various novel treatments for CD (Lu et al., 2017, Riebold et al., 2015, Páez-Pereda et al., 2001). This mouse model could be adapted to study the suppressive effects of the POMC ASOs on ACTH production *in vivo*.

In addition to examining the effects of POMC ASOs on *Pomc* expression, an animal model would be useful to analyse any off-target and immunogenic effects of POMC ASOs as well as any other toxicities that can result in unwanted side-effects such as thrombocytopenia (Frazier, 2015). Detailed pharmacodynamics and pharmacokinetics of POMC ASO application could also be evaluated.

7.3.4 Delivery methods

The delivery of ASOs to the required tissue is one of several challenges to overcome when using such molecules as a therapeutic. Cell surface receptors with restricted expression provide one possible way by which ASOs can be directed to their required target cell. The ASOs themselves require conjugation to a relevant peptide ligand or to an antibody against the specific receptor (Arnold et al., 2018, Juliano et al., 2011). For example, an ASO-antibody conjugate has been used to downregulate key genes in their target glioblastoma stem cells (Arnold et al., 2018).

In the case of CD, the CRHR1 (Aguilera et al., 2004) is one possible target for cell-specific POMC ASO delivery. The receptor is expressed specifically on corticotrophs in the anterior pituitary and is endocytosed on binding to its ligand CRH (dos Santos Claro et al., 2019, Tu et al., 2007). This may allow POMC ASOs conjugated to CRH or to an anti-CRHR1 antibody to be delivered to and internalised by the required cells.

Earlier studies revealed that CRHR1 was expressed in AtT-20 cells, as detected by RT-PCR and CRH-stimulation experiments (Alzahrani, 2021). As a model for

investigating the delivery of POMC ASOs as CRH-conjugates or as conjugates with a specific anti-CRHR1 antibody, it is likely that AtT-20 cells would therefore prove useful.

7.3.5 Human studies and clinical trials

To date, many clinical trials have been completed or are in progress involving different ASO therapeutics and encompassing diseases such as cancer and conditions affecting the cardiovascular and neurological systems (Tables 1.3 and 1.4) (Crooke et al., 2021b). So far, nine ASOs have been given final approval for clinical use (Table 1.3) (Crooke et al., 2021b). Most commonly, the approved ASOs are PS-MOE-modified gapmers, including Nusinersen, Inotersen, Mipomersen, and Volanesorsen, and PMOs, such as Viltolarsen, Golodirsen, Casimersen, and Eteplirsen. The two main mechanisms of action for the approved ASOs are RNase H-mediated degradation of mRNA, e.g., Fomivirsen, or an exon-skipping process, e.g., Nusinersen (Crooke et al., 2021b).

The trials of approved ASOs have indicated that clinically relevant means of delivery such as systemic or subcutaneous administration are possible (Crooke et al., 2021b). In general, PS-modified ASOs can be delivered by almost all routes of administration, including orally. They have been demonstrated to be effective when given intrathecally for treating neurological diseases, and by aerosol administration to treat pulmonary disease (Crooke et al., 2021b). In addition, both PS-modified and PS-MOE-modified ASOs can be administered by almost all parenteral routes, as well as by mouth. Amongst the approved ASOs, Viltolarsen, Golodirsen, Eteplirsen, and Casimersen are injected intravenously, whilst others are given subcutaneously, including Mipomersen, Inotersen, and Volanesorsen. Furthermore, Nusinersen is given intrathecally and Fomivirsen by intravitreal injection.

Following administration, approved PMOs and PS-MOE-modified ASOs have been shown to exhibit rapid and complete absorption (Crooke et al., 2020, Cirak et al., 2011). After systemic application, PS-modified ASOs distribute generally through the body, with the liver, bone marrow, kidneys, fat, and spleen accumulating the highest concentrations. On increasing doses, the ASOs then tend to accumulate in other tissues. Studies have revealed that PMOs have a tissue elimination half-life of 7-14 days, being eliminated via urine as complete molecules (Cirak et al., 2011), whilst PS-

MOE-modified ASOs have a half-life of 2-4 weeks (Crooke et al., 2020). Again, elimination is via urine, but the PS-MOE-modified ASOs are cleared as fragments due to nuclease digestion (Crooke et al., 2020). All approved PMOs have been shown to have excellent safety profiles (De Vivo et al., 2019), but treatment with Inotersen and Volanesorsen, both PS-MOE-modified ASOs, have been associated with severe thrombocytopenia in a small number of patients (Witztum et al., 2019, Benson et al., 2018).

Interestingly, ASO technology has shown initial applicability for the treatment of the endocrine condition acromegaly, in a randomised, parallel-group, open-label phase 2 study (Trainer et al., 2018). The disease is caused by hyper-secretion of growth hormone from a pituitary adenoma, which in turn results in increased circulating levels of insulin-like growth factor-1 (IGF-1). The ASO, ATL1103, was designed to prevent growth hormone receptor mRNA translation by a RNase H mechanism, and consisted of a PS-backbone and OMe-modifications in a gapmer structure. Twenty-six patients with acromegaly were randomised and given a 200-mg dose of ATL1103 subcutaneously either once or twice per week for 13 weeks. The baseline median IGF-

1 serum levels were 447 and 649 ng/ml in the once- and twice-weekly groups, respectively. At week 14, twice-weekly application of ATL1103 resulted in a median fall in IGF-1 levels of 27.8% (P = 0.0002), indicating that the ASO treatment significantly lowered IGF-1 in acromegalic patients. In a similar way, the treatment of CD using ASOs could reduce the high levels of ACTH secretion that elevate blood cortisol.

7.4 Final Conclusions

Overall, in this project, detailed in vitro research has been undertaken to investigate the possibility of using specific ASOs to target the mouse *Pomc* gene and reduce ACTH secretion from AtT-20 cells. This is a first step in determining if POMC ASOs could be a potential treatment for CD, which is caused by an excess of cortisol resulting from an ACTH-hyper-secreting pituitary adenoma. The immediate future work would be to move the research into an *in vivo* animal model, before the investigation of POMC ASOs in clinical trials with human participants would be possible.

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Appendices

Appendix I: Full output data from *S*fold/*S*oligo for design of POMC antisense oligonucleotide

Column 1: target position (starting - ending) Column 2: target sequence (5' -> 3')
Column 3: antisense oligo (5' -> 3') Column 4: GC content (%)
Column 5: oligo binding energy (kcal/mol)
Column 6: GGGG indicator = 1 for at least one GGGG in the target sequence; GGGG indicator = 0 for otherwise.

1-	19	GGGACCAAACGGGAGGCGA	TCGCCTCCCGTTTGGTCCC	68.4%	-3.2	0
2-	20	GGACCAAACGGGAGGCGAC	GTCGCCTCCCGTTTGGTCC	68.4%	-3.3	0
3-	21	GACCAAACGGGAGGCGACG	CGTCGCCTCCCGTTTGGTC	68.4%	-3.3	0
4-	22	ACCAAACGGGAGGCGACGG	CCGTCGCCTCCCGTTTGGT	68.4%	-5.9	0
5-	23	CCAAACGGGAGGCGACGGA	TCCGTCGCCTCCCGTTTGG	68.4%	-7.0	0
6-	24	CAAACGGGAGGCGACGGAA	TTCCGTCGCCTCCCGTTTG	63.2%	-7.8	0
7-	25	AAACGGGAGGCGACGGAAG	CTTCCGTCGCCTCCCGTTT	63.2%	-7.0	0
8-	26	AACGGGAGGCGACGGAAGA	TCTTCCGTCGCCTCCCGTT	63.2%	-6.2	0
9-	27	ACGGGAGGCGACGGAAGAG	CTCTTCCGTCGCCTCCCGT	68.4%	-7.3	0
10-	28	CGGGAGGCGACGGAAGAGA	TCTCTTCCGTCGCCTCCCG	68.4%	-7.5	0
11-	29	GGGAGGCGACGGAAGAGAA	TTCTCTTCCGTCGCCTCCC	63.2%	-7.9	0
12-	30	GGAGGCGACGGAAGAGAAA	TTTCTCTTCCGTCGCCTCC	57.9%	-8.4	0
13-	31	GAGGCGACGGAAGAGAAAA	TTTTCTCTTCCGTCGCCTC	52.6%	-8.6	0
14-	32	AGGCGACGGAAGAGAAAAG	CTTTTCTCTTCCGTCGCCT	52.6%	-8.5	0
15-	33	GGCGACGGAAGAGAAAAGA	TCTTTTCTCTTCCGTCGCC	52.6%	-8.5	0
16-	34	GCGACGGAAGAGAAAAGAG	CTCTTTTCTCTTCCGTCGC	52.6%	-8.5	0
17-	35	CGACGGAAGAGAAAAGAGG	CCTCTTTTCTCTTCCGTCG	52.6%	-8.4	0
18-	36	GACGGAAGAGAAAAGAGGU	ACCTCTTTTCTCTTCCGTC	47.4%	-8.4	0
19-	37	ACGGAAGAGAAAAGAGGUU	AACCTCTTTTCTCTTCCGT	42.1%	-7.1	0
20-	38	CGGAAGAGAAAAGAGGUUA	TAACCTCTTTTCTCTTCCG	42.1%	-7.0	0
21-	39	GGAAGAGAAAAGAGGUUAA	TTAACCTCTTTTCTCTTCC	36.8%	-7.0	0
22-	40	GAAGAGAAAAGAGGUUAAG	CTTAACCTCTTTTCTCTTC	36.8%	-6.0	0
23-	41	AAGAGAAAAGAGGUUAAGA	TCTTAACCTCTTTTCTCTT	31.6%	-4.9	0
24-	42	AGAGAAAAGAGGUUAAGAG	CTCTTAACCTCTTTTCTCT	36.8%	-4.1	0
25-	43	GAGAAAAGAGGUUAAGAGC	GCTCTTAACCTCTTTTCTC	42.1%	-4.2	0
26-	44	AGAAAAGAGGUUAAGAGCA	TGCTCTTAACCTCTTTTCT	36.8%	-4.1	0
27-	45	GAAAAGAGGUUAAGAGCAG	CTGCTCTTAACCTCTTTTC	42.1%	-3.1	0
28-	46	AAAAGAGGUUAAGAGCAGU	ACTGCTCTTAACCTCTTTT	36.8%	-2.3	0
29-	47	AAAGAGGUUAAGAGCAGUG	CACTGCTCTTAACCTCTTT	42.1%	-2.1	0
30-	48	AAGAGGUUAAGAGCAGUGA	TCACTGCTCTTAACCTCTT	42.1%	-1.6	0
31-	49	AGAGGUUAAGAGCAGUGAC	GTCACTGCTCTTAACCTCT	47.4%	-2.5	0
32-	50	GAGGUUAAGAGCAGUGACU	AGTCACTGCTCTTAACCTC	47.4%	-3.0	0
33-	51	AGGUUAAGAGCAGUGACUA	TAGTCACTGCTCTTAACCT	42.1%	-3.4	0
34-	52	GGUUAAGAGCAGUGACUAA	TTAGTCACTGCTCTTAACC	42.1%	-4.2	0
35-	53	GUUAAGAGCAGUGACUAAG	CTTAGTCACTGCTCTTAAC	42.1%	-5.3	0
36-	54	UUAAGAGCAGUGACUAAGA	TCTTAGTCACTGCTCTTAA	36.8%	-6.1	0
37-	55	UAAGAGCAGUGACUAAGAG	CTCTTAGTCACTGCTCTTA	42.1%	-6.3	0
38-	56	AAGAGCAGUGACUAAGAGA	TCTCTTAGTCACTGCTCTT	42.1%	-6.4	0
39-	57	AGAGCAGUGACUAAGAGAG	CTCTCTTAGTCACTGCTCT	47.4%	-6.5	0
40-	58	GAGCAGUGACUAAGAGAGG	CCTCTCTTAGTCACTGCTC	52.6%	-5.3	0
41-	59	AGCAGUGACUAAGAGAGGC	GCCTCTCTTAGTCACTGCT	52.6%	-5.2	0
42-	60	GCAGUGACUAAGAGAGGCC	GGCCTCTCTTAGTCACTGC	57.9%	-5.1	0
43-	61	CAGUGACUAAGAGAGGCCA	TGGCCTCTCTTAGTCACTG	52.6%	-5.1	0
44-	62	AGUGACUAAGAGAGGCCAC	GTGGCCTCTCTTAGTCACT	52.6%	-5.3	0
45-	63	GUGACUAAGAGAGGCCACU	AGTGGCCTCTCTTAGTCAC	52.6%	-4.7	0
46-	64	UGACUAAGAGAGGCCACUG	CAGTGGCCTCTCTTAGTCA	52.6%	-4.6	0
47-	65	GACUAAGAGAGGCCACUGA	TCAGTGGCCTCTCTTAGTC	52.6%	-4.2	0

48-	66	ACUAAGAGAGGCCACUGAA	TTCAGTGGCCTCTCTTAGT	47.4%	-4.1	0
49-	67	CUAAGAGAGGCCACUGAAC	GTTCAGTGGCCTCTCTTAG	52.6%	-4.0	0
50-	68	UAAGAGAGGCCACUGAACA	TGTTCAGTGGCCTCTCTTA	47.4%	-3.9	0
51-	69	AAGAGAGGCCACUGAACAU	ATGTTCAGTGGCCTCTCTT	47.4%	-4.2	0
52-	70	AGAGAGGCCACUGAACAUC	GATGTTCAGTGGCCTCTCT	52.6%	-3.7	0
53-	71	GAGAGGCCACUGAACAUCU	AGATGTTCAGTGGCCTCTC	52.6%	-2.7	0
54-	72	AGAGGCCACUGAACAUCUU	AAGATGTTCAGTGGCCTCT	47.4%	-1.9	0
55-	73	GAGGCCACUGAACAUCUUU	AAAGATGTTCAGTGGCCTC	47.4%	-1.7	0
56-	74	AGGCCACUGAACAUCUUUG	CAAAGATGTTCAGTGGCCT	47.4%	-1.8	0
57-	75	GGCCACUGAACAUCUUUGU	ACAAAGATGTTCAGTGGCC	47.4%	-1.7	0
58-	76	GCCACUGAACAUCUUUGUC	GACAAAGATGTTCAGTGGC	47.4%	-1.3	0
59-	77	CCACUGAACAUCUUUGUCC	GGACAAAGATGTTCAGTGG	47.4%	-1.3	0
60-	78	CACUGAACAUCUUUGUCCC	GGGACAAAGATGTTCAGTG	47.4%	-1.3	0
61-	79	ACUGAACAUCUUUGUCCCC	GGGGACAAAGATGTTCAGT	47.4%	-1.3	0
62-	80	CUGAACAUCUUUGUCCCCA	TGGGGACAAAGATGTTCAG	47.4%	-1.3	0
63-	81	UGAACAUCUUUGUCCCCAG	CTGGGGACAAAGATGTTCA	47.4%	-2.9	0
64-	82	GAACAUCUUUGUCCCCAGA	TCTGGGGACAAAGATGTTC	47.4%	-3.8	0
65-	83	AACAUCUUUGUCCCCAGAG	CTCTGGGGACAAAGATGTT	47.4%	-5.2	0
66-	84	ACAUCUUUGUCCCCAGAGA	TCTCTGGGGACAAAGATGT	47.4%	-5.9	0
67-	85	CAUCUUUGUCCCCAGAGAG	CTCTCTGGGGACAAAGATG	52.6%	-5.0	0
68-	86	AUCUUUGUCCCCAGAGAGC	GCTCTCTGGGGACAAAGAT	52.6%	-4.7	0
69-	87	UCUUUGUCCCCAGAGAGCU	AGCTCTCTGGGGACAAAGA	52.6%	-3.9	0
70-	88	CUUUGUCCCCAGAGAGCUG	CAGCTCTCTGGGGACAAAG	57.9%	-3.6	0
71-	89	UUUGUCCCCAGAGAGCUGC	GCAGCTCTCTGGGGACAAA	57.9%	-4.6	0
72-	90	UUGUCCCCAGAGAGCUGCC	GGCAGCTCTCTGGGGACAA	63.2%	-4.6	0
73-	91	UGUCCCCAGAGAGCUGCCU	AGGCAGCTCTCTGGGGACA	63.2%	-4.6	0
74-	92	GUCCCCAGAGAGCUGCCUU	AAGGCAGCTCTCTGGGGAC	63.2%	-4.5	0
75-	93	UCCCCAGAGAGCUGCCUUU	AAAGGCAGCTCTCTGGGGA	57.9%	-4.4	0
76-	94	CCCCAGAGAGCUGCCUUUC	GAAAGGCAGCTCTCTGGGG	63.2%	-4.4	0
77-	95	CCCAGAGAGCUGCCUUUCC	GGAAAGGCAGCTCTCTGGG	63.2%	-4.3	0
78-	96	CCAGAGAGCUGCCUUUCCG	CGGAAAGGCAGCTCTCTGG	63.2%	-4.4	0
79-	97	CAGAGAGCUGCCUUUCCGC	GCGGAAAGGCAGCTCTCTG	63.2%	-5.9	0
80-	98	AGAGAGCUGCCUUUCCGCG	CGCGGAAAGGCAGCTCTCT	63.2%	-6.2	0
81-	99	GAGAGCUGCCUUUCCGCGA	TCGCGGAAAGGCAGCTCTC	63.2%	-4.6	0
82-	100	AGAGCUGCCUUUCCGCGAC	GTCGCGGAAAGGCAGCTCT	63.2%	-3.6	0
83-	101	GAGCUGCCUUUCCGCGACA	TGTCGCGGAAAGGCAGCTC	63.2%	-1.9	0
84-	102	AGCUGCCUUUCCGCGACAG	CTGTCGCGGAAAGGCAGCT	63.2%	-0.9	0
85-	103	GCUGCCUUUCCGCGACAGG	CCTGTCGCGGAAAGGCAGC	68.4%	-3.6	0
86-	104	CUGCCUUUCCGCGACAGGC	GCCTGTCGCGGAAAGGCAG	68.4%	-3.6	0
87-	105	UGCCUUUCCGCGACAGGCA	TGCCTGTCGCGGAAAGGCA	63.2%	-3.6	0
88-	106	GCCUUUCCGCGACAGGCAG	CTGCCTGTCGCGGAAAGGC	68.4%	-3.6	0
89-	107	CCUUUCCGCGACAGGCAGG	CCTGCCTGTCGCGGAAAGG	68.4%	-2.5	0
90-	108	CUUUCCGCGACAGGCAGGA	TCCTGCCTGTCGCGGAAAG	63.2%	-3.8	0
91-	109	UUUCCGCGACAGGCAGGAG	CTCCTGCCTGTCGCGGAAA	63.2%	-5.6	0
92-	110	UUCCGCGACAGGCAGGAGA	TCTCCTGCCTGTCGCGGAA	63.2%	-6.9	0
93-	111	UCCGCGACAGGCAGGAGAC	GTCTCCTGCCTGTCGCGGA	68.4%	-6.9	0
94-	112	CCGCGACAGGCAGGAGACU	AGTCTCCTGCCTGTCGCGG	68.4%	-6.8	0
95-	113	CGCGACAGGCAGGAGACUG	CAGTCTCCTGCCTGTCGCG	68.4%	-6.8	0
96-	114	GCGACAGGCAGGAGACUGA	TCAGTCTCCTGCCTGTCGC	63.2%	-6.7	0
97-	115	CGACAGGCAGGAGACUGAA	TTCAGTCTCCTGCCTGTCG	57.9%	-6.2	0
98-	116	GACAGGCAGGAGACUGAAC	GTTCAGTCTCCTGCCTGTC	57.9%	-7.6	0
99-	117	ACAGGCAGGAGACUGAACA	TGTTCAGTCTCCTGCCTGT	52.6%	-8.3	0
100-	118	CAGGCAGGAGACUGAACAU	ATGTTCAGTCTCCTGCCTG	52.6%	-8.2	0
101-	119	AGGCAGGAGACUGAACAUG	CATGTTCAGTCTCCTGCCT	52.6%	-8.2	0
102-	120	GGCAGGAGACUGAACAUGU	ACATGTTCAGTCTCCTGCC	52.6%	-8.0	0
103-	121	GCAGGAGACUGAACAUGUU	AACATGTTCAGTCTCCTGC	47.4%	-5.3	0
104-	122	CAGGAGACUGAACAUGUUG	CAACATGTTCAGTCTCCTG	47.4%	-5.3	0
105-	123	AGGAGACUGAACAUGUUGG	CCAACATGTTCAGTCTCCT	47.4%	-5.3	0
106-	124	GGAGACUGAACAUGUUGGA	TCCAACATGTTCAGTCTCC	47.4%	-5.3	0
107-	125	GAGACUGAACAUGUUGGAA	TTCCAACATGTTCAGTCTC	42.1%	-5.3	0

108- 126	AGACUGAACAUGUUGGAAA	TTTCCAACATGTTCAGTCT	36.8%	-4.0	0
109- 127	GACUGAACAUGUUGGAAAG	CTTTCCAACATGTTCAGTC	42.1%	-2.3	0
110- 128	ACUGAACAUGUUGGAAAGA	TCTTTCCAACATGTTCAGT	36.8%	-1.0	0
111- 129	CUGAACAUGUUGGAAAGAU	ATCTTTCCAACATGTTCAG	36.8%	-1.4	0
112- 130	UGAACAUGUUGGAAAGAUA	TATCTTTCCAACATGTTCA	31.6%	-1.4	0
113- 131	GAACAUGUUGGAAAGAUAG	CTATCTTTCCAACATGTTC	36.8%	-1.4	0
114- 132	AACAUGUUGGAAAGAUAGC	GCTATCTTTCCAACATGTT	36.8%	-1.4	0
115- 133	ACAUGUUGGAAAGAUAGCG	CGCTATCTTTCCAACATGT	42.1%	-0.4	0
116- 134	CAUGUUGGAAAGAUAGCGG	CCGCTATCTTTCCAACATG	47.4%	1.6	0
117- 135	AUGUUGGAAAGAUAGCGGG	CCCGCTATCTTTCCAACAT	47.4%	2.4	0
118- 136	UGUUGGAAAGAUAGCGGGA	TCCCGCTATCTTTCCAACA	47.4%	2.5	0
119- 137	GUUGGAAAGAUAGCGGGAG	CTCCCGCTATCTTTCCAAC	52.6%	2.5	0
120- 138	UUGGAAAGAUAGCGGGAGA	TCTCCCGCTATCTTTCCAA	47.4%	2.5	0
121- 139	UGGAAAGAUAGCGGGAGAG	CTCTCCCGCTATCTTTCCA	52.6%	2.5	0
122- 140	GGAAAGAUAGCGGGAGAGA	TCTCTCCCGCTATCTTTCC	52.6%	2.4	0
123- 141	GAAAGAUAGCGGGAGAGAA	TTCTCTCCCGCTATCTTTC	47.4%	2.3	0
124- 142	AAAGAUAGCGGGAGAGAAA	TTTCTCTCCCGCTATCTTT	42.1%	2.0	0
125- 143	AAGAUAGCGGGAGAGAAAG	CTTTCTCTCCCGCTATCTT	47.4%	1.8	0
126- 144	AGAUAGCGGGAGAGAAAGC	GCTTTCTCTCCCGCTATCT	52.6%	1.5	0
127- 145	GAUAGCGGGAGAGAAAGCC	GGCTTTCTCTCCCGCTATC	57.9%	0.4	0
128- 146	AUAGCGGGAGAGAAAGCCG	CGGCTTTCTCTCCCGCTAT	57.9%	-0.4	0
129- 147	UAGCGGGAGAGAAAGCCGA	TCGGCTTTCTCTCCCGCTA	57.9%	-0.2	0
130- 148	AGCGGGAGAGAAAGCCGAG	CTCGGCTTTCTCTCCCGCT	63.2%	-0.2	0
131- 149	GCGGGAGAGAAAGCCGAGU	ACTCGGCTTTCTCTCCCGC	63.2%	-0.2	0
132- 150	CGGGAGAGAAAGCCGAGUC	GACTCGGCTTTCTCTCCCG	63.2%	-0.5	0
133- 151	GGGAGAGAAAGCCGAGUCA	TGACTCGGCTTTCTCTCCC	57.9%	-0.6	0
134- 152	GGAGAGAAAGCCGAGUCAC	GTGACTCGGCTTTCTCTCC	57.9%	-1.1	0
135- 153	GAGAGAAAGCCGAGUCACA	TGTGACTCGGCTTTCTCTC	52.6%	-1.2	0
136- 154	AGAGAAAGCCGAGUCACAA	TTGTGACTCGGCTTTCTCT	47.4%	-1.5	0
137- 155	GAGAAAGCCGAGUCACAAU	ATTGTGACTCGGCTTTCTC	47.4%	-1.6	0
138- 156	AGAAAGCCGAGUCACAAUA	TATTGTGACTCGGCTTTCT	42.1%	-1.7	0
139- 157	GAAAGCCGAGUCACAAUAA	TTATTGTGACTCGGCTTTC	42.1%	-1.9	0
140- 158	AAAGCCGAGUCACAAUAAA	TTTATTGTGACTCGGCTTT	36.8%	-1.9	0
141- 159	AAGCCGAGUCACAAUAAAC	GTTTATTGTGACTCGGCTT	42.1%	-1.8	0
142- 160	AGCCGAGUCACAAUAAACU	AGTTTATTGTGACTCGGCT	42.1%	-1.4	0
143- 161	GCCGAGUCACAAUAAACUC	GAGTTTATTGTGACTCGGC	47.4%	-1.3	0
144- 162	CCGAGUCACAAUAAACUCC	GGAGTTTATTGTGACTCGG	47.4%	-1.0	0
145- 163	CGAGUCACAAUAAACUCCU	AGGAGTTTATTGTGACTCG	42.1%	0.0	0
146- 164	GAGUCACAAUAAACUCCUA	TAGGAGTTTATTGTGACTC	36.8%	0.2	0
147- 165	AGUCACAAUAAACUCCUAA	TTAGGAGTTTATTGTGACT	31.6%	-0.6	0
148- 166	GUCACAAUAAACUCCUAAU	ATTAGGAGTTTATTGTGAC	31.6%	-1.4	0
149- 167	UCACAAUAAACUCCUAAUG	CATTAGGAGTTTATTGTGA	31.6%	-2.7	0
150- 168	CACAAUAAACUCCUAAUGG	CCATTAGGAGTTTATTGTG	36.8%	-4.8	0
151- 169	ACAAUAAACUCCUAAUGGU	ACCATTAGGAGTTTATTGT	31.6%	-5.5	0
152- 170	CAAUAAACUCCUAAUGGUG	CACCATTAGGAGTTTATTG	36.8%	-5.0	0
153- 171	AAUAAACUCCUAAUGGUGG	CCACCATTAGGAGTTTATT	36.8%	-4.8	0
154- 172	AUAAACUCCUAAUGGUGGA	TCCACCATTAGGAGTTTAT	36.8%	-4.6	0
155- 173	UAAACUCCUAAUGGUGGAG	CTCCACCATTAGGAGTTTA	42.1%	-4.8	0
156- 174	AAACUCCUAAUGGUGGAGU	ACTCCACCATTAGGAGTTT	42.1%	-4.7	0
157- 175	AACUCCUAAUGGUGGAGUU	AACTCCACCATTAGGAGTT	42.1%	-4.5	0
158- 176	ACUCCUAAUGGUGGAGUUC	GAACTCCACCATTAGGAGT	47.4%	-5.1	0
159- 177	CUCCUAAUGGUGGAGUUCA	TGAACTCCACCATTAGGAG	47.4%	-5.8	0
160- 178	UCCUAAUGGUGGAGUUCAU	ATGAACTCCACCATTAGGA	42.1%	-6.5	0
161- 179	CCUAAUGGUGGAGUUCAUU	AATGAACTCCACCATTAGG	42.1%	-6.5	0
162- 180	CUAAUGGUGGAGUUCAUUU	AAATGAACTCCACCATTAG	36.8%	-6.5	0
163- 181	UAAUGGUGGAGUUCAUUUG	CAAATGAACTCCACCATTA	36.8%	-6.4	0
164- 182	AAUGGUGGAGUUCAUUUGU	ACAAATGAACTCCACCATT	36.8%	-5.9	0
165- 183	AUGGUGGAGUUCAUUUGUU	AACAAATGAACTCCACCAT	36.8%	-4.9	0
166- 184	UGGUGGAGUUCAUUUGUUG	CAACAAATGAACTCCACCA	42.1%	-4.4	0
167- 185	GGUGGAGUUCAUUUGUUGU	ACAACAAATGAACTCCACC	42.1%	-3.1	0

168-	186	GUGGAGUUCAUUUGUUGUU	AACAACAAATGAACTCCAC	36.8%	-0.8 0
169-	187	UGGAGUUCAUUUGUUGUUG	CAACAACAAATGAACTCCA	36.8%	-0.2 0
170-	188	GGAGUUCAUUUGUUGUUGC	GCAACAACAAATGAACTCC	42.1%	-0.2 0
171-	189	GAGUUCAUUUGUUGUUGCU	AGCAACAACAAATGAACTC	36.8%	-0.2 0
172-	190	AGUUCAUUUGUUGUUGCUG	CAGCAACAACAAATGAACT	36.8%	-0.4 0
173-	191	GUUCAUUUGUUGUUGCUGU	ACAGCAACAACAAATGAAC	36.8%	-0.6 0
174-	192	UUCAUUUGUUGUUGCUGUA	TACAGCAACAACAAATGAA	31.6%	-0.9 0
175-	193	UCAUUUGUUGUUGCUGUAG	CTACAGCAACAACAAATGA	36.8%	-2.0 0
176-	194	CAUUUGUUGUUGCUGUAGA	TCTACAGCAACAACAAATG	36.8%	-2.1 0
177-	195	AUUUGUUGUUGCUGUAGAC	GTCTACAGCAACAACAAAT	36.8%	-2.5 0
178-	196	UUUGUUGUUGCUGUAGACG	CGTCTACAGCAACAACAAA	42.1%	-2.0 0
179-	197	UUGUUGUUGCUGUAGACGU	ACGTCTACAGCAACAACAA	42.1%	-1.9 0
180-	198	UGUUGUUGCUGUAGACGUC	GACGTCTACAGCAACAACA	47.4%	-2.2 0
181-	199	GUUGUUGCUGUAGACGUCC	GGACGTCTACAGCAACAAC	52.6%	-2.5 0
182-	200	UUGUUGCUGUAGACGUCCA	TGGACGTCTACAGCAACAA	47.4%	-2.5 0
183-	201	UGUUGCUGUAGACGUCCAA	TTGGACGTCTACAGCAACA	47.4%	-3.2 0
184-	202	GUUGCUGUAGACGUCCAAA	TTTGGACGTCTACAGCAAC	47.4%	-3.7 0
185-	203	UUGCUGUAGACGUCCAAAC	GTTTGGACGTCTACAGCAA	47.4%	-3.9 0
186-	204	UGCUGUAGACGUCCAAACC	GGTTTGGACGTCTACAGCA	52.6%	-4.2 0
187-	205	GCUGUAGACGUCCAAACCC	GGGTTTGGACGTCTACAGC	57.9%	-4.4 0
188-	206	CUGUAGACGUCCAAACCCU	AGGGTTTGGACGTCTACAG	52.6%	-4.6 0
189-	207	UGUAGACGUCCAAACCCUC	GAGGGTTTGGACGTCTACA	52.6%	-4.9 0
190-	208	GUAGACGUCCAAACCCUCG	CGAGGGTTTGGACGTCTAC	57.9%	-4.7 0
191-	209	UAGACGUCCAAACCCUCGU	ACGAGGGTTTGGACGTCTA	52.6%	-4.2 0
192-	210	AGACGUCCAAACCCUCGUU	AACGAGGGTTTGGACGTCT	52.6%	-3.9 0
193-	211	GACGUCCAAACCCUCGUUU	AAACGAGGGTTTGGACGTC	52.6%	-2.9 0
194-	212	ACGUCCAAACCCUCGUUUC	GAAACGAGGGTTTGGACGT	52.6%	-2.8 0
195-	213	CGUCCAAACCCUCGUUUCU	AGAAACGAGGGTTTGGACG	52.6%	-1.7 0
196-	214	GUCCAAACCCUCGUUUCUC	GAGAAACGAGGGTTTGGAC	52.6%	-1.6 0
197-	215	UCCAAACCCUCGUUUCUCU	AGAGAAACGAGGGTTTGGA	47.4%	-1.5 0
198-	216	CCAAACCCUCGUUUCUCUG	CAGAGAAACGAGGGTTTGG	52.6%	-1.2 0
199-	217	CAAACCCUCGUUUCUCUGC	GCAGAGAAACGAGGGTTTG	52.6%	-1.0 0
200-	218	AAACCCUCGUUUCUCUGCG	CGCAGAGAAACGAGGGTTT	52.6%	-0.9 0
201-	219	AACCCUCGUUUCUCUGCGC	GCGCAGAGAAACGAGGGTT	57.9%	-0.8 0
202-	220	ACCCUCGUUUCUCUGCGCA	TGCGCAGAGAAACGAGGGT	57.9%	-0.3 0
203-	221	CCCUCGUUUCUCUGCGCAU	ATGCGCAGAGAAACGAGGG	57.9%	0.0 0
204-	222	CCUCGUUUCUCUGCGCAUC	GATGCGCAGAGAAACGAGG	57.9%	0.3 0
205-	223	CUCGUUUCUCUGCGCAUCU	AGATGCGCAGAGAAACGAG	52.6%	0.7 0
206-	224	UCGUUUCUCUGCGCAUCUU	AAGATGCGCAGAGAAACGA	47.4%	0.9 0
207-	225	CGUUUCUCUGCGCAUCUUA	TAAGATGCGCAGAGAAACG	47.4%	1.2 0
208-	226	GUUUCUCUGCGCAUCUUAG	CTAAGATGCGCAGAGAAAC	47.4%	1.2 0
209-	227	UUUCUCUGCGCAUCUUAGC	GCTAAGATGCGCAGAGAAA	47.4%	1.3 0
210-	228	UUCUCUGCGCAUCUUAGCA	TGCTAAGATGCGCAGAGAA	47.4%	1.3 0
211-	229	UCUCUGCGCAUCUUAGCAG	CTGCTAAGATGCGCAGAGA	52.6%	1.3 0
212-	230	CUCUGCGCAUCUUAGCAGA	TCTGCTAAGATGCGCAGAG	52.6%	2.0 0
213-	231	UCUGCGCAUCUUAGCAGAU	ATCTGCTAAGATGCGCAGA	47.4%	1.1 0
214-	232	CUGCGCAUCUUAGCAGAUC	GATCTGCTAAGATGCGCAG	52.6%	-0.3 0
215-	233	UGCGCAUCUUAGCAGAUCU	AGATCTGCTAAGATGCGCA	47.4%	-0.4 0
216-	234	GCGCAUCUUAGCAGAUCUG	CAGATCTGCTAAGATGCGC	52.6%	-0.4 0
217-	235	CGCAUCUUAGCAGAUCUGG	CCAGATCTGCTAAGATGCG	52.6%	-0.4 0
218-	236	GCAUCUUAGCAGAUCUGGG	CCCAGATCTGCTAAGATGC	52.6%	-0.4 0
219-	237	CAUCUUAGCAGAUCUGGGG	CCCCAGATCTGCTAAGATG	52.6%	0.3 1
220-	238	AUCUUAGCAGAUCUGGGGU	ACCCCAGATCTGCTAAGAT	47.4%	0.5 1
221-	239	UCUUAGCAGAUCUGGGGUG	CACCCCAGATCTGCTAAGA	52.6%	0.5 1
222-	240	CUUAGCAGAUCUGGGGUGG	CCACCCCAGATCTGCTAAG	57.9%	0.2 1
223-	241	UUAGCAGAUCUGGGGUGGU	ACCACCCCAGATCTGCTAA	52.6%	0.0 1
224-	242	UAGCAGAUCUGGGGUGGUU	AACCACCCCAGATCTGCTA	52.6%	0.0 1
225-	243	AGCAGAUCUGGGGUGGUUG	CAACCACCCCAGATCTGCT	57.9%	0.0 1
226-	244	GCAGAUCUGGGGUGGUUGC	GCAACCACCCCAGATCTGC	63.2%	0.0 1
227-	245	CAGAUCUGGGGUGGUUGCA	TGCAACCACCCCAGATCTG	57.9%	0.0 1

228- 246	AGAUCUGGGGUGGUUGCAU	ATGCAACCACCCCAGATCT	52.6%	-0.4	1
229- 247	GAUCUGGGGUGGUUGCAUU	AATGCAACCACCCCAGATC	52.6%	-0.5	1
230- 248	AUCUGGGGUGGUUGCAUUG	CAATGCAACCACCCCAGAT	52.6%	-0.9	1
231- 249	UCUGGGGUGGUUGCAUUGU	ACAATGCAACCACCCCAGA	52.6%	-0.3	1
232- 250	CUGGGGUGGUUGCAUUGUG	CACAATGCAACCACCCCAG	57.9%	0.8	1
233- 251	UGGGGUGGUUGCAUUGUGA	TCACAATGCAACCACCCCA	52.6%	0.5	1
234- 252	GGGGUGGUUGCAUUGUGAU	ATCACAATGCAACCACCCC	52.6%	0.2	1
235- 253	GGGUGGUUGCAUUGUGAUA	TATCACAATGCAACCACCC	47.4%	0.0	0
236- 254	GGUGGUUGCAUUGUGAUAA	TTATCACAATGCAACCACC	42.1%	-1.0	0
237- 255	GUGGUUGCAUUGUGAUAAU	ATTATCACAATGCAACCAC	36.8%	-1.8	0
238- 256	UGGUUGCAUUGUGAUAAUU	AATTATCACAATGCAACCA	31.6%	-2.0	0
239- 257	GGUUGCAUUGUGAUAAUUA	TAATTATCACAATGCAACC	31.6%	-2.5	0
240- 258	GUUGCAUUGUGAUAAUUAC	GTAATTATCACAATGCAAC	31.6%	-4.1	0
241- 259	UUGCAUUGUGAUAAUUACG	CGTAATTATCACAATGCAA	31.6%	-4.8	0
242- 260	UGCAUUGUGAUAAUUACGU	ACGTAATTATCACAATGCA	31.6%	-5.5	0
243- 261	GCAUUGUGAUAAUUACGUG	CACGTAATTATCACAATGC	36.8%	-5.9	0
244- 262	CAUUGUGAUAAUUACGUGG	CCACGTAATTATCACAATG	36.8%	-5.9	0
245- 263	AUUGUGAUAAUUACGUGGG	CCCACGTAATTATCACAAT	36.8%	-5.9	0
246- 264	UUGUGAUAAUUACGUGGGU	ACCCACGTAATTATCACAA	36.8%	-5.7	0
247- 265	UGUGAUAAUUACGUGGGUU	AACCCACGTAATTATCACA	36.8%	-5.6	0
248- 266	GUGAUAAUUACGUGGGUUA	TAACCCACGTAATTATCAC	36.8%	-5.5	0
249- 267	UGAUAAUUACGUGGGUUAU	ATAACCCACGTAATTATCA	31.6%	-5.8	0
250- 268	GAUAAUUACGUGGGUUAUA	TATAACCCACGTAATTATC	31.6%	-5.7	0
251- 269	AUAAUUACGUGGGUUAUAG	CTATAACCCACGTAATTAT	31.6%	-5.5	0
252- 270	UAAUUACGUGGGUUAUAGG	CCTATAACCCACGTAATTA	36.8%	-5.3	0
253- 271	AAUUACGUGGGUUAUAGGA	TCCTATAACCCACGTAATT	36.8%	-5.0	0
254- 272	AUUACGUGGGUUAUAGGAC	GTCCTATAACCCACGTAAT	42.1%	-4.1	0
255- 273	UUACGUGGGUUAUAGGACA	TGTCCTATAACCCACGTAA	42.1%	-3.5	0
256- 274	UACGUGGGUUAUAGGACAG	CTGTCCTATAACCCACGTA	47.4%	-3.4	0
257- 275	ACGUGGGUUAUAGGACAGG	CCTGTCCTATAACCCACGT	52.6%	-2.8	0
258- 276	CGUGGGUUAUAGGACAGGA	TCCTGTCCTATAACCCACG	52.6%	-0.9	0
259- 277	GUGGGUUAUAGGACAGGAC	GTCCTGTCCTATAACCCAC	52.6%	0.0	0
260- 278	UGGGUUAUAGGACAGGACG	CGTCCTGTCCTATAACCCA	52.6%	0.0	0
261- 279	GGGUUAUAGGACAGGACGG	CCGTCCTGTCCTATAACCC	57.9%	0.5	0
262- 280	GGUUAUAGGACAGGACGGG	CCCGTCCTGTCCTATAACC	57.9%	0.5	0
263- 281	GUUAUAGGACAGGACGGGG	CCCCGTCCTGTCCTATAAC	57.9%	-0.5	1
264- 282	UUAUAGGACAGGACGGGGU	ACCCCGTCCTGTCCTATAA	52.6%	-0.9	1
265- 283	UAUAGGACAGGACGGGGUC	GACCCCGTCCTGTCCTATA	57.9%	-1.9	1
266- 284	AUAGGACAGGACGGGGUCC	GGACCCCGTCCTGTCCTAT	63.2%	-2.4	1
267- 285	UAGGACAGGACGGGGUCCC	GGGACCCCGTCCTGTCCTA	68.4%	-2.7	1
268- 286	AGGACAGGACGGGGUCCCU	AGGGACCCCGTCCTGTCCT	68.4%	-2.4	1
269- 287	GGACAGGACGGGGUCCCUC	GAGGGACCCCGTCCTGTCC	73.7%	-2.3	1
270- 288	GACAGGACGGGGUCCCUCC	GGAGGGACCCCGTCCTGTC	73.7%	-2.5	1
271- 289	ACAGGACGGGGUCCCUCCA	TGGAGGGACCCCGTCCTGT	68.4%	-2.6	1
272- 290	CAGGACGGGGUCCCUCCAA	TTGGAGGGACCCCGTCCTG	68.4%	-3.4	1
273- 291	AGGACGGGGUCCCUCCAAU	ATTGGAGGGACCCCGTCCT	63.2%	-3.3	1
274- 292	GGACGGGGUCCCUCCAAUC	GATTGGAGGGACCCCGTCC	68.4%	-3.7	1
275- 293	GACGGGGUCCCUCCAAUCU	AGATTGGAGGGACCCCGTC	63.2%	-3.9	1
276- 294	ACGGGGUCCCUCCAAUCUU	AAGATTGGAGGGACCCCGT	57.9%	-3.9	1
277- 295	CGGGGUCCCUCCAAUCUUG	CAAGATTGGAGGGACCCCG	63.2%	-4.0	1
278- 296	GGGGUCCCUCCAAUCUUGU	ACAAGATTGGAGGGACCCC	57.9%	-3.5	1
279- 297	GGGUCCCUCCAAUCUUGUU	AACAAGATTGGAGGGACCC	52.6%	-3.5	0
280- 298	GGUCCCUCCAAUCUUGUUU	AAACAAGATTGGAGGGACC	47.4%	-3.6	0
281- 299	GUCCCUCCAAUCUUGUUUG	CAAACAAGATTGGAGGGAC	47.4%	-2.7	0
282- 300	UCCCUCCAAUCUUGUUUGC	GCAAACAAGATTGGAGGGA	47.4%	-2.4	0
283- 301	CCCUCCAAUCUUGUUUGCC	GGCAAACAAGATTGGAGGG	52.6%	-1.4	0
284- 302	CCUCCAAUCUUGUUUGCCU	AGGCAAACAAGATTGGAGG	47.4%	-0.7	0
285- 303	CUCCAAUCUUGUUUGCCUC	GAGGCAAACAAGATTGGAG	47.4%	-0.2	0
286- 304	UCCAAUCUUGUUUGCCUCU	AGAGGCAAACAAGATTGGA	42.1%	-0.7	0
287- 305	CCAAUCUUGUUUGCCUCUG	CAGAGGCAAACAAGATTGG	47.4%	-0.7	0

288- 306	CAAUCUUGUUUGCCUCUGC	GCAGAGGCAAACAAGATTG	47.4%	-0.6	0
289- 307	AAUCUUGUUUGCCUCUGCA	TGCAGAGGCAAACAAGATT	42.1%	-0.6	0
290- 308	AUCUUGUUUGCCUCUGCAG	CTGCAGAGGCAAACAAGAT	47.4%	0.2	0
291- 309	UCUUGUUUGCCUCUGCAGA	TCTGCAGAGGCAAACAAGA	47.4%	0.3	0
292- 310	CUUGUUUGCCUCUGCAGAG	CTCTGCAGAGGCAAACAAG	52.6%	0.6	0
293- 311	UUGUUUGCCUCUGCAGAGA	TCTCTGCAGAGGCAAACAA	47.4%	0.6	0
294- 312	UGUUUGCCUCUGCAGAGAC	GTCTCTGCAGAGGCAAACA	52.6%	0.5	0
295- 313	GUUUGCCUCUGCAGAGACU	AGTCTCTGCAGAGGCAAAC	52.6%	0.5	0
296- 314	UUUGCCUCUGCAGAGACUA	TAGTCTCTGCAGAGGCAAA	47.4%	0.6	0
297- 315	UUGCCUCUGCAGAGACUAG	CTAGTCTCTGCAGAGGCAA	52.6%	0.6	0
298- 316	UGCCUCUGCAGAGACUAGG	CCTAGTCTCTGCAGAGGCA	57.9%	0.7	0
299- 317	GCCUCUGCAGAGACUAGGC	GCCTAGTCTCTGCAGAGGC	63.2%	0.8	0
300- 318	CCUCUGCAGAGACUAGGCC	GGCCTAGTCTCTGCAGAGG	63.2%	0.9	0
301- 319	CUCUGCAGAGACUAGGCCU	AGGCCTAGTCTCTGCAGAG	57.9%	0.9	0
302- 320	UCUGCAGAGACUAGGCCUG	CAGGCCTAGTCTCTGCAGA	57.9%	1.0	0
303- 321	CUGCAGAGACUAGGCCUGA	TCAGGCCTAGTCTCTGCAG	57.9%	0.2	0
304- 322	UGCAGAGACUAGGCCUGAC	GTCAGGCCTAGTCTCTGCA	57.9%	0.5	0
305- 323	GCAGAGACUAGGCCUGACA	TGTCAGGCCTAGTCTCTGC	57.9%	0.5	0
306- 324	CAGAGACUAGGCCUGACAC	GTGTCAGGCCTAGTCTCTG	57.9%	-1.3	0
307- 325	AGAGACUAGGCCUGACACG	CGTGTCAGGCCTAGTCTCT	57.9%	-2.9	0
308- 326	GAGACUAGGCCUGACACGU	ACGTGTCAGGCCTAGTCTC	57.9%	-3.8	0
309- 327	AGACUAGGCCUGACACGUG	CACGTGTCAGGCCTAGTCT	57.9%	-3.8	0
310- 328	GACUAGGCCUGACACGUGG	CCACGTGTCAGGCCTAGTC	63.2%	-3.9	0
311- 329	ACUAGGCCUGACACGUGGA	TCCACGTGTCAGGCCTAGT	57.9%	-4.8	0
312- 330	CUAGGCCUGACACGUGGAA	TTCCACGTGTCAGGCCTAG	57.9%	-4.7	0
313- 331	UAGGCCUGACACGUGGAAG	CTTCCACGTGTCAGGCCTA	57.9%	-4.9	0
314- 332	AGGCCUGACACGUGGAAGA	TCTTCCACGTGTCAGGCCT	57.9%	-5.0	0
315- 333	GGCCUGACACGUGGAAGAU	ATCTTCCACGTGTCAGGCC	57.9%	-5.9	0
316- 334	GCCUGACACGUGGAAGAUG	CATCTTCCACGTGTCAGGC	57.9%	-6.1	0
317- 335	CCUGACACGUGGAAGAUGC	GCATCTTCCACGTGTCAGG	57.9%	-6.3	0
318- 336	CUGACACGUGGAAGAUGCC	GGCATCTTCCACGTGTCAG	57.9%	-6.4	0
319- 337	UGACACGUGGAAGAUGCCG	CGGCATCTTCCACGTGTCA	57.9%	-6.3	0
320- 338	GACACGUGGAAGAUGCCGA	TCGGCATCTTCCACGTGTC	57.9%	-6.3	0
321- 339	ACACGUGGAAGAUGCCGAG	CTCGGCATCTTCCACGTGT	57.9%	-5.3	0
322- 340	CACGUGGAAGAUGCCGAGA	TCTCGGCATCTTCCACGTG	57.9%	-5.2	0
323- 341	ACGUGGAAGAUGCCGAGAU	ATCTCGGCATCTTCCACGT	52.6%	-5.3	0
324- 342	CGUGGAAGAUGCCGAGAUU	AATCTCGGCATCTTCCACG	52.6%	-3.4	0
325- 343	GUGGAAGAUGCCGAGAUUC	GAATCTCGGCATCTTCCAC	52.6%	-1.8	0
326- 344	UGGAAGAUGCCGAGAUUCU	AGAATCTCGGCATCTTCCA	47.4%	-0.9	0
327- 345	GGAAGAUGCCGAGAUUCUG	CAGAATCTCGGCATCTTCC	52.6%	-0.8	0
328- 346	GAAGAUGCCGAGAUUCUGC	GCAGAATCTCGGCATCTTC	52.6%	-0.7	0
329- 347	AAGAUGCCGAGAUUCUGCU	AGCAGAATCTCGGCATCTT	47.4%	0.3	0
330- 348	AGAUGCCGAGAUUCUGCUA	TAGCAGAATCTCGGCATCT	47.4%	-0.1	0
331- 349	GAUGCCGAGAUUCUGCUAC	GTAGCAGAATCTCGGCATC	52.6%	-1.6	0
332- 350	AUGCCGAGAUUCUGCUACA	TGTAGCAGAATCTCGGCAT	47.4%	-2.1	0
333- 351	UGCCGAGAUUCUGCUACAG	CTGTAGCAGAATCTCGGCA	52.6%	-1.4	0
334- 352	GCCGAGAUUCUGCUACAGU	ACTGTAGCAGAATCTCGGC	52.6%	-1.3	0
335- 353	CCGAGAUUCUGCUACAGUC	GACTGTAGCAGAATCTCGG	52.6%	-1.5	0
336- 354	CGAGAUUCUGCUACAGUCG	CGACTGTAGCAGAATCTCG	52.6%	-1.6	0
337- 355	GAGAUUCUGCUACAGUCGC	GCGACTGTAGCAGAATCTC	52.6%	-1.8	0
338- 356	AGAUUCUGCUACAGUCGCU	AGCGACTGTAGCAGAATCT	47.4%	-1.8	0
339- 357	GAUUCUGCUACAGUCGCUC	GAGCGACTGTAGCAGAATC	52.6%	-2.9	0
340- 358	AUUCUGCUACAGUCGCUCA	TGAGCGACTGTAGCAGAAT	47.4%	-3.5	0
341- 359	UUCUGCUACAGUCGCUCAG	CTGAGCGACTGTAGCAGAA	52.6%	-3.3	0
342- 360	UCUGCUACAGUCGCUCAGG	CCTGAGCGACTGTAGCAGA	57.9%	-3.3	0
343- 361	CUGCUACAGUCGCUCAGGG	CCCTGAGCGACTGTAGCAG	63.2%	-3.3	0
344- 362	UGCUACAGUCGCUCAGGGG	CCCCTGAGCGACTGTAGCA	63.2%	-3.3	1
345- 363	GCUACAGUCGCUCAGGGGC	GCCCCTGAGCGACTGTAGC	68.4%	-3.3	1
346- 364	CUACAGUCGCUCAGGGGCC	GGCCCCTGAGCGACTGTAG	68.4%	-3.2	1
347- 365	UACAGUCGCUCAGGGGCCC	GGGCCCCTGAGCGACTGTA	68.4%	-3.1	1

348- 366	ACAGUCGCUCAGGGGCCCU	AGGGCCCCTGAGCGACTGT	68.4%	-2.6	1
349- 367	CAGUCGCUCAGGGGCCCUG	CAGGGCCCCTGAGCGACTG	73.7%	-0.8	1
350- 368	AGUCGCUCAGGGGCCCUGU	ACAGGGCCCCTGAGCGACT	68.4%	-0.2	1
351- 369	GUCGCUCAGGGGCCCUGUU	AACAGGGCCCCTGAGCGAC	68.4%	-0.2	1
352- 370	UCGCUCAGGGGCCCUGUUG	CAACAGGGCCCCTGAGCGA	68.4%	-0.1	1
353- 371	CGCUCAGGGGCCCUGUUGC	GCAACAGGGCCCCTGAGCG	73.7%	0.2	1
354- 372	GCUCAGGGGCCCUGUUGCU	AGCAACAGGGCCCCTGAGC	68.4%	0.4	1
355- 373	CUCAGGGGCCCUGUUGCUG	CAGCAACAGGGCCCCTGAG	68.4%	0.6	1
356- 374	UCAGGGGCCCUGUUGCUGG	CCAGCAACAGGGCCCCTGA	68.4%	0.7	1
357- 375	CAGGGGCCCUGUUGCUGGC	GCCAGCAACAGGGCCCCTG	73.7%	1.4	1
358- 376	AGGGGCCCUGUUGCUGGCC	GGCCAGCAACAGGGCCCCT	73.7%	2.1	1
359- 377	GGGGCCCUGUUGCUGGCCC	GGGCCAGCAACAGGGCCCC	78.9%	2.1	1
360- 378	GGGCCCUGUUGCUGGCCCU	AGGGCCAGCAACAGGGCCC	73.7%	2.0	0
361- 379	GGCCCUGUUGCUGGCCCUC	GAGGGCCAGCAACAGGGCC	73.7%	2.0	0
362- 380	GCCCUGUUGCUGGCCCUCC	GGAGGGCCAGCAACAGGGC	73.7%	2.0	0
363- 381	CCCUGUUGCUGGCCCUCCU	AGGAGGGCCAGCAACAGGG	68.4%	2.0	0
364- 382	CCUGUUGCUGGCCCUCCUG	CAGGAGGGCCAGCAACAGG	68.4%	1.8	0
365- 383	CUGUUGCUGGCCCUCCUGC	GCAGGAGGGCCAGCAACAG	68.4%	1.8	0
366- 384	UGUUGCUGGCCCUCCUGCU	AGCAGGAGGGCCAGCAACA	63.2%	1.8	0
367- 385	GUUGCUGGCCCUCCUGCUU	AAGCAGGAGGGCCAGCAAC	63.2%	1.8	0
368- 386	UUGCUGGCCCUCCUGCUUC	GAAGCAGGAGGGCCAGCAA	63.2%	1.9	0
369- 387	UGCUGGCCCUCCUGCUUCA	TGAAGCAGGAGGGCCAGCA	63.2%	2.0	0
370- 388	GCUGGCCCUCCUGCUUCAG	CTGAAGCAGGAGGGCCAGC	68.4%	2.1	0
371- 389	CUGGCCCUCCUGCUUCAGA	TCTGAAGCAGGAGGGCCAG	63.2%	2.1	0
372- 390	UGGCCCUCCUGCUUCAGAC	GTCTGAAGCAGGAGGGCCA	63.2%	0.4	0
373- 391	GGCCCUCCUGCUUCAGACC	GGTCTGAAGCAGGAGGGCC	68.4%	0.4	0
374- 392	GCCCUCCUGCUUCAGACCU	AGGTCTGAAGCAGGAGGGC	63.2%	0.4	0
375- 393	CCCUCCUGCUUCAGACCUC	GAGGTCTGAAGCAGGAGGG	63.2%	0.9	0
376- 394	CCUCCUGCUUCAGACCUCC	GGAGGTCTGAAGCAGGAGG	63.2%	0.9	0
377- 395	CUCCUGCUUCAGACCUCCA	TGGAGGTCTGAAGCAGGAG	57.9%	0.8	0
378- 396	UCCUGCUUCAGACCUCCAU	ATGGAGGTCTGAAGCAGGA	52.6%	0.8	0
379- 397	CCUGCUUCAGACCUCCAUA	TATGGAGGTCTGAAGCAGG	52.6%	0.8	0
380- 398	CUGCUUCAGACCUCCAUAG	CTATGGAGGTCTGAAGCAG	52.6%	0.2	0
381- 399	UGCUUCAGACCUCCAUAGA	TCTATGGAGGTCTGAAGCA	47.4%	-1.0	0
382- 400	GCUUCAGACCUCCAUAGAU	ATCTATGGAGGTCTGAAGC	47.4%	-1.6	0
383- 401	CUUCAGACCUCCAUAGAUG	CATCTATGGAGGTCTGAAG	47.4%	-3.1	0
384- 402	UUCAGACCUCCAUAGAUGU	ACATCTATGGAGGTCTGAA	42.1%	-3.4	0
385- 403	UCAGACCUCCAUAGAUGUG	CACATCTATGGAGGTCTGA	47.4%	-3.4	0
386- 404	CAGACCUCCAUAGAUGUGU	ACACATCTATGGAGGTCTG	47.4%	-3.4	0
387- 405	AGACCUCCAUAGAUGUGUG	CACACATCTATGGAGGTCT	47.4%	-3.4	0
388- 406	GACCUCCAUAGAUGUGUGG	CCACACATCTATGGAGGTC	52.6%	-3.4	0
389- 407	ACCUCCAUAGAUGUGUGGA	TCCACACATCTATGGAGGT	47.4%	-3.3	0
390- 408	CCUCCAUAGAUGUGUGGAG	CTCCACACATCTATGGAGG	52.6%	-1.6	0
391- 409	CUCCAUAGAUGUGUGGAGC	GCTCCACACATCTATGGAG	52.6%	-1.5	0
392- 410	UCCAUAGAUGUGUGGAGCU	AGCTCCACACATCTATGGA	47.4%	-1.5	0
393- 411	CCAUAGAUGUGUGGAGCUG	CAGCTCCACACATCTATGG	52.6%	-1.5	0
394- 412	CAUAGAUGUGUGGAGCUGG	CCAGCTCCACACATCTATG	52.6%	-1.6	0
395- 413	AUAGAUGUGUGGAGCUGGU	ACCAGCTCCACACATCTAT	47.4%	-1.8	0
396- 414	UAGAUGUGUGGAGCUGGUG	CACCAGCTCCACACATCTA	52.6%	-1.8	0
397- 415	AGAUGUGUGGAGCUGGUGC	GCACCAGCTCCACACATCT	57.9%	-1.8	0
398- 416	GAUGUGUGGAGCUGGUGCC	GGCACCAGCTCCACACATC	63.2%	-1.2	0
399- 417	AUGUGUGGAGCUGGUGCCU	AGGCACCAGCTCCACACAT	57.9%	-0.7	0
400- 418	UGUGUGGAGCUGGUGCCUG	CAGGCACCAGCTCCACACA	63.2%	0.2	0
401- 419	GUGUGGAGCUGGUGCCUGG	CCAGGCACCAGCTCCACAC	68.4%	1.7	0
402- 420	UGUGGAGCUGGUGCCUGGA	TCCAGGCACCAGCTCCACA	63.2%	2.0	0
403- 421	GUGGAGCUGGUGCCUGGAG	CTCCAGGCACCAGCTCCAC	68.4%	2.0	0
404- 422	UGGAGCUGGUGCCUGGAGA	TCTCCAGGCACCAGCTCCA	63.2%	1.6	0
405- 423	GGAGCUGGUGCCUGGAGAG	CTCTCCAGGCACCAGCTCC	68.4%	1.0	0
406- 424	GAGCUGGUGCCUGGAGAGC	GCTCTCCAGGCACCAGCTC	68.4%	0.1	0
407- 425	AGCUGGUGCCUGGAGAGCA	TGCTCTCCAGGCACCAGCT	63.2%	-0.5	0

408- 426	GCUGGUGCCUGGAGAGCAG	CTGCTCTCCAGGCACCAGC	68.4%	-0.5	0
409- 427	CUGGUGCCUGGAGAGCAGC	GCTGCTCTCCAGGCACCAG	68.4%	-0.5	0
410- 428	UGGUGCCUGGAGAGCAGCC	GGCTGCTCTCCAGGCACCA	68.4%	-0.5	0
411- 429	GGUGCCUGGAGAGCAGCCA	TGGCTGCTCTCCAGGCACC	68.4%	-0.5	0
412- 430	GUGCCUGGAGAGCAGCCAG	CTGGCTGCTCTCCAGGCAC	68.4%	-0.5	0
413- 431	UGCCUGGAGAGCAGCCAGU	ACTGGCTGCTCTCCAGGCA	63.2%	-0.2	0
414- 432	GCCUGGAGAGCAGCCAGUG	CACTGGCTGCTCTCCAGGC	68.4%	-0.5	0
415- 433	CCUGGAGAGCAGCCAGUGC	GCACTGGCTGCTCTCCAGG	68.4%	-2.8	0
416- 434	CUGGAGAGCAGCCAGUGCC	GGCACTGGCTGCTCTCCAG	68.4%	-2.9	0
417- 435	UGGAGAGCAGCCAGUGCCA	TGGCACTGGCTGCTCTCCA	63.2%	-2.3	0
418- 436	GGAGAGCAGCCAGUGCCAG	CTGGCACTGGCTGCTCTCC	68.4%	-2.3	0
419- 437	GAGAGCAGCCAGUGCCAGG	CCTGGCACTGGCTGCTCTC	68.4%	-2.3	0
420- 438	AGAGCAGCCAGUGCCAGGA	TCCTGGCACTGGCTGCTCT	63.2%	-2.3	0
421- 439	GAGCAGCCAGUGCCAGGAC	GTCCTGGCACTGGCTGCTC	68.4%	-3.0	0
422- 440	AGCAGCCAGUGCCAGGACC	GGTCCTGGCACTGGCTGCT	68.4%	-2.6	0
423- 441	GCAGCCAGUGCCAGGACCU	AGGTCCTGGCACTGGCTGC	68.4%	-2.0	0
424- 442	CAGCCAGUGCCAGGACCUC	GAGGTCCTGGCACTGGCTG	68.4%	-1.1	0
425- 443	AGCCAGUGCCAGGACCUCA	TGAGGTCCTGGCACTGGCT	63.2%	-0.5	0
426- 444	GCCAGUGCCAGGACCUCAC	GTGAGGTCCTGGCACTGGC	68.4%	-0.9	0
427- 445	CCAGUGCCAGGACCUCACC	GGTGAGGTCCTGGCACTGG	68.4%	-1.2	0
428- 446	CAGUGCCAGGACCUCACCA	TGGTGAGGTCCTGGCACTG	63.2%	-1.6	0
429- 447	AGUGCCAGGACCUCACCAC	GTGGTGAGGTCCTGGCACT	63.2%	-2.3	0
430- 448	GUGCCAGGACCUCACCACG	CGTGGTGAGGTCCTGGCAC	68.4%	-3.0	0
431- 449	UGCCAGGACCUCACCACGG	CCGTGGTGAGGTCCTGGCA	68.4%	-3.3	0
432- 450	GCCAGGACCUCACCACGGA	TCCGTGGTGAGGTCCTGGC	68.4%	-3.2	0
433- 451	CCAGGACCUCACCACGGAG	CTCCGTGGTGAGGTCCTGG	68.4%	-1.1	0
434- 452	CAGGACCUCACCACGGAGA	TCTCCGTGGTGAGGTCCTG	63.2%	-1.8	0
435- 453	AGGACCUCACCACGGAGAG	CTCTCCGTGGTGAGGTCCT	63.2%	-1.8	0
436- 454	GGACCUCACCACGGAGAGC	GCTCTCCGTGGTGAGGTCC	68.4%	-1.8	0
437- 455	GACCUCACCACGGAGAGCA	TGCTCTCCGTGGTGAGGTC	63.2%	-2.0	0
438- 456	ACCUCACCACGGAGAGCAA	TTGCTCTCCGTGGTGAGGT	57.9%	-2.3	0
439- 457	CCUCACCACGGAGAGCAAC	GTTGCTCTCCGTGGTGAGG	63.2%	-1.7	0
440- 458	CUCACCACGGAGAGCAACC	GGTTGCTCTCCGTGGTGAG	63.2%	-1.7	0
441- 459	UCACCACGGAGAGCAACCU	AGGTTGCTCTCCGTGGTGA	57.9%	-1.7	0
442- 460	CACCACGGAGAGCAACCUG	CAGGTTGCTCTCCGTGGTG	63.2%	-2.6	0
443- 461	ACCACGGAGAGCAACCUGC	GCAGGTTGCTCTCCGTGGT	63.2%	-4.2	0
444- 462	CCACGGAGAGCAACCUGCU	AGCAGGTTGCTCTCCGTGG	63.2%	-4.3	0
445- 463	CACGGAGAGCAACCUGCUG	CAGCAGGTTGCTCTCCGTG	63.2%	-4.1	0
446- 464	ACGGAGAGCAACCUGCUGG	CCAGCAGGTTGCTCTCCGT	63.2%	-3.7	0
447- 465	CGGAGAGCAACCUGCUGGC	GCCAGCAGGTTGCTCTCCG	68.4%	-2.9	0
448- 466	GGAGAGCAACCUGCUGGCU	AGCCAGCAGGTTGCTCTCC	63.2%	-2.3	0
449- 467	GAGAGCAACCUGCUGGCUU	AAGCCAGCAGGTTGCTCTC	57.9%	-2.0	0
450- 468	AGAGCAACCUGCUGGCUUG	CAAGCCAGCAGGTTGCTCT	57.9%	-1.8	0
451- 469	GAGCAACCUGCUGGCUUGC	GCAAGCCAGCAGGTTGCTC	63.2%	-1.7	0
452- 470	AGCAACCUGCUGGCUUGCA	TGCAAGCCAGCAGGTTGCT	57.9%	-0.9	0
453- 471	GCAACCUGCUGGCUUGCAU	ATGCAAGCCAGCAGGTTGC	57.9%	-1.0	0
454- 472	CAACCUGCUGGCUUGCAUC	GATGCAAGCCAGCAGGTTG	57.9%	-1.2	0
455- 473	AACCUGCUGGCUUGCAUCC	GGATGCAAGCCAGCAGGTT	57.9%	-1.0	0
456- 474	ACCUGCUGGCUUGCAUCCG	CGGATGCAAGCCAGCAGGT	63.2%	-0.7	0
457- 475	CCUGCUGGCUUGCAUCCGG	CCGGATGCAAGCCAGCAGG	68.4%	-0.7	0
458- 476	CUGCUGGCUUGCAUCCGGG	CCCGGATGCAAGCCAGCAG	68.4%	-0.8	0
459- 477	UGCUGGCUUGCAUCCGGGC	GCCCGGATGCAAGCCAGCA	68.4%	-0.8	0
460- 478	GCUGGCUUGCAUCCGGGCU	AGCCCGGATGCAAGCCAGC	68.4%	-0.4	0
461- 479	CUGGCUUGCAUCCGGGCUU	AAGCCCGGATGCAAGCCAG	63.2%	1.1	0
462- 480	UGGCUUGCAUCCGGGCUUG	CAAGCCCGGATGCAAGCCA	63.2%	1.6	0
463- 481	GGCUUGCAUCCGGGCUUGC	GCAAGCCCGGATGCAAGCC	68.4%	1.7	0
464- 482	GCUUGCAUCCGGGCUUGCA	TGCAAGCCCGGATGCAAGC	63.2%	1.7	0
465- 483	CUUGCAUCCGGGCUUGCAA	TTGCAAGCCCGGATGCAAG	57.9%	1.5	0
466- 484	UUGCAUCCGGGCUUGCAAA	TTTGCAAGCCCGGATGCAA	52.6%	1.3	0
467- 485	UGCAUCCGGGCUUGCAAAC	GTTTGCAAGCCCGGATGCA	57.9%	-0.2	0

468-	486	GCAUCCGGGCUUGCAAACU	AGTTTGCAAGCCCGGATGC	57.9%	-0.9 0
469-	487	CAUCCGGGCUUGCAAACUC	GAGTTTGCAAGCCCGGATG	57.9%	-2.0 0
470-	488	AUCCGGGCUUGCAAACUCG	CGAGTTTGCAAGCCCGGAT	57.9%	-3.3 0
471-	489	UCCGGGCUUGCAAACUCGA	TCGAGTTTGCAAGCCCGGA	57.9%	-3.2 0
472-	490	CCGGGCUUGCAAACUCGAC	GTCGAGTTTGCAAGCCCGG	63.2%	-3.1 0
473-	491	CGGGCUUGCAAACUCGACC	GGTCGAGTTTGCAAGCCCG	63.2%	-3.0 0
474-	492	GGGCUUGCAAACUCGACCU	AGGTCGAGTTTGCAAGCCC	57.9%	-3.0 0
475-	493	GGCUUGCAAACUCGACCUC	GAGGTCGAGTTTGCAAGCC	57.9%	-3.0 0
476-	494	GCUUGCAAACUCGACCUCU	AGAGGTCGAGTTTGCAAGC	52.6%	-2.9 0
477-	495	CUUGCAAACUCGACCUCUC	GAGAGGTCGAGTTTGCAAG	52.6%	-2.9 0
478-	496	UUGCAAACUCGACCUCUCG	CGAGAGGTCGAGTTTGCAA	52.6%	-2.4 0
479-	497	UGCAAACUCGACCUCUCGC	GCGAGAGGTCGAGTTTGCA	57.9%	-2.3 0
480-	498	GCAAACUCGACCUCUCGCU	AGCGAGAGGTCGAGTTTGC	57.9%	-2.3 0
481-	499	CAAACUCGACCUCUCGCUG	CAGCGAGAGGTCGAGTTTG	57.9%	-2.3 0
482-	500	AAACUCGACCUCUCGCUGG	CCAGCGAGAGGTCGAGTTT	57.9%	-2.3 0
483-	501	AACUCGACCUCUCGCUGGA	TCCAGCGAGAGGTCGAGTT	57.9%	-2.1 0
484-	502	ACUCGACCUCUCGCUGGAG	CTCCAGCGAGAGGTCGAGT	63.2%	-1.9 0
485-	503	CUCGACCUCUCGCUGGAGA	TCTCCAGCGAGAGGTCGAG	63.2%	-0.5 0
486-	504	UCGACCUCUCGCUGGAGAC	GTCTCCAGCGAGAGGTCGA	63.2%	0.1 0
487-	505	CGACCUCUCGCUGGAGACG	CGTCTCCAGCGAGAGGTCG	68.4%	1.3 0
488-	506	GACCUCUCGCUGGAGACGC	GCGTCTCCAGCGAGAGGTC	68.4%	2.7 0
489-	507	ACCUCUCGCUGGAGACGCC	GGCGTCTCCAGCGAGAGGT	68.4%	2.8 0
490-	508	CCUCUCGCUGGAGACGCCC	GGGCGTCTCCAGCGAGAGG	73.7%	2.8 0
491-	509	CUCUCGCUGGAGACGCCCG	CGGGCGTCTCCAGCGAGAG	73.7%	2.2 0
492-	510	UCUCGCUGGAGACGCCCGU	ACGGGCGTCTCCAGCGAGA	68.4%	1.9 0
493-	511	CUCGCUGGAGACGCCCGUG	CACGGGCGTCTCCAGCGAG	73.7%	1.9 0
494-	512	UCGCUGGAGACGCCCGUGU	ACACGGGCGTCTCCAGCGA	68.4%	1.7 0
495-	513	CGCUGGAGACGCCCGUGUU	AACACGGGCGTCTCCAGCG	68.4%	1.6 0
496-	514	GCUGGAGACGCCCGUGUUU	AAACACGGGCGTCTCCAGC	63.2%	1.6 0
497-	515	CUGGAGACGCCCGUGUUUC	GAAACACGGGCGTCTCCAG	63.2%	1.6 0
498-	516	UGGAGACGCCCGUGUUUCC	GGAAACACGGGCGTCTCCA	63.2%	1.6 0
499-	517	GGAGACGCCCGUGUUUCCU	AGGAAACACGGGCGTCTCC	63.2%	1.6 0
500-	518	GAGACGCCCGUGUUUCCUG	CAGGAAACACGGGCGTCTC	63.2%	1.6 0
501-	519	AGACGCCCGUGUUUCCUGG	CCAGGAAACACGGGCGTCT	63.2%	1.6 0
502-	520	GACGCCCGUGUUUCCUGGC	GCCAGGAAACACGGGCGTC	68.4%	-1.0 0
503-	521	ACGCCCGUGUUUCCUGGCA	TGCCAGGAAACACGGGCGT	63.2%	-1.8 0
504-	522	CGCCCGUGUUUCCUGGCAA	TTGCCAGGAAACACGGGCG	63.2%	-2.8 0
505-	523	GCCCGUGUUUCCUGGCAAC	GTTGCCAGGAAACACGGGC	63.2%	-2.8 0
506-	524	CCCGUGUUUCCUGGCAACG	CGTTGCCAGGAAACACGGG	63.2%	-2.8 0
507-	525	CCGUGUUUCCUGGCAACGG	CCGTTGCCAGGAAACACGG	63.2%	-2.8 0
508-	526	CGUGUUUCCUGGCAACGGA	TCCGTTGCCAGGAAACACG	57.9%	-2.7 0
509-	527	GUGUUUCCUGGCAACGGAG	CTCCGTTGCCAGGAAACAC	57.9%	-2.3 0
510-	528	UGUUUCCUGGCAACGGAGA	TCTCCGTTGCCAGGAAACA	52.6%	-2.1 0
511-	529	GUUUCCUGGCAACGGAGAU	ATCTCCGTTGCCAGGAAAC	52.6%	-2.2 0
512-	530	UUUCCUGGCAACGGAGAUG	CATCTCCGTTGCCAGGAAA	52.6%	-2.6 0
513-	531	UUCCUGGCAACGGAGAUGA	TCATCTCCGTTGCCAGGAA	52.6%	-3.6 0
514-	532	UCCUGGCAACGGAGAUGAA	TTCATCTCCGTTGCCAGGA	52.6%	-4.2 0
515-	533	CCUGGCAACGGAGAUGAAC	GTTCATCTCCGTTGCCAGG	57.9%	-5.5 0
516-	534	CUGGCAACGGAGAUGAACA	TGTTCATCTCCGTTGCCAG	52.6%	-6.0 0
517-	535	UGGCAACGGAGAUGAACAG	CTGTTCATCTCCGTTGCCA	52.6%	-6.0 0
518-	536	GGCAACGGAGAUGAACAGC	GCTGTTCATCTCCGTTGCC	57.9%	-6.0 0
519-	537	GCAACGGAGAUGAACAGCC	GGCTGTTCATCTCCGTTGC	57.9%	-6.0 0
520-	538	CAACGGAGAUGAACAGCCC	GGGCTGTTCATCTCCGTTG	57.9%	-3.3 0
521-	539	AACGGAGAUGAACAGCCCC	GGGGCTGTTCATCTCCGTT	57.9%	-2.4 0
522-	540	ACGGAGAUGAACAGCCCCU	AGGGGCTGTTCATCTCCGT	57.9%	-1.5 0
523-	541	CGGAGAUGAACAGCCCCUG	CAGGGGCTGTTCATCTCCG	63.2%	-1.5 0
524-	542	GGAGAUGAACAGCCCCUGA	TCAGGGGCTGTTCATCTCC	57.9%	-1.5 0
525-	543	GAGAUGAACAGCCCCUGAC	GTCAGGGGCTGTTCATCTC	57.9%	-1.6 0
526-	544	AGAUGAACAGCCCCUGACU	AGTCAGGGGCTGTTCATCT	52.6%	-1.7 0
527-	545	GAUGAACAGCCCCUGACUG	CAGTCAGGGGCTGTTCATC	57.9%	-1.5 0

528- 546 AUGAACAGCCCCUGACUGA TCAGTCAGGGGCTGTTCAT 52.6% -2.6 0
529- 547 UGAACAGCCCCUGACUGAA TTCAGTCAGGGGCTGTTCA 52.6% -3.5 0
530- 548 GAACAGCCCCUGACUGAAA TTTCAGTCAGGGGCTGTTC 52.6% -3.8 0
531- 549 AACAGCCCCUGACUGAAAA TTTTCAGTCAGGGGCTGTT 47.4% -3.8 0
532- 550 ACAGCCCCUGACUGAAAAC GTTTTCAGTCAGGGGCTGT 52.6% -3.3 0
533- 551 CAGCCCCUGACUGAAAACC GGTTTTCAGTCAGGGGCTG 57.9% -2.0 0
534- 552 AGCCCCUGACUGAAAACCC GGGTTTTCAGTCAGGGGCT 57.9% -1.4 0
535- 553 GCCCCUGACUGAAAACCCC GGGGTTTTCAGTCAGGGGC 63.2% -1.4 0
536- 554 CCCCUGACUGAAAACCCCC GGGGGTTTTCAGTCAGGGG 63.2% -1.5 0
537- 555 CCCUGACUGAAAACCCCCG CGGGGGTTTTCAGTCAGGG 63.2% -1.5 0
538- 556 CCUGACUGAAAACCCCCGG CCGGGGGTTTTCAGTCAGG 63.2% -1.5 0
539- 557 CUGACUGAAAACCCCCGGA TCCGGGGGTTTTCAGTCAG 57.9% -1.5 0
540- 558 UGACUGAAAACCCCCGGAA TTCCGGGGGTTTTCAGTCA 52.6% -1.4 0
541- 559 GACUGAAAACCCCCGGAAG CTTCCGGGGGTTTTCAGTC 57.9% -1.4 0
542- 560 ACUGAAAACCCCCGGAAGU ACTTCCGGGGGTTTTCAGT 52.6% -1.3 0
543- 561 CUGAAAACCCCCGGAAGUA TACTTCCGGGGGTTTTCAG 52.6% -1.2 0
544- 562 UGAAAACCCCCGGAAGUAC GTACTTCCGGGGGTTTTCA 52.6% -1.5 0
545- 563 GAAAACCCCCGGAAGUACG CGTACTTCCGGGGGTTTTC 57.9% -1.7 0
546- 564 AAAACCCCCGGAAGUACGU ACGTACTTCCGGGGGTTTT 52.6% -1.3 0
547- 565 AAACCCCCGGAAGUACGUC GACGTACTTCCGGGGGTTT 57.9% -0.4 0
548- 566 AACCCCCGGAAGUACGUCA TGACGTACTTCCGGGGGTT 57.9% 0.6 0
549- 567 ACCCCCGGAAGUACGUCAU ATGACGTACTTCCGGGGGT 57.9% -1.6 0
550- 568 CCCCCGGAAGUACGUCAUG CATGACGTACTTCCGGGGG 63.2% -1.6 0
551- 569 CCCCGGAAGUACGUCAUGG CCATGACGTACTTCCGGGG 63.2% -1.6 0
552- 570 CCCGGAAGUACGUCAUGGG CCCATGACGTACTTCCGGG 63.2% -1.6 0
553- 571 CCGGAAGUACGUCAUGGGU ACCCATGACGTACTTCCGG 57.9% -1.6 0
554- 572 CGGAAGUACGUCAUGGGUC GACCCATGACGTACTTCCG 57.9% -1.6 0
555- 573 GGAAGUACGUCAUGGGUCA TGACCCATGACGTACTTCC 52.6% -1.6 0
556- 574 GAAGUACGUCAUGGGUCAC GTGACCCATGACGTACTTC 52.6% -1.5 0
557- 575 AAGUACGUCAUGGGUCACU AGTGACCCATGACGTACTT 47.4% -1.3 0
558- 576 AGUACGUCAUGGGUCACUU AAGTGACCCATGACGTACT 47.4% -1.3 0
559- 577 GUACGUCAUGGGUCACUUC GAAGTGACCCATGACGTAC 52.6% -1.3 0
560- 578 UACGUCAUGGGUCACUUCC GGAAGTGACCCATGACGTA 52.6% -1.3 0
561- 579 ACGUCAUGGGUCACUUCCG CGGAAGTGACCCATGACGT 57.9% -1.3 0
562- 580 CGUCAUGGGUCACUUCCGC GCGGAAGTGACCCATGACG 63.2% -1.4 0
563- 581 GUCAUGGGUCACUUCCGCU AGCGGAAGTGACCCATGAC 57.9% -1.6 0
564- 582 UCAUGGGUCACUUCCGCUG CAGCGGAAGTGACCCATGA 57.9% -1.6 0
565- 583 CAUGGGUCACUUCCGCUGG CCAGCGGAAGTGACCCATG 63.2% 0.7 0
566- 584 AUGGGUCACUUCCGCUGGG CCCAGCGGAAGTGACCCAT 63.2% -0.2 0
567- 585 UGGGUCACUUCCGCUGGGA TCCCAGCGGAAGTGACCCA 63.2% -0.2 0
568- 586 GGGUCACUUCCGCUGGGAC GTCCCAGCGGAAGTGACCC 68.4% -0.4 0
569- 587 GGUCACUUCCGCUGGGACC GGTCCCAGCGGAAGTGACC 68.4% -0.5 0
570- 588 GUCACUUCCGCUGGGACCG CGGTCCCAGCGGAAGTGAC 68.4% -0.5 0
571- 589 UCACUUCCGCUGGGACCGC GCGGTCCCAGCGGAAGTGA 68.4% -0.7 0
572- 590 CACUUCCGCUGGGACCGCU AGCGGTCCCAGCGGAAGTG 68.4% -0.9 0
573- 591 ACUUCCGCUGGGACCGCUU AAGCGGTCCCAGCGGAAGT 63.2% -0.9 0
574- 592 CUUCCGCUGGGACCGCUUC GAAGCGGTCCCAGCGGAAG 68.4% -1.1 0
575- 593 UUCCGCUGGGACCGCUUCG CGAAGCGGTCCCAGCGGAA 68.4% -1.0 0
576- 594 UCCGCUGGGACCGCUUCGG CCGAAGCGGTCCCAGCGGA 73.7% -3.0 0
577- 595 CCGCUGGGACCGCUUCGGC GCCGAAGCGGTCCCAGCGG 78.9% -3.1 0
578- 596 CGCUGGGACCGCUUCGGCC GGCCGAAGCGGTCCCAGCG 78.9% -3.1 0
579- 597 GCUGGGACCGCUUCGGCCC GGGCCGAAGCGGTCCCAGC 78.9% -3.0 0
580- 598 CUGGGACCGCUUCGGCCCC GGGGCCGAAGCGGTCCCAG 78.9% -2.9 0
581- 599 UGGGACCGCUUCGGCCCCA TGGGGCCGAAGCGGTCCCA 73.7% -2.9 0
582- 600 GGGACCGCUUCGGCCCCAG CTGGGGCCGAAGCGGTCCC 78.9% -3.3 0
583- 601 GGACCGCUUCGGCCCCAGG CCTGGGGCCGAAGCGGTCC 78.9% -4.2 0
584- 602 GACCGCUUCGGCCCCAGGA TCCTGGGGCCGAAGCGGTC 73.7% -4.5 0
585- 603 ACCGCUUCGGCCCCAGGAA TTCCTGGGGCCGAAGCGGT 68.4% -5.5 0
586- 604 CCGCUUCGGCCCCAGGAAC GTTCCTGGGGCCGAAGCGG 73.7% -5.4 0
587- 605 CGCUUCGGCCCCAGGAACA TGTTCCTGGGGCCGAAGCG 68.4% -5.2 0

588-	606	GCUUCGGCCCCAGGAACAG	CTGTTCCTGGGGCCGAAGC	68.4%	-5.3	0
589-	607	CUUCGGCCCCAGGAACAGC	GCTGTTCCTGGGGCCGAAG	68.4%	-5.0	0
590-	608	UUCGGCCCCAGGAACAGCA	TGCTGTTCCTGGGGCCGAA	63.2%	-4.8	0
591-	609	UCGGCCCCAGGAACAGCAG	CTGCTGTTCCTGGGGCCGA	68.4%	-5.4	0
592-	610	CGGCCCCAGGAACAGCAGC	GCTGCTGTTCCTGGGGCCG	73.7%	-7.8	0
593-	611	GGCCCCAGGAACAGCAGCA	TGCTGCTGTTCCTGGGGCC	68.4%	-8.5	0
594-	612	GCCCCAGGAACAGCAGCAG	CTGCTGCTGTTCCTGGGGC	68.4%	-8.3	0
595-	613	CCCCAGGAACAGCAGCAGU	ACTGCTGCTGTTCCTGGGG	63.2%	-8.7	0
596-	614	CCCAGGAACAGCAGCAGUG	CACTGCTGCTGTTCCTGGG	63.2%	-8.7	0
597-	615	CCAGGAACAGCAGCAGUGC	GCACTGCTGCTGTTCCTGG	63.2%	-8.7	0
598-	616	CAGGAACAGCAGCAGUGCU	AGCACTGCTGCTGTTCCTG	57.9%	-8.6	0
599-	617	AGGAACAGCAGCAGUGCUG	CAGCACTGCTGCTGTTCCT	57.9%	-8.6	0
600-	618	GGAACAGCAGCAGUGCUGG	CCAGCACTGCTGCTGTTCC	63.2%	-7.4	0
601-	619	GAACAGCAGCAGUGCUGGC	GCCAGCACTGCTGCTGTTC	63.2%	-7.9	0
602-	620	AACAGCAGCAGUGCUGGCA	TGCCAGCACTGCTGCTGTT	57.9%	-7.5	0
603-	621	ACAGCAGCAGUGCUGGCAG	CTGCCAGCACTGCTGCTGT	63.2%	-6.6	0
604-	622	CAGCAGCAGUGCUGGCAGC	GCTGCCAGCACTGCTGCTG	68.4%	-6.6	0
605-	623	AGCAGCAGUGCUGGCAGCG	CGCTGCCAGCACTGCTGCT	68.4%	-6.5	0
606-	624	GCAGCAGUGCUGGCAGCGC	GCGCTGCCAGCACTGCTGC	73.7%	-6.5	0
607-	625	CAGCAGUGCUGGCAGCGCG	CGCGCTGCCAGCACTGCTG	73.7%	-6.5	0
608-	626	AGCAGUGCUGGCAGCGCGG	CCGCGCTGCCAGCACTGCT	73.7%	-6.5	0
609-	627	GCAGUGCUGGCAGCGCGGC	GCCGCGCTGCCAGCACTGC	78.9%	-5.9	0
610-	628	CAGUGCUGGCAGCGCGGCG	CGCCGCGCTGCCAGCACTG	78.9%	-3.3	0
611-	629	AGUGCUGGCAGCGCGGCGC	GCGCCGCGCTGCCAGCACT	78.9%	-4.9	0
612-	630	GUGCUGGCAGCGCGGCGCA	TGCGCCGCGCTGCCAGCAC	78.9%	-4.0	0
613-	631	UGCUGGCAGCGCGGCGCAG	CTGCGCCGCGCTGCCAGCA	78.9%	-5.3	0
614-	632	GCUGGCAGCGCGGCGCAGA	TCTGCGCCGCGCTGCCAGC	78.9%	-6.6	0
615-	633	CUGGCAGCGCGGCGCAGAG	CTCTGCGCCGCGCTGCCAG	78.9%	-6.8	0
616-	634	UGGCAGCGCGGCGCAGAGG	CCTCTGCGCCGCGCTGCCA	78.9%	-6.8	0
617-	635	GGCAGCGCGGCGCAGAGGC	GCCTCTGCGCCGCGCTGCC	84.2%	-6.8	0
618-	636	GCAGCGCGGCGCAGAGGCG	CGCCTCTGCGCCGCGCTGC	84.2%	-6.8	0
619-	637	CAGCGCGGCGCAGAGGCGU	ACGCCTCTGCGCCGCGCTG	78.9%	-4.5	0
620-	638	AGCGCGGCGCAGAGGCGUG	CACGCCTCTGCGCCGCGCT	78.9%	-3.8	0
621-	639	GCGCGGCGCAGAGGCGUGC	GCACGCCTCTGCGCCGCGC	84.2%	-3.6	0
622-	640	CGCGGCGCAGAGGCGUGCG	CGCACGCCTCTGCGCCGCG	84.2%	-3.6	0
623-	641	GCGGCGCAGAGGCGUGCGG	CCGCACGCCTCTGCGCCGC	84.2%	-4.3	0
624-	642	CGGCGCAGAGGCGUGCGGA	TCCGCACGCCTCTGCGCCG	78.9%	-4.7	0
625-	643	GGCGCAGAGGCGUGCGGAG	CTCCGCACGCCTCTGCGCC	78.9%	-4.7	0
626-	644	GCGCAGAGGCGUGCGGAGG	CCTCCGCACGCCTCTGCGC	78.9%	-4.7	0
627-	645	CGCAGAGGCGUGCGGAGGA	TCCTCCGCACGCCTCTGCG	73.7%	-4.7	0
628-	646	GCAGAGGCGUGCGGAGGAA	TTCCTCCGCACGCCTCTGC	68.4%	-5.4	0
629-	647	CAGAGGCGUGCGGAGGAAG	CTTCCTCCGCACGCCTCTG	68.4%	-2.9	0
630-	648	AGAGGCGUGCGGAGGAAGA	TCTTCCTCCGCACGCCTCT	63.2%	-2.1	0
631-	649	GAGGCGUGCGGAGGAAGAG	CTCTTCCTCCGCACGCCTC	68.4%	-0.3	0
632-	650	AGGCGUGCGGAGGAAGAGG	CCTCTTCCTCCGCACGCCT	68.4%	0.9	0
633-	651	GGCGUGCGGAGGAAGAGGC	GCCTCTTCCTCCGCACGCC	73.7%	1.1	0
634-	652	GCGUGCGGAGGAAGAGGCG	CGCCTCTTCCTCCGCACGC	73.7%	1.1	0
635-	653	CGUGCGGAGGAAGAGGCGG	CCGCCTCTTCCTCCGCACG	73.7%	0.9	0
636-	654	GUGCGGAGGAAGAGGCGGU	ACCGCCTCTTCCTCCGCAC	68.4%	0.8	0
637-	655	UGCGGAGGAAGAGGCGGUG	CACCGCCTCTTCCTCCGCA	68.4%	0.8	0
638-	656	GCGGAGGAAGAGGCGGUGU	ACACCGCCTCTTCCTCCGC	68.4%	0.5	0
639-	657	CGGAGGAAGAGGCGGUGUG	CACACCGCCTCTTCCTCCG	68.4%	0.5	0
640-	658	GGAGGAAGAGGCGGUGUGG	CCACACCGCCTCTTCCTCC	68.4%	0.4	0
641-	659	GAGGAAGAGGCGGUGUGGG	CCCACACCGCCTCTTCCTC	68.4%	1.1	0
642-	660	AGGAAGAGGCGGUGUGGGG	CCCCACACCGCCTCTTCCT	68.4%	1.4	1
643-	661	GGAAGAGGCGGUGUGGGGA	TCCCCACACCGCCTCTTCC	68.4%	1.2	1
644-	662	GAAGAGGCGGUGUGGGGAG	CTCCCCACACCGCCTCTTC	68.4%	0.7	1
645-	663	AAGAGGCGGUGUGGGGAGA	TCTCCCCACACCGCCTCTT	63.2%	0.1	1
646-	664	AGAGGCGGUGUGGGGAGAU	ATCTCCCCACACCGCCTCT	63.2%	0.7	1
647-	665	GAGGCGGUGUGGGGAGAUG	CATCTCCCCACACCGCCTC	68.4%	0.7	1

648-	666	AGGCGGUGUGGGGAGAUGG	CCATCTCCCCACACCGCCT	68.4%	0.7	1
649-	667	GGCGGUGUGGGGAGAUGGC	GCCATCTCCCCACACCGCC	73.7%	0.6	1
650-	668	GCGGUGUGGGGAGAUGGCA	TGCCATCTCCCCACACCGC	68.4%	0.6	1
651-	669	CGGUGUGGGGAGAUGGCAG	CTGCCATCTCCCCACACCG	68.4%	-0.7	1
652-	670	GGUGUGGGGAGAUGGCAGU	ACTGCCATCTCCCCACACC	63.2%	-1.5	1
653-	671	GUGUGGGGAGAUGGCAGUC	GACTGCCATCTCCCCACAC	63.2%	-2.4	1
654-	672	UGUGGGGAGAUGGCAGUCC	GGACTGCCATCTCCCCACA	63.2%	-4.3	1
655-	673	GUGGGGAGAUGGCAGUCCA	TGGACTGCCATCTCCCCAC	63.2%	-5.1	1
656-	674	UGGGGAGAUGGCAGUCCAG	CTGGACTGCCATCTCCCCA	63.2%	-5.4	1
657-	675	GGGGAGAUGGCAGUCCAGA	TCTGGACTGCCATCTCCCC	63.2%	-5.8	1
658-	676	GGGAGAUGGCAGUCCAGAG	CTCTGGACTGCCATCTCCC	63.2%	-5.8	0
659-	677	GGAGAUGGCAGUCCAGAGC	GCTCTGGACTGCCATCTCC	63.2%	-5.8	0
660-	678	GAGAUGGCAGUCCAGAGCC	GGCTCTGGACTGCCATCTC	63.2%	-5.7	0
661-	679	AGAUGGCAGUCCAGAGCCG	CGGCTCTGGACTGCCATCT	63.2%	-5.5	0
662-	680	GAUGGCAGUCCAGAGCCGA	TCGGCTCTGGACTGCCATC	63.2%	-6.0	0
663-	681	AUGGCAGUCCAGAGCCGAG	CTCGGCTCTGGACTGCCAT	63.2%	-5.9	0
664-	682	UGGCAGUCCAGAGCCGAGU	ACTCGGCTCTGGACTGCCA	63.2%	-5.9	0
665-	683	GGCAGUCCAGAGCCGAGUC	GACTCGGCTCTGGACTGCC	68.4%	-5.8	0
666-	684	GCAGUCCAGAGCCGAGUCC	GGACTCGGCTCTGGACTGC	68.4%	-5.8	0
667-	685	CAGUCCAGAGCCGAGUCCA	TGGACTCGGCTCTGGACTG	63.2%	-5.8	0
668-	686	AGUCCAGAGCCGAGUCCAC	GTGGACTCGGCTCTGGACT	63.2%	-7.2	0
669-	687	GUCCAGAGCCGAGUCCACG	CGTGGACTCGGCTCTGGAC	68.4%	-7.0	0
670-	688	UCCAGAGCCGAGUCCACGC	GCGTGGACTCGGCTCTGGA	68.4%	-8.1	0
671-	689	CCAGAGCCGAGUCCACGCG	CGCGTGGACTCGGCTCTGG	73.7%	-8.2	0
672-	690	CAGAGCCGAGUCCACGCGA	TCGCGTGGACTCGGCTCTG	68.4%	-7.2	0
673-	691	AGAGCCGAGUCCACGCGAG	CTCGCGTGGACTCGGCTCT	68.4%	-6.5	0
674-	692	GAGCCGAGUCCACGCGAGG	CCTCGCGTGGACTCGGCTC	73.7%	-5.9	0
675-	693	AGCCGAGUCCACGCGAGGG	CCCTCGCGTGGACTCGGCT	73.7%	-5.5	0
676-	694	GCCGAGUCCACGCGAGGGC	GCCCTCGCGTGGACTCGGC	78.9%	-5.5	0
677-	695	CCGAGUCCACGCGAGGGCA	TGCCCTCGCGTGGACTCGG	73.7%	-5.5	0
678-	696	CGAGUCCACGCGAGGGCAA	TTGCCCTCGCGTGGACTCG	68.4%	-6.5	0
679-	697	GAGUCCACGCGAGGGCAAG	CTTGCCCTCGCGTGGACTC	68.4%	-7.1	0
680-	698	AGUCCACGCGAGGGCAAGC	GCTTGCCCTCGCGTGGACT	68.4%	-7.1	0
681-	699	GUCCACGCGAGGGCAAGCG	CGCTTGCCCTCGCGTGGAC	73.7%	-6.7	0
682-	700	UCCACGCGAGGGCAAGCGC	GCGCTTGCCCTCGCGTGGA	73.7%	-6.8	0
683-	701	CCACGCGAGGGCAAGCGCU	AGCGCTTGCCCTCGCGTGG	73.7%	-6.8	0
684-	702	CACGCGAGGGCAAGCGCUC	GAGCGCTTGCCCTCGCGTG	73.7%	-6.8	0
685-	703	ACGCGAGGGCAAGCGCUCC	GGAGCGCTTGCCCTCGCGT	73.7%	-6.7	0
686-	704	CGCGAGGGCAAGCGCUCCU	AGGAGCGCTTGCCCTCGCG	73.7%	-5.9	0
687-	705	GCGAGGGCAAGCGCUCCUA	TAGGAGCGCTTGCCCTCGC	68.4%	-5.1	0
688-	706	CGAGGGCAAGCGCUCCUAC	GTAGGAGCGCTTGCCCTCG	68.4%	-4.6	0
689-	707	GAGGGCAAGCGCUCCUACU	AGTAGGAGCGCTTGCCCTC	63.2%	-4.0	0
690-	708	AGGGCAAGCGCUCCUACUC	GAGTAGGAGCGCTTGCCCT	63.2%	-3.0	0
691-	709	GGGCAAGCGCUCCUACUCC	GGAGTAGGAGCGCTTGCCC	68.4%	-3.0	0
692-	710	GGCAAGCGCUCCUACUCCA	TGGAGTAGGAGCGCTTGCC	63.2%	-3.0	0
693-	711	GCAAGCGCUCCUACUCCAU	ATGGAGTAGGAGCGCTTGC	57.9%	-3.0	0
694-	712	CAAGCGCUCCUACUCCAUG	CATGGAGTAGGAGCGCTTG	57.9%	-3.0	0
695-	713	AAGCGCUCCUACUCCAUGG	CCATGGAGTAGGAGCGCTT	57.9%	-2.9	0
696-	714	AGCGCUCCUACUCCAUGGA	TCCATGGAGTAGGAGCGCT	57.9%	-2.8	0
697-	715	GCGCUCCUACUCCAUGGAG	CTCCATGGAGTAGGAGCGC	63.2%	-2.4	0
698-	716	CGCUCCUACUCCAUGGAGC	GCTCCATGGAGTAGGAGCG	63.2%	-1.8	0
699-	717	GCUCCUACUCCAUGGAGCA	TGCTCCATGGAGTAGGAGC	57.9%	-1.7	0
700-	718	CUCCUACUCCAUGGAGCAC	GTGCTCCATGGAGTAGGAG	57.9%	-1.6	0
701-	719	UCCUACUCCAUGGAGCACU	AGTGCTCCATGGAGTAGGA	52.6%	-1.6	0
702-	720	CCUACUCCAUGGAGCACUU	AAGTGCTCCATGGAGTAGG	52.6%	-1.6	0
703-	721	CUACUCCAUGGAGCACUUC	GAAGTGCTCCATGGAGTAG	52.6%	-1.6	0
704-	722	UACUCCAUGGAGCACUUCC	GGAAGTGCTCCATGGAGTA	52.6%	-1.0	0
705-	723	ACUCCAUGGAGCACUUCCG	CGGAAGTGCTCCATGGAGT	57.9%	-0.7	0
706-	724	CUCCAUGGAGCACUUCCGC	GCGGAAGTGCTCCATGGAG	63.2%	0.7	0
707-	725	UCCAUGGAGCACUUCCGCU	AGCGGAAGTGCTCCATGGA	57.9%	0.4	0

708- 726	CCAUGGAGCACUUCCGCUG	CAGCGGAAGTGCTCCATGG	63.2%	0.5	0
709- 727	CAUGGAGCACUUCCGCUGG	CCAGCGGAAGTGCTCCATG	63.2%	0.5	0
710- 728	AUGGAGCACUUCCGCUGGG	CCCAGCGGAAGTGCTCCAT	63.2%	0.5	0
711- 729	UGGAGCACUUCCGCUGGGG	CCCCAGCGGAAGTGCTCCA	68.4%	0.5	1
712- 730	GGAGCACUUCCGCUGGGGC	GCCCCAGCGGAAGTGCTCC	73.7%	0.2	1
713- 731	GAGCACUUCCGCUGGGGCA	TGCCCCAGCGGAAGTGCTC	68.4%	-0.6	1
714- 732	AGCACUUCCGCUGGGGCAA	TTGCCCCAGCGGAAGTGCT	63.2%	0.2	1
715- 733	GCACUUCCGCUGGGGCAAG	CTTGCCCCAGCGGAAGTGC	68.4%	0.3	1
716- 734	CACUUCCGCUGGGGCAAGC	GCTTGCCCCAGCGGAAGTG	68.4%	0.5	1
717- 735	ACUUCCGCUGGGGCAAGCC	GGCTTGCCCCAGCGGAAGT	68.4%	0.5	1
718- 736	CUUCCGCUGGGGCAAGCCG	CGGCTTGCCCCAGCGGAAG	73.7%	0.5	1
719- 737	UUCCGCUGGGGCAAGCCGG	CCGGCTTGCCCCAGCGGAA	73.7%	0.5	1
720- 738	UCCGCUGGGGCAAGCCGGU	ACCGGCTTGCCCCAGCGGA	73.7%	0.5	1
721- 739	CCGCUGGGGCAAGCCGGUG	CACCGGCTTGCCCCAGCGG	78.9%	0.5	1
722- 740	CGCUGGGGCAAGCCGGUGG	CCACCGGCTTGCCCCAGCG	78.9%	0.5	1
723- 741	GCUGGGGCAAGCCGGUGGG	CCCACCGGCTTGCCCCAGC	78.9%	0.5	1
724- 742	CUGGGGCAAGCCGGUGGGC	GCCCACCGGCTTGCCCCAG	78.9%	0.6	1
725- 743	UGGGGCAAGCCGGUGGGCA	TGCCCACCGGCTTGCCCCA	73.7%	1.5	1
726- 744	GGGGCAAGCCGGUGGGCAA	TTGCCCACCGGCTTGCCCC	73.7%	0.5	1
727- 745	GGGCAAGCCGGUGGGCAAG	CTTGCCCACCGGCTTGCCC	73.7%	-1.2	0
728- 746	GGCAAGCCGGUGGGCAAGA	TCTTGCCCACCGGCTTGCC	68.4%	-2.4	0
729- 747	GCAAGCCGGUGGGCAAGAA	TTCTTGCCCACCGGCTTGC	63.2%	-3.3	0
730- 748	CAAGCCGGUGGGCAAGAAA	TTTCTTGCCCACCGGCTTG	57.9%	-3.6	0
731- 749	AAGCCGGUGGGCAAGAAAC	GTTTCTTGCCCACCGGCTT	57.9%	-4.0	0
732- 750	AGCCGGUGGGCAAGAAACG	CGTTTCTTGCCCACCGGCT	63.2%	-3.9	0
733- 751	GCCGGUGGGCAAGAAACGG	CCGTTTCTTGCCCACCGGC	68.4%	-3.8	0
734- 752	CCGGUGGGCAAGAAACGGC	GCCGTTTCTTGCCCACCGG	68.4%	-3.7	0
735- 753	CGGUGGGCAAGAAACGGCG	CGCCGTTTCTTGCCCACCG	68.4%	-3.7	0
736- 754	GGUGGGCAAGAAACGGCGC	GCGCCGTTTCTTGCCCACC	68.4%	-3.7	0
737- 755	GUGGGCAAGAAACGGCGCC	GGCGCCGTTTCTTGCCCAC	68.4%	-3.6	0
738- 756	UGGGCAAGAAACGGCGCCC	GGGCGCCGTTTCTTGCCCA	68.4%	-3.6	0
739- 757	GGGCAAGAAACGGCGCCCG	CGGGCGCCGTTTCTTGCCC	73.7%	-3.6	0
740- 758	GGCAAGAAACGGCGCCCGG	CCGGGCGCCGTTTCTTGCC	73.7%	-3.6	0
741- 759	GCAAGAAACGGCGCCCGGU	ACCGGGCGCCGTTTCTTGC	68.4%	-3.6	0
742- 760	CAAGAAACGGCGCCCGGUG	CACCGGGCGCCGTTTCTTG	68.4%	-3.6	0
743- 761	AAGAAACGGCGCCCGGUGA	TCACCGGGCGCCGTTTCTT	63.2%	-3.6	0
744- 762	AGAAACGGCGCCCGGUGAA	TTCACCGGGCGCCGTTTCT	63.2%	-2.7	0
745- 763	GAAACGGCGCCCGGUGAAG	CTTCACCGGGCGCCGTTTC	68.4%	-1.0	0
746- 764	AAACGGCGCCCGGUGAAGG	CCTTCACCGGGCGCCGTTT	68.4%	0.2	0
747- 765	AACGGCGCCCGGUGAAGGU	ACCTTCACCGGGCGCCGTT	68.4%	1.1	0
748- 766	ACGGCGCCCGGUGAAGGUG	CACCTTCACCGGGCGCCGT	73.7%	1.6	0
749- 767	CGGCGCCCGGUGAAGGUGU	ACACCTTCACCGGGCGCCG	73.7%	2.5	0
750- 768	GGCGCCCGGUGAAGGUGUA	TACACCTTCACCGGGCGCC	68.4%	2.5	0
751- 769	GCGCCCGGUGAAGGUGUAC	GTACACCTTCACCGGGCGC	68.4%	2.5	0
752- 770	CGCCCGGUGAAGGUGUACC	GGTACACCTTCACCGGGCG	68.4%	2.5	0
753- 771	GCCCGGUGAAGGUGUACCC	GGGTACACCTTCACCGGGC	68.4%	2.5	0
754- 772	CCCGGUGAAGGUGUACCCC	GGGGTACACCTTCACCGGG	68.4%	2.5	0
755- 773	CCGGUGAAGGUGUACCCCA	TGGGGTACACCTTCACCGG	63.2%	2.5	0
756- 774	CGGUGAAGGUGUACCCCAA	TTGGGGTACACCTTCACCG	57.9%	2.4	0
757- 775	GGUGAAGGUGUACCCCAAC	GTTGGGGTACACCTTCACC	57.9%	0.4	0
758- 776	GUGAAGGUGUACCCCAACG	CGTTGGGGTACACCTTCAC	57.9%	-1.3	0
759- 777	UGAAGGUGUACCCCAACGU	ACGTTGGGGTACACCTTCA	52.6%	-1.4	0
760- 778	GAAGGUGUACCCCAACGUU	AACGTTGGGGTACACCTTC	52.6%	-1.4	0
761- 779	AAGGUGUACCCCAACGUUG	CAACGTTGGGGTACACCTT	52.6%	-1.4	0
762- 780	AGGUGUACCCCAACGUUGC	GCAACGTTGGGGTACACCT	57.9%	-1.4	0
763- 781	GGUGUACCCCAACGUUGCU	AGCAACGTTGGGGTACACC	57.9%	-1.3	0
764- 782	GUGUACCCCAACGUUGCUG	CAGCAACGTTGGGGTACAC	57.9%	-1.3	0
765- 783	UGUACCCCAACGUUGCUGA	TCAGCAACGTTGGGGTACA	52.6%	-1.3	0
766- 784	GUACCCCAACGUUGCUGAG	CTCAGCAACGTTGGGGTAC	57.9%	-1.2	0
767- 785	UACCCCAACGUUGCUGAGA	TCTCAGCAACGTTGGGGTA	52.6%	-0.8	0

768-	786	ACCCCAACGUUGCUGAGAA	TTCTCAGCAACGTTGGGGT	52.6%	-0.9	0
769-	787	CCCCAACGUUGCUGAGAAC	GTTCTCAGCAACGTTGGGG	57.9%	-3.0	0
770-	788	CCCAACGUUGCUGAGAACG	CGTTCTCAGCAACGTTGGG	57.9%	-4.3	0
771-	789	CCAACGUUGCUGAGAACGA	TCGTTCTCAGCAACGTTGG	52.6%	-5.2	0
772-	790	CAACGUUGCUGAGAACGAG	CTCGTTCTCAGCAACGTTG	52.6%	-5.4	0
773-	791	AACGUUGCUGAGAACGAGU	ACTCGTTCTCAGCAACGTT	47.4%	-5.4	0
774-	792	ACGUUGCUGAGAACGAGUC	GACTCGTTCTCAGCAACGT	52.6%	-5.4	0
775-	793	CGUUGCUGAGAACGAGUCG	CGACTCGTTCTCAGCAACG	57.9%	-3.4	0
776-	794	GUUGCUGAGAACGAGUCGG	CCGACTCGTTCTCAGCAAC	57.9%	-1.8	0
777-	795	UUGCUGAGAACGAGUCGGC	GCCGACTCGTTCTCAGCAA	57.9%	-1.7	0
778-	796	UGCUGAGAACGAGUCGGCG	CGCCGACTCGTTCTCAGCA	63.2%	-1.9	0
779-	797	GCUGAGAACGAGUCGGCGG	CCGCCGACTCGTTCTCAGC	68.4%	-4.2	0
780-	798	CUGAGAACGAGUCGGCGGA	TCCGCCGACTCGTTCTCAG	63.2%	-5.2	0
781-	799	UGAGAACGAGUCGGCGGAG	CTCCGCCGACTCGTTCTCA	63.2%	-5.3	0
782-	800	GAGAACGAGUCGGCGGAGG	CCTCCGCCGACTCGTTCTC	68.4%	-5.4	0
783-	801	AGAACGAGUCGGCGGAGGC	GCCTCCGCCGACTCGTTCT	68.4%	-5.4	0
784-	802	GAACGAGUCGGCGGAGGCC	GGCCTCCGCCGACTCGTTC	73.7%	-5.4	0
785-	803	AACGAGUCGGCGGAGGCCU	AGGCCTCCGCCGACTCGTT	68.4%	-5.4	0
786-	804	ACGAGUCGGCGGAGGCCUU	AAGGCCTCCGCCGACTCGT	68.4%	-5.4	0
787-	805	CGAGUCGGCGGAGGCCUUU	AAAGGCCTCCGCCGACTCG	68.4%	-3.3	0
788-	806	GAGUCGGCGGAGGCCUUUC	GAAAGGCCTCCGCCGACTC	68.4%	-2.0	0
789-	807	AGUCGGCGGAGGCCUUUCC	GGAAAGGCCTCCGCCGACT	68.4%	-1.0	0
790-	808	GUCGGCGGAGGCCUUUCCC	GGGAAAGGCCTCCGCCGAC	73.7%	-1.1	0
791-	809	UCGGCGGAGGCCUUUCCCC	GGGGAAAGGCCTCCGCCGA	73.7%	-1.3	0
792-	810	CGGCGGAGGCCUUUCCCCU	AGGGGAAAGGCCTCCGCCG	73.7%	-1.3	0
793-	811	GGCGGAGGCCUUUCCCCUA	TAGGGGAAAGGCCTCCGCC	68.4%	-1.4	0
794-	812	GCGGAGGCCUUUCCCCUAG	CTAGGGGAAAGGCCTCCGC	68.4%	-2.4	0
795-	813	CGGAGGCCUUUCCCCUAGA	TCTAGGGGAAAGGCCTCCG	63.2%	-2.7	0
796-	814	GGAGGCCUUUCCCCUAGAG	CTCTAGGGGAAAGGCCTCC	63.2%	-2.8	0
797-	815	GAGGCCUUUCCCCUAGAGU	ACTCTAGGGGAAAGGCCTC	57.9%	-0.6	0
798-	816	AGGCCUUUCCCCUAGAGUU	AACTCTAGGGGAAAGGCCT	52.6%	0.4	0
799-	817	GGCCUUUCCCCUAGAGUUC	GAACTCTAGGGGAAAGGCC	57.9%	0.3	0
800-	818	GCCUUUCCCCUAGAGUUCA	TGAACTCTAGGGGAAAGGC	52.6%	0.2	0
801-	819	CCUUUCCCCUAGAGUUCAA	TTGAACTCTAGGGGAAAGG	47.4%	-0.5	0
802-	820	CUUUCCCCUAGAGUUCAAG	CTTGAACTCTAGGGGAAAG	47.4%	-1.9	0
803-	821	UUUCCCCUAGAGUUCAAGA	TCTTGAACTCTAGGGGAAA	42.1%	-2.9	0
804-	822	UUCCCCUAGAGUUCAAGAG	CTCTTGAACTCTAGGGGAA	47.4%	-4.0	0
805-	823	UCCCCUAGAGUUCAAGAGG	CCTCTTGAACTCTAGGGGA	52.6%	-5.9	0
806-	824	CCCCUAGAGUUCAAGAGGG	CCCTCTTGAACTCTAGGGG	57.9%	-5.9	0
807-	825	CCCUAGAGUUCAAGAGGGA	TCCCTCTTGAACTCTAGGG	52.6%	-6.0	0
808-	826	CCUAGAGUUCAAGAGGGAG	CTCCCTCTTGAACTCTAGG	52.6%	-5.9	0
809-	827	CUAGAGUUCAAGAGGGAGC	GCTCCCTCTTGAACTCTAG	52.6%	-5.7	0
810-	828	UAGAGUUCAAGAGGGAGCU	AGCTCCCTCTTGAACTCTA	47.4%	-5.7	0
811-	829	AGAGUUCAAGAGGGAGCUG	CAGCTCCCTCTTGAACTCT	52.6%	-5.8	0
812-	830	GAGUUCAAGAGGGAGCUGG	CCAGCTCCCTCTTGAACTC	57.9%	-6.5	0
813-	831	AGUUCAAGAGGGAGCUGGA	TCCAGCTCCCTCTTGAACT	52.6%	-7.0	0
814-	832	GUUCAAGAGGGAGCUGGAA	TTCCAGCTCCCTCTTGAAC	52.6%	-7.0	0
815-	833	UUCAAGAGGGAGCUGGAAG	CTTCCAGCTCCCTCTTGAA	52.6%	-7.0	0
816-	834	UCAAGAGGGAGCUGGAAGG	CCTTCCAGCTCCCTCTTGA	57.9%	-7.1	0
817-	835	CAAGAGGGAGCUGGAAGGC	GCCTTCCAGCTCCCTCTTG	63.2%	-7.0	0
818-	836	AAGAGGGAGCUGGAAGGCG	CGCCTTCCAGCTCCCTCTT	63.2%	-6.9	0
819-	837	AGAGGGAGCUGGAAGGCGA	TCGCCTTCCAGCTCCCTCT	63.2%	-6.4	0
820-	838	GAGGGAGCUGGAAGGCGAG	CTCGCCTTCCAGCTCCCTC	68.4%	-5.4	0
821-	839	AGGGAGCUGGAAGGCGAGC	GCTCGCCTTCCAGCTCCCT	68.4%	-5.1	0
822-	840	GGGAGCUGGAAGGCGAGCG	CGCTCGCCTTCCAGCTCCC	73.7%	-4.2	0
823-	841	GGAGCUGGAAGGCGAGCGG	CCGCTCGCCTTCCAGCTCC	73.7%	-2.4	0
824-	842	GAGCUGGAAGGCGAGCGGC	GCCGCTCGCCTTCCAGCTC	73.7%	-2.3	0
825-	843	AGCUGGAAGGCGAGCGGCC	GGCCGCTCGCCTTCCAGCT	73.7%	-2.3	0
826-	844	GCUGGAAGGCGAGCGGCCA	TGGCCGCTCGCCTTCCAGC	73.7%	-2.2	0
827-	845	CUGGAAGGCGAGCGGCCAU	ATGGCCGCTCGCCTTCCAG	68.4%	-2.5	0

828- 846 UGGAAGGCGAGCGGCCAUU AATGGCCGCTCGCCTTCCA 63.2% -2.5 0
829- 847 GGAAGGCGAGCGGCCAUUA TAATGGCCGCTCGCCTTCC 63.2% -2.5 0
830- 848 GAAGGCGAGCGGCCAUUAG CTAATGGCCGCTCGCCTTC 63.2% -0.6 0
831- 849 AAGGCGAGCGGCCAUUAGG CCTAATGGCCGCTCGCCTT 63.2% 0.0 0
832- 850 AGGCGAGCGGCCAUUAGGC GCCTAATGGCCGCTCGCCT 68.4% 0.3 0
833- 851 GGCGAGCGGCCAUUAGGCU AGCCTAATGGCCGCTCGCC 68.4% 0.4 0
834- 852 GCGAGCGGCCAUUAGGCUU AAGCCTAATGGCCGCTCGC 63.2% 0.5 0
835- 853 CGAGCGGCCAUUAGGCUUG CAAGCCTAATGGCCGCTCG 63.2% 0.5 0
836- 854 GAGCGGCCAUUAGGCUUGG CCAAGCCTAATGGCCGCTC 63.2% 0.1 0
837- 855 AGCGGCCAUUAGGCUUGGA TCCAAGCCTAATGGCCGCT 57.9% -0.7 0
838- 856 GCGGCCAUUAGGCUUGGAG CTCCAAGCCTAATGGCCGC 63.2% -1.8 0
839- 857 CGGCCAUUAGGCUUGGAGC GCTCCAAGCCTAATGGCCG 63.2% -1.9 0
840- 858 GGCCAUUAGGCUUGGAGCA TGCTCCAAGCCTAATGGCC 57.9% -1.7 0
841- 859 GCCAUUAGGCUUGGAGCAG CTGCTCCAAGCCTAATGGC 57.9% -1.6 0
842- 860 CCAUUAGGCUUGGAGCAGG CCTGCTCCAAGCCTAATGG 57.9% -1.6 0
843- 861 CAUUAGGCUUGGAGCAGGU ACCTGCTCCAAGCCTAATG 52.6% -1.6 0
844- 862 AUUAGGCUUGGAGCAGGUC GACCTGCTCCAAGCCTAAT 52.6% -1.6 0
845- 863 UUAGGCUUGGAGCAGGUCC GGACCTGCTCCAAGCCTAA 57.9% -1.2 0
846- 864 UAGGCUUGGAGCAGGUCCU AGGACCTGCTCCAAGCCTA 57.9% -1.2 0
847- 865 AGGCUUGGAGCAGGUCCUG CAGGACCTGCTCCAAGCCT 63.2% -1.1 0
848- 866 GGCUUGGAGCAGGUCCUGG CCAGGACCTGCTCCAAGCC 68.4% -1.3 0
849- 867 GCUUGGAGCAGGUCCUGGA TCCAGGACCTGCTCCAAGC 63.2% -1.9 0
850- 868 CUUGGAGCAGGUCCUGGAG CTCCAGGACCTGCTCCAAG 63.2% -2.3 0
851- 869 UUGGAGCAGGUCCUGGAGU ACTCCAGGACCTGCTCCAA 57.9% -2.5 0
852- 870 UGGAGCAGGUCCUGGAGUC GACTCCAGGACCTGCTCCA 63.2% -2.4 0
853- 871 GGAGCAGGUCCUGGAGUCC GGACTCCAGGACCTGCTCC 68.4% -2.4 0
854- 872 GAGCAGGUCCUGGAGUCCG CGGACTCCAGGACCTGCTC 68.4% -1.9 0
855- 873 AGCAGGUCCUGGAGUCCGA TCGGACTCCAGGACCTGCT 63.2% -1.1 0
856- 874 GCAGGUCCUGGAGUCCGAC GTCGGACTCCAGGACCTGC 68.4% 0.2 0
857- 875 CAGGUCCUGGAGUCCGACG CGTCGGACTCCAGGACCTG 68.4% 1.0 0
858- 876 AGGUCCUGGAGUCCGACGC GCGTCGGACTCCAGGACCT 68.4% 0.5 0
859- 877 GGUCCUGGAGUCCGACGCG CGCGTCGGACTCCAGGACC 73.7% 0.3 0
860- 878 GUCCUGGAGUCCGACGCGG CCGCGTCGGACTCCAGGAC 73.7% -0.3 0
861- 879 UCCUGGAGUCCGACGCGGA TCCGCGTCGGACTCCAGGA 68.4% -0.6 0
862- 880 CCUGGAGUCCGACGCGGAG CTCCGCGTCGGACTCCAGG 73.7% -0.7 0
863- 881 CUGGAGUCCGACGCGGAGA TCTCCGCGTCGGACTCCAG 68.4% -0.8 0
864- 882 UGGAGUCCGACGCGGAGAA TTCTCCGCGTCGGACTCCA 63.2% -0.9 0
865- 883 GGAGUCCGACGCGGAGAAG CTTCTCCGCGTCGGACTCC 68.4% -1.9 0
866- 884 GAGUCCGACGCGGAGAAGG CCTTCTCCGCGTCGGACTC 68.4% -2.0 0
867- 885 AGUCCGACGCGGAGAAGGA TCCTTCTCCGCGTCGGACT 63.2% -1.5 0
868- 886 GUCCGACGCGGAGAAGGAC GTCCTTCTCCGCGTCGGAC 68.4% -1.5 0
869- 887 UCCGACGCGGAGAAGGACG CGTCCTTCTCCGCGTCGGA 68.4% -1.6 0
870- 888 CCGACGCGGAGAAGGACGA TCGTCCTTCTCCGCGTCGG 68.4% -2.1 0
871- 889 CGACGCGGAGAAGGACGAC GTCGTCCTTCTCCGCGTCG 68.4% -2.4 0
872- 890 GACGCGGAGAAGGACGACG CGTCGTCCTTCTCCGCGTC 68.4% -2.6 0
873- 891 ACGCGGAGAAGGACGACGG CCGTCGTCCTTCTCCGCGT 68.4% -2.4 0
874- 892 CGCGGAGAAGGACGACGGG CCCGTCGTCCTTCTCCGCG 73.7% -2.3 0
875- 893 GCGGAGAAGGACGACGGGC GCCCGTCGTCCTTCTCCGC 73.7% -2.4 0
876- 894 CGGAGAAGGACGACGGGCC GGCCCGTCGTCCTTCTCCG 73.7% -3.1 0
877- 895 GGAGAAGGACGACGGGCCC GGGCCCGTCGTCCTTCTCC 73.7% -4.0 0
878- 896 GAGAAGGACGACGGGCCCU AGGGCCCGTCGTCCTTCTC 68.4% -3.9 0
879- 897 AGAAGGACGACGGGCCCUA TAGGGCCCGTCGTCCTTCT 63.2% -4.0 0
880- 898 GAAGGACGACGGGCCCUAC GTAGGGCCCGTCGTCCTTC 68.4% -5.4 0
881- 899 AAGGACGACGGGCCCUACC GGTAGGGCCCGTCGTCCTT 68.4% -6.9 0
882- 900 AGGACGACGGGCCCUACCG CGGTAGGGCCCGTCGTCCT 73.7% -6.7 0
883- 901 GGACGACGGGCCCUACCGG CCGGTAGGGCCCGTCGTCC 78.9% -5.7 0
884- 902 GACGACGGGCCCUACCGGG CCCGGTAGGGCCCGTCGTC 78.9% -5.4 0
885- 903 ACGACGGGCCCUACCGGGU ACCCGGTAGGGCCCGTCGT 73.7% -5.2 0
886- 904 CGACGGGCCCUACCGGGUG CACCCGGTAGGGCCCGTCG 78.9% -4.8 0
887- 905 GACGGGCCCUACCGGGUGG CCACCCGGTAGGGCCCGTC 78.9% -4.5 0

888- 906 ACGGGCCCUACCGGGUGGA TCCACCCGGTAGGGCCCGT 73.7% -4.1 0
889- 907 CGGGCCCUACCGGGUGGAG CTCCACCCGGTAGGGCCCG 78.9% -4.4 0
890- 908 GGGCCCUACCGGGUGGAGC GCTCCACCCGGTAGGGCCC 78.9% -5.2 0
891- 909 GGCCCUACCGGGUGGAGCA TGCTCCACCCGGTAGGGCC 73.7% -5.8 0
892- 910 GCCCUACCGGGUGGAGCAC GTGCTCCACCCGGTAGGGC 73.7% -6.7 0
893- 911 CCCUACCGGGUGGAGCACU AGTGCTCCACCCGGTAGGG 68.4% -6.7 0
894- 912 CCUACCGGGUGGAGCACUU AAGTGCTCCACCCGGTAGG 63.2% -5.5 0
895- 913 CUACCGGGUGGAGCACUUC GAAGTGCTCCACCCGGTAG 63.2% -4.7 0
896- 914 UACCGGGUGGAGCACUUCC GGAAGTGCTCCACCCGGTA 63.2% -4.8 0
897- 915 ACCGGGUGGAGCACUUCCG CGGAAGTGCTCCACCCGGT 68.4% -5.7 0
898- 916 CCGGGUGGAGCACUUCCGC GCGGAAGTGCTCCACCCGG 73.7% -6.6 0
899- 917 CGGGUGGAGCACUUCCGCU AGCGGAAGTGCTCCACCCG 68.4% -5.8 0
900- 918 GGGUGGAGCACUUCCGCUG CAGCGGAAGTGCTCCACCC 68.4% -6.3 0
901- 919 GGUGGAGCACUUCCGCUGG CCAGCGGAAGTGCTCCACC 68.4% -6.3 0
902- 920 GUGGAGCACUUCCGCUGGA TCCAGCGGAAGTGCTCCAC 63.2% -6.4 0
903- 921 UGGAGCACUUCCGCUGGAG CTCCAGCGGAAGTGCTCCA 63.2% -6.4 0
904- 922 GGAGCACUUCCGCUGGAGC GCTCCAGCGGAAGTGCTCC 68.4% -6.6 0
905- 923 GAGCACUUCCGCUGGAGCA TGCTCCAGCGGAAGTGCTC 63.2% -7.3 0
906- 924 AGCACUUCCGCUGGAGCAA TTGCTCCAGCGGAAGTGCT 57.9% -8.2 0
907- 925 GCACUUCCGCUGGAGCAAC GTTGCTCCAGCGGAAGTGC 63.2% -8.9 0
908- 926 CACUUCCGCUGGAGCAACC GGTTGCTCCAGCGGAAGTG 63.2% -8.0 0
909- 927 ACUUCCGCUGGAGCAACCC GGGTTGCTCCAGCGGAAGT 63.2% -7.4 0
910- 928 CUUCCGCUGGAGCAACCCG CGGGTTGCTCCAGCGGAAG 68.4% -6.6 0
911- 929 UUCCGCUGGAGCAACCCGC GCGGGTTGCTCCAGCGGAA 68.4% -6.4 0
912- 930 UCCGCUGGAGCAACCCGCC GGCGGGTTGCTCCAGCGGA 73.7% -6.4 0
913- 931 CCGCUGGAGCAACCCGCCC GGGCGGGTTGCTCCAGCGG 78.9% -6.1 0
914- 932 CGCUGGAGCAACCCGCCCA TGGGCGGGTTGCTCCAGCG 73.7% -5.5 0
915- 933 GCUGGAGCAACCCGCCCAA TTGGGCGGGTTGCTCCAGC 68.4% -5.3 0
916- 934 CUGGAGCAACCCGCCCAAG CTTGGGCGGGTTGCTCCAG 68.4% -3.2 0
917- 935 UGGAGCAACCCGCCCAAGG CCTTGGGCGGGTTGCTCCA 68.4% -3.0 0
918- 936 GGAGCAACCCGCCCAAGGA TCCTTGGGCGGGTTGCTCC 68.4% -2.9 0
919- 937 GAGCAACCCGCCCAAGGAC GTCCTTGGGCGGGTTGCTC 68.4% -3.4 0
920- 938 AGCAACCCGCCCAAGGACA TGTCCTTGGGCGGGTTGCT 63.2% -3.6 0
921- 939 GCAACCCGCCCAAGGACAA TTGTCCTTGGGCGGGTTGC 63.2% -4.3 0
922- 940 CAACCCGCCCAAGGACAAG CTTGTCCTTGGGCGGGTTG 63.2% -4.9 0
923- 941 AACCCGCCCAAGGACAAGC GCTTGTCCTTGGGCGGGTT 63.2% -5.6 0
924- 942 ACCCGCCCAAGGACAAGCG CGCTTGTCCTTGGGCGGGT 68.4% -4.7 0
925- 943 CCCGCCCAAGGACAAGCGU ACGCTTGTCCTTGGGCGGG 68.4% -3.3 0
926- 944 CCGCCCAAGGACAAGCGUU AACGCTTGTCCTTGGGCGG 63.2% -3.3 0
927- 945 CGCCCAAGGACAAGCGUUA TAACGCTTGTCCTTGGGCG 57.9% -3.3 0
928- 946 GCCCAAGGACAAGCGUUAC GTAACGCTTGTCCTTGGGC 57.9% -4.0 0
929- 947 CCCAAGGACAAGCGUUACG CGTAACGCTTGTCCTTGGG 57.9% -4.1 0
930- 948 CCAAGGACAAGCGUUACGG CCGTAACGCTTGTCCTTGG 57.9% -4.1 0
931- 949 CAAGGACAAGCGUUACGGU ACCGTAACGCTTGTCCTTG 52.6% -4.1 0
932- 950 AAGGACAAGCGUUACGGUG CACCGTAACGCTTGTCCTT 52.6% -4.1 0
933- 951 AGGACAAGCGUUACGGUGG CCACCGTAACGCTTGTCCT 57.9% -3.1 0
934- 952 GGACAAGCGUUACGGUGGC GCCACCGTAACGCTTGTCC 63.2% -2.7 0
935- 953 GACAAGCGUUACGGUGGCU AGCCACCGTAACGCTTGTC 57.9% -2.1 0
936- 954 ACAAGCGUUACGGUGGCUU AAGCCACCGTAACGCTTGT 52.6% -1.8 0
937- 955 CAAGCGUUACGGUGGCUUC GAAGCCACCGTAACGCTTG 57.9% -1.3 0
938- 956 AAGCGUUACGGUGGCUUCA TGAAGCCACCGTAACGCTT 52.6% -1.1 0
939- 957 AGCGUUACGGUGGCUUCAU ATGAAGCCACCGTAACGCT 52.6% -0.9 0
940- 958 GCGUUACGGUGGCUUCAUG CATGAAGCCACCGTAACGC 57.9% -0.8 0
941- 959 CGUUACGGUGGCUUCAUGA TCATGAAGCCACCGTAACG 52.6% -0.2 0
942- 960 GUUACGGUGGCUUCAUGAC GTCATGAAGCCACCGTAAC 52.6% -1.4 0
943- 961 UUACGGUGGCUUCAUGACC GGTCATGAAGCCACCGTAA 52.6% -2.7 0
944- 962 UACGGUGGCUUCAUGACCU AGGTCATGAAGCCACCGTA 52.6% -3.2 0
945- 963 ACGGUGGCUUCAUGACCUC GAGGTCATGAAGCCACCGT 57.9% -4.1 0
946- 964 CGGUGGCUUCAUGACCUCC GGAGGTCATGAAGCCACCG 63.2% -4.6 0
947- 965 GGUGGCUUCAUGACCUCCG CGGAGGTCATGAAGCCACC 63.2% -5.0 0

948- 966	GUGGCUUCAUGACCUCCGA	TCGGAGGTCATGAAGCCAC	57.9%	-5.6	0
949- 967	UGGCUUCAUGACCUCCGAG	CTCGGAGGTCATGAAGCCA	57.9%	-5.7	0
950- 968	GGCUUCAUGACCUCCGAGA	TCTCGGAGGTCATGAAGCC	57.9%	-5.6	0
951- 969	GCUUCAUGACCUCCGAGAA	TTCTCGGAGGTCATGAAGC	52.6%	-5.7	0
952- 970	CUUCAUGACCUCCGAGAAG	CTTCTCGGAGGTCATGAAG	52.6%	-5.8	0
953- 971	UUCAUGACCUCCGAGAAGA	TCTTCTCGGAGGTCATGAA	47.4%	-5.9	0
954- 972	UCAUGACCUCCGAGAAGAG	CTCTTCTCGGAGGTCATGA	52.6%	-5.9	0
955- 973	CAUGACCUCCGAGAAGAGC	GCTCTTCTCGGAGGTCATG	57.9%	-5.8	0
956- 974	AUGACCUCCGAGAAGAGCC	GGCTCTTCTCGGAGGTCAT	57.9%	-5.7	0
957- 975	UGACCUCCGAGAAGAGCCA	TGGCTCTTCTCGGAGGTCA	57.9%	-5.6	0
958- 976	GACCUCCGAGAAGAGCCAG	CTGGCTCTTCTCGGAGGTC	63.2%	-6.2	0
959- 977	ACCUCCGAGAAGAGCCAGA	TCTGGCTCTTCTCGGAGGT	57.9%	-6.4	0
960- 978	CCUCCGAGAAGAGCCAGAC	GTCTGGCTCTTCTCGGAGG	63.2%	-5.5	0
961- 979	CUCCGAGAAGAGCCAGACG	CGTCTGGCTCTTCTCGGAG	63.2%	-4.3	0
962- 980	UCCGAGAAGAGCCAGACGC	GCGTCTGGCTCTTCTCGGA	63.2%	-3.9	0
963- 981	CCGAGAAGAGCCAGACGCC	GGCGTCTGGCTCTTCTCGG	68.4%	-3.1	0
964- 982	CGAGAAGAGCCAGACGCCC	GGGCGTCTGGCTCTTCTCG	68.4%	-2.5	0
965- 983	GAGAAGAGCCAGACGCCCC	GGGGCGTCTGGCTCTTCTC	68.4%	-3.2	0
966- 984	AGAAGAGCCAGACGCCCCU	AGGGGCGTCTGGCTCTTCT	63.2%	-3.3	0
967- 985	GAAGAGCCAGACGCCCCUG	CAGGGGCGTCTGGCTCTTC	68.4%	-3.6	0
968- 986	AAGAGCCAGACGCCCCUGG	CCAGGGGCGTCTGGCTCTT	68.4%	-3.6	0
969- 987	AGAGCCAGACGCCCCUGGU	ACCAGGGGCGTCTGGCTCT	68.4%	-3.6	0
970- 988	GAGCCAGACGCCCCUGGUG	CACCAGGGGCGTCTGGCTC	73.7%	-3.6	0
971- 989	AGCCAGACGCCCCUGGUGA	TCACCAGGGGCGTCTGGCT	68.4%	-3.7	0
972- 990	GCCAGACGCCCCUGGUGAC	GTCACCAGGGGCGTCTGGC	73.7%	-5.4	0
973- 991	CCAGACGCCCCUGGUGACG	CGTCACCAGGGGCGTCTGG	73.7%	-5.6	0
974- 992	CAGACGCCCCUGGUGACGC	GCGTCACCAGGGGCGTCTG	73.7%	-5.6	0
975- 993	AGACGCCCCUGGUGACGCU	AGCGTCACCAGGGGCGTCT	68.4%	-5.1	0
976- 994	GACGCCCCUGGUGACGCUC	GAGCGTCACCAGGGGCGTC	73.7%	-3.8	0
977- 995	ACGCCCCUGGUGACGCUCU	AGAGCGTCACCAGGGGCGT	68.4%	-2.9	0
978- 996	CGCCCCUGGUGACGCUCUU	AAGAGCGTCACCAGGGGCG	68.4%	-2.6	0
979- 997	GCCCCUGGUGACGCUCUUC	GAAGAGCGTCACCAGGGGC	68.4%	-2.4	0
980- 998	CCCCUGGUGACGCUCUUCA	TGAAGAGCGTCACCAGGGG	63.2%	-2.2	0
981- 999	CCCUGGUGACGCUCUUCAA	TTGAAGAGCGTCACCAGGG	57.9%	-3.0	0
982-1000	CCUGGUGACGCUCUUCAAG	CTTGAAGAGCGTCACCAGG	57.9%	-3.1	0
983-1001	CUGGUGACGCUCUUCAAGA	TCTTGAAGAGCGTCACCAG	52.6%	-2.4	0
984-1002	UGGUGACGCUCUUCAAGAA	TTCTTGAAGAGCGTCACCA	47.4%	-2.4	0
985-1003	GGUGACGCUCUUCAAGAAC	GTTCTTGAAGAGCGTCACC	52.6%	-2.5	0
986-1004	GUGACGCUCUUCAAGAACG	CGTTCTTGAAGAGCGTCAC	52.6%	-2.7	0
987-1005	UGACGCUCUUCAAGAACGC	GCGTTCTTGAAGAGCGTCA	52.6%	-3.0	0
988-1006	GACGCUCUUCAAGAACGCC	GGCGTTCTTGAAGAGCGTC	57.9%	-3.2	0
989-1007	ACGCUCUUCAAGAACGCCA	TGGCGTTCTTGAAGAGCGT	52.6%	-3.7	0
990-1008	CGCUCUUCAAGAACGCCAU	ATGGCGTTCTTGAAGAGCG	52.6%	-2.1	0
991-1009	GCUCUUCAAGAACGCCAUC	GATGGCGTTCTTGAAGAGC	52.6%	-2.2	0
992-1010	CUCUUCAAGAACGCCAUCA	TGATGGCGTTCTTGAAGAG	47.4%	-2.4	0
993-1011	UCUUCAAGAACGCCAUCAU	ATGATGGCGTTCTTGAAGA	42.1%	-3.2	0
994-1012	CUUCAAGAACGCCAUCAUC	GATGATGGCGTTCTTGAAG	47.4%	-4.6	0
995-1013	UUCAAGAACGCCAUCAUCA	TGATGATGGCGTTCTTGAA	42.1%	-5.4	0
996-1014	UCAAGAACGCCAUCAUCAA	TTGATGATGGCGTTCTTGA	42.1%	-6.4	0
997-1015	CAAGAACGCCAUCAUCAAG	CTTGATGATGGCGTTCTTG	47.4%	-7.2	0
998-1016	AAGAACGCCAUCAUCAAGA	TCTTGATGATGGCGTTCTT	42.1%	-7.4	0
999-1017	AGAACGCCAUCAUCAAGAA	TTCTTGATGATGGCGTTCT	42.1%	-6.8	0
1000-1018	GAACGCCAUCAUCAAGAAC	GTTCTTGATGATGGCGTTC	47.4%	-7.6	0
1001-1019	AACGCCAUCAUCAAGAACG	CGTTCTTGATGATGGCGTT	47.4%	-7.3	0
1002-1020	ACGCCAUCAUCAAGAACGC	GCGTTCTTGATGATGGCGT	52.6%	-6.9	0
1003-1021	CGCCAUCAUCAAGAACGCG	CGCGTTCTTGATGATGGCG	57.9%	-6.5	0
1004-1022	GCCAUCAUCAAGAACGCGC	GCGCGTTCTTGATGATGGC	57.9%	-7.0	0
1005-1023	CCAUCAUCAAGAACGCGCA	TGCGCGTTCTTGATGATGG	52.6%	-7.3	0
1006-1024	CAUCAUCAAGAACGCGCAC	GTGCGCGTTCTTGATGATG	52.6%	-8.0	0
1007-1025	AUCAUCAAGAACGCGCACA	TGTGCGCGTTCTTGATGAT	47.4%	-7.8	0

1008-1026	UCAUCAAGAACGCGCACAA	TTGTGCGCGTTCTTGATGA	47.4%	-8.6	0
1009-1027	CAUCAAGAACGCGCACAAG	CTTGTGCGCGTTCTTGATG	52.6%	-9.3	0
1010-1028	AUCAAGAACGCGCACAAGA	TCTTGTGCGCGTTCTTGAT	47.4%	-9.7	0
1011-1029	UCAAGAACGCGCACAAGAA	TTCTTGTGCGCGTTCTTGA	47.4%	-9.2	0
1012-1030	CAAGAACGCGCACAAGAAG	CTTCTTGTGCGCGTTCTTG	52.6%	-8.4	0
1013-1031	AAGAACGCGCACAAGAAGG	CCTTCTTGTGCGCGTTCTT	52.6%	-8.3	0
1014-1032	AGAACGCGCACAAGAAGGG	CCCTTCTTGTGCGCGTTCT	57.9%	-7.4	0
1015-1033	GAACGCGCACAAGAAGGGC	GCCCTTCTTGTGCGCGTTC	63.2%	-6.6	0
1016-1034	AACGCGCACAAGAAGGGCC	GGCCCTTCTTGTGCGCGTT	63.2%	-6.3	0
1017-1035	ACGCGCACAAGAAGGGCCA	TGGCCCTTCTTGTGCGCGT	63.2%	-6.1	0
1018-1036	CGCGCACAAGAAGGGCCAG	CTGGCCCTTCTTGTGCGCG	68.4%	-5.1	0
1019-1037	GCGCACAAGAAGGGCCAGU	ACTGGCCCTTCTTGTGCGC	63.2%	-5.0	0
1020-1038	CGCACAAGAAGGGCCAGUG	CACTGGCCCTTCTTGTGCG	63.2%	-4.8	0
1021-1039	GCACAAGAAGGGCCAGUGA	TCACTGGCCCTTCTTGTGC	57.9%	-5.1	0
1022-1040	CACAAGAAGGGCCAGUGAG	CTCACTGGCCCTTCTTGTG	57.9%	-4.4	0
1023-1041	ACAAGAAGGGCCAGUGAGG	CCTCACTGGCCCTTCTTGT	57.9%	-3.7	0
1024-1042	CAAGAAGGGCCAGUGAGGG	CCCTCACTGGCCCTTCTTG	63.2%	-2.7	0
1025-1043	AAGAAGGGCCAGUGAGGGU	ACCCTCACTGGCCCTTCTT	57.9%	-2.2	0
1026-1044	AGAAGGGCCAGUGAGGGUG	CACCCTCACTGGCCCTTCT	63.2%	-1.6	0
1027-1045	GAAGGGCCAGUGAGGGUGC	GCACCCTCACTGGCCCTTC	68.4%	-1.5	0
1028-1046	AAGGGCCAGUGAGGGUGCA	TGCACCCTCACTGGCCCTT	63.2%	-1.5	0
1029-1047	AGGGCCAGUGAGGGUGCAG	CTGCACCCTCACTGGCCCT	68.4%	-1.8	0
1030-1048	GGGCCAGUGAGGGUGCAGG	CCTGCACCCTCACTGGCCC	73.7%	-1.3	0
1031-1049	GGCCAGUGAGGGUGCAGGG	CCCTGCACCCTCACTGGCC	73.7%	-0.5	0
1032-1050	GCCAGUGAGGGUGCAGGGG	CCCCTGCACCCTCACTGGC	73.7%	-0.5	1
1033-1051	CCAGUGAGGGUGCAGGGGU	ACCCCTGCACCCTCACTGG	68.4%	-0.5	1
1034-1052	CAGUGAGGGUGCAGGGGUC	GACCCCTGCACCCTCACTG	68.4%	-0.5	1
1035-1053	AGUGAGGGUGCAGGGGUCU	AGACCCCTGCACCCTCACT	63.2%	-0.4	1
1036-1054	GUGAGGGUGCAGGGGUCUU	AAGACCCCTGCACCCTCAC	63.2%	0.1	1
1037-1055	UGAGGGUGCAGGGGUCUUC	GAAGACCCCTGCACCCTCA	63.2%	0.1	1
1038-1056	GAGGGUGCAGGGGUCUUCU	AGAAGACCCCTGCACCCTC	63.2%	-0.3	1
1039-1057	AGGGUGCAGGGGUCUUCUC	GAGAAGACCCCTGCACCCT	63.2%	-1.0	1
1040-1058	GGGUGCAGGGGUCUUCUCA	TGAGAAGACCCCTGCACCC	63.2%	-1.5	1
1041-1059	GGUGCAGGGGUCUUCUCAU	ATGAGAAGACCCCTGCACC	57.9%	-2.3	1
1042-1060	GUGCAGGGGUCUUCUCAUU	AATGAGAAGACCCCTGCAC	52.6%	-2.4	1
1043-1061	UGCAGGGGUCUUCUCAUUC	GAATGAGAAGACCCCTGCA	52.6%	-3.6	1
1044-1062	GCAGGGGUCUUCUCAUUCC	GGAATGAGAAGACCCCTGC	57.9%	-4.8	1
1045-1063	CAGGGGUCUUCUCAUUCCA	TGGAATGAGAAGACCCCTG	52.6%	-4.2	1
1046-1064	AGGGGUCUUCUCAUUCCAA	TTGGAATGAGAAGACCCCT	47.4%	-3.8	1
1047-1065	GGGGUCUUCUCAUUCCAAG	CTTGGAATGAGAAGACCCC	52.6%	-3.6	1
1048-1066	GGGUCUUCUCAUUCCAAGG	CCTTGGAATGAGAAGACCC	52.6%	-3.7	0
1049-1067	GGUCUUCUCAUUCCAAGGC	GCCTTGGAATGAGAAGACC	52.6%	-3.8	0
1050-1068	GUCUUCUCAUUCCAAGGCC	GGCCTTGGAATGAGAAGAC	52.6%	-3.8	0
1051-1069	UCUUCUCAUUCCAAGGCCC	GGGCCTTGGAATGAGAAGA	52.6%	-4.0	0
1052-1070	CUUCUCAUUCCAAGGCCCC	GGGGCCTTGGAATGAGAAG	57.9%	-4.4	0
1053-1071	UUCUCAUUCCAAGGCCCCC	GGGGGCCTTGGAATGAGAA	57.9%	-4.8	0
1054-1072	UCUCAUUCCAAGGCCCCCU	AGGGGGCCTTGGAATGAGA	57.9%	-5.2	0
1055-1073	CUCAUUCCAAGGCCCCCUC	GAGGGGGCCTTGGAATGAG	63.2%	-5.6	0
1056-1074	UCAUUCCAAGGCCCCCUCC	GGAGGGGGCCTTGGAATGA	63.2%	-5.5	0
1057-1075	CAUUCCAAGGCCCCCUCCC	GGGAGGGGGCCTTGGAATG	68.4%	-5.2	0
1058-1076	AUUCCAAGGCCCCCUCCCU	AGGGAGGGGGCCTTGGAAT	63.2%	-4.8	0
1059-1077	UUCCAAGGCCCCCUCCCUG	CAGGGAGGGGGCCTTGGAA	68.4%	-4.2	0
1060-1078	UCCAAGGCCCCCUCCCUGC	GCAGGGAGGGGGCCTTGGA	73.7%	-4.5	0
1061-1079	CCAAGGCCCCCUCCCUGCA	TGCAGGGAGGGGGCCTTGG	73.7%	-3.5	0
1062-1080	CAAGGCCCCCUCCCUGCAU	ATGCAGGGAGGGGGCCTTG	68.4%	-2.2	0
1063-1081	AAGGCCCCCUCCCUGCAUG	CATGCAGGGAGGGGGCCTT	68.4%	-2.2	0
1064-1082	AGGCCCCCUCCCUGCAUGG	CCATGCAGGGAGGGGGCCT	73.7%	-2.0	0
1065-1083	GGCCCCCUCCCUGCAUGGG	CCCATGCAGGGAGGGGGCC	78.9%	-1.7	0
1066-1084	GCCCCCUCCCUGCAUGGGC	GCCCATGCAGGGAGGGGGC	78.9%	-1.6	0
1067-1085	CCCCCUCCCUGCAUGGGCG	CGCCCATGCAGGGAGGGGG	78.9%	-1.6	0

1068-1086	CCCCUCCCUGCAUGGGCGA	TCGCCCATGCAGGGAGGGG	73.7%	-2.4	0
1069-1087	CCCUCCCUGCAUGGGCGAG	CTCGCCCATGCAGGGAGGG	73.7%	-2.6	0
1070-1088	CCUCCCUGCAUGGGCGAGC	GCTCGCCCATGCAGGGAGG	73.7%	-2.5	0
1071-1089	CUCCCUGCAUGGGCGAGCU	AGCTCGCCCATGCAGGGAG	68.4%	-2.2	0
1072-1090	UCCCUGCAUGGGCGAGCUG	CAGCTCGCCCATGCAGGGA	68.4%	-1.9	0
1073-1091	CCCUGCAUGGGCGAGCUGA	TCAGCTCGCCCATGCAGGG	68.4%	-2.6	0
1074-1092	CCUGCAUGGGCGAGCUGAU	ATCAGCTCGCCCATGCAGG	63.2%	-2.2	0
1075-1093	CUGCAUGGGCGAGCUGAUG	CATCAGCTCGCCCATGCAG	63.2%	-1.6	0
1076-1094	UGCAUGGGCGAGCUGAUGA	TCATCAGCTCGCCCATGCA	57.9%	-1.5	0
1077-1095	GCAUGGGCGAGCUGAUGAC	GTCATCAGCTCGCCCATGC	63.2%	-1.4	0
1078-1096	CAUGGGCGAGCUGAUGACC	GGTCATCAGCTCGCCCATG	63.2%	-1.0	0
1079-1097	AUGGGCGAGCUGAUGACCU	AGGTCATCAGCTCGCCCAT	57.9%	-0.9	0
1080-1098	UGGGCGAGCUGAUGACCUC	GAGGTCATCAGCTCGCCCA	63.2%	-0.6	0
1081-1099	GGGCGAGCUGAUGACCUCU	AGAGGTCATCAGCTCGCCC	63.2%	-0.4	0
1082-1100	GGCGAGCUGAUGACCUCUA	TAGAGGTCATCAGCTCGCC	57.9%	-0.5	0
1083-1101	GCGAGCUGAUGACCUCUAG	CTAGAGGTCATCAGCTCGC	57.9%	-0.4	0
1084-1102	CGAGCUGAUGACCUCUAGC	GCTAGAGGTCATCAGCTCG	57.9%	-0.4	0
1085-1103	GAGCUGAUGACCUCUAGCC	GGCTAGAGGTCATCAGCTC	57.9%	-0.3	0
1086-1104	AGCUGAUGACCUCUAGCCU	AGGCTAGAGGTCATCAGCT	52.6%	0.5	0
1087-1105	GCUGAUGACCUCUAGCCUC	GAGGCTAGAGGTCATCAGC	57.9%	0.8	0
1088-1106	CUGAUGACCUCUAGCCUCU	AGAGGCTAGAGGTCATCAG	52.6%	1.1	0
1089-1107	UGAUGACCUCUAGCCUCUU	AAGAGGCTAGAGGTCATCA	47.4%	1.2	0
1090-1108	GAUGACCUCUAGCCUCUUA	TAAGAGGCTAGAGGTCATC	47.4%	1.4	0
1091-1109	AUGACCUCUAGCCUCUUAG	CTAAGAGGCTAGAGGTCAT	47.4%	2.4	0
1092-1110	UGACCUCUAGCCUCUUAGA	TCTAAGAGGCTAGAGGTCA	47.4%	2.5	0
1093-1111	GACCUCUAGCCUCUUAGAG	CTCTAAGAGGCTAGAGGTC	52.6%	2.6	0
1094-1112	ACCUCUAGCCUCUUAGAGU	ACTCTAAGAGGCTAGAGGT	47.4%	2.6	0
1095-1113	CCUCUAGCCUCUUAGAGUU	AACTCTAAGAGGCTAGAGG	47.4%	2.8	0
1096-1114	CUCUAGCCUCUUAGAGUUA	TAACTCTAAGAGGCTAGAG	42.1%	2.8	0
1097-1115	UCUAGCCUCUUAGAGUUAC	GTAACTCTAAGAGGCTAGA	42.1%	0.7	0
1098-1116	CUAGCCUCUUAGAGUUACC	GGTAACTCTAAGAGGCTAG	47.4%	0.7	0
1099-1117	UAGCCUCUUAGAGUUACCU	AGGTAACTCTAAGAGGCTA	42.1%	0.7	0
1100-1118	AGCCUCUUAGAGUUACCUG	CAGGTAACTCTAAGAGGCT	47.4%	0.8	0
1101-1119	GCCUCUUAGAGUUACCUGU	ACAGGTAACTCTAAGAGGC	47.4%	0.8	0
1102-1120	CCUCUUAGAGUUACCUGUG	CACAGGTAACTCTAAGAGG	47.4%	0.9	0
1103-1121	CUCUUAGAGUUACCUGUGU	ACACAGGTAACTCTAAGAG	42.1%	0.9	0
1104-1122	UCUUAGAGUUACCUGUGUU	AACACAGGTAACTCTAAGA	36.8%	0.7	0
1105-1123	CUUAGAGUUACCUGUGUUA	TAACACAGGTAACTCTAAG	36.8%	0.3	0
1106-1124	UUAGAGUUACCUGUGUUAG	CTAACACAGGTAACTCTAA	36.8%	-0.9	0
1107-1125	UAGAGUUACCUGUGUUAGG	CCTAACACAGGTAACTCTA	42.1%	-0.9	0
1108-1126	AGAGUUACCUGUGUUAGGA	TCCTAACACAGGTAACTCT	42.1%	-1.0	0
1109-1127	GAGUUACCUGUGUUAGGAA	TTCCTAACACAGGTAACTC	42.1%	-1.1	0
1110-1128	AGUUACCUGUGUUAGGAAA	TTTCCTAACACAGGTAACT	36.8%	-1.3	0
1111-1129	GUUACCUGUGUUAGGAAAU	ATTTCCTAACACAGGTAAC	36.8%	-1.7	0
1112-1130	UUACCUGUGUUAGGAAAUA	TATTTCCTAACACAGGTAA	31.6%	-2.3	0
1113-1131	UACCUGUGUUAGGAAAUAA	TTATTTCCTAACACAGGTA	31.6%	-3.3	0
1114-1132	ACCUGUGUUAGGAAAUAAA	TTTATTTCCTAACACAGGT	31.6%	-4.2	0
1115-1133	CCUGUGUUAGGAAAUAAAA	TTTTATTTCCTAACACAGG	31.6%	-3.2	0
1116-1134	CUGUGUUAGGAAAUAAAAC	GTTTTATTTCCTAACACAG	31.6%	-4.9	0
1117-1135	UGUGUUAGGAAAUAAAACC	GGTTTTATTTCCTAACACA	31.6%	-6.7	0
1118-1136	GUGUUAGGAAAUAAAACCU	AGGTTTTATTTCCTAACAC	31.6%	-6.8	0
1119-1137	UGUUAGGAAAUAAAACCUU	AAGGTTTTATTTCCTAACA	26.3%	-6.8	0
1120-1138	GUUAGGAAAUAAAACCUUU	AAAGGTTTTATTTCCTAAC	26.3%	-6.8	0
1121-1139	UUAGGAAAUAAAACCUUUC	GAAAGGTTTTATTTCCTAA	26.3%	-7.1	0
1122-1140	UAGGAAAUAAAACCUUUCA	TGAAAGGTTTTATTTCCTA	26.3%	-7.1	0
1123-1141	AGGAAAUAAAACCUUUCAG	CTGAAAGGTTTTATTTCCT	31.6%	-8.3	0
1124-1142	GGAAAUAAAACCUUUCAGA	TCTGAAAGGTTTTATTTCC	31.6%	-8.2	0
1125-1143	GAAAUAAAACCUUUCAGAU	ATCTGAAAGGTTTTATTTC	26.3%	-8.9	0
1126-1144	AAAUAAAACCUUUCAGAUU	AATCTGAAAGGTTTTATTT	21.1%	-9.0	0
1127-1145	AAUAAAACCUUUCAGAUUU	AAATCTGAAAGGTTTTATT	21.1%	-9.1	0

1128-1146	AUAAAACCUUUCAGAUUUC	GAAATCTGAAAGGTTTTAT	26.3%	-8.9	0
1129-1147	UAAAACCUUUCAGAUUUCA	TGAAATCTGAAAGGTTTTA	26.3%	-8.5	0
1130-1148	AAAACCUUUCAGAUUUCAC	GTGAAATCTGAAAGGTTTT	31.6%	-8.0	0
1131-1149	AAACCUUUCAGAUUUCACA	TGTGAAATCTGAAAGGTTT	31.6%	-7.0	0
1132-1150	AACCUUUCAGAUUUCACAG	CTGTGAAATCTGAAAGGTT	36.8%	-6.0	0
1133-1151	ACCUUUCAGAUUUCACAGU	ACTGTGAAATCTGAAAGGT	36.8%	-5.0	0
1134-1152	CCUUUCAGAUUUCACAGUC	GACTGTGAAATCTGAAAGG	42.1%	-4.7	0
1135-1153	CUUUCAGAUUUCACAGUCG	CGACTGTGAAATCTGAAAG	42.1%	-3.0	0
1136-1154	UUUCAGAUUUCACAGUCGG	CCGACTGTGAAATCTGAAA	42.1%	-2.9	0
1137-1155	UUCAGAUUUCACAGUCGGC	GCCGACTGTGAAATCTGAA	47.4%	-2.9	0
1138-1156	UCAGAUUUCACAGUCGGCU	AGCCGACTGTGAAATCTGA	47.4%	-2.8	0
1139-1157	CAGAUUUCACAGUCGGCUC	GAGCCGACTGTGAAATCTG	52.6%	-2.6	0
1140-1158	AGAUUUCACAGUCGGCUCU	AGAGCCGACTGTGAAATCT	47.4%	-2.4	0
1141-1159	GAUUUCACAGUCGGCUCUG	CAGAGCCGACTGTGAAATC	52.6%	-0.8	0
1142-1160	AUUUCACAGUCGGCUCUGA	TCAGAGCCGACTGTGAAAT	47.4%	0.0	0
1143-1161	UUUCACAGUCGGCUCUGAU	ATCAGAGCCGACTGTGAAA	47.4%	0.2	0
1144-1162	UUCACAGUCGGCUCUGAUC	GATCAGAGCCGACTGTGAA	52.6%	-1.0	0
1145-1163	UCACAGUCGGCUCUGAUCU	AGATCAGAGCCGACTGTGA	52.6%	-1.6	0
1146-1164	CACAGUCGGCUCUGAUCUU	AAGATCAGAGCCGACTGTG	52.6%	-1.7	0
1147-1165	ACAGUCGGCUCUGAUCUUC	GAAGATCAGAGCCGACTGT	52.6%	-3.1	0
1148-1166	CAGUCGGCUCUGAUCUUCA	TGAAGATCAGAGCCGACTG	52.6%	-3.9	0
1149-1167	AGUCGGCUCUGAUCUUCAA	TTGAAGATCAGAGCCGACT	47.4%	-4.9	0
1150-1168	GUCGGCUCUGAUCUUCAAU	ATTGAAGATCAGAGCCGAC	47.4%	-5.8	0
1151-1169	UCGGCUCUGAUCUUCAAUA	TATTGAAGATCAGAGCCGA	42.1%	-6.4	0
1152-1170	CGGCUCUGAUCUUCAAUAA	TTATTGAAGATCAGAGCCG	42.1%	-5.9	0
1153-1171	GGCUCUGAUCUUCAAUAAA	TTTATTGAAGATCAGAGCC	36.8%	-6.8	0
1154-1172	GCUCUGAUCUUCAAUAAAA	TTTTATTGAAGATCAGAGC	31.6%	-7.8	0
1155-1173	CUCUGAUCUUCAAUAAAAA	TTTTTATTGAAGATCAGAG	26.3%	-8.7	0
1156-1174	UCUGAUCUUCAAUAAAAAC	GTTTTTATTGAAGATCAGA	26.3%	-10.6	0
1157-1175	CUGAUCUUCAAUAAAAACU	AGTTTTTATTGAAGATCAG	26.3%	-11.3	0
1158-1176	UGAUCUUCAAUAAAAACUG	CAGTTTTTATTGAAGATCA	26.3%	-12.6	0
1159-1177	GAUCUUCAAUAAAAACUGC	GCAGTTTTTATTGAAGATC	31.6%	-13.6	0
1160-1178	AUCUUCAAUAAAAACUGCG	CGCAGTTTTTATTGAAGAT	31.6%	-14.1	0
1161-1179	UCUUCAAUAAAAACUGCGU	ACGCAGTTTTTATTGAAGA	31.6%	-14.0	0
1162-1180	CUUCAAUAAAAACUGCGUA	TACGCAGTTTTTATTGAAG	31.6%	-12.9	0
1163-1181	UUCAAUAAAAACUGCGUAA	TTACGCAGTTTTTATTGAA	26.3%	-13.1	0
1164-1182	UCAAUAAAAACUGCGUAAA	TTTACGCAGTTTTTATTGA	26.3%	-13.9	0
1165-1183	CAAUAAAAACUGCGUAAAU	ATTTACGCAGTTTTTATTG	26.3%	-13.4	0
1166-1184	AAUAAAAACUGCGUAAAUA	TATTTACGCAGTTTTTATT	21.1%	-13.1	0
1167-1185	AUAAAAACUGCGUAAAUAA	TTATTTACGCAGTTTTTAT	21.1%	-13.1	0
1168-1186	UAAAAACUGCGUAAAUAAA	TTTATTTACGCAGTTTTTA	21.1%	-13.1	0
1169-1187	AAAAACUGCGUAAAUAAAG	CTTTATTTACGCAGTTTTT	26.3%	-13.2	0
1170-1188	AAAACUGCGUAAAUAAAGU	ACTTTATTTACGCAGTTTT	26.3%	-12.6	0
1171-1189	AAACUGCGUAAAUAAAGUC	GACTTTATTTACGCAGTTT	31.6%	-12.4	0
1172-1190	AACUGCGUAAAUAAAGUCA	TGACTTTATTTACGCAGTT	31.6%	-12.3	0
1173-1191	ACUGCGUAAAUAAAGUCAA	TTGACTTTATTTACGCAGT	31.6%	-12.4	0
1174-1192	CUGCGUAAAUAAAGUCAAA	TTTGACTTTATTTACGCAG	31.6%	-11.5	0
1175-1193	UGCGUAAAUAAAGUCAAAA	TTTTGACTTTATTTACGCA	26.3%	-11.6	0
1176-1194	GCGUAAAUAAAGUCAAAAC	GTTTTGACTTTATTTACGC	31.6%	-11.8	0
1177-1195	CGUAAAUAAAGUCAAAACA	TGTTTTGACTTTATTTACG	26.3%	-11.2	0
1178-1196	GUAAAUAAAGUCAAAACAC	GTGTTTTGACTTTATTTAC	26.3%	-12.0	0
1179-1197	UAAAUAAAGUCAAAACACA	TGTGTTTTGACTTTATTTA	21.1%	-12.1	0
1180-1198	AAAUAAAGUCAAAACACAA	TTGTGTTTTGACTTTATTT	21.1%	-12.7	0
1181-1199	AAUAAAGUCAAAACACAAC	GTTGTGTTTTGACTTTATT	26.3%	-13.6	0
1182-1200	AUAAAGUCAAAACACAACU	AGTTGTGTTTTGACTTTAT	26.3%	-13.4	0
1183-1201	UAAAGUCAAAACACAACUG	CAGTTGTGTTTTGACTTTA	31.6%	-12.5	0
1184-1202	AAAGUCAAAACACAACUGU	ACAGTTGTGTTTTGACTTT	31.6%	-12.0	0
1185-1203	AAGUCAAAACACAACUGUC	GACAGTTGTGTTTTGACTT	36.8%	-11.1	0
1186-1204	AGUCAAAACACAACUGUCC	GGACAGTTGTGTTTTGACT	42.1%	-10.2	0
1187-1205	GUCAAAACACAACUGUCCA	TGGACAGTTGTGTTTTGAC	42.1%	-9.6	0

```
1188-1206 UCAAAACACAACUGUCCAG CTGGACAGTTGTGTTTTGA  42.1%  -10.8  0
1189-1207 CAAAACACAACUGUCCAGU ACTGGACAGTTGTGTTTTG  42.1%  -10.9  0
1190-1208 AAAACACAACUGUCCAGUU AACTGGACAGTTGTGTTTT  36.8%  -10.2  0
1191-1209 AAACACAACUGUCCAGUUA TAACTGGACAGTTGTGTTT  36.8%   -9.8  0
1192-1210 AACACAACUGUCCAGUUAC GTAACTGGACAGTTGTGTT  42.1%  -10.9  0
1193-1211 ACACAACUGUCCAGUUACA TGTAACTGGACAGTTGTGT  42.1%  -11.0  0
1194-1212 CACAACUGUCCAGUUACAC GTGTAACTGGACAGTTGTG  47.4%  -11.4  0
1195-1213 ACAACUGUCCAGUUACACU AGTGTAACTGGACAGTTGT  42.1%  -11.8  0
1196-1214 CAACUGUCCAGUUACACUA TAGTGTAACTGGACAGTTG  42.1%  -10.9  0
```

Appendix II: Homo sapiens *POMC* transcript

RefSeq accession number: NM_000939.4

GGCGGCGAAGGAGGGGAAGAAGAGCCGCGACCGAGAGAGGCCGCCGAGCGTCCCCGCCCTCAGAGAGCAG
CCTCCCGAGACAGAGCCTCAGCCTGCCTGGAAGATGCCGAGATCGTGCTGCAGCCGCTCGGGGGCCCTGT
TGCTGGCCTTGCTGCTTCAGGCCTCCATGGAAGTGCGTGGCTGGTGCCTGGAGAGCAGCCAGTGTCAGGA
CCTCACCACGGAAAGCAACCTGCTGGAGTGCATCCGGGCCTGCAAGCCCGACCTCTCGGCCGAGACTCCC
ATGTTCCCGGGAAATGGCGACGAGCAGCCTCTGACCGAGAACCCCCGGAAGTACGTCATGGGCCACTTCC
GCTGGGACCGATTCGGCCGCCGCAACAGCAGCAGCAGCGGCAGCAGCGGCGCAGGGCAGAAGCGCGAGGA
CGTCTCAGCGGGCGAAGACTGCGGCCCGCTGCCTGAGGGCGGCCCCGAGCCCCGCAGCGATGGTGCCAAG
CCGGGCCCGCGCGAGGGCAAGCGCTCCTACTCCATGGAGCACTTCCGCTGGGGCAAGCCGGTGGGCAAGA
AGCGGCGCCCAGTGAAGGTGTACCCTAACGGCGCCGAGGACGAGTCGGCCGAGGCCTTCCCCCTGGAGTT
CAAGAGGGAGCTGACTGGCCAGCGACTCCGGGAGGGAGATGGCCCCGACGGCCCTGCCGATGACGGCGCA
GGGGCCCAGGCCGACCTGGAGCACAGCCTGCTGGTGGCGGCCGAGAAGAAGGACGAGGGCCCCTACAGGA
TGGAGCACTTCCGCTGGGGCAGCCCGCCCAAGGACAAGCGCTACGGCGGTTTCATGACCTCCGAGAAGAG
CCAGACGCCCCTGGTGACGCTGTTCAAAAACGCCATCATCAAGAACGCCTACAAGAAGGGCGAGTGAGGG
CACAGCGGGGCCCCAGGGCTACCCTCCCCCAGGAGGTCGACCCCAAAGCCCCTTGCTCTCCCCTGCCCTG
CTGCCGCCTCCCAGCCTGGGGGGTCGTGGCAGATAATCAGCCTCTTAAAGCTGCCTGTAGTTAGGAAATA
AAACCTTTCAAATTTCACATCCACCTCTGACTTTGAATGTAAACTGTGTGAATAAAGTAAAAATACGTAG
CCGTCAAA

Appendix III: Comparison of human *POMC* and mouse *Pomc* transcripts

Top row = Human *POMC* gene transcript
Bottom row = Mouse *Pomc* gene transcript
POMC ASO targets are highlighted in yellow

```
EMBOSS_001       568 CCCAGTGAAGGTGTACCCTAACGGCGCCGAGGACGAGTCGGCCGAGGCCT    617
                     |||.|||||||||||||||.||||..||.|||.||||||||||.|||||||
EMBOSS_001       754 CCCGGTGAAGGTGTACCCCAACGTTGCTGAGAACGAGTCGGCGGAGGCCT    803

EMBOSS_001       618 TCCCCCTGGAGTTCAAGAGGGAGCTGACTGGCCAGCGACTCCGGGAGGGA    667
                     |.||||||||||||||||||||
EMBOSS_001       804 TTCCCCTAGAGTTCAAGAGGGAGCTG------------------------    829

EMBOSS_001       668 GATGGCCCCGACGGCCCTGCCGATGACGGCGCAGGGGCC--CAGGCCGAC    715
                                           ||.|||| ||.||||  .|||    |
EMBOSS_001       830 ------------------------GAAGGCG-AGCGGCCATTAGG----C    850

EMBOSS_001       716 CTGGAGCACAGCCTGCTGGTGGCGGCCGAGAAGAAGGACGAGGGCCCCTA    765
                     .|||||||   ..|.||||.|.|.|.||.|.||||||||||.||.|||||
EMBOSS_001       851 TTGGAGCA---GGTCCTGGAGTCCGACGCGGAGAAGGACGACGGGCCCTA    897

EMBOSS_001       766 CAGGATGGAGCACTTCCGCTGGGGCAGCCCGCCCAAGGACAAGCGCTACG    815
                     |.||.||||||||||||||||.|||.||||||||||||||||||.||||
EMBOSS_001       898 CCGGGTGGAGCACTTCCGCTGGAGCAACCCGCCCAAGGACAAGCGTTACG    947

EMBOSS_001       816 GCGGTTTCATGACCTCCGAGAAGAGCCAGACGCCCCTGGTGACGCTGTTC    865
                     |.||.||||||||||||||||||||||||||||||||||||||||.|||
EMBOSS_001       948 GTGGCTTCATGACCTCCGAGAAGAGCCAGACGCCCCTGGTGACGCTCTTC    997

EMBOSS_001       866 AAAAACGCCATCATCAAGAACGCCTACAAGAAGGGCGAGTGAGGGCACAG    915
                     ||.||||||||||||||||||..||||||||||.||||||||..|||
EMBOSS_001       998 AAGAACGCCATCATCAAGAACGCGCACAAGAAGGGCCAGTGAGGGTGCAG   1047

EMBOSS_001       916 CGGGGCCCCAGGG-CTACCCTCCCCCAGGAGGTCGACCCCAAAGCCCCTT    964
                               ||| ||.|.|               |..||||.||||
EMBOSS_001      1048 ----------GGGTCTTCTC---------------ATTCCAAGGCCC---   1069

EMBOSS_001       965 GCTCTCCCCTGCCCTGCTGCCGCCTCCCAGCCTGGGGGGTCGTGGCAGAT   1014
                                         ||||||.||.||||.|     .||.|||
EMBOSS_001      1070 ----------------------CCTCCCTGCATGGGCG-----AGCTGAT   1092

EMBOSS_001      1015 AATC---AGCCTCTTAAAGCTGCCTGTAGTTAGGAAATAAAACCTTTCAA   1061
                     .|.|   |||||||||.||.|.|||||| ||||||||||||||||||||.
EMBOSS_001      1093 GACCTCTAGCCTCTTAGAGTTACCTGT-GTTAGGAAATAAAACCTTTCAG   1141

EMBOSS_001      1062 ATTTCACATCCACCTCTGACTTTGAATGTAAACTGTGTGAATAAAGTAAA   1111
                     ||||||||..|..|||||||..||.|||..||||||.||.||||||||.||
EMBOSS_001      1142 ATTTCACAGTCGGCTCTGATCTTCAATAAAAACTGCGTAAATAAAGTCAA   1191

EMBOSS_001      1112 AATACGTAGCCGTCAAA--------   1128
                     ||.||  |.|.|||.|.
EMBOSS_001      1192 AACAC--AACTGTCCAGTTACACTA   1214
```