

The Effects of Hypoglycaemia on Inflammation and Atherosclerosis

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Abstract

Hypoglycaemia is associated with cardiovascular (CV) events in diabetes. The mechanisms through which hypoglycaemia may increase CV risk, however, remain to be elucidated. I hypothesised that hypoglycaemia induces activation of the innate immune system, through: monocyte mobilisation and activation, increased platelet reactivity and platelet-leukocyte interactions and therefore accelerates pre-established atherosclerosis through inflammatory pathways.

I conducted studies in both man and mouse. In a novel human experimental model of combined hypoglycaemia and low-dose endotoxin challenge, I studied 24 healthy participants that underwent either a hyperinsulinaemic-hypoglycaemic (2.5 mmol/l) (n=8), euglycaemic (6.0 mmol/l) (n=8) or sham-saline clamp (n=8) (normoglycaemic conditions). To determine if antecedent hypoglycaemia modified innate immune responses, all participants then received a low dose (0.3 ng/kg) intravenous endotoxin challenge 48 hours later. I studied *in vivo* monocyte mobilisation and monocyte-platelet interactions. In comparison to controls, hypoglycaemia increased total leukocytes and significantly mobilised pro-inflammatory CD16⁺ monocytes. Platelet aggregation to agonist, and formation of monocyte-platelet aggregates, increased following hypoglycaemia with significant aggregation of CD16⁺ monocytes and platelets. Compared to euglycaemia, hypoglycaemia caused greater leukocyte mobilisation in response to endotoxin stimulation 48 hours later.

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In mice, I developed a unique model of recurrent insulin induced hypoglycaemia (n=10) or sham-saline injection (n=10) in high fat diet fed Apolipoprotein E deficient mice (ApoE ^{-/-}) that had pre-established atherosclerotic plaques. I show that 8 episodes of a modest recurrent hypoglycaemic stimulus over 4 weeks compared to sham-saline injection resulted in a trend towards increased total atherosclerotic burden in whole aortae.

I conclude that hypoglycaemia mobilises monocytes, increases platelet reactivity, promotes interaction between platelets and pro-inflammatory monocyte subsets, and changes the subsequent immune response to endotoxin in humans. In mice, data suggest pro-atherosclerotic effects of recurrent hypoglycaemia that abolish anti-atherogenic effects of insulin. Collectively, these data highlight mechanisms whereby hypoglycaemia may increase CV risk.

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Declaration

This thesis takes the form of an alternative format thesis. Here, I have combined traditional thesis chapters with material from a publication upon which I am first author and primary contributor (chapter 3).

<https://www.ncbi.nlm.nih.gov/pubmed/?term=A+IQBAL+JCEM+2019>

I confirm that the work submitted is my own and that due credit has been given for data that was not acquired by me within the thesis where applicable. Specifically, I have obtained permission from original copyright owners to reproduce or adapt material presented herein. My contribution to both human and murine studies is explicitly indicated below.

Human studies: I conducted all human studies. I drafted all study experimental protocols and with the help of my supervisors (Professor Sabroe and Professor Heller) obtained ethics and local trust (NHS) approval. I was responsible for recruiting all participants, screening participants and then performing all trial visits until each participant successfully exited the study. Thus, I independently performed glucose clamp studies and intravenous endotoxin challenge with support from a broader research team that included experienced nursing staff and occasionally a clinical colleague. It is necessary for these complex clinical studies to be performed safely within the support of a clinical team. I learnt, performed and optimised all experimental assays myself prior to the first trial visit. As the nature

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of the trial demanded, I clinically supervise participants at all times during the trial visit to ensure their safety (during hypoglycaemic and euglycaemic clamps) and following intravenous endotoxin challenge, I received support in preparing whole blood for flow cytometry and platelet assays during trial visits. This support was provided by two Sheffield Hallam University placement students (Danielle Lambert and Lewis Birch) over the course of the study, that I trained and supervised. Additional trial support was provided during the demanding endotoxin visits (x 4 time points) by a member of the departmental technical team (Fiona Wright or Linda Kay). I performed flow cytometry for all samples from each time point across the study. I also collected Sysmex full blood count data myself. I analysed all data myself.

Murine studies: I obtained a home office personal licence and conducted all experiments myself. I was responsible for monitoring animals on procedure and regularly weighing animals and dispensing Western diet. I received assistance from Carl Wright and Jessica Willis from the departmental technical team in these duties over the course of the experiments. I received training and assistance from Dr Mark Ariaans and Carl Wright in animal cardiac puncture prior to performing these independently. I performed animal sacrifice and dissection independently. I received instruction in sectioning and assistance in sectioning of animal tissue from Fiona Wright. I performed all experimental assays unless otherwise stated in chapter 2, including immunohistochemistry for which I received supervision from Fiona Wright and Dr Jessica Johnston. I collected and analysed all data.

Publications and conference presentations

Published articles related to this work:

1. **Iqbal, A.**, Prince, L. R., Novodvorsky, P., Bernjak, A., Thomas, M. R., Birch, L., Lambert, D., Kay, L. J., Wright, F. J., Macdonald, I. A., Jacques, R. M., Storey, R. F., McCrimmon, R. J., Francis, S., Heller, S. R. and Sabroe, I. (2019) 'Effect of Hypoglycemia on Inflammatory Responses and the Response to Low-Dose Endotoxemia in Humans', *The Journal of Clinical Endocrinology & Metabolism*, 104(4), pp. 1187–1199. doi: 10.1210/jc.2018-01168.
2. **Iqbal, A.**, Novodvorsky, P. and Heller, S. R. (2018) 'Recent updates on type 1 diabetes mellitus management for clinicians', *Diabetes and Metabolism Journal*, pp. 3–18. doi: 10.4093/dmj.2018.42.1.3.
3. **Iqbal, A.** and Heller, S. R. (2018) 'The role of structured education in the management of hypoglycaemia', *Diabetologia*, 61(4), pp. 751–760. doi: 10.1007/s00125-017-4334-z.
4. **Iqbal, A.** and Heller, S. (2016) 'Managing Hypoglycaemia', *Best Practice & Research Clinical Endocrinology & Metabolism*. doi:10.1016/j.beem.2016.06.004.

Conference abstracts related to this work:

1. **Iqbal, A.**, Prince L, Novodvorsky P, Bernjak A, Thomas M, Birch L, Lambert D, Kay L, Jacques R, Storey R, McCrimmon R, Francis S, Sabroe I & Heller S. "Acute Hypoglycaemia Results in Increased Activation of Responses to An Inflammatory Challenge Induced 48 hours Later in Human Subjects." In *Diabetologia*, 61:1-620, **2018**. doi: 10.1007/s00125-018-4693-0. **European Association for the Study of Diabetes meeting, Berlin. Moderated poster presentation.**
2. **Iqbal, A.**, Thomas M, Novodvorsky P, Bernjak A, Birch L, Lambert D, Kay L, Wright F, Prince L, Francis S, Storey R, Sabroe I & Heller S. "Hypoglycaemia Increases Platelet Reactivity and Promotes Formation of Proatherogenic Platelet-Leucocyte Aggregates." In *Diabetologia*, 60:1–608, **2017**. doi:10.1007/s00125-017-4350-z. **European Association for the Study of Diabetes meeting, Lisbon. Moderated poster presentation.**
3. **Iqbal, A.**, Thomas M, Novodvorsky P, Bernjak A, Birch L, Lambert D, Kay L, Wright F, Prince L, Francis S, Storey R, Sabroe I & Heller SR. "Effect of Hypoglycemia on Platelet Reactivity and Platelet-Leukocyte Kinetics." In *Diabetes*, 66:A101–73. American Diabetes Association, **2017**.

doi:10.2337/db17-381-663. **American Diabetes Association meeting, 77th Scientific Sessions, San Diego. Oral presentation.**

4. **Iqbal A**, Novodvorsky P, Bernjak A, Chow E, Lambert D, Birch L, Kay L, Wright F, Prince L, Francis S, Sabroe I, and Heller SR. "Hypoglycemia Increases Circulating Proatherogenic Intermediate Monocytes in Healthy Volunteers." In *Diabetes*, 65:A101–69. American Diabetes Association, **2016**. doi:10.2337/db16-382-651. **American Diabetes Association meeting, 76th Scientific Sessions, New Orleans. Moderated poster presentation.**

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List of abbreviations

- **ACCORD** Action to Control Cardiovascular Risk in Diabetes
- **ACS** Acute coronary syndrome
- **ADA** American Diabetes Association
- **ADP** Adenosine diphosphate
- **ADVANCE** Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation
- **AMI** Acute Myocardial Infarction
- **ANCOVA** Analysis of covariance
- **ANOVA** Analysis of variance
- **APC** Allophycocyanin
- **ApoE** ^{-/-} Apolipoprotein E deficient mice
- **AR1** Autoregressive correlation structure
- **BMI** Body mass index
- **CAD** Coronary artery disease
- **CCL2** C-C motif chemokine ligand 2
- **CCR2** C-C motif chemokine receptor 2
- **CGM** Continuous glucose monitoring
- **CM** Classical monocyte
- **CM-MPA** Classical monocyte-platelet aggregate
- **CSA** Cross-sectional area
- **CSII** Continuous subcutaneous insulin infusion
- **CV** Cardiovascular
- **CVA** Cerebrovascular accident
- **CVD** Cardiovascular disease
- **CX₃CL1** CX₃C chemokine ligand 1
- **CX₃CR1** CX₃C chemokine receptor 1
- **CXCL8** C-X-C motif chemokine ligand 8
- **DC** Dendritic cell
- **DCCT** Diabetes Control and Complications Trial
- **DFU** Diabetic foot ulcer
- **EASD** European Association for the Study of Diabetes
- **ECG** Electrocardiography
- **EDIC** Epidemiology of Diabetes Interventions and Complications
- **EDTA** Ethylenediaminetetraacetic acid
- **EEL** External elastic lamina
- **ELISA** Enzyme-linked immunosorbent assay
- **FACS** Fluorescence-activated cell sorting
- **FFA** Free fatty acids
- **FITC** Fluorescein isothiocyanate
- **FMO** Fluorescence minus one
- **GM-CSF** Granulocyte-macrophage colony stimulating factor
- **HAAF** Hypoglycaemia associated autonomic failure
- **HbA1c** Glycated haemoglobin A1c
- **HSC** Haematopoietic stem cell

- **i.p.** Intraperitoneal
- **IAH** Impaired awareness of hypoglycaemia
- **ICAM-1** Intracellular adhesion molecule-1
- **IM** Intermediate monocyte
- **IM-MPA** Intermediate monocyte-platelet aggregate
- **IRS** Insulin receptor substrate-1
- **ISO** International Organisation for Standardisation
- **LFA-1** Lymphocyte function associated antigen-1
- **LPS** Lipopolysaccharide
- **M-CSF** Macrophage-colony stimulating factor
- **MACE** Major adverse cardiovascular events
- **MAPK** Mitogen-activated protein kinase
- **MHC** Major histocompatibility complex
- **MPA** Monocyte platelet aggregate
- **MVG** Modified Van Gieson
- **NCM** Non-classical monocyte
- **NCM-MPA** Non-classical monocyte platelet aggregate
- **NK** Natural killer cell
- **NO** Nitric oxide
- **NPA** Neutrophil platelet aggregate
- **NSTEMI** Non-ST elevation myocardial infarction
- **PBS** Phosphate buffered saline
- **PE** Phycoerythrin
- **PECy7** Phycoerythrin-Cy7
- **PerCP** Peridinin chlorophyll protein
- **PI3K** Phosphatidylinositol-3-kinase
- **PRR** Pattern recognition receptors
- **PSGL-1** P-selectin glycoprotein ligand 1
- **ROS** Reactive oxygen species
- **STEMI** ST-elevation myocardial infarction
- **STH** Sheffield Teaching Hospitals
- **T1D** Type I Diabetes
- **T2D** Type 2 Diabetes
- **TLR** Toll-like receptors
- **TMB** 3,3',5,5' tetramethylbenzidine
- **TREM** Triggering receptors expressed on myeloid cells
- **UA** Unstable angina
- **UKPDS** United Kingdom Prospective Diabetes Study Group
- **VADT** Veteran Administration Diabetes Trial
- **VCAM-1** Vascular cell adhesion molecule-1
- **VLA-4** Very late antigen-4
- **VSMC** Vascular smooth muscle cell
- **WBC** White blood cell
- **WHO** World Health Organisation

Chapter 1- Introduction

The earliest accurate description of diabetes mellitus arguably comes from Aretaeus of Cappadocia (80-138 AD). Aretaeus astutely observed that patients with diabetes had excessive thirst and urinated frequently. The Persian polymath Avicenna (980-1037) gave a more detailed clinical account of diabetes including a peculiar sweetness to urine from patients with diabetes in his influential text the *Canon of Medicine*. Thomas Crawley in 1788 first suggested that the pancreas gland was implicated in the pathophysiology of diabetes. It was not until 1921, however, that Canadian scientists Banting and Best first extracted insulin from dogs to successfully treat diabetes (Banting, Campbell and Fletcher, 1923).

Today, diabetes poses a significant global threat to human health, wellbeing and prosperity. The number of adults with diabetes has risen from 108 million in 1980 to 422 million in 2014 and alarmingly this number is projected to exceed 500 million by 2035 (Mathers *et al.*, 2006). There are 3.6 million people living with confirmed diabetes in the UK (Diabetes UK, 2016). Diabetes is ordinarily dichotomised into type 1 diabetes (T1D) and type 2 diabetes (T2D). The diagnostic criteria for both T1D and T2D as set by the World Health Organization (WHO) and adopted by learned societies including the American Diabetes Association (ADA) is the presence of typical diabetes symptoms (polyuria, polydipsia and unexplained weight loss for T1D) plus: a random venous plasma glucose ≥ 11.1 mmol/l or a fasting plasma glucose concentration ≥ 7.0 mmol/l or a two hour plasma glucose concentration ≥ 11.1 mmol/l following a 75g anhydrous glucose challenge in an oral glucose tolerance test (American Diabetes Association, 2010). In 2011, the

WHO added a further criteria of using HbA1c in the diagnosis of diabetes whereby an HbA1c of $> 6.5\%$ (48 mmol/mol) is sufficient for the diagnosis of diabetes (WHO, 2011). T1D is characterised by an absolute deficiency of insulin. In T2D, which accounts for between 90-95% of diabetes, a combined resistance to insulin both in skeletal muscle and the liver, in addition to defective insulin production by pancreatic β cells is present (DeFronzo, 2004). The underlying biochemical abnormality in both T1D and T2D is persistent hyperglycaemia, which leads to a myriad of complications affecting both small and large blood vessels in the body termed micro and macrovascular complications respectively. Within macrovascular complications, the leading cause of death for all patients with diabetes is cardiovascular disease (CVD), usually due to acute myocardial infarction (AMI) or cerebrovascular accident (CVA) (Morrish *et al.*, 2001; Laing *et al.*, 2003; Tu *et al.*, 2008).

Much investigation has focussed on hyperglycaemia and the benefit of good glycaemic control in reducing micro and macrovascular complications in diabetes. Whilst the role of tight glycaemic control in reducing microvascular complications is well established (UK Prospective Diabetes Study Group, 1998 a,b), the contribution of tight glucose control in reducing macrovascular complications remains less certain. Three large randomised controlled trials: Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE study) and the Veteran's Affairs Diabetes Trial (VADT) (Gerstein *et al.*, 2008; Patel *et al.*, 2008; Duckworth *et al.*, 2009) have recently tested the effects of intensive glucose control on major cardiovascular (CV) end points in T2D.

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Participants underwent randomisation to achieve intensive glucose control, defined by a glycated haemoglobin A1c (HbA1c) of <6.0% in ACCORD and VADT and <6.5% in the ADVANCE trial, versus more lax standard glycaemic control. In none of the trials was there a reduction in overall CV risk in those randomised to intensive glucose control. In the ACCORD trial, a significant excess CV mortality was noted in the intensive glucose arm compared with standard treatment prompting early trial closure after 3.5 years. It is noteworthy, that severe hypoglycaemia rates were more common in the ACCORD group treated more intensively. These developments raised significant alarm and instigated intense debate in the diabetes community providing me with the impetus to pursue this research.

In this introductory chapter, I will first discuss the epidemiology, physiology and clinical consequences of hypoglycaemia. As an association between severe hypoglycaemia and increased CV mortality is more consistently reported in T2D, I will then provide a critical review of trials of intensive glycaemic control in T2D in light of hypoglycaemia emerging as a potential causal factor for CV events from these data. Existing literature on mechanistic links between hypoglycaemia and CVD will then be reviewed. I will then detail the aetiology of atherosclerosis focusing on the role of the innate immune system prior to discussing the use of intravenous endotoxin challenge as a model of sterile inflammation in humans. Finally, I aim to summarise lacunae in the field and present my hypotheses and aims.

1.2 Hypoglycaemia

1.2.1 Epidemiology of hypoglycaemia

An ADA working group (American Diabetes Association 2005) has defined symptomatic hypoglycaemia as the presence of typical symptoms of hypoglycaemia with a glucose measurement < 3.9 mmol/l and severe hypoglycaemia as any episode requiring the assistance of another person to recover. These definitions have limitations, however, and universal application is difficult. For example, paediatricians generally include hypoglycaemic episodes that cause coma or seizures in their definition for severe hypoglycaemia as young children always need external assistance. Furthermore, many studies report hypoglycaemic episodes at a glucose level lower than 3.9 mmol/l, often on the basis that they are more clinically relevant. This heterogeneity in hypoglycaemia classification makes it difficult to compare different studies and interventions. In particular, episodes of hypoglycaemia that are associated with mortality are not recorded systematically according to an agreed classification.

Recently, the ADA and the European Association for the Study of Diabetes (EASD) agreed a position statement that proposes the inclusion of a third glucose level, denoting 'clinically important' (in addition to < 3.9 mmol/l and severe hypoglycaemia) at < 3.0 mmol/l for the classification of hypoglycaemia (The International Hypoglycaemia Study Group 2017). However, these new proposed criteria for hypoglycaemia have yet to be agreed more widely. Thus, in this section I will generally confine my comments to severe hypoglycaemia in adults, the definition of which (severe hypoglycaemia: requiring assistance of another person

to recover) is currently widely accepted. It is important to note that data from clinical trials report rates of hypoglycaemia, including severe, which are many times lower than rates collected from observational data. In the Diabetes Control and Complications Trial (DCCT), which is considered to have resulted in an epidemic of severe hypoglycaemia in the intensive arm, reported rates expressed in episodes per patient year were considerably lower than rates from observational studies (Diabetes Control and Complications Trial Research Group *et al.*, 1993). More recent clinical trials have reported even lower rates of severe hypoglycaemia (Bergenstal *et al.*, 2010). In contrast, data reported in observational studies have shown virtually no reduction in rates of severe hypoglycaemia over the last 20 years, despite the introduction of insulin analogues and continuous subcutaneous insulin infusion (CSII) (Table 1.1)(MacLeod *et al.* 1993; ter Braak *et al.* 2000; Pedersen-Bjergaard *et al.* 2004; UK Hypoglycaemia Study Group 2007; Kristensen *et al.* 2012). These data suggest that clinicians are either failing to exploit technological developments or that other factors often determine hypoglycaemic risk. Prospective population based studies which have examined hypoglycaemic rates indicate that rates of severe hypoglycaemia in insulin treated T2D patients are generally a third of the rates seen in patients with T1D (Donnelly *et al.*, 2005). Interestingly, comparable rates of hypoglycaemia have been reported in T1D and T2D once patients were matched for duration of insulin treatment (Hepburn *et al.*, 1993). Data from another prospective study also showed that severe hypoglycaemia rates rise in both T1D and T2D with increasing duration of treatment (UK Hypoglycaemia Study Group 2007). These findings may be explained by a progressive decline in endogenous insulin production leading to both diminished physiological protection from glucagon release and more variable

free insulin levels. Finally, as T2D is much more common than T1D with an increasing incidence predicted in the coming years (Hossain, Kawar and El Nahas, 2007), management of hypoglycaemia in T2D in terms of numbers of cases is at least as important as in T1D.

Table 1.1 A summary of observational studies investigating the frequency of severe hypoglycaemia in diabetes adapted from (Iqbal and Heller, 2018).

Study	Number of patients	Age (years) median (range) or mean \pm SD	Follow up (months)	Frequency of hypoglycaemia (episodes/per person/year)	Proportion affected (%)
MacLeod, 1993 (Scotland)	600	41 (14-79)	12	1.6	29
ter Braak 2000 (Denmark)	195	41 \pm 14	12	1.5	41
Pedersen-Bjergaard 2004 (Denmark)	1076	40 (18-81)	12	1.3	37
UK Hypoglycaemia Study Group 2007 (United Kingdom)	100 (46<5 years; 54>15 years)	<5y 41 \pm 13 >15y: 53 \pm 10	9-12	1.1	22
				3.2	46
Kristensen 2012 (Denmark)	3861	48 \pm 15	12	1.2	31

1.2.2 Physiology of hypoglycaemia

The brain relies on glucose as an obligate fuel and this explains both the vulnerability of patients to hypoglycaemia and the array of defences that have evolved as stress responses. These physiological responses are termed counter-regulatory (opposing the regulatory effect of insulin)(Figure 1.1). Two key responses are triggered by hypoglycaemia: 1) Increased endogenous glucose production via glycogenolysis and gluconeogenesis; 2) A behavioural response

prompting the individual to consume food. In individuals without diabetes, the initial response to glucose values falling below 4.6 mmol/l is a reduction in endogenous insulin secretion (Cryer, Davis and Shamoon, 2003). Since individuals with T1D and insulin-treated T2D depend on injected insulin boluses in the absence of endogenous insulin secretion, their ability to defend themselves against hypoglycaemia requires additional mechanisms. Initially, as glucose concentrations fall below 3.8 mmol/l, endogenous glucagon secretion from pancreatic α cells induces both glycogenolysis and gluconeogenesis (Cryer, 2008). As blood glucose falls further, the adrenal medulla secretes adrenaline (and to a lesser extent also noradrenaline) which promotes hepatic glucose release through similar mechanisms. Raised cortisol and growth hormone concentrations stimulate gluconeogenesis but since subsequent increases in blood glucose lag in time, these response are less relevant acutely (Cryer, 1994).

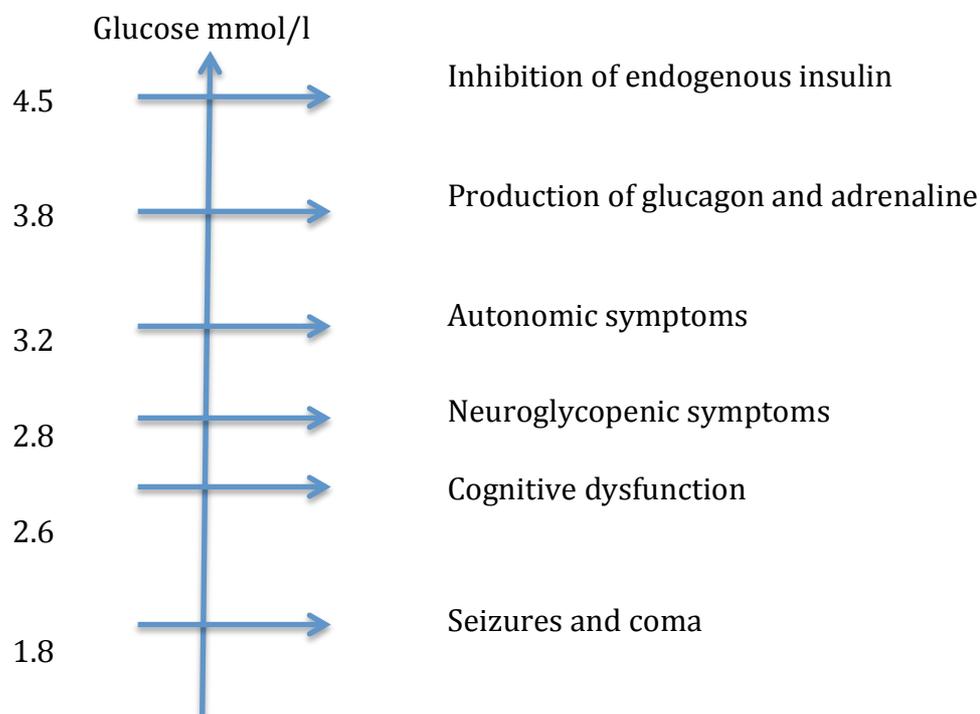


Figure 1-1 Counter-regulatory response thresholds and accompanying symptoms in response to hypoglycaemia in individuals without diabetes.

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Experimental data indicate that counter-regulatory responses to hypoglycaemia whilst intact at diagnosis of T1D, are gradually lost with increasing disease duration (Bolli *et al.*, 1983; White *et al.*, 1983). Counter-regulatory responses to hypoglycaemia have been less extensively studied in individuals with T2D but the same drivers which increase vulnerability in T1D may also operate in longstanding T2D, but the precise mechanisms are not known (Spyer *et al.*, 2000; Segel, Paramore and Cryer, 2002).

As demonstrated by Heller and Cryer, and others, the impaired sympatho-adrenal response to hypoglycaemia is a consequence, at least in part, of repeated episodes of iatrogenic hypoglycaemia which attenuate the autonomic response to subsequent hypoglycaemia (Amiel *et al.*, 1988; Heller and Cryer, 1991; Davis *et al.*, 2009). Recurrent, episodic hypoglycaemic events of sufficient depth and duration progressively blunt and impair normal counter-regulatory responses to hypoglycaemia predisposing patients to a vicious cycle of ever more frequent hypoglycaemic episodes with a falling glucose threshold to trigger counter-regulation (Cryer, 1993). Cryer has termed this sequence of events hypoglycaemia-associated autonomic failure (HAAF)(Cryer, 1992).

There is, however, good evidence to suggest that reduced neuroendocrine and symptomatic responses to hypoglycaemia even in long-standing impaired awareness of hypoglycaemia (IAH), can be reversed with meticulous avoidance of further hypoglycaemia (Fanelli *et al.*, 1993; Cranston *et al.*, 1994).

1.3 Evidence for hypoglycaemia as a risk factor for cardiovascular mortality in clinical studies

The UKPDS study was an influential prospective, multicentre, randomised controlled trial that showed that intensive glycaemic control {average HbA1c of 7% (52 mmol/mol)} in newly diagnosed T2D significantly reduced microvascular complications with a non-significant reduction in the relative risk of myocardial infarction ($p = 0.052$) (UK Prospective Diabetes Study Group, 1998 a,b). A 10 year follow-up of the UKPDS cohort examined if intensive glycaemic control had effects on macrovascular outcomes in the longer term despite HbA1c levels which were comparable between the intensive and the control arms of the original trial in the additional 10 years of follow up (Holman *et al.*, 2008). In this study, a significant reduction in the risk of myocardial infarction ($p = 0.01$) was observed.

Three, multicentre, randomised controlled trials (ACCORD, ADVANCE, VADT) aimed to definitively test the hypothesis that intensive glycaemic control reduces CV mortality in T2D (Gerstein *et al.*, 2008; Patel *et al.*, 2008; Duckworth *et al.*, 2009). Collectively, the above studies recruited 23,182 participants globally with known T2D and pre-existing CVD or established cardiovascular (CV) risk factors. In all trials no significant reduction in major CV events was observed. In the ACCORD trial, 5,128 participants were randomised to intensive glucose control (HbA1c < 6.0% (42.1 mmol/mol) and 5,123 participants randomised to the standard treatment arm (HbA1c 7-7.9% (53-62.8 mmol/mol) (Gerstein *et al.*, 2008). Surprisingly, significant excess mortality (257 deaths) was noted in the intensive glucose arm compared to standard treatments (203 deaths) (hazard ratio 1.22; 95% CI, 1.01 to 1.46; $p = 0.01$). This observation led to early trial closure at 3.5

years of follow-up. Possible explanations for this result include, weight gain, specific medications and the play of chance. A statistically significant excess CV mortality was not noted in the ADVANCE and VADT cohorts.

Interestingly, in ACCORD, ADVANCE and VADT an increased incidence of hypoglycaemia in the intensive glycaemic control arms was seen (Gerstein *et al.*, 2008; Patel *et al.*, 2008; Duckworth *et al.*, 2009). Participants in the intensive treatment arms of the ACCORD and VADT trials had higher rates of hypoglycaemia compared to standard treatment (ACCORD 16.2% vs. 5.1%; VADT 21.2% vs. 9.9%) in comparison to ADVANCE (2.7% vs. 1.5%) (Figure 1.2). The finding of excess mortality in the ACCORD trial but not in ADVANCE and VADT is intriguing and is likely explained by intrinsic differences in the study participants such as weight, differences in side effects to different diabetes drugs and pre-existing duration of diabetes (Table 1.2). In the ACCORD trial, severe hypoglycaemia was associated with increased mortality across both the intensive and standard treatment arms (Gerstein *et al.*, 2008). CV death accounted for a nearly a third of all deaths in the ACCORD study (Gerstein *et al.*, 2008). In the intensive treatment arm of ACCORD, annual unadjusted mortality was 2.8% in those that had one or more episode of severe hypoglycaemia compared to 1.2% in those with no episodes of severe hypoglycaemia (hazard ratio, 1.41; 95% CI, 1.03-1.93)(Bonds *et al.*, 2010). However, the risk of death following hypoglycaemia was comparatively lower in the intensive glucose control arm compared to standard control (hazard ratio, 1.41; 95% CI, 1.03-1.93 vs. hazard ratio, 2.30; 95% CI 1.46-3.65). Furthermore, there is a dichotomy in the ACCORD data in that overall fatal CV deaths were significantly higher in the intensive arm but the rates of non-fatal AMI were in fact lower in the

intensive glucose control arm (hazard ratio, 0.76, 95% CI, 0.67-0.92; 0.004). In a meta-analysis of thirteen trials (n=34,533) studying intensive glycaemic control in T2D a similar trend towards a reduction in the risk of non-fatal AMI in those randomised to intensive glucose control was noted (Boussageon *et al.*, 2011). In summary, trial data suggest that intensive glucose control is potentially cardioprotective against non-fatal AMI and this protection appears to be preferentially conferred to those that have a low CVD burden to begin with. CV benefits of intensive glucose control in some, however, appear to be counterbalanced by a significant increase in the risk of fatal AMI in others with intensive glucose control being associated with a much higher risk of severe hypoglycaemia.

It is extremely challenging to confirm or refute a causal link between hypoglycaemia and observed CV mortality. One reason is that these trials were not originally designed to examine the relationship between hypoglycaemia and CV outcomes. The ACCORD investigators have argued that there is scant evidence linking severe hypoglycaemia to increased CV mortality in the intensive glycaemic control arm of their trial, since they observed no direct relationship between severe hypoglycaemic episodes and subsequent deaths (Riddle *et al.*, 2010). Nevertheless, the potential role of hypoglycaemia in exacerbating CV risk in patients randomised to intensive glucose control in ACCORD was underestimated (Frier, Schernthaner and Heller, 2011). Hypoglycaemia may have been under-reported in the intensive treatment arm since repeated episodes of hypoglycaemia lead to reduced hypoglycaemia awareness. Furthermore, a causal hypoglycaemic event may have led to pathophysiological changes leading to increased mortality

downstream of the event. Indeed, it is striking that in all three of these major CV outcome trials, a severe hypoglycaemic event predicted mortality, not at the time but weeks or months after the event (Gerstein *et al.*, 2008; Patel *et al.*, 2008; Duckworth *et al.*, 2009; Bonds *et al.*, 2010; Zoungas *et al.*, 2010). Furthermore, some excess deaths in the intensive treatment arm of ACCORD may have been erroneously coded as being due to coronary events with no possibility of glucose measurement post-mortem (Frier, Schernthaner and Heller, 2011). Also, it is noteworthy that only severe hypoglycaemic events were measured in these large studies as such events are much easier to record than non-severe hypoglycaemia.

Whilst it is challenging to establish causation rather than association, due to potential confounding, hypoglycaemia is probably associated with excess CV mortality in those with comorbidities such as liver disease, chronic kidney disease and general frailty that are likely to lead to hypoglycaemic events (Zoungas *et al.*, 2010). Hence, hypoglycaemia might merely be a marker for susceptibility, as opposed to being a direct cause of mortality. However, a recent large systematic review and meta-analysis using specific statistical adjustments, concluded that it was unlikely that comorbidities alone explained the relationship between hypoglycaemia and CV disease (Goto *et al.*, 2013). The authors studied the relationship in studies involving nearly a million participants and demonstrated that severe hypoglycaemia was strongly associated with CV disease (relative risk 2.05, 95% confidence interval 1.74 to 2.42; $p < 0.001$).

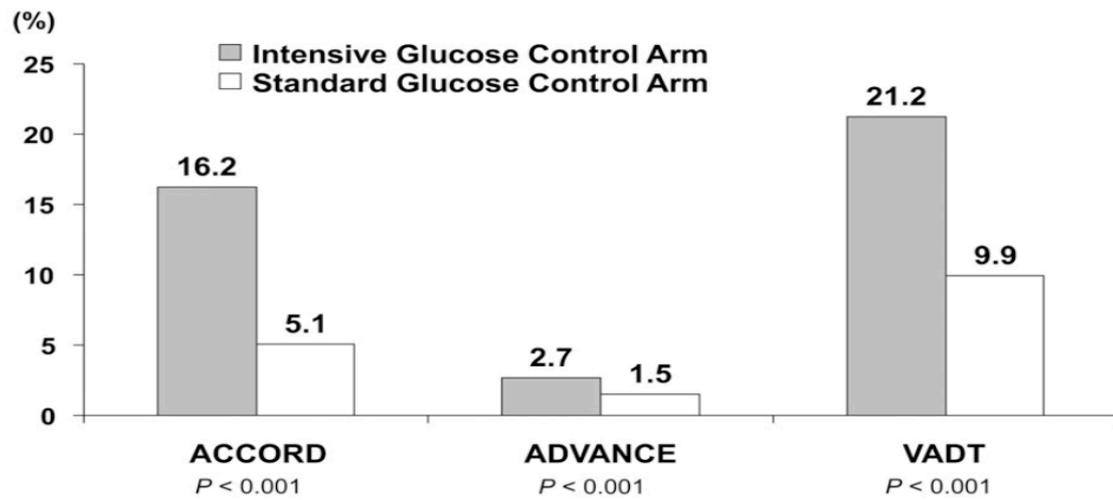


Figure 1-2 Percentage of participants with severe hypoglycaemic events in the ACCORD, ADVANCE and VADT trials adapted from (Frier, Schernthaner and Heller, 2011). Abbreviations: ACCORD, action to control cardiovascular risk in diabetes; ADVANCE, action in diabetes and vascular disease: preterax and diamicron modified release controlled evaluation; VADT, veteran's affairs diabetes trial.

Table 1.2 Clinical characteristics and differences in primary CVD endpoint, overall mortality and CV mortality between intensive glycaemic control and standard arms of ACCORD, ADVANCE and VADT adapted from (Frier, Schernthaner and Heller, 2011). Abbreviations: ACCORD, action to control cardiovascular risk in diabetes; ADVANCE, action in diabetes and vascular disease: preterax and diamicron modified release controlled evaluation; VADT, veteran’s affairs diabetes trial, BMI, body mass index; CVD, cardiovascular disease; CV, cardiovascular; ns, non-significant.

	ACCORD	ADVANCE	VADT
n	10,251	11,140	1,791
Age (years)	62	66	60
Men/Women (%)	61/39	58/42	97/3
Duration of study (years)	3.5	5.0	5.6
BMI (kg/m²)	32.2 ± 5.5	28.0 ± 5.0	31.3 ± 3.5
Duration of diabetes (years)	10	8	11.5
CVD (%)	35	32	40
Primary CVD end point	↓10% (p=0.16)	↓6% (p=0.37)	↓13% (p=0.12)
Mortality (overall)	↑22% (p=0.04)	↓7% (p=NS)	↑6.5% (p=ns)
CV mortality	↑35% (p=0.02)	↓12% (p=ns)	↑25% (p=ns)

1.4 Mechanistic links between hypoglycaemia and CVD

In the previous section I have highlighted clinical trial data suggesting hypoglycaemia may be causally related to CV risk. This is biologically plausible owing to a broad array of hypoglycaemia mediated effects on the CV system which will be discussed below.

1.4.1 Cardiac ischaemia

Adrenaline acting via β_1 adrenoreceptors increases heart rate and agonism of α and β_2 adrenoreceptors increases blood pressure (Lohmann, Loesment and Kaehler, 1990). In healthy volunteers, a study using radionuclide ventriculography demonstrated that hypoglycaemia associated catecholamine release increased cardiac output via increased myocardial contractility thus increasing myocardial oxygen demand (Fisher *et al.*, 1987). In those with diabetes and pre-existing coronary artery disease (CAD) or established CV risk factors, increased myocardial oxygen demand may outstrip supply, resulting in cardiac tissue ischaemia and eventually infarction. Also, in healthy individuals undergoing acute hypoglycaemia, the arterial tree demonstrates a degree of elasticity which ensures that the blood flow wave generated from increased myocardial contraction during systole returns to the heart during early diastole thus enhancing coronary artery perfusion which occurs mainly in diastole (Sommerfield *et al.*, 2007). In individuals with T1D for more than 15 years, a loss of vessel wall elasticity due to progressive stiffening means that blood flow generated during early systole returns back to the heart in late systole as opposed to early diastole thus potentially compromising coronary artery perfusion (Sommerfield *et al.*, 2007). Interestingly, in a study using myocardial contrast echocardiography, acute hypoglycaemia also reduced myocardial blood flow in 19 healthy volunteers and 28 subjects with T1D without CAD (Rana *et al.*, 2011). The exact mechanisms are not clear, but the authors speculated that the effects of coronary vasoconstrictors could potentially explain their findings.

Electrocardiographic (ECG) changes of cardiac ischaemia have been previously reported in a patient with hypoglycaemia induced coma (Markel, Keidar and Yasin, 1994). Others have reported hypoglycaemia induced angina pectoris in a patient with diabetes and CAD (Duh and Feinglos, 1994). In a study of 6 patients with T2D without known cardiac disease, induction of hypoglycaemia resulted in cardiac ischaemia in 5 patients as evidenced by ST segment depression and T-wave flattening (Lindström *et al.*, 1992). Another study enrolled 21 patients with both CAD and T2D and performed simultaneous continuous glucose monitoring (CGM) and cardiac Holter monitoring over a continuous 72 hour period (Desouza *et al.*, 2003). In the 54 recorded episodes of hypoglycaemia (< 3.9 mmol/l), 10 episodes were associated with symptoms of chest pain of which 4 had clear ECG changes of ischaemia whilst neither chest pain nor ECG changes were documented in normoglycaemia (Desouza *et al.*, 2003). For ethical reasons, there is a paucity of experimental data prospectively verifying case reports and relatively small observational studies of hypoglycaemia induced cardiac ischaemia. However, it remains plausible that hypoglycaemia and subsequent adrenaline mediated haemodynamic effects may progress CVD.

1.4.2 Hypoglycaemia and cardiac arrhythmogenesis

Hypoglycaemia can be pro-arrhythmogenic via a number of mechanisms and hypoglycaemia has thus been implicated in sudden cardiac death. Nocturnal sudden death in young patients with T1D that are previously well is well documented and termed 'dead-in-bed-syndrome' (Campbell, 1991; Tattersall and Gill, 1991). It has been postulated that nocturnal hypoglycaemia generated arrhythmias may be responsible for the dead-in-bed syndrome. There is

circumstantial evidence linking hypoglycaemia to sudden death in a patient with T1D who was having CGM and insulin administration via an insulin pump (Tanenberg et al. 2010). The subject studied by Tanenberg et al. had a propensity to nocturnal hypoglycaemia and was found dead after an overnight dip of interstitial blood glucose to 1.8 mmol/l. In addition, a number of epidemiological studies have supported the existence of the dead-in-bed phenomenon (Sartor and Dahlquist, 1995; Dahlquist and Källén, 2005; Skrivarhaug *et al.*, 2006), In particular, a retrospective review of autopsy reports in those T1D aged less than 40 found unexpected sudden death to be more than 4 fold higher in comparison to a non-diabetic population (Tu *et al.*, 2008).

Hypoglycaemia may predispose to tachyarrhythmias, through effects on QT interval (usually expressed as QT interval corrected for heart rate - QTc) and on electrolytes. Mechanistically, hypoglycaemia is known to cause QT interval prolongation and increase QT dispersion (QTd) during cardiac electrical repolarisation (Robinson *et al.*, 2003; Koivikko *et al.*, 2008). QT changes during hypoglycaemia can account for ventricular tachycardia and atrial fibrillation (Collier *et al.*, 1987; Chelliah, 2000). QTc prolongation in hypoglycaemia is mediated by catecholamines, hypokalaemia (caused by catecholamine and insulin mediated shift of potassium into the intracellular compartment) and direct inhibitory effect of hypoglycaemia on the current through the human ether-a-go-go related gene channel (Zhang *et al.*, 2003; Nordin, 2010). Out of these three mechanisms, catecholamine effect is thought to be the most important one as evidenced by the fact that selective β blockade with atenolol has been shown to attenuate QTc prolongation associated with hypoglycaemia in T1D (Lee *et al.*,

2005). There is evidence to suggest that the adrenergic response to hypoglycaemia can result in increased myocardial calcium which may lead to ventricular tachycardia (Nordin, 2010).

Hypoglycaemia may also predispose to bradyarrhythmias (Navarro-Gutiérrez *et al.*, 2003; Gill *et al.*, 2009). Data from our group has suggested that an increased vagal tone following counter-regulation to hypoglycaemia may account for observed bradyarrhythmias (Chow *et al.*, 2014). Chow *et al.* also found that bradycardia and atrial and ventricular ectopic beats occurred more frequently during nocturnal hypoglycaemia in ambulatory patients with T2D. We have recently recapitulated these diurnal differences in a cohort of young subjects with T1D (Novodvorsky *et al.*, 2017). These observations strengthen the notion that nocturnal hypoglycaemia is particularly deleterious. Nocturnal hypoglycaemia has also been shown to cause QTc prolongation (Gill *et al.*, 2009; Chow *et al.*, 2014). In addition, sleep appears to modulate the protective counter-regulatory response to hypoglycaemia by reducing the threshold of glucose for initiation of counter-regulation in early sleep with late nocturnal sleep reducing the induction of counter-regulatory hormones (Jones *et al.*, 1998). Taken together, these data imply that hypoglycaemia mediated changes in cardiac rate, rhythm, autonomic function and repolarisation may increase the risk of sudden CV death.

1.4.3 Effects of hypoglycaemia on platelets

Platelets play an important role in thrombotic cardiac complications associated with diabetes. Hypoglycaemia can influence both platelet activation and aggregation (Hutton *et al.*, 1979; Wright *et al.*, 2010). In those with T1D,

hypoglycaemia has been shown to reduce platelet counts (Dalsgaard-Nielsen, Madsbad and Hilsted, 1982). Increased platelet activation *in vivo* during hypoglycaemia has been demonstrated by increased soluble P-selectin (Gogitidze Joy *et al.*, 2010; Joy *et al.*, 2015), β thromboglobulin (Trovati *et al.*, 1986; Takeda *et al.*, 1988) and increased monocyte platelet aggregates (MPAs) (Wright *et al.*, 2010). Additionally, increased plasma sCD40L concentrations have been shown in response to acute hypoglycaemia (Wright *et al.*, 2010). Hypoglycaemia increases platelet reactivity to agonists *in vitro* (Trovati *et al.*, 1986). Increased platelet activation and aggregation are thought to be mediated by an adrenergic response to hypoglycaemia (Hutton *et al.*, 1979). Recent work from our group has also shown that in T2D, hypoglycaemia increases platelet reactivity but these changes are short lived and last up to 24 hours following hyperinsulinaemic hypoglycaemia (Chow *et al.*, 2018). Interestingly, experimental hypoglycaemia in our cohort was also associated with pro-thrombotic changes in fibrin clot density that lasted up to 7 days after moderate hypoglycaemia.

1.4.4 Effects of hypoglycaemia on inflammation

Systemic inflammation is critical to the aetiology of atherosclerosis. Acute hypoglycaemia induced by an insulin bolus in healthy volunteers resulted in an increase in the pro-inflammatory cytokines: TNF- α , IL-1 β , IL-6 and IL-8 (Razavi Nematollahi *et al.*, 2009). There was also an increase in markers of lipid peroxidation, reactive oxygen species (ROS) and a significant leukocytosis in response to hypoglycaemia (Razavi Nematollahi *et al.*, 2009). Acute leukocytosis and increased monocyte CD40 expression in response to insulin induced hypoglycaemia have also been shown by others (Wright *et al.*, 2010; Ratter *et al.*,

2017). Changes in total leukocyte numbers and other indices of inflammation were accompanied by a simultaneous increase in the counter-regulatory hormones adrenaline, noradrenaline and cortisol, implying hormonal mediation of pro-inflammatory changes in response to hypoglycaemia (Razavi Nematollahi *et al.*, 2009). These data are limited, however, as they are not representative of hypoglycaemia as encountered in clinical practice on account of supra-physiological doses of insulin used to rapidly lower blood glucose. Latterly, the effects of acute hypoglycaemia on pro-inflammatory markers in healthy individuals and those with T1D have been investigated more robustly by using hyperinsulinaemic hypoglycaemic and euglycaemic clamps that allowed the investigators to control for the anti-inflammatory effects of insulin (Dotson *et al.*, 2008; Gogitidze Joy *et al.*, 2010). Moderate hypoglycaemia at 2.9 mmol/l for 120 minutes increased levels of IL-6 in both healthy volunteers and in those with T1D (Gogitidze Joy *et al.*, 2010). Levels of IL-6 also increased significantly following a hypoglycaemic stimulus at 2.7 mmol/l for 110 minutes in healthy volunteers (Dotson *et al.*, 2008). The acute phase C reactive protein (CRP) has also been implicated as a predictor of acute coronary syndrome (ACS) in epidemiological studies (Ridker *et al.*, 2002; Danesh *et al.*, 2004). In a study of healthy volunteers and those with T1D, hypoglycaemia increased CRP levels for up to 24 hours (Galloway *et al.*, 2000). A more recent study however demonstrated that an increase in CRP following hypoglycaemia was more acute and only confined to healthy volunteers (Wright *et al.*, 2010).

Cell adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin orchestrate interactions

between a damaged vascular endothelium and leukocytes, an initial step in plaque formation and atherosclerosis (Chi & Melendez et al. 2007). Significantly higher levels of ICAM-1, VCAM-1 and E-selectin have been demonstrated during hypoglycaemia compared to euglycaemia in both healthy volunteers and T1D patients (Gogitidze Joy *et al.*, 2010; Joy *et al.*, 2015). Furthermore, in a rat model of T2D, recurrent hypoglycaemia (once every 3 days for 5 weeks) upregulated endothelial expression of ICAM-1 and VCAM-1 and increased the number of monocytes adhering to the vascular endothelium via catecholamine mediated nuclear translocation of NF- κ B (Jin *et al.*, 2011).

Suggestion of a link between hypoglycaemia and atherogenesis is also derived from *in vitro* studies demonstrating a rapid reduction in nitric oxide (NO) availability in endothelial cells following hypoglycaemia (Wang *et al.*, 2012). Also, recently generated evidence suggests that endogenous NO mediated endothelial vasodilation is reduced following an episode of acute hypoglycaemia in man (Joy *et al.*, 2015). Moreover, repeated episodes of hypoglycaemia further impaired vascular function by reducing endogenous and exogenous NO mediated endothelial function (Joy *et al.*, 2015). In addition, evidence is emerging to support the notion that rebound hyperglycaemia during recovery from hypoglycaemia may exacerbate endothelial dysfunction, inflammation and oxidative stress generation (Ceriello *et al.*, 2012). Together, these findings support an earlier observation that recurrent hypoglycaemia aggravates pre-clinical atherosclerosis in T1D compared to matched controls that do not suffer recurrent hypoglycaemia (Giménez *et al.*, 2011). A summary of the mechanistic links between hypoglycaemia and CVD is illustrated in Figure 1.3.

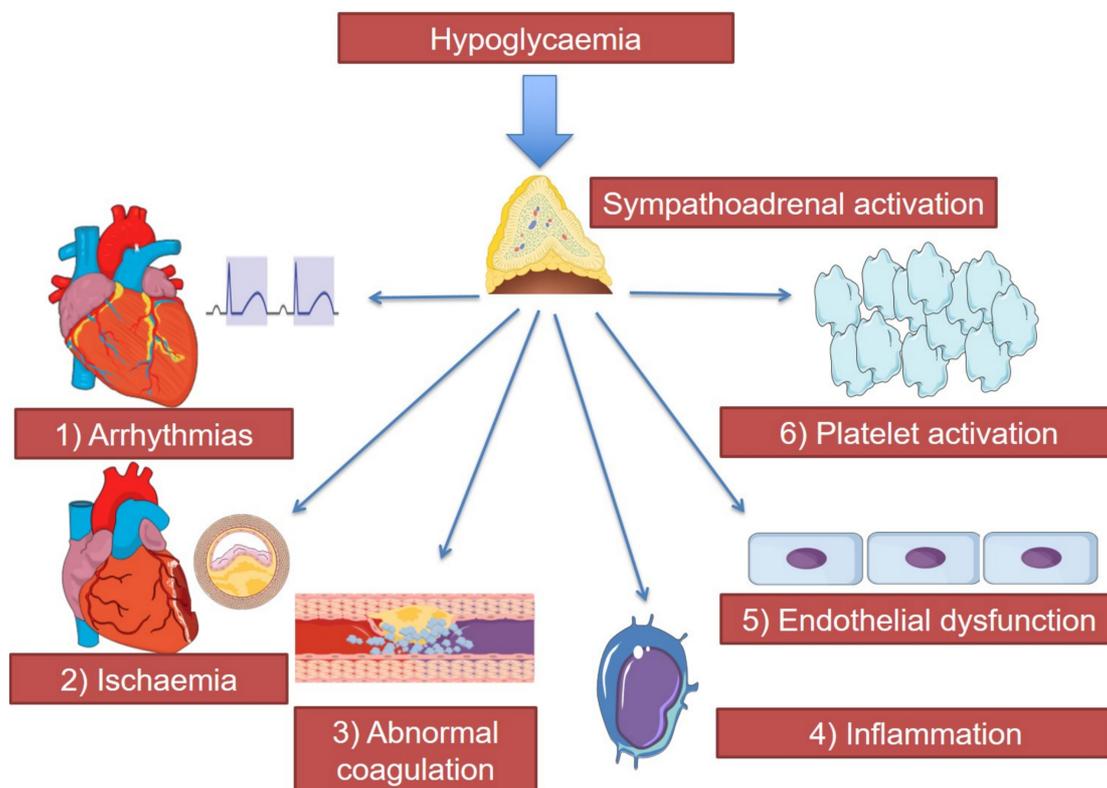


Figure 1-3 Putative mechanisms linking hypoglycaemia and CVD. Hypoglycaemia is characterised by sympho-adrenal activation. Abnormal cardiac repolarisation and deranged autonomic function as well as the acute haemodynamic consequences of hypoglycaemia can induce CV events via arrhythmias (1) and cardiac ischaemia (2). Enhanced coagulation and impaired fibrinolysis (3) in addition to platelet activation and aggregation (6) promotes atherothrombosis. An acute leukocytosis and production of pro-inflammatory cytokines (4) as well as upregulation of cell adhesion molecules (5) is likely to encourage atherogenesis in an incremental fashion. Adapted from (Iqbal, Novodvorsky and Heller, 2018).

1.4 Atherosclerosis

Atherosclerosis gives rise to CV disease and CAD which are the leading causes of death worldwide (Weber and Noels, 2011). The aetiology of atherosclerosis is complex, and multifactorial, but at its core involves a progressive narrowing of the lumen in large and medium-sized arteries due to accumulation of lipids and fibrous material (Lusis, 2000). The aetiological processes are chronic as in humans early

atherosclerotic lesions known as 'fatty streaks' start to appear within human foetal aorta (Napoli *et al.*, 1997). The pathology is driven by chronic inflammation which appears at preferential sites in the arterial tree such as points of bifurcation as a likely consequence of cellular responses to altered fluid dynamics at these sites (Moore and Tabas, 2011). Injury to vessels through risk factors such as hypercholesterolaemia and hypertension initiates the process as it allows accumulation of low-density lipoproteins (LDL) in the subendothelial matrix (Lusis, 2000). Within the arterial intima, LDL particles undergo oxidation leading to the release of phospholipids including lysophosphatidylcholine resulting in endothelial cell activation (Weber and Noels, 2011). Endothelial activation then facilitates leukocyte capture, activation and firm arrest at the site of vessel injury prior to transendothelial diapedesis in a sequence of events termed the leukocyte adhesion cascade (Ley *et al.*, 2007). The various leukocytes recruited in this inflammatory cascade include: monocytes, neutrophils, dendritic cells, T cells, B cells and mast cells (Weber, Zernecke and Libby, 2008).

E-selectin, L-selectin and P-selectin bind rapidly to respective ligands thereby mediating capture and rolling of leukocytes and platelets on the vascular endothelium (Alon *et al.*, 1995). Initial capture or tethering is mediated via P-selectin which is expressed on the endothelium or L-selectin which is ubiquitously expressed on leukocytes and binds to its membrane bound ligand CD34 on the endothelium (Baumhueter *et al.*, 1994).

Firm adhesion of leukocytes to the activated endothelium is mediated by integrins and their counter ligands. The activated endothelium is characterised by

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expression of adhesion molecules including VCAM-1 and ICAM-1 which bind to leukocyte integrins (Kume *et al.*, 1992). Adherent leukocytes then, under the influence of an array of chemokines, migrate into the arterial intima. Whilst beyond the scope of this introduction to describe in detail, monocytes, T and B lymphocytes are attracted by chemokines including monocyte chemoattractant protein-1 (MCP-1 or CCL2) on account of these cells expression the c-c motif chemokine receptor type 2 (CCR2) (Boring *et al.*, 1998). Other chemokines including c-c motif chemokine ligand 5 (CCL5 or RANTES) and IFN-inducible T cell α -chemoattractant (I-TAC or CXCL11) recruit T lymphocytes to human atherosclerotic lesions by binding to CCR1 and CXCR3 respectively (Mach *et al.*, 1999). Notably, fractalkine (CX₃CL1) which is a membrane bound chemokine, plays a key role in atherogenesis by recruitment of CX₃CR1 expressing leukocytes including monocytes, T cells and natural killer (NK) cells (Imai *et al.*, 1997; Lesnik, Haskell and Charo, 2003). Monocyte traffic and recruitment into the atherosclerotic plaque is discussed in more detail in section 1.4.2.

In addition to leukocytes, platelets also adhere to activated endothelium and contribute to the initiation of the atherosclerotic lesion formation as a key player (Massberg *et al.*, 2002). Modification of LDL in the vessel wall is not only confined to oxidation, but also includes lipolysis, proteolysis and aggregation (Lusis, 2000). Lipid oxidised LDL (oxLDL) appears to be particularly important in driving inflammatory cell infiltration into arterial intima (Lusis, 2000). Under the influence of macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) produced by endothelial and several other cells, monocytes in the intima differentiate into macrophages (Hansson, Robertson and

Söderberg-Nauclér, 2006; Gisterå and Hansson, 2017). In addition, smooth muscle cells can also differentiate into macrophage-like cells (Gisterå and Hansson, 2017). Macrophage formation is a critical step in the pathogenesis of atherosclerosis as macrophages internalise oxLDL to form foam cells leading to production of pro-inflammatory cytokines including IL-1 β and this augments upregulation of cell adhesion molecules with greater leukocyte traffic into the lesion (Hansson, Robertson and Söderberg-Nauclér, 2006). In due course, a more mature plaque lesion develops which contains a central necrotic core covered by a fibrous cap and regions infiltrated by inflammatory cells producing pro-inflammatory mediators (Hansson, Robertson and Söderberg-Nauclér, 2006). Rupture of atherosclerotic plaques leads to exposure of the necrotic core resulting in atherothrombosis and the clinical syndromes of AMI and CVA. Unstable plaque in the presence of activated platelets may also be more likely to result in coronary artery occlusion and the presence of autonomic dysfunction in people with diabetes makes them particularly vulnerable (Hess, Marx and Lehrke, 2012). Figure 1.4 summarises the pathophysiology of atherosclerosis and plaque rupture.

As the focus of this thesis is to investigate the effects of hypoglycaemia on inflammation as a driver for atherosclerosis, I will now describe the role of the immune system in pathogenesis of atherosclerosis. In particular, I will concentrate on monocytes as plaque macrophages that are derived from circulating monocytes constitute the majority of leukocytes in the atherosclerotic plaque (Lundberg and Hansson, 2010) and then discuss the 'vulnerable' atherosclerotic plaque and atherothrombosis.

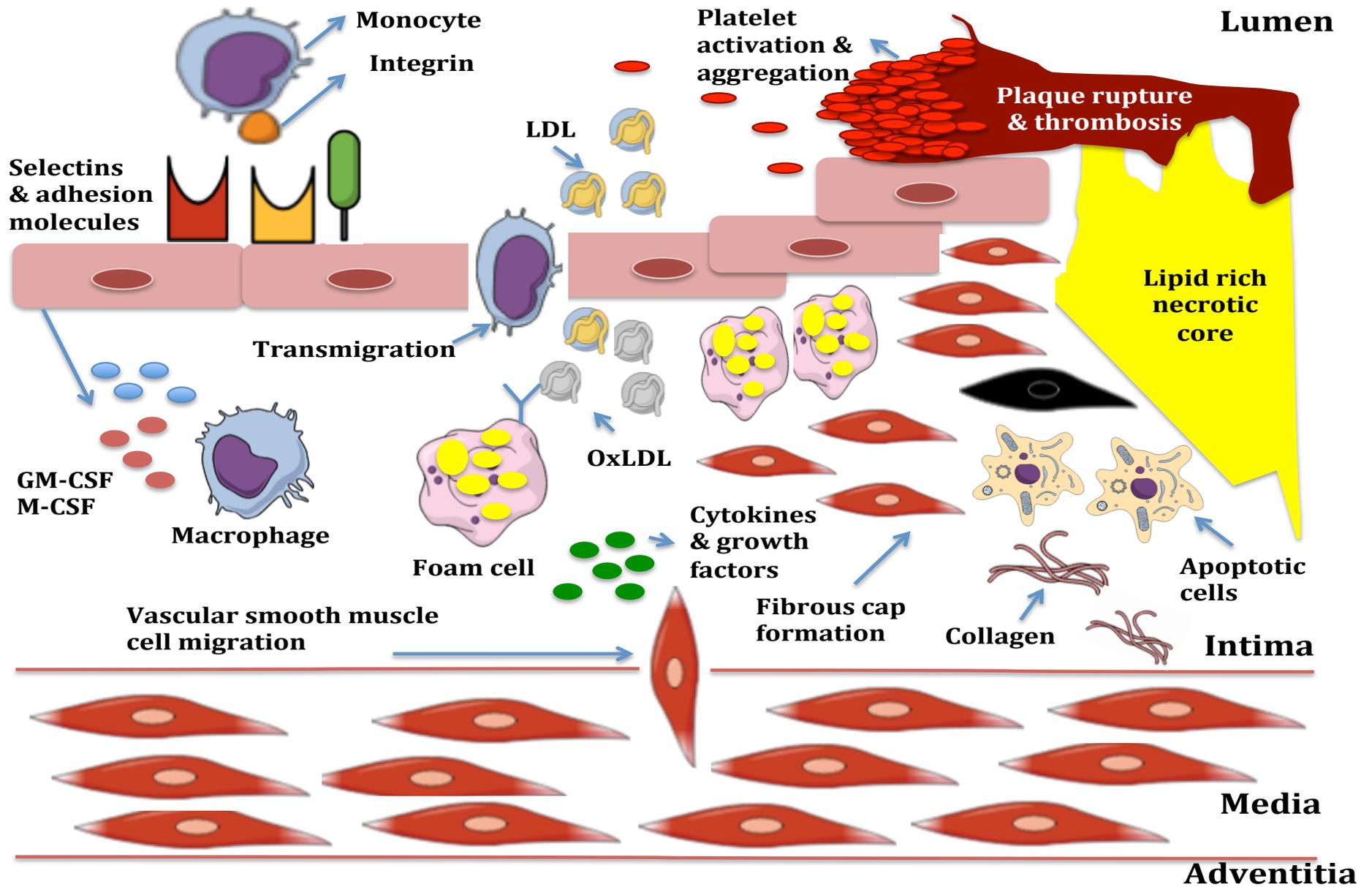


Figure 1-4 Summarises the pathophysiology of lesion formation in atherosclerosis. Upon injury to the vessel wall, such as due to high circulating levels of LDL cholesterol, there is subendothelial accumulation of lipoproteins, resulting in endothelial cell activation. The activated endothelium expresses selectins and upregulates cell adhesion molecules. These facilitate the capture and arrest of leukocytes including monocytes onto the endothelium through adhesion cell molecule-integrin interactions. Under the influence of chemokines, monocytes transmigrate into the subendothelial space. Infiltrated monocytes then differentiate into macrophages in a process facilitated by GM-CSF and M-CSF which are produced by activated endothelial and other cells. Within the arterial intima, LDL particles undergo oxidation to form OxLDL that are phagocytosed by macrophages via scavenger receptors resulting in the formation of foam cells. Pro-inflammatory cytokines and growth factors produced by foam cells encourage migration of vascular smooth muscle cells from the tunica media into the intima. Over time, an accumulation of lipid rich foam cells and vascular smooth muscle cells in addition to the synthesis of collagen and extracellular matrix causes the intima to thicken and bulge towards the arterial lumen. Furthermore, apoptosis and necrosis of foam cells and vascular smooth muscle cells encourages the formation of a lipid rich necrotic core and release of matrix metalloproteinases which destabilise the plaque. Erosion and fissuring of the fibrous plaque allows the highly thrombogenic contents of the necrotic core to come into direct contact with circulating blood. Platelets rapidly activate and aggregate at this site resulting in the formation of a thrombus and this can significantly narrow the vessel lumen thus leading to an acute myocardial infarction. Abbreviations: LDL, low density lipoproteins; oxLDL, oxidised low density lipoprotein; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage-colony stimulating factor.

1.4.1 The innate immune system in atherosclerosis

The innate system also has a major role in the pathogenesis of atherosclerosis as it launches a defence of arteries challenged by a host of inflammatory stimuli. Activation of the innate immune system is dependent on a number of pattern-recognition receptors (PRR) (Lundberg and Hansson, 2010). Broadly, PRR can be sub-classified into endocytic receptors and signalling receptors. Endocytic receptors largely mediate antigen uptake with ligand binding to these receptors not usually inducing pro-inflammatory changes (Lundberg and Hansson, 2010). In contrast, when ligated, a second distinct group of signalling receptors activates pro-

inflammatory pathways by upregulation of a number of pro-inflammatory genes (Lundberg and Hansson, 2010). In atherosclerosis, endocytic scavenger receptors that recognise oxLDL and mediate its internalisation include: CL-P1, LOX-1, CXCL16, CD36, MARCO, SRA-1 and SRA-II (Greaves and Gordon, 2009). Thus, by being involved in clearance of oxLDL, endocytic PRR directly contribute to the formation of foam cells.

Toll-like receptors (TLRs) are highly evolutionarily conserved and are the principal signalling PRR. TLR expression occurs on a number cells which are present in the developing atherosclerotic lesion (Akira, Uematsu and Takeuchi, 2006; Lundberg and Hansson, 2010). In cells expressing TLR, stimulation with the respective TLR ligands triggers intracellular signalling pathways including mitogen activated protein kinase (MAPK). This culminates in the activation of a number of pro-inflammatory transcription factors including nuclear factor (NF) κ B, activating protein (AP)-1 and interferon regulating factors (IRF) (Lundberg and Hansson, 2010). In both normal and atherosclerotic human vasculature, expression of a wide repertoire of TLR including TLR1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 has been demonstrated (Edfeldt *et al.*, 2002; Curtiss and Tobias, 2009). Evidence from human specimens suggests that TLR2 and TLR4 play a dominant role in orchestrating macrovascular inflammation (Pryshchep *et al.*, 2008).

In addition to membrane bound PRR such as TLR, intracellular signalling PRR exist in innate immune cells. These include intracellular TLRs, but also a range of other proteins that can organise into high molecular weight inflammasomes (Hansson and Hermansson, 2011). The latter have been described as 'molecular platforms'

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and upon activation trigger secretion of pro-inflammatory cytokines including IL-1 β and IL-18 (Schroder and Tschopp, 2010). The nucleotide binding domain, leucine rich containing family, pyrin domain containing 3 (NLRP3) inflammasome has been shown to be activated by cholesterol crystals in phagocytes *in vitro* in a process involving phago-lysosomal damage (Duewell *et al.*, 2010). In parallel *in vivo* experiments, the latter authors also reported that mice deficient in NLRP3 developed less atherosclerosis. Recently, a randomised double-blinded placebo controlled trial investigated the efficacy of IL-1 β targeting in reducing CV events in 10,061 patients with established CVD (Ridker *et al.*, 2017). Using canakinumab, a therapeutic monoclonal antibody targeting IL-1 β , the investigators demonstrated a significant reduction in recurrent CV events compared to placebo. There was, however, a high incidence of fatal infection associated with canakinumab use, but all cause mortality was not significantly different between treatment and placebo (Ridker *et al.*, 2017).

The different groups of PRR do not operate in isolation and significant cross-talk exists in the developing atherosclerotic lesion to generate a broader innate immune response to injury (Figure 1.5).

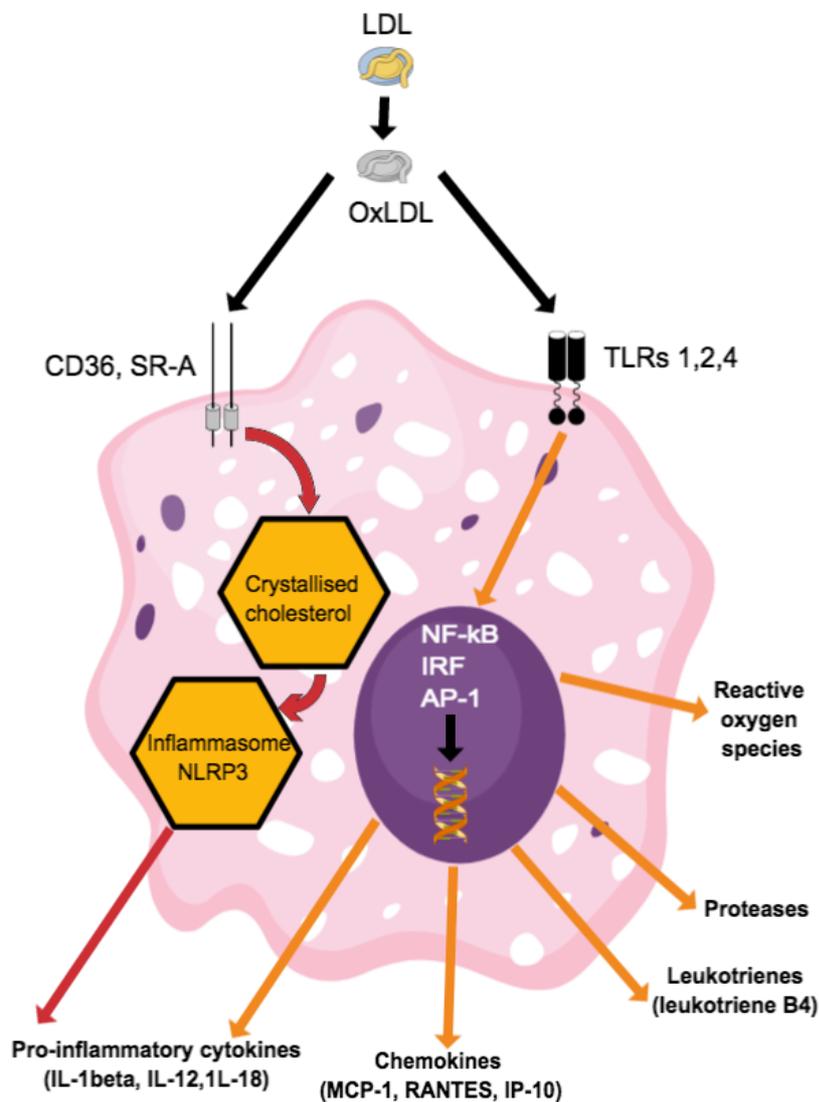


Figure 1-5 Activation of the innate immune system in atherosclerosis. Innate immune cells including macrophages and dendritic cells express a variety of pattern recognition receptors including TLRs, CD36, SRA that can induce pro-atherosclerotic changes through different inflammatory pathways. Ligation of TLRs with OxLDL activates intracellular signalling pathways that culminate in activation of the transcription factors NF- κ B, IRF and AP-1 which then drive up regulation of genes encoding pro-inflammatory cytokines, chemokines, proteases (collagenases, elastases, cathepsins), reactive oxygen and nitrogen species and eicosanoids, principally leukotriene B4 all of which collectively promote inflammation and atherosclerosis. OxLDL can also be internalised by scavenger receptors including CD36 and SRA which results in accumulation of intracellular cholesterol crystals promoting macrophages into foam cells. Cholesterol crystals can trigger activation of inflammasomes including NLRP3 that further augment inflammation by producing pro-inflammatory cytokines including IL-1 β . Abbreviations: TLR, toll-like receptors; SRA, scavenger receptor class A; oxLDL, oxidised low density lipoprotein; NF- κ B, nuclear factor κ B; IRF, interferon regulating factors; AP-1, activating protein-1; NLRP3, nucleotide binding domain, leucine rich containing, family pyrin domain containing 3.

1.4.2 Monocytes in atherosclerosis

Monocytes are a subset of circulating leukocytes and differentiate into macrophages and dendritic cells (DC) in tissues (Shi and Pamer, 2011). Monocytes have been studied extensively in the context of atherosclerosis both in murine models and humans given their role as major players in the innate immune system with key functional roles in phagocytosis, antigen presentation and cytokine production (Ziegler-Heitbrock, 2015).

1. Monocyte subsets

Monocytes comprise 4% and 10% of all blood leukocytes in mice and humans, respectively (Dutta and Nahrendorf, 2015). Considerable heterogeneity exists within and between species in circulating blood monocytes based on chemokine receptor expression and presence of cell surface molecules (Shi and Pamer, 2011; Wong *et al.*, 2012). In mice, the main subset of CD115⁺ monocytes express high levels of Ly-6c and CCR2, but low levels of CX₃CR1 and are termed Ly-6c^{high} monocytes (Shi and Pamer, 2011). The latter have been termed 'inflammatory' monocytes based on data demonstrating their rapid recruitment to sites of inflammation and production of pro-inflammatory cytokines including IL-1 β and TNF- α (Dutta and Nahrendorf, 2015). A second major subset of monocytes identified in mice express low levels of Ly-6c and CCR2, but high levels of CX₃CR1 and are termed Ly-6c^{low} monocytes (Dutta and Nahrendorf, 2015). Less prevalent than Ly-6c^{high} cells, Ly-6c^{low} monocytes are thought to reside in the blood stream and patrol the endothelium through long-range crawling allowing these cells to scavenge lipids, cellular debris and microorganisms (Auffray *et al.*, 2007). Recent studies have determined that human monocytes can be classified into 3 distinct

subsets based on surface expression of CD14 (LPS and apoptotic cell receptor) and CD16 (Fc γ receptor type III) termed classical monocytes (CM: CD14⁺⁺ CD16⁻, 'Mon1'), intermediate monocytes (IM: CD14⁺⁺ CD16⁺, 'Mon2') and non-classical monocytes (NCM: CD14⁺ CD16⁺⁺, 'Mon3') (Ziegler-Heitbrock *et al.*, 2010; Weber *et al.*, 2016). Classical monocytes comprise 85% of circulating human monocytes, the remaining 15% being comprised of intermediate and non-classical monocytes (Wong *et al.*, 2012). Classical monocytes in addition to being most abundant also express CCR2 and are thought to be homologous to mouse Ly-6c^{high} cells (Shi and Pamer, 2011). Intermediate monocytes have pro-inflammatory roles and non-classical monocytes, on account of having *in vivo* patrolling behaviour involved in tissue surveillance, are thought to resemble murine Ly-6c^{low} cells (Cros *et al.*, 2010; Dutta and Nahrendorf, 2015). Both IMs and NCMs have traditionally been clustered together under the umbrella of CD16⁺ monocytes. This is especially true for earlier studies (Passlick, Flieger and Loms Ziegler-Heitbrock, 1989) before the current nomenclature was devised and when less sophisticated flow cytometric analyses were available thus limiting the ability to dissect these populations. Once stimulated with TLR ligands, CD16⁺ cells produce pro-inflammatory cytokines including TNF- α (Grage-Griebenow *et al.*, 2001). Furthermore, selective depletion of these cells both *in vitro* and *in vivo* by the anti-inflammatory effects of high dose glucocorticoids has traditionally justified their pro-inflammatory categorisation (Dayyani *et al.*, 2003).

A number of detailed gene expression profiling studies have furthered our knowledge of human monocyte biology beyond what is possible within the analytical constraints of even contemporary multi-colour flow cytometry (Mobley

et al., 2007; Ancuta *et al.*, 2009; Zhao *et al.*, 2009; Wong *et al.*, 2011). Certain defining features that are subset specific emerge from these data. CMs possess the widest repertoire of sensing receptor genes and genes involved in tissue repair and immune responses (Wong *et al.*, 2011). The relatively higher expression of pro-inflammatory genes including *S100A8/9* and *S100A12* genes which encode the phagocyte-specific S100 proteins may underpin the ability of CMs to orchestrate inflammation (Roth *et al.*, 2003). The transcription factor AP-1 is also most highly expressed by CMs at a gene level (Wong *et al.*, 2011). This is perhaps reflected in the ability of CMs to produce the widest array of chemokines and cytokines in response to LPS stimulation *in vitro* including: G-CSF, CCL2, RANTES, IL-6, IL-8, IL-1 β , TNF- α and IL-10 (Wong *et al.*, 2011). Microarray studies suggest a close resemblance in the expressed subsets of genome of IMs and NCMs (Wong *et al.*, 2011). There are, however, genetic differences between these monocyte subsets. Major histocompatibility complex (MHC) class II expression is for example higher in IMs relative to NCMs (Wong *et al.*, 2011). These findings support an earlier observation that IMs have a greater role in antigen processing and presentation, specifically to T-cells in addition to IL-12 production capacity (Grage-Griebenow *et al.*, 2001). In addition, IMs have an enrichment of genes related to angiogenesis (*TIE2*, *CD105*) (Zawada *et al.*, 2011). At least one study has, however, suggested that upon LPS stimulation *in vitro*, IMs are the primary producers of the anti-inflammatory cytokine IL-10 (Skrzeczyńska-Moncznik *et al.*, 2008) contrary to a more recent investigation (Wong *et al.*, 2011) demonstrating that CMs were unequivocally the most prolific producers of IL-10 following LPS. Discrepant reporting on the cytokine production capacity of human monocyte subsets is a likely reflection of methodological differences. For example, isolation of human

monocytes subsets can be performed using a variety of clones of the anti-CD14 monoclonal antibody. There exist clonal differences in that with certain anti-CD14 clones, for example ME52, LPS stimulation of CD14 is significantly inhibited (Power *et al.*, 2004). Arguably, intracellular staining for cytokines in whole blood which avoids the pitfalls of cellular activation and inhibition due to isolation techniques yields the most reliable data (Wong *et al.*, 2011). In contrast to IMs, NCMs have a greater expression of genes involved in re-arrangement of the cellular cytoskeleton (Wong *et al.*, 2011). This supports the role of these cells in *in vivo* patrolling (Cros *et al.*, 2010). In response to LPS *in vitro*, NCMs have also been shown to be the highest producers of pro-inflammatory cytokines TNF- α and IL-1 β (Belge *et al.*, 2002; Wong *et al.*, 2011). These data indicate that NCMs have a pro-inflammatory role that extends beyond tissue patrolling and surveillance of nucleic acids and viruses via TLR7 and TLR8 as previously reported (Cros *et al.*, 2010). Table 1.3 summarises the functional and phenotypic features of the three human monocyte subsets.

Table 1.3 Functional and phenotypic characteristics of the three human monocyte subsets adapted from (Wong *et al.*, 2012). Abbreviations: CCR2, c-c motif chemokine receptor type 2; CX₃CR1, C-X3-C motif chemokine receptor type 1; CD62L, L-selectin; HLA-DR, human leukocyte antigen D related; SLAN, 6-sulfo LacNAc; G-CSF, granulocyte colony stimulating factor; IL-10, interleukin 10; RANTES, regulated on activation, normal T cell expressed and secreted; IL-6, interleukin 6; IL-8, interleukin 8; TNF- α , tumour necrosis factor alpha; IL-1 β , interleukin 1 beta; LPS, lipopolysaccharide; ROS, reactive oxygen species.

Characteristic	Classical monocyte <i>CD14⁺⁺ CD16⁻</i> 'Mon1'	Intermediate monocyte <i>CD14⁺⁺ CD16⁺</i> 'Mon2'	Non-classical monocyte <i>CD14⁺ CD16⁺⁺</i> 'Mon3'
Percentage of total circulating monocytes	85%	5%	10%
Key chemokine receptors	CCR2 ^{high} CX ₃ CR1 ^{low} CD62L	CCR2 ^{low} CX ₃ CR1 ^{high} HLA-DR	CCR2 ^{low} CX ₃ CR1 ^{high} SLAN
Cytokine response to LPS	IL-10, G-CSF, RANTES, IL-6, IL-8	IL-6, IL-8	TNF- α , IL-1 β , IL-6, IL-8
Functions	Superior phagocytosis	T-cell proliferation and stimulation ROS production Angiogenesis Pro-inflammatory	In vivo patrolling behaviour Antiviral roles Likely pro-inflammatory role

2. Monocyte production and replenishment

Our current understanding of monocyte origin, kinetics and fate under homeostasis and inflammation are largely derived from studies in rodents. Within the bone marrow, haematopoietic stem cells (HSC) give rise to common myeloid progenitors, granulocyte/macrophage progenitors and macrophage and dendritic cell progenitors (MDP) (Dutta and Nahrendorf, 2015). Monocyte production in the bone marrow is under the control of M-CSF and targeted disruption of the M-CSF-1

receptor gene in mice results in diminished monocyte counts and reduced atherosclerosis (Rajavashisth *et al.*, 1998; Dai *et al.*, 2002). Egress of Ly-6c^{high} monocytes from the bone marrow into the blood stream is dependent on CCR2 (Shi and Pamer, 2011) and in ApoE^{-/-} mice, deficiency of CCR2 decreases atherosclerotic lesion formation (Boring *et al.*, 1998). Upon emigration from the bone marrow, Ly-6c^{high} monocytes are recruited to sites of inflammation where they can differentiate into inflammatory DCs or inflammatory macrophages (Shi and Pamer, 2011). Interestingly, Ly-6c^{high} monocytes can generate Ly-6c^{low} monocytes under steady state conditions and the latter ordinarily patrol the endothelium with a half life of approximately 2.5 days, although may differentiate into alternatively activated macrophages with an anti-inflammatory phenotype and play a part in wound healing (Yona *et al.*, 2013). In the absence of inflammation, Ly-6c^{high} monocytes can return to the bone marrow and have a short life span with a typical half life of < 1 day whilst in circulation (Shi and Pamer, 2011). The spleen acts an important reservoir of Ly-6c^{high} monocytes and can rapidly deploy these cells to sites of inflammation (Swirski *et al.*, 2009). It appears that in addition to hosting bone marrow derived monocytes, the spleen plays a part in active production of monocytes from HSC in splenic niche sites (Robbins *et al.*, 2012). Monocytes originating in the spleen through extramedullary haematopoiesis have been shown to contribute directly to the atherosclerotic lesion via fate mapping studies (Robbins *et al.*, 2012). The latter authors also report that functionally, spleen derived monocytes displayed an inflammatory profile resembling bone marrow derived cells characterised by production of pro-inflammatory cytokines, generation of ROS and production of proteases. Furthermore, these cells ultimately formed foam cells through ingesting lipids. In ApoE^{-/-} mice, Dutta *et al.*

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demonstrated that an episode of AMI accelerates pre-established atherosclerosis (Dutta *et al.*, 2012). The authors showed a sustained systemic monocytosis in response to AMI that was mediated by an increased sympathetic drive. Sympathetic nervous signaling in the bone marrow via β_3 adrenergic receptors released HSC. The latter seeded the spleen resulting in increased monocyte numbers via extramedullary haematopoiesis. Spleen derived monocytes then trafficked to the plaque and continued to promote plaque instability in the days following an acute CV event (Dutta *et al.*, 2012).

There is a paucity of data detailing the fate and life-span of human monocyte subsets. Nevertheless, approaches involving radio-isotope labelling of human leukocytes *in vivo* have advanced our understanding in this field (Busch *et al.*, 2007). In a recent study, Patel *et al.* using *in vivo* deuterium labelling studied human monocyte subset kinetics in 30 healthy volunteers (Patel *et al.*, 2017). The authors report that under homeostatic conditions, CMs arise from monocyte precursors in the bone marrow and circulate systemically for approximately 1 day. Within the peripheral circulation, CMs sequentially transition into IMs and the latter ultimately transition into NCMs before leaving the circulation.

3 Monocyte recruitment in atherosclerosis

A classical cascade involving selectins, cell adhesion molecules and chemokines orchestrates monocyte traffic and recruitment into the atherosclerotic plaque. During atherosclerosis, recruitment of both Ly-6c^{high} and Ly-6c^{low} monocytes in the plaque has been reported with comparatively less evidence to characterise recruitment of the latter (Woollard and Geissmann, 2010). The selectin families

have been shown to play a key role in mediating monocyte traffic into the plaque. Initially, monocytes undergo P-selectin and E-selectin dependent rolling on the endothelium (Shi and Pamer, 2011). P-selectin is expressed on activated endothelium and platelets and can bind neutrophils, monocytes and lymphocytes via its ligand P-selectin glycoprotein ligand 1 (PSGL1) which is expressed by these cells. E-selectin also interacts with PSGL1 (Katayama *et al.*, 2003) and notably LDLR^{-/-} mice with a combined deficiency of P-selectin and E-selectin had significant protection from atherosclerosis (Dong *et al.*, 1998). Ly-6c^{high} monocytes preferentially home into sites of developing atherosclerotic lesions as a consequence of highly expressing PSGL1 with subsequent P-selectin or E-selectin mediated recruitment (An *et al.*, 2008).

Firm adhesion of monocytes onto the activated endothelium is facilitated by integrins on the surface of monocytes that bind to endothelial cell adhesion molecules (Ley *et al.*, 2007). The very late antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1) are highly expressed on circulating monocytes and interact with VCAM-1 and ICAM-1 respectively (Woollard and Geissmann, 2010). Human monocytes have been shown to express the integrin CD11c which is not present on murine monocytes (Geissmann, Jung and Littman, 2003), however, genetic analysis of human NCMs has revealed upregulation of patrolling associated integrins including LFA-1 (G. Thomas *et al.*, 2015). Several studies have highlighted an important role for both VCAM-1 and ICAM-1 in monocyte traffic into the plaque (Patel *et al.*, 1998; Cybulsky *et al.*, 2001; Shi and Pamer, 2011)

Chemokines which are secreted by endothelial cells, smooth muscle cells, fibroblasts and macrophages interact with their respective receptors on monocytes to facilitate extravasation of monocytes into the atherosclerotic plaque. In Ly-6c^{high} monocytes, CCR2, CX₃CR1 and CCR5 direct traffic into lesions (Woollard and Geissmann, 2010). In ApoE^{-/-} mice, combined inhibition of CCL2, CX₃CR1 and CCR5 led to a 90% reduction in atherosclerosis with an abrogation of monocytosis normally associated with hyperlipidaemia in these animals (Combadiere *et al.*, 2008). In CD16⁺ monocytes, CX₃CR1 expression has been shown to preferentially mediate arrest and migration of these pro-inflammatory cells on the vessel wall (Ancuta *et al.*, 2003).

1.4.3 Clinical studies of human monocyte subsets and CVD

A number of observational studies have suggested that leukocytosis is a poor prognostic indicator in stable CAD and following AMI and CVA (Brown, Giles and Croft, 2001; Madjid *et al.*, 2004; Ohira *et al.*, 2006). Epidemiological studies investigating an association between total monocyte counts and risk of acute CV events have yielded conflicting results that can possibly be explained by methodological differences and confounding factors. Some studies suggest that a higher total monocyte count is a strong independent predictor of future CV events (Horne *et al.*, 2005; Dragu *et al.*, 2008). Others have reported no significant relationship between monocyte counts and CV mortality (Gurm *et al.*, 2003; Rana *et al.*, 2007).

A number of observational studies have also examined the relationship between different monocyte subsets and CV risk. Whilst differences in flow cytometric

gating strategies between studies makes it challenging to directly compare the absolute numbers and percentages of individual subsets, an association between CD16⁺ IMs and NCMs and CV risk is emerging (Schlitt *et al.*, 2004; Heine *et al.*, 2008; Wildgruber *et al.*, 2009; Poitou *et al.*, 2011; Rogacev *et al.*, 2011; Hilgendorf and Swirski, 2012). One of the largest observational studies investigating the role of monocyte subsets in predicting CV events prospectively studied 951 patients referred for elective coronary angiography over a mean follow-up of 2.6 years (Rogacev *et al.*, 2012). In this high-risk population, the primary endpoint, defined a priori as first occurrence of CV death, AMI or non-haemorrhagic stroke was independently predicted by total numbers of IMs but not total monocyte counts or numbers of CMs and NCMs.

Relatively small (< 100 subjects) observational studies have also investigated the relationship between monocyte subsets and coronary artery plaque stability in patients with stable angina, unstable angina and those with recent AMI treated with intra-coronary stents (Imanishi *et al.*, 2010; Kashiwagi *et al.*, 2010; Liu *et al.*, 2010). Collectively, these studies demonstrated that higher levels of peripheral CD16⁺ monocytes positively correlated with plaque instability and stent failure. Importantly, there is also epidemiological evidence to suggest that CD14⁺⁺ CD16⁻ CMs may increase CV risk (Hristov *et al.*, 2010). In a study of 700 subjects from a general population both a higher absolute number and percentage of CMs independently predicted future CV events (Berg *et al.*, 2012).

There is good evidence from murine models that a systemically stressful event such as AMI mobilises monocytes (Nahrendorf *et al.*, 2007; Dutta *et al.*, 2012). Others

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have also investigated monocyte subset kinetics in man and demonstrated a preferential expansion of the IM population during the first 72 hours following a ST-elevation myocardial infarction (STEMI)(Tapp *et al.*, 2012; Zhou *et al.*, 2016). Moreover, these dynamic changes in IMs adversely predicted CV outcomes at up to 2 years of follow-up (Zhou *et al.*, 2016) . Mobilisation of monocytes in the context of AMI in mice has been shown to be catecholamine mediated (Dutta *et al.*, 2012). In humans, exercise that generates a catecholamine response of a similar order of magnitude to acute CV events also selectively mobilises CD16⁺ cells in a catecholamine dependent fashion (Birgit Steppich *et al.*, 2000). The rapidity (within 5 minutes) with which CD16⁺ monocytes appear in the circulation suggests mobilisation from the marginal pool where these cells preferentially home on account of expressing the adhesion molecules CD11d and VLA-4 (Birgit Steppich *et al.*, 2000). In healthy volunteers, intravenous adrenaline infusion also rapidly mobilised CD16⁺ monocytes that had characteristic a CD11a^{high} and CXCR1^{high} expression allowing them to attach to the vascular endothelium and then demarginate under the influence of adrenaline agonism of surface β 2 adrenoreceptors (Dimitrov, Lange and Born, 2010).

Overall, data from observational studies lends credibility to the notion that NCMs and IMs are particularly important in the pathogenesis of atherosclerosis and are preferentially mobilised following acute CV events in a catecholamine dependent fashion. These studies are, however, limited by their observational nature, relatively small number of participants and a non-uniform methodological approach to identifying and enumerating human monocyte subsets which makes direct comparison between studies challenging.

1.4.4 Macrophages in atherosclerosis

Macrophages play a critical role in the aetiology of atherosclerosis. Traditionally, macrophage subsets have been divided into M1 and M2 phenotypes. M1 macrophages have been characterised as being 'pro-inflammatory' on account of these cells being activated by a "classical" stimulus such as LPS or other TLR ligands with subsequent production of pro-inflammatory cytokines including IL-1 β , TNF- α and IL-12 (Moore, Sheedy and Fisher, 2013). M1 macrophages have been implicated in plaque evolution and destabilisation (Moore, Sheedy and Fisher, 2013). In contrast, M2 macrophages are thought to be activated "alternatively" by IL-13 and IL-4 and deemed 'anti-inflammatory' based on these cells secreting anti-inflammatory cytokines including IL-10 and IL-1 receptor antagonist (Moore, Sheedy and Fisher, 2013). M2 macrophages display high endocytic activity and secrete tissue repair factors including collagen (Moore, Sheedy and Fisher, 2013). The transcription factors NR4A1 (NUR77) and Krüppel-like factor 4 (KLF4) are critical regulators of macrophage polarisation and inhibit M1 polarisation whilst favouring the M2 phenotype (Liao *et al.*, 2011; Hamers *et al.*, 2012; Hanna *et al.*, 2012).

A heterogeneous distribution of macrophage populations has been reported in human atherosclerotic plaques with M1 macrophages localising to lipid rich areas in contrast to M2 macrophages that locate far from the lipid core of the lesion and contain smaller lipid droplets in comparison to M1 cells (Chinetti-Gbaguidi *et al.*, 2011). Data from ApoE $^{-/-}$ mice suggests an early overrepresentation of M2 macrophages in the developing plaque but with lesional progression, a dominance of M1 over the M2 phenotype has been reported (Khallou-Laschet *et al.*, 2010). M1

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macrophages in the aged plaque are most likely derived from M2 cells that predominate in the nascent lesion giving rise to the notion of macrophage plasticity in atherosclerosis (Khallou-Laschet *et al.*, 2010).

The dichotomisation of macrophages into M1 pro-inflammatory and M2 anti-inflammatory phenotypes has, however, recently been questioned (Moore, Sheedy and Fisher, 2013). It has been argued that *in vivo*, lesional macrophages are likely to encounter a multitude of signals meaning that a phenotypic spectrum of macrophages exists depending on the prevailing conditions within the atherosclerotic plaque. Specifically, the presence of oxidised lipids (Gallardo-Soler *et al.*, 2008) and other signals in the plaque can trigger modulation of macrophages and a considerable heterogeneity exists as a result of functional plasticity (Gleissner *et al.*, 2010).

1.4.5 Platelets in innate immunity and atherosclerosis

It is now increasingly recognised that platelets play a key role in generating the immune responses driving atherosclerosis.

Upon activation, platelets release the contents of their α granules including adenosine diphosphate (ADP) which in turn acts on platelet P2Y₁ and P2Y₁₂ receptors promoting further platelet activation and release of ADP in a positive amplification loop (Thomas and Storey, 2015). Platelet α granules contain a whole host of mediators that drive inflammation via releasing chemokines which control leukocyte traffic and function and expressing receptors that directly interact with

leukocytes to induce pro-inflammatory effects (Thomas and Storey, 2015). Platelet factor 4 (PF4 or CXCL4) is abundantly expressed in α granules and plays a key role in atherosclerosis as elimination of PF4 in ApoE $^{-/-}$ mice dramatically reduced atherosclerotic burden by 73% (Sachais *et al.*, 2007). RANTES (CCL5), a chemokine also secreted by α granules plays a key part in atherosclerosis by encouraging monocyte recruitment to the atherosclerotic endothelium (Mause *et al.*, 2005). Activated platelets also promoted endothelial expression of ICAM-1 and CCL2 via IL-1 dependent mechanisms encouraging monocyte arrest on the activated endothelium, a key step in early atherogenesis (Gawaz *et al.*, 2000).

In addition to production of chemokines, activated platelets modulate expression of adhesion molecules on their surface allowing interactions between platelets, leukocytes and endothelial cells. Activated platelets release P-selectin (CD62P) from their α granules which is expressed on the platelet plasma membrane and facilitates links between thrombosis and inflammation (Stenberg *et al.*, 1985). A variety of innate immune cells including neutrophils, monocytes and CD34⁺ haematopoietic progenitor cells possess the corresponding P-selectin glycoprotein ligand-1 (PSGL1, CD162) (Spertini *et al.*, 1996) allowing direct interaction between platelets and immune cells which will be discussed below.

1.4.6 Platelet-leukocyte interactions

Platelets initially bind to leukocytes via PSGL1 and this interaction triggers more firm interactions between leukocyte β 2 integrin CD11b/CD18 and a number of platelet ligands including glycoprotein 1b, platelet bound fibrinogen or platelet

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ICAM-2 (Evangelista *et al.*, 1999). In addition, P-selectin expressed by immobilised platelets on the vessel wall also promotes adhesion of leukocytes to endothelial cells via P-selectin-PSGL1 mediated interactions thus promoting leukocyte extravasation to sites of injury (Mine *et al.*, 2001). Platelet P-selectin mediated interactions with leukocytes are critical to the development of atherosclerosis as in ApoE^{-/-} mice with selective disruption of platelet P-selectin being strongly atheroprotective (Burger and Wagner, 2003).

The binding of leukocytes to platelets to form circulating monocyte and neutrophil platelet-aggregates (MPAs and NPAs) has functional consequences for leukocytes that are important in the pathogenesis of atherosclerosis. In monocytes, platelet binding via P-selectin-PSGL1 increased expression and activity of the $\alpha 4$ - $\beta 1$ and αM - $\beta 2$ (CD11b/CD18) integrins and increased monocyte adhesion to VCAM-1, ICAM-1 and fibronectin (Martins *et al.*, 2005). Platelet adhesion to monocytes via P-selectin also modulated cytokine production by enhancing nuclear translocation of NF- κ B with an increased secretion of pro-inflammatory cytokines CCL2 (MCP-1) and TNF- α (Weyrich *et al.*, 1995). Notably, monocyte-platelet interactions *in vitro* upregulated CD16 expression on monocytes shifting the phenotype of CMs (CD14⁺⁺ CD16⁻) towards the pro-inflammatory IMs (CD14⁺⁺ CD16⁺) (Passacuale *et al.*, 2011). In human neutrophils, P-selectin binding to PSGL-1 induced activation of αM - $\beta 2$ integrins promoting the adhesive properties of these cells (Ma, Plow and Geng, 2004).

Platelet-leukocyte interactions also occur through P-selectin-PSGL1 independent mechanisms including triggering receptors expressed on myeloid cells (TREM)

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(Klesney-Tait, Turnbull and Colonna, 2006). Moreover, interactions between the CD40 receptor and its ligand CD40L (CD154) have been shown to be important in the pathogenesis of atherothrombosis (Anand et al. 2003). Platelets express CD40L on their surface which induces tissue factor expression on whole blood monocytes (Lindmark, Tenno and Siegbahn, 2000). Activated platelets are also a major source of soluble CD40L (sCD40L) which has been shown to activate human endothelial cells by increased production of IL-6 and increased expression of endothelial P-selectin and E-selectin (Déchanet *et al.*, 1997). These changes promote leukocyte traffic and adhesion at sites of evolving atherosclerotic plaque. Figure 1.6 summarises schematically interactions between monocyte and platelets to form MPAs.

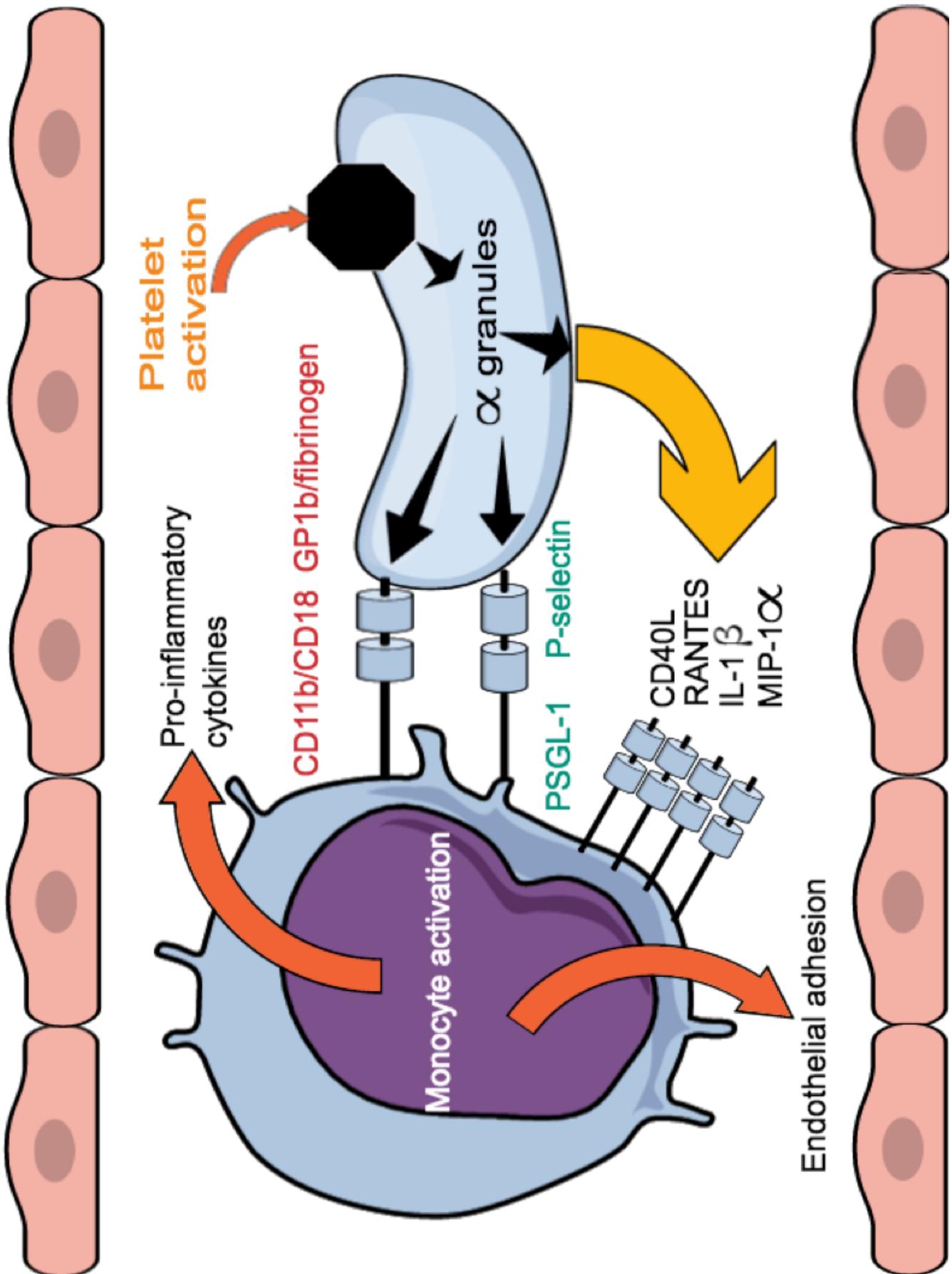


Figure 1-6 Interactions between monocytes and platelets to form monocyte-platelet aggregates. Platelets can be activated by a number of agonists including: adrenaline, thromboxane A₂, thrombin, collagen and platelet activating factor. Upon activation, platelet α granules degranulate releasing a number of mediators including: CD40L, IL-1 β , RANTES, PF4, and MIP-1 α . Action of these inflammatory mediators on corresponding monocyte receptors (CD40-CD40L, IL-1R-IL-1 β , CCR5-RANTES, CCR3-RANTES, CCR1-RANTES, CCR1-MIP-1 α and PF4 receptor-PF4) induces pro-inflammatory changes in monocytes characterised by the production of MCP-1/CCL2, IL-6 and TNF- α . Platelet membrane bound P-selectin interacts with PSGL1 on monocytes to form monocyte-platelet aggregates. This initial attachment is strengthened by interactions between monocyte expressed integrins CD11b/CD18 and platelet glycoprotein 1b, platelet bound fibrinogen or platelet ICAM-2. Monocyte-platelet aggregate formation activates monocytes and upregulates expression of monocyte adhesion molecules facilitating recruitment to atherosclerotic lesions. Also, monocyte activation creates a pro-inflammatory milieu by augmenting pro-inflammatory cytokine production through induction of a number of pro-inflammatory transcription factors including NF- κ B. Monocyte activation also results in synthesis and release of platelet agonists including platelet activating factor thus contributing to further platelet activation in a vicious cycle. Abbreviations: CD40L, CD40 ligand; IL-1 β , interleukin 1 beta; RANTES, regulated on activation, normal T cell expressed and secreted; PF4, platelet factor 4; MIP-1 α , macrophage inflammatory protein 1 alpha; IL-1R, interleukin 1 receptor; CCR5, c-c motif chemokine receptor type 5; CCR3, c-c motif chemokine receptor type 3; CCR1, c-c motif chemokine receptor type 1; MCP-1, monocyte chemoattractant protein-1; CCL2, c-c motif chemokine ligand 2; IL-6, interleukin 6; TNF- α , tumour necrosis factor alpha; PSGL1, P-selectin glycoprotein ligand 1; ICAM-2, intracellular adhesion molecule-2; NF- κ B, nuclear factor κ B.

1.4.7 Clinical studies of monocyte-platelet aggregates in CVD

Early observational studies suggested that MPA formation is increased in CAD with enhanced MPA formation specifically in the context of T1D and T2D related angiopathy (Furman *et al.*, 1998; Kaplar *et al.*, 2001). MPA formation is also important in the pathogenesis of acute CV events with elevated MPA counts noted post AMI (Furman *et al.*, 2001; Sarma *et al.*, 2002). Circulating MPAs are a very sensitive, early marker of AMI, and have been shown to be superior to P-selectin as a marker of platelet activation *in vivo* (Furman *et al.*, 2001; Michelson *et al.*, 2001). MPA formation in ACS may have a prognostic implication as levels of MPAs correlate with troponin elevation and are strongly related to risk of in-hospital cardiac events including death (Zhang *et al.*, 2007). Recently, Tapp *et al.* enumerated total MPA formation but also phenotyped MPAs by monocyte subsets in patients with STEMI (n=40), stable CAD (n=40) and healthy volunteers (n=40) (Tapp *et al.*, 2012). They reported a significant increase (80%) in total MPA count following STEMI compared to controls and interestingly this increase persisted at up to day 30 of longitudinal follow-up despite anti-platelet therapy and a normalisation of the total monocyte count. Within monocyte subsets, IMs had the highest proportion (approximately 20%) of aggregation with platelets followed by NCMs-platelets (14.5%) and CMs-platelets (13.5%). More recent studies of patients with unstable angina (UA) and STEMI support these findings with an enhanced formation of IM specific MPAs increasing future CV risk (Zeng *et al.*, 2014; Zhou *et al.*, 2016).

1.4.8 Clinical studies of neutrophil-platelet aggregates in CVD

Clinical observational studies have also specifically examined neutrophil-platelet interactions. An early study of 25 patients with UA compared to 25 control patients with stable angina reported a near 5 fold increased NPA formation as well as neutrophil activation demonstrated by increased CD11b expression and L-selectin shedding in the UA group (Ott *et al.*, 1996) These data were supported by a later study of 71 patients with UA and 22 patients with stable CAD (Murasaki *et al.*, 2007). In addition to a significantly higher NPA formation and neutrophil CD11b expression, there was a higher expression of platelet P-selectin in patients with UA compared to stable CAD controls. In addition to UA, excess PNAs (> 1 fold increase compared to controls) have also been reported in 23 patients with a non-ST-segment elevation myocardial infarction (NSTEMI) (Xiao and Thérroux, 2004). Interestingly, antagonism of the platelet P2Y₁₂ receptor with clopidogrel attenuated NPA formation in this cohort.

Overall, relatively small, case-control studies have demonstrated increased MPA and NPA counts in both stable CAD and following acute CV events. However, the precise mechanistic roles of leukocyte-platelet aggregates in the pathophysiology of both stable and acute CV pathology have not been addressed *in vivo*. Nevertheless, cross-talk between leukocytes and platelets to form aggregate complexes is likely to have important biological consequences in atherosclerosis.

1.4.9 The advanced atherosclerotic plaque and foam cells

1 The advanced plaque

The development of the atherosclerotic plaque is complex and involves a number of stages as described in Figure 1.4. Progression of the atherosclerotic plaque from the initial fatty streak to intermediate lesions is also defined by an accumulation of lipid rich largely acellular material in the intimal layer (Stary *et al.*, 1994). In some advanced lesions, lipid pools enlarge and are invaded by plaque macrophages giving rise to consolidated necrotic cores (Bentzon *et al.*, 2014). More advanced lesions contain larger necrotic cores that completely lack matrix but have greater macrophage infiltration (Kolodgie *et al.*, 2003). In the advanced atherosclerotic lesion, the necrotic core is covered by an overlying fibrous cap (Bentzon *et al.*, 2014). The strength and stability of the fibrous cap is largely determined by the tensile strength of collagen within it (Libby, 2013). Rupturing of the fibrous cap exposes highly thrombogenic material in the necrotic core leading to acute CV events.

2 Mechanisms of foam cell formation

Foam cells are generated through the uptake of modified LDL and cholesterol ester in macrophages (Lusis, 2000). Modification of LDL into oxLDL is critical to the formation of foam cells as unmodified LDL particles found in the peripheral blood of healthy volunteers does not induce foam cell formation in macrophages *in vitro* (Lusis, 2000; Chistiakov *et al.*, 2017). Lipid uptake, processing and efflux by macrophages under homeostatic conditions and dysregulation of these

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mechanisms in atherosclerosis resulting in foam cell formation is summarised in Figure 1.7.

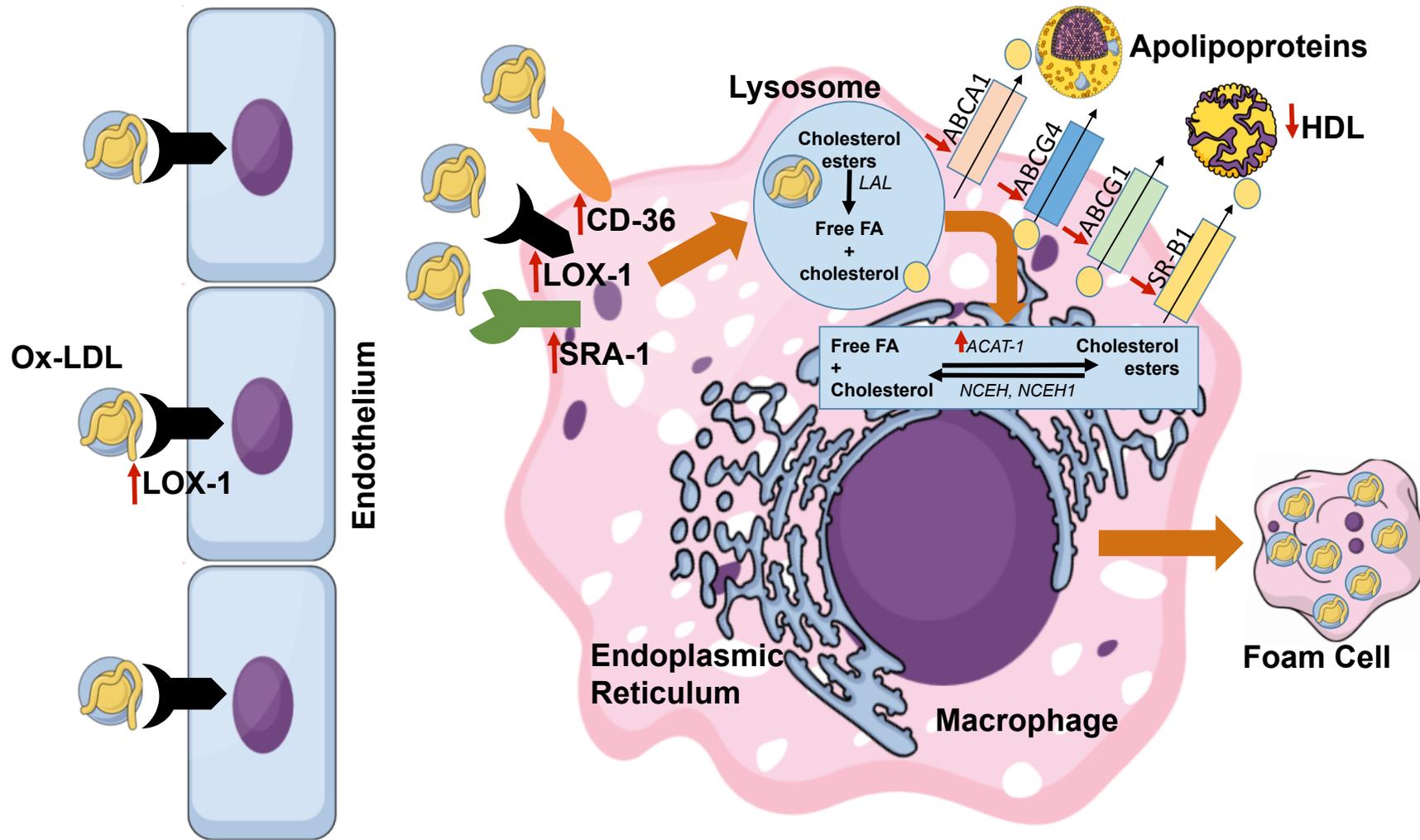


Figure 1-7 Mechanisms of foam cell formation. OxLDL binds LOX-1 which is expressed on endothelial cells. OxLDL is then trafficked into the arterial intimal layer, which is infiltrated by macrophages in atherosclerosis. Macrophages internalise oxLDL via CD36, SR-A1 and LOX-1. Relatively stable cholesterol esters upon entering macrophage lysosomes are converted to free fatty acids and free cholesterol under the action of LAL. Excess free cholesterol then traffics to the endoplasmic reticulum where under the action of ACAT1 it is re-esterified into cholesterol esters. Within the endoplasmic reticulum, NCEH directly opposes the action of ACAT1 by degrading cholesterol esters back into fatty acids and free cholesterol thus preventing cholesterol ester accumulation in the endoplasmic reticulum, a number of transporters, including ABCA1, ABCG1 and SR-B1, efflux free cholesterol out of macrophages in a process called reverse transport. HDL and apolipoproteins act as free cholesterol acceptors transporting these molecules for processing in the liver. Lipid handling by macrophages is dysregulated in atherosclerosis (red arrows). Under the influence of inflammatory mediators such as IFN- γ , scavenger receptor expression on the endothelium and macrophages is increased leading to increased oxLDL uptake. ACAT-1 activity is enhanced through the actions of IFN- γ and TNF- α whilst there is a down regulation of NCEH activity. The pro-inflammatory cytokines IL-12, IL-18, IL-1 β , IFN- γ downregulate cholesterol efflux. An accumulation of lipids in macrophages via these mechanisms transforms them into foam cells. Abbreviations: oxLDL, oxidised low density lipoprotein; LOX-1, oxidised low density lipoprotein receptor-1 (LOX-1); SR-A1, scavenger receptor A1; LAL, lysosomal acid lipase; ACAT1, acyl coenzyme A: cholesterol acyltransferase-1; NCEH, neutral cholesterol ester hydrolase; ABCA1, adenosine triphosphate (ATP)-binding cassette (ABC) transporter A1; ABCG1, adenosine triphosphate (ATP)-binding cassette (ABC) transporter G1; SR-B1, macrophage scavenger receptor class B type 1; HDL, high density lipoprotein, IFN- γ , interferon gamma; TNF- α , tumour necrosis factor alpha; IL-12, interleukin 12; IL-18, interleukin 18; IL-1 β ; interleukin 1 beta.

1.4.10 Inflammatory mechanisms driving plaque rupture and thrombosis

Acute CV events such as ACS can be caused by two broad mechanisms: either plaque rupture or plaque erosion. Fibrous plaque rupture is implicated in most fatal cases of ACS (Libby *et al.*, 2014). There is also extensive evidence that plaque rupture is driven by inflammation with certain characteristics of the plaque including: a thin fibrous cap, large necrotic core, numerous macrophages, spotty calcification and remodelling being associated with rupture and thrombosis (Bentzon *et al.*, 2014).

Ruptured plaques are associated with a thin fibrous cap which has been quantified in the range of 60-70 μm in post-mortem studies in humans (Libby *et al.*, 2014). A critical determinant of the integrity of the fibrous cap is its collagen content which is modulated through effects of the immune system on both collagen production and degradation (Libby *et al.*, 2014). In the atherosclerotic plaque, CD4⁺ T-helper (T_{H1}) subset lymphocytes which produce the pro-inflammatory cytokines IFN- γ and TNF increase plaque vulnerability by inhibiting collagen synthesis (Amento *et al.*, 1991; Hansson, Robertson and Söderberg-Nauclér, 2006; Libby *et al.*, 2014).

The susceptibility of a fibrous cap to thinning and subsequent rupture is also dependent upon collagen degradation by collagenases (Libby, 2013). The interaction of CD40 with its ligand CD40L (CD154) is also important in the evolution of fibrous plaques towards rupture (Schönbeck, Sukhova, *et al.*, 2000). In the atherosclerotic plaque, cross talk exists between the adaptive immune system (T cells) and the innate immune system (macrophages). Ligation of the CD40

receptor on macrophages with CD40L expressed by T cells induces production of inflammatory cytokines including IFN- γ and upregulates expression of adhesion molecules promoting further leukocyte traffic to the plaque thereby increasing instability (Bentzon *et al.*, 2014).

Rupturing of a thin fibrous cap can be a slow incremental process but there is good evidence to suggest that emotional and physical stimuli that induce activation of the sympathetic nervous system such as physical activity, anxiety, infections and cocaine use can trigger plaque rupture and ACS (Mittleman and Mostofsky, 2011). This is an important observation in light of hypoglycaemia also triggering a sympathetic response. Furthermore, sympathetic activation in the context of an AMI has been shown to aggravate inflammation in the atherosclerotic plaque in part through increased protease activity (Dutta *et al.*, 2012).

A larger necrotic core also confers an increased CV risk (Libby *et al.*, 2014). Inflammation within the plaque determines necrotic core formation and worsening necrosis in the core drives further inflammation and plaque progression towards rupture (Tabas, 2010; Moore and Tabas, 2011).

Calcification within the atherosclerotic plaque is associated with plaque rupture and ACS (Camici *et al.*, 2012; Maldonado *et al.*, 2012). Plaque calcification may have an added relevance in the pathogenesis of ACS in those with T2D. In a study of 581 patients with T2D, carotid plaque calcification predicted major adverse cardiovascular events (MACE) independent of confounders (Vigili de Kreutzenberg

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et al., 2015). In addition, remodelling of coronary arteries characterised by enlargement is an important predictor of the future risk of acute CV events (Clarkson *et al.*, 1994) and is governed by inflammatory mediators in the plaque with IL-1 signalling being particularly important (Alexander *et al.*, 2012).

Thrombosis occurs when the fibrous cap ruptures allowing blood to come into direct contact with thrombogenic material present in the plaques core (Libby, 2013). The thrombogenicity of the plaque is determined in part through tissue factor expression in macrophages and VSMCs. CD40-CD40L interactions have been shown to augment tissue factor expression in both plaque macrophages and VSCMs (Mach *et al.*, 1997; Schönbeck, Mach, *et al.*, 2000).

1.4.11 The infection, inflammation, atherosclerosis paradigm

As inflammation has gained recognition as a central pathophysiological process in atherosclerosis, research has focussed on triggers for inflammation including low-grade infection as an additional risk factor for atherosclerosis.

Historic, anecdotal observations, have supported the notion that acute infection can lead to AMI. Subsequently, pathogens have been found in atherosclerotic plaques including *Chlamydia pneumoniae*, and cytomegalovirus (CMV) (Adam *et al.*, 1987; Kuo *et al.*, 1993). Other seroepidemiological studies, however, did not demonstrate that previous infection with a number of organisms increased subsequent CV risk (Ridker *et al.*, 1999). As debate has existed around the concept of a causative relationship between infectious pathogens and atherosclerosis, research has focused on proof-of-concept animal studies. In ApoE ^{-/-} mice that underwent

induction of chronic infections with cytomegalovirus and *Chlamydia pneumoniae* an increase in atherosclerotic lesion size was demonstrated (Hsich *et al.*, 2001; Ezzahiri *et al.*, 2003). A number of mechanisms have been suggested through which infectious pathogens may contribute to atherosclerosis. These include the systemic host response to infection that is pro-atherogenic including: production of pro-inflammatory cytokines (chemokines and non-chemokines) and upregulation of vascular adhesion molecules (Epstein *et al.*, 2009). In addition, activation of monocytes and T-cells and cross-reactivity with self-antigens resulting in an autoimmune response may contribute to atherosclerosis (Epstein *et al.*, 2009).

Patients with diabetes are more prone to infections when compared to those without diabetes (Geerlings and Hoepelman, 1999). The underlying reasons for this propensity to infection in diabetes is thought to be complex but is in part due to abnormalities in both the innate and adaptive immune responses. A common infection encountered in diabetes is a diabetic foot ulcer (DFU). Interestingly, epidemiological data has linked chronic low-grade infection such as in chronic DFU with an increased CV mortality (Brownrigg *et al.*, 2012). Furthermore, diabetes is a major risk factor for periodontitis which is characterised by chronic inflammation (P M Preshaw *et al.*, 2012). In those with diabetes, risk of CV mortality is nearly 3 fold higher in those with severe periodontitis compared to those without (P M Preshaw *et al.*, 2012). As low-grade infection results in chronic inflammation, one explanation for an increased CV risk in the context of DFU and severe periodontitis maybe through progression of atherosclerosis. Further, it may be that there is a synergistic effect of hypoglycaemia and low-grade sepsis in driving chronic

inflammation that serves to aggravate atherosclerosis thus opposing the benefits of keeping glucose close to target.

1.6 *In vivo* endotoxin challenge as a model of sterile inflammation

Endotoxin or Gram-negative bacterial LPS has been administered to humans under differing circumstances over the past century (Suffredini and Noveck, 2014). In 1976, a large quantity of endotoxin was extracted from *E. coli* O:113 for use in human experimental studies. This endotoxin preparation has since been recognised by the WHO as a standard for use in various assays (Suffredini and Noveck, 2014). Research over the last 30 years has highlighted the complexity of the innate immune system as a highly conserved system of defence against infections. Soon after encountering recognised molecular motifs such as endotoxin, ligand binding with microbial structures results in activation of lymphoid and myeloid cells in addition to humoral proteins. A cascade of inflammatory effects ensues which involves recruitment of inflammatory cells and proteins to the site of infection resulting in microbial clearance and tissue repair. Adaptive immune responses follow on from the initial innate immune response allowing the organism to build a repertoire of defence against specific infectious agents if encountered prospectively. Following administration of intravenous endotoxin in healthy human volunteers, a constellation of inflammatory effects is observed which is dose-dependent and resembles the systemic inflammatory response syndrome seen in sepsis (Suffredini, Hochstein and McMahon, 1999). Depending on the dose of endotoxin, a fever develops 3-5 hours after injection of endotoxin. Peripheral blood leukocyte changes occur between 3-5 hours with an initial decline in the absolute number of mononuclear cells and a corresponding neutrophilia

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(Suffredini, Hochstein and McMahon, 1999). Neutrophils demonstrate an upregulation in the activation marker CD11b and release granule constituents (Pajkrt *et al.*, 1997). Further, neutrophil traffic to the pulmonary microcirculation is increased but the alveolar space itself appears to be protected from the stimulatory effects of endotoxin challenge (Boujoukos *et al.*, 1993). Levels of pro-inflammatory cytokines (TNF- α , IL-6, IL-8 and IL-1 β) appear to peak between 1-3 hours following endotoxin (Martich, Boujoukos and Suffredini, 1993). Intravenous endotoxin challenge is also a potent stimulator of the hypothalamo-pituitary-adrenal axis and an increase in the concentration of adrenocorticotrophic hormone (ACTH), cortisol and dihydroepiandrosterone (DHEA) occurs within 3 hours of injection (Fiuza and Suffredini, 2001). Changes in DHEA levels are particularly interesting, as DHEA antagonises glucocorticoid action and enhances mononuclear cell responses (Fiuza and Suffredini, 2001). Ultra-purified endotoxin is therefore particularly useful in experimental models of human inflammation given its ability to induce short-lived, sterile inflammation in a safe fashion which can be reproduced scientifically.

1.7 Hypothesis and aims

Diabetes is common and its prevalence is rising with CVD as a leading cause of death in those with diabetes. Hypoglycaemia is commonly encountered in the management of diabetes. Data from recent large randomised trials suggests that hypoglycaemia as a consequence of intensive glucose control may be associated with excess CV mortality. Whilst evidence from post-hoc analyses of trials suggests that hypoglycaemia may not be causally implicated, there is a growing body of evidence highlighting a number of mechanisms whereby hypoglycaemia may lead

to CV events. A number of studies have reported abnormal cardiac repolarisation and arrhythmias in both spontaneous and experimental hypoglycaemia. Acute and recurrent hypoglycaemia have also been shown to be pro-inflammatory and pro-thrombotic thus potentially promoting increased CV risk. These studies have investigated both healthy volunteers and those with diabetes but have, however, been mainly descriptive in nature. Whilst changes in pro-inflammatory cytokines, endothelial cell adhesion molecules, total leukocyte kinetics and endothelial-leukocyte interactions have been offered as plausible explanations for increased CV risk, in depth study of the various components of the inflammatory response to hypoglycaemia is yet to be performed. Specifically, the precise inflammatory pathways through which hypoglycaemia may accelerate atherosclerosis remain to be dissected.

Monocytes and macrophages are critical to the development and progression of the atherosclerotic plaque, yet there is a distinct paucity of data on these cells. In addition, interactions of monocytes and neutrophils with platelets to form circulating pro-atherogenic aggregates have not been comprehensively assessed. Platelet aggregation and activation in itself is a well recognised risk factor for macrovascular events especially in the context of the unstable plaque and ACS and needs further evaluation in the context of hypoglycaemia. Within monocyte subsets, the CD16⁺ NCMs and IMs have been shown in human observational studies to be prospectively linked with future CV risk. There is little data examining the effects of hypoglycaemia on monocyte subset kinetics and activation. Furthermore, most human experimental studies have concentrated on the acute inflammatory effects of hypoglycaemia. Data from clinical trials, however, indicate that

hypoglycaemia predicts CV mortality downstream from the event (Duckworth *et al.*, 2009; Bonds *et al.*, 2010). Lastly, there is a dearth of experimental data from murine models combining hypoglycaemia and atherosclerosis. As ethical constraints limit human studies of experimental hypoglycaemia in those with diabetes and CVD, animal experimentation is crucial to exploring these mechanistic links.

I hypothesised that hypoglycaemia primes the innate immune response and results in: monocyte mobilisation and activation, increased platelet reactivity and increased leukocyte-platelet interactions.

I aimed to:

- a) Develop a novel experimental model to explore the effects of acute hypoglycaemia on innate immune responses and platelet biology in healthy human volunteers**
- b) Develop a robust murine model of recurrent hypoglycaemia and atherosclerosis**
- c) Explore the consequences of hypoglycaemia on plaque biology in the murine model**

To probe how hypoglycaemia may modulate monocyte function in the human *in vivo*, I chose to combine a classical hypoglycaemic stimulus with a subsequent *in vivo* systemic stimulus of the innate immune system. To achieve this, I combined hyperinsulinaemic-hypoglycaemic, hyperinsulinaemic-euglycaemic and sham-

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saline (normoglycaemic) clamps with low-dose intravenous endotoxin challenge 48 hours later in healthy volunteers. This model allowed me to probe the durable effects of hypoglycaemia on the innate immune system. In addition, endotoxaemia added relevance to those with diabetes that have chronic low-grade infections associated with increased CV risk. I chose a low dose of endotoxin in view of future translatability of my novel model in more vulnerable participants with diabetes.

In mice, using an ApoE ^{-/-} model of atherosclerosis, I aimed to confirm or refute a causal relationship between antecedent recurrent hypoglycaemia and a worsening of pre-established atherosclerosis through inflammatory effects already demonstrated in humans.

Chapter 2- Research Governance, Materials and Methods

In this chapter I will detail the experimental methods used in the both human and murine studies with justification of approach in light of ethical and scientific reasons.

2.1 Human Studies: effects of experimental hypoglycaemia and low dose *in vivo* endotoxin challenge on innate immune responses in healthy volunteers

2.1.1 Study overview

The effects of hypoglycaemia on monocyte biology have received very little attention in humans. I aimed to examine the extent to which hypoglycaemia modulates innate immune function principally employing multi-colour flow cytometric analysis to enumerate monocyte subsets and platelet-leukocyte aggregates as well as studying established monocyte activation markers. All participants then received low dose intravenous endotoxin challenge 48 hours later to allow me to probe the effects of hypoglycaemia on these aspects of innate immune function.

Human experimental models of hypoglycaemia and endotoxin challenge had been developed by my research group, but had never been used in combination either by us, or by others. Since both models study mechanisms which may increase CV risk, it was important to first establish their safety when combined in healthy volunteers before seeking to use these in people with diabetes. In addition, as I was establishing a novel model, it was important to study inflammatory responses in healthy humans so that these could be used as a direct comparator for those with diabetes in potential future studies. To increase the safety of these models, I also adopted a new low dose endotoxin challenge model.

2.1.2 Research governance

This study received favourable approval from the local ethics committee (REC 14/YH/1264) and all participants provided written informed consent. All experiments were conducted in accordance with the principles enshrined in the Declaration of Helsinki. I investigated the inflammatory consequences of hypoglycaemia using the hyperinsulinaemic hypoglycaemic clamp. The latter is a well established technique for the experimental study of hypoglycaemia (DeFronzo, Tobin and Andres, 1979). The hyperinsulinaemic clamp allows precise control of blood glucose in an experimental environment. Furthermore, unlike hypoglycaemia encountered as a consequence of treatment for diabetes, higher concentrations of insulin are used in clamp experiments with greater hormonal counter-regulation to hypoglycaemia as a consequence. However, pharmacological hyperinsulinaemia necessary for precise glucose control during clamps may introduce artefact by suppression of inflammation (Chaudhuri *et al.*, 2004; Dandona *et al.*, 2010).

The main ethical issue with clamp studies is the potential of cardiac arrhythmias during hypoglycaemia. The occurrence of arrhythmias with hypoglycaemia however, is exceedingly rare and generally is reported at severe hypoglycaemia (< 2mmol/l glucose) whilst I aimed to achieve a nadir of 2.5 mmol/l glucose in a controlled fashion. In addition, my group had previously performed over 500 hyperinsulinaemic clamps in our unit on healthy volunteers and patients with diabetes, including those with T2D, and had reported no serious adverse events. I performed ECG monitoring throughout the hyperinsulinaemic clamp along with blood pressure monitoring. In the event of abnormalities in heart rhythm during

the hypoglycaemia clamp or if the participant wished to stop the experiment, blood sugars can be normalised rapidly using intravenous dextrose.

Over the past 20 years, approximately a thousand healthy volunteers have taken part in similar studies involving an endotoxin challenge *in vivo* to study the resulting immune response. The endotoxin administered does not actually contain bacteria and cannot cause an infection. A high dose endotoxin challenge (4 ng/kg) given to volunteers characteristically produces varying degrees of fever, headache, joint aches, muscle aches, and occasionally chills. The degree of fever varies somewhat among people, but on average, oral temperature will rise to 38°C. Previous experience within our group of using 2 ng/kg intravenous endotoxin indicated various side effects may occur (M. R. Thomas *et al.*, 2015). The peak temperature rise occurred at 1-2 hours following endotoxin and then temperature returned to normal by the end of the study day. Rarely participants experienced nausea and vomiting. I administered a much lower endotoxin dose (0.3 ng/kg) hence the above effects were not observed in this study. This was in keeping with other studies in healthy volunteers using a low dose (0.3-0.4 ng/kg) intravenous endotoxin challenge (Taudorf *et al.*, 2007; Hutchinson *et al.*, 2013). The final dose of endotoxin was established after conducting a pilot low dose endotoxin challenge in two subjects to demonstrate changes in total peripheral white blood cell kinetics in keeping with known dose responses (Suffredini, Hochstein and McMahon, 1999). Timing of sampling following endotoxin injection was based on published changes in: circulating immune cells, cell surface markers and hormonal changes consistent with activation of the hypothalamo-pituitary-adrenal axis in response to endotoxin challenge in man (Boujoukos *et al.*, 1993; Martich, Boujoukos and Suffredini, 1993;

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Fiuza and Suffredini, 2001). I continuously monitored heart rhythm and rate (ECG) and was prepared to treat any significant cardiac abnormality. Temperature, pulse and blood pressure were measured regularly during the endotoxin visit.

2.1.3 Study design

This was a single-blinded, prospective study of three independent parallel groups (hyperinsulinaemic-hypoglycaemia (2.5 mmol/l) /euglycaemia (6 mmol/l) and sham-saline controls) conducted in a random group order at the NIHR Sheffield Clinical Research Facility, Northern General Hospital, Sheffield, United Kingdom between January 2015 and April 2016. The sham-saline control arm controlled for direct immunological actions of insulin. Clamp visits and endotoxin challenge were separated by 48 hours to minimise potential carryover effects, principally the impact of insulin signalling on leukocyte function and to demonstrate potential prolonged effects of glycaemic variation on inflammatory parameters.

In the initial visit (visit 1), all volunteers were screened for clinical symptoms and signs of CVD and diabetes (Figure 2.1). Eligible volunteers were randomly allocated to 3 experimental groups (8 subjects/group in total): group 1 = experimental hypoglycaemia and endotoxin challenge, group 2 = experimental euglycaemia and endotoxin challenge and group 3 = sham-saline clamp and endotoxin challenge. Table 2.1 summarises the various measurements obtained at each trial visit.

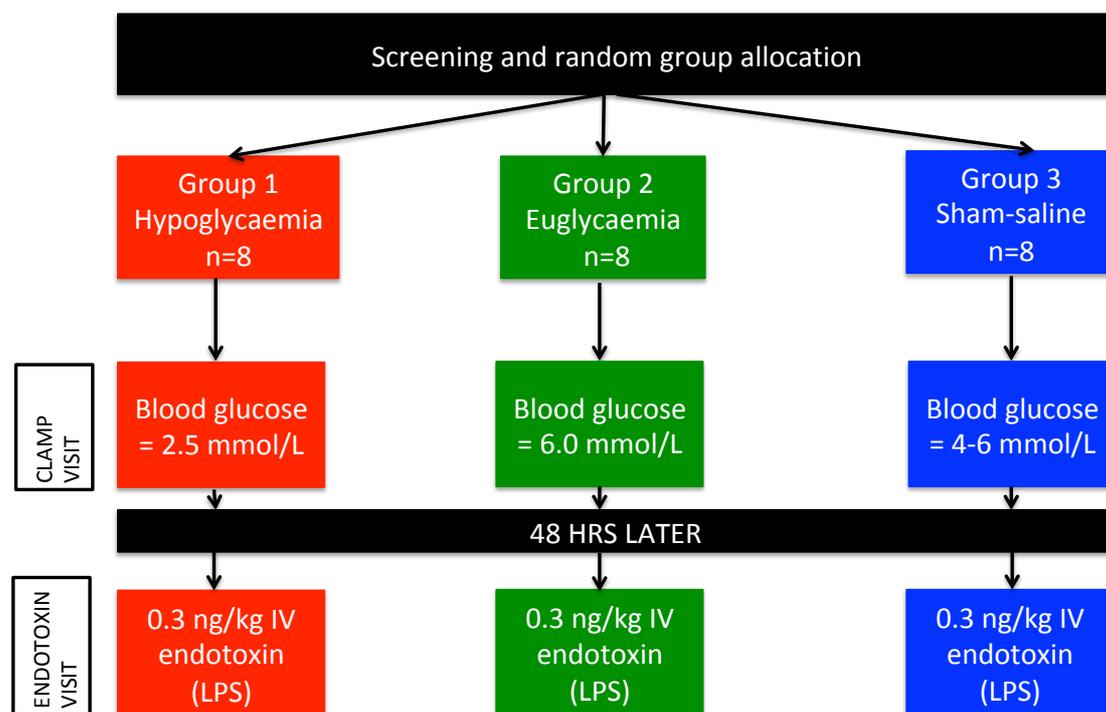


Figure 2-1 Overview of study visits. Interested volunteers underwent a formal screening visit to establish eligibility as per the study inclusion and exclusion criteria prior to random allocation to one of three study groups. Clamp and endotoxin visits were separated by 48 hours.

Table 2.1 Physical, biochemical and inflammatory parameters that were assessed over the course of experiments. Measurements for sham-saline control group (group 3) were identical to the hypoglycaemia and euglycaemia groups. Abbreviations: FBC, full blood count; LFT, liver function test; UE, urea and electrolytes; HR, heart rate; BP, blood pressure; GLU, glucose; EU, euglycaemia; HYPO, hypoglycaemia; hsCRP, high-sensitivity c reactive protein; ECG, electrocardiogram and HbA1c, glycated haemoglobin A1c.

S= screening D= day	S	EU clamp				HYPO clamp				D 1	Endotoxin challenge			
Time (min)		0	30	60	Recovery	0	30	60	Recovery		0	1 2 0	2 4 0	3 6 0
HR,BP,ECG	•	•	•	•	•	•	•	•	•	•	•	•	•	•
UE	•	•		•		•		•		•	•			•
GLU	•	Every 15 mins	Every 5 mins	Every 5 mins	Every 5- 15 mins	Every 15 mins	Every 5 mins	Every 5 mins	Every 5- 15 mins	•	•			•
Monocyte mobilisation and activation		•		•		•		•		•	•	•	•	•
Cytokines											•	•	•	•
Platelet studies		•		•		•		•		•	•	•	•	•
hsCRP		•		•		•		•		•	•	•	•	•
HbA1c,lipids,FBC, LFT	•													
Cortisol		•		•		•		•		•	•	•	•	•
Growth hormone		•		•		•		•		•	•	•	•	•
Catecholamines		•		•		•		•		•	•			•
Free insulin		•	•	•		•	•	•						

2.1.4 Identification of volunteers

Eligible healthy volunteers were recruited from students and staff at the University of Sheffield. Emails advertising the study were sent using a broad University of Sheffield email distribution list. I recruited healthy individuals without diabetes and with no history of CVD between the ages of 18-65. Specific inclusion and exclusion criteria are outlined below to ensure a safe and scientifically robust study.

Inclusion criteria:

- Healthy male subjects, or non-pregnant female subjects.
- Age between 18 and 65 years inclusive.
- Non-diabetic: HbA1c less than 48 mmol/mol (6.5%) as defined by the WHO.
- Non smokers.
- Body mass index (BMI) between 18 and 28 kg/m² inclusive, with a body weight between 60-100 kg.
- Subjects in good health as determined by a medical history, physical examination, vital signs and clinical laboratory test results including renal, liver function and full blood count.
- Women using effective contraception if sexually active. The study period was short, and a negative pregnancy test was also required where appropriate (premenopausal women).
- Subjects have given their informed consent before any trial-related activity.

Exclusion criteria:

- Documented myocardial infarction, ischaemic heart disease, cardiac arrhythmia, heart failure, peripheral vascular disease, stroke, visual impairment due to retinopathy, or nephropathy as demonstrated by elevated serum creatinine accompanied by proteinuria and microalbuminuria.
- Abnormal resting 12 lead ECG and specifically a resting heart rate outside the range of 50-100 beats per minute.
- Significant symptoms suggestive of CVD.
- Pregnancy.
- Known untreated hyperthyroidism.
- Epilepsy or previous seizures.
- Patients on beta-blockers or medications that affect the QT interval.
- Patients on anticoagulants or antiplatelet therapy.
- Cardiac autonomic neuropathy.
- Serious intercurrent illness including infections within the last 4 weeks.
- Previous history of deep venous thrombosis or pulmonary embolus.
- Inability to communicate in English or mental incapacity (prerequisite for informed consent).
- Family history of sudden death.
- In the opinion of the investigator, subjects with, or a history of, cancer, diabetes or clinically significant CV, respiratory, metabolic, renal, hepatic, gastrointestinal, haematological, dermatological, neurological, psychiatric, or other major disorders.

- Any clinically significant abnormal laboratory test results at screening.
- Subjects who have a supine blood pressure at screening, after resting for 5 minutes, higher than 150/90 mmHg or lower than 105/65 mmHg.

2.1.5 Study visits

The study visit schedule is outlined in Figure 2.1.

Screening (visit 1)

Screening included collection of demographic information, full history (including enquiry into symptoms of diabetes and CVD), and a thorough clinical examination including measurement of resting heart rate, blood pressure and tympanic body temperature. A drug history was obtained including specific enquiry into allergies, over the counter medication and recreational drug use. Blood was drawn for full blood count, urea and electrolytes, random glucose, lipid profile, liver function test and HbA1c. A baseline 12 lead ECG was performed. I allowed for a 13% (1/8) screen failure rate, as guided by experience from previous clamp studies carried out within our group.

A negative urinary pregnancy test was required for premenopausal women at the start of visit 2 and visit 4, regardless of contraception history.

Experimental euglycaemia (visit 2 EU CLAMP)

1. Participants attended the Clinical Research Facility at 8 am having fasted from midnight. Participants were advised to avoid caffeine on the day of the clamp and vigorous exercise in the previous 24 hours.
2. Baseline physiological measurement and a 12 lead ECG were obtained.
3. An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm.
4. A second retrograde cannula was inserted following application of local anaesthetic cream (EMLA, Astra Zeneca, UK) to the dorsal hand vein of the non-dominant hand. This hand was then placed in a heated chamber (The Sheffield Hand Warmer, UK) at 55°C to allow arterialisation of venous blood for glucose sampling (Liu *et al.*, 1992). Placing the hand in a heated chamber allows mixing of some arterial blood with the venous circulatory beds thus allowing determination of the arterialised blood glucose values. A slow infusion of 0.9% sodium chloride was used to keep this cannula patent.
5. A third cannula was inserted in the contralateral antecubital fossa for biochemical and thrombosis measurements. Measurements for urea and electrolytes, hormones (including cortisol, growth hormone and catecholamines), thrombosis and inflammatory markers were taken at baseline and 60 minutes.
6. **Test period:** Baseline glucose was assessed by capillary blood glucose testing. For the hyperinsulinaemic clamp procedure, a primed continuous infusion of insulin (Human Actrapid, Novo Nordisk Pharmaceuticals Ltd, Crawley, West Sussex, UK) was administered at a rate of 90 mU/m²/minute (clamp insulin infusion) calculated using the Dubois formula (Body Surface

area (m²) = 0.007184 × (patient height in cm) 0.725 × (patient weight in kg)

0.425. The same rate of insulin infusion was used between the euglycaemia and hypoglycaemia groups in order to ensure parity in insulin levels.

7. A 20% dextrose (Baxter, Baxter Healthcare Ltd, Norfolk, UK) infusion administered via an infusion pump (Alaris ® GP plus, Cardinal Health, 1180 Rolle, Switzerland) was adjusted according to arterialised blood glucose concentrations measured every 5 minutes using a bedside glucose analyser (Yellow Spring Instruments, Ohio, USA). Blood glucose was titrated from baseline to a target of 6 mmol/l over 30 minutes. Blood glucose was maintained at the target level for 60 minutes. The time when target glucose of 6 mmol/l is reached is designated time 0. Volunteers were blinded to glucose levels.
8. Heart rate, blood pressure and high resolution ECG monitoring were performed.
9. **Recovery:** At the end of the 60 minute test period, the clamp insulin infusion was switched off. A 20% dextrose infusion was continued for the next 20 minutes to maintain a blood glucose level of at least 6 mmol/l. Glucose was monitored every 5 minutes for the first 10 minutes during recovery, thereafter every 10 minutes. Volunteers then received a standardised meal. Physical and biochemical measurements were taken as described in Table 2.1.

Day 1 post euglycaemic clamp (visit 3 day 1)

1. Participants were asked to return in the morning following the clamp visit. Participants were advised to avoid caffeine intake on the day and vigorous exertion in the previous 24 hours.
2. Heart rate and blood pressure were checked.
3. An ECG was performed.
4. Blood was taken for measurements including urea and electrolytes, glucose, hormones, thrombosis and inflammatory markers.

Endotoxin (visit 4)

1. Volunteers returned 48 hours following the clamp visit. Volunteers were advised to avoid caffeine intake on the day and vigorous exertion in the previous 24 hours.
2. Heart rate and blood pressure were checked.
3. An ECG was performed.
4. One intravenous cannula was inserted into the antecubital fossa of the non-dominant arm. An additional intravenous cannula was inserted into the contralateral antecubital fossa for blood sampling throughout the study visit and kept patent using a slow infusion of 0.9% sodium chloride.
5. *Escherichia coli* O:113 LPS (obtained from Dr Anthony Suffredini, Clinical Centre Reference Endotoxin National Institutes of Health, Bethesda, Maryland, USA) was used. Endotoxin powder was reconstituted in 1 ml of sterile 0.9% sodium chloride to form a solution at a concentration of 1000 ng/ml, which was vortexed for 60 minutes. The weight-adjusted dose of

endotoxin was obtained from this solution, added to 5 ml of 0.9% sodium chloride and administered as a slow bolus injection over 1 minute.

6. Vital signs (heart rate, blood pressure and temperature) were measured at 30 minute intervals for 360 minutes post endotoxin injection (designated time 0).
7. A continuous intravenous infusion of 125 ml/hr of 0.9% NaCl was administered for 240 minutes following endotoxin challenge through the cannula used for endotoxin injection to avoid hypotension.
8. Blood was taken for urea and electrolytes, glucose, hormones, thrombosis and inflammatory markers at baseline (0) and at 120, 240 and 360 minutes following endotoxin challenge. All laboratory measurements were performed by staff blinded to glucose group allocation.

Hypoglycaemic clamp (visit 2 HYPO clamp)

The protocol was identical to the euglycaemic visit except the target glucose was 2.5 mmol/l. Volunteers underwent a hypoglycaemic clamp of 60 minutes duration. An infusion of 20% dextrose was titrated to maintain the target glucose. At the end of hypoglycaemic clamps, the insulin infusion was switched off and 20% dextrose continued for at least 20 minutes until euglycaemia was reached. Volunteers received a standardised meal and had blood glucose monitored for at least 1 hour after the end of clamps to ensure blood glucose in the euglycaemic range. All participants were advised to be wary of increased risk of hypoglycaemia in the next 48 hours, although the risk was deemed to be small in healthy volunteers.

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Participants were not allowed to drive following clamp and endotoxin visits and had a taxi arranged for them.

Visit 3 (day 1 post HYPO clamp), visit 4 (endotoxin)

The protocol was identical to follow up visits following the hypoglycaemia clamp.

Sham-saline clamp (visit 2)

Volunteers allocated to the sham (no insulin or dextrose) clamp underwent exactly the same pre-visit preparation as those allocated to the euglycaemia and hypoglycaemia groups. The experimental protocol including intravenous cannulation and blood sampling for the sham clamp visits was identical to the euglycaemia and hypoglycaemia arms except no glucose manipulation occurred and in place of insulin and dextrose subjects received a slow infusion of 0.9% sodium chloride for 60 minutes at a standardised rate.

Visit 3 (day 1 post sham clamp), visit 4 (endotoxin)

The protocol was identical to follow up visits following the hypoglycaemia and euglycaemia clamps.

2.1.5 Biochemical analyses

Arterialised whole blood Glucose

Glucose was measured from 1 ml of arterialised whole blood from the retrograde cannula on a bedside glucose analyser (Yellow springs Instrument 2300 STAT, Ohio, USA) every 5 minutes. The glucose analyser utilises a glucose oxidase method for quantification of blood glucose. Arterialised blood glucose is measured as this is

unaffected by peripheral tissue consumption of glucose as occurs in the venous circulation.

Catecholamines (adrenaline and noradrenaline)

Venous forearm blood (3.5 ml) was collected into lithium heparin tubes on ice containing 50 microlitres of EGTA/glutathione preservative and centrifuged at 4°C, 1000g for 10 minutes. The resulting supernatant was stored at -80°C until assayed by high performance liquid chromatography. Samples were sent for batch analysis at the University of Nottingham. The coefficient of variation (standard deviation/mean expressed as percentage) was 9.7% for noradrenaline and 11.4% for adrenaline.

Cortisol and Growth Hormone

Blood (5 ml) was collected into a serum separator tube (SST) (BD Vacutainer® SST™ II Advance) and samples were allowed to clot for 30 minutes. The samples were centrifuged and the serum frozen at -20°C until measured using a commercial immunoradiometric assay (Cobas system, Roche Diagnostics, Burgess Hill, West Sussex, UK). This assay was performed in Sheffield Teaching Hospitals (STH) clinical laboratories. Sample collection for cortisol and growth hormone was controlled for time of day across the three study groups.

Urea and electrolytes

Blood (5 ml) was collected into a SST tube (BD Vacutainer® SST™ II Advance) with a clot accelerator and separation gel. The sample was centrifuged at 3000g at room temperature for 10 minutes. Assays on the resulting serum were performed at STH laboratories using an automated system (SYNCHRON LX® System, Beckman Coulter, Inc, High Wycombe, UK).

Glycated haemoglobin A1c (HbA1c)

Blood (2 ml) was collected into EDTA (BD Vacutainer™ K3E) tubes and analysed using an ion exchange high performance liquid chromatography technique at STH laboratories.

Free insulin

Blood (1ml) was collected into EDTA (BD Vacutainer™ K3E) for each sample prior to centrifugation at 3000g for 10 minutes at room temperature. The resulting plasma was stored at -80°C. Samples were sent for batch analysis at the Royal Devon and Exeter NHS trust laboratories using a dedicated immunoassay (Cobas insulin electrochemiluminescence immunoassay, Roche Diagnostics, Burgess Hill, West Sussex, UK). Briefly, this assay employed a mouse monoclonal anti-insulin antibody labeled with ruthenium and a second mouse monoclonal antibody coupled to paramagnetic particles using a sandwich principle.

2.1.6 Cell counts and flow cytometry

Inflammatory cytokines, adhesion molecules & selectins

Blood (5 ml) was collected into EDTA tubes (BD Vacutainer™ K3E) and plasma obtained by centrifugation at 3000g for 25 minutes at room temperature. Aliquots of plasma were stored at -80°C for analysis by cytometric bead array in the core flow cytometry facility at the University of Sheffield. Analysis using cytokine bead array was employed as it allowed multiple analytes to be assayed at the same time using fewer dilutions and small sample sizes. Cross-reactivity between analytes was avoided by strictly adhering to the cytokine combination specified by the manufacturer. A panel was designed to measure the following human soluble proteins: IL-6, IL-8, MCP-1, CD62P, CD62E, VCAM-1, RANTES, CX3CL1 (fractalkine). As part of the assay, standards were serially diluted 1:2 to 1:256 for use as controls. The dynamic range of the assay was 10-1,250 pg/ml. An initial run demonstrated that samples for VCAM-1 and RANTES were significantly above the upper detection limit (1,250 pg/ml) of the assay and thus these samples had to be diluted 1:50 and 1:10 respectively. The remaining samples were run neat. The assay was performed in small flow cytometry tubes. To each tube, 25 µL of capture bead master mix and 25 µL of the sample or standard was added and mixed. The tubes were then incubated in the dark for 1 hour at room temperature. Following this, 25 µL of detection master mix was added to each tube and the tubes incubated in the dark at room temperature for 2 hours. Wash buffer (500 µL) was added to each tube using repeat pipetting and the samples centrifuged at 200g for 5 minutes. The supernatant was then aspirated and the pellet resuspended in 200 µL of wash buffer prior to transferring samples to a 96 well plate which was run on an Attune flow cytometer (Thermo Fisher Scientific, Altrincham, Greater Manchester, UK)

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with data analysed using the instruments software. Standard curves for each analyte was constructed using five parameter logistic equations from which median fluorescence values were converted into cytokine concentrations in pg/mL for each sample.

Cell counts

Total and differential white blood cell (WBC) and platelet counts in 1 ml of EDTA-anticoagulated blood were determined using an automated clinical grade Sysmex cell counter (XN-9000, Sysmex, Milton Keynes, UK). For the clamp visit, alternative WBC counting methodologies were piloted for the first two subjects in each group but these were later deemed less accurate than the Sysmex cell counter.

Monocyte-platelet aggregates

MPAs were studied by drawing 5 ml of whole blood into 0.5 ml of trisodium citrate dihydrate (3.13% w/v) with care to avoid agitation and thus activation. Citrate anticoagulated whole blood (480µl) was added to four plastic round base tubes to neatly fit into a heat block at 37 °C. A stirrer bar was added to one tube but not others to promote agitation and thus formation of MPAs acting as a positive control. Blood was incubated in the heat block for 10 minutes at 37 °C. At the end of incubation, 100 µl of blood was taken from each tube and pipetted into corresponding pre-labelled plastic tubes on a rack containing 2 ml of 1 in 10 fluorescence-activates cell sorting (FACS) lyse solution (BD, Oxford, UK) (diluted in sterile water) and left undisturbed for 10 minutes. Addition of blood to the FACS lysis solution resulted in lysis of erythrocytes. Following FACS lysis, samples were centrifuged at 300g for 5 minutes. Once the supernatant had been carefully removed using a transfer pipette in 200 µl aliquots, the resultant pellet was

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resuspended in 50 µl phosphate buffered saline (PBS) and 10% bovine serum albumin. The resulting suspension was stained with target antibody cocktails and isotype cocktails. Staining with antibodies was performed using FITC-conjugated CD16 (302006, BioLegend, London, UK, diluted 1 in 10 in PBS), APC-conjugated CD14 (325608, BioLegend, London, UK, diluted 1 in 10 in PBS) and PE-conjugated CD42a (558819, BD Biosciences, Oxford, UK diluted 1 in 3 in PBS). Isotype staining was performed using FITC Mouse IgG1, κ isotype Ctrl Antibody (400108, BioLegend, London, UK), APC Mouse IgG1, κ isotype Ctrl (FC) Antibody (400122, BioLegend, London, UK) and Mouse IgG1 κ isotype Control PE (555749, BD Biosciences, Oxford, UK). Isotype controls were diluted in PBS to exactly match the target antibody concentrations used. Following staining with target antibodies and isotypes, samples were incubated in the dark at room temperature for 20 minutes. The final step in the assay was to fix cells using 200 µl of FACSFix (BD, Oxford, UK). Flow cytometric analysis was performed immediately using a BD C6 Accuri multi-colour flow cytometer (BD, Oxford, UK). Monocytes were gated based on forward and side scatter morphology and positively selected using CD14 expression. Neutrophils were gated using forward and side scatter morphology and through exclusion of monocytes. Monocyte-platelet aggregation was determined by measuring monocyte median fluorescence of the platelet specific marker CD42a. Flow cytometry data were analysed using Flow Jo (FlowJo, version 10, Ashland, Oregon, USA).

Monocyte phenotyping, enumeration and activation status

Monocyte mobilisation and activation was studied using whole blood flow cytometry, focusing on CM, IM and NCM subsets. Flow cytometry was carried out at

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the core flow cytometry facility at the University of Sheffield using the LSRII flow cytometer (BD Biosciences, New Jersey, USA). FITC-conjugated CD16 (302006, BioLegend, London, UK,) and APC-conjugated CD14 (325608, BioLegend, London, UK,) were used as monocyte phenotyping markers. PE-conjugated CD66c (551478, BD Biosciences, Oxford, UK) was used as a neutrophil exclusion marker. PE-Cy7-conjugated CD11b (301322, BioLegend, London, UK) and (PerCP)-eFluor® 710-conjugated CX₃CR1 (46-6099-42, eBioscience, Thermo Fisher Scientific, Manchester, UK) were used as monocyte activation markers. Table 2.2 summarises the antibody panel used to determine monocyte phenotype and activation status including the lasers employed.

Table 2.2 Summary of human cell markers against which target antibodies are directed, conjugated fluorochromes used and combination of lasers employed in the LSRII flow cytometer. Abbreviations: FSC, forward scatter; SSC, side scatter; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; PECy7, phycoerythrin-Cy7; (PerCP)-eFluor®710, peridinin chlorophyll protein.

Human cell marker	Fluorochrome	Emission wavelength
-	-	FSC
-	-	SSC
CD16	FITC	Blue 530/30
CD66c	PE	Blue 575/26
CD14	APC	Red 660/20
CD11b	PE Cy7	Blue 780/60
CX₃CR1	(PerCP)-eFluor® 710	Blue 695/40

Traditional isotype controls for the antibodies used were as follows: FITC Mouse IgG1, κ isotype Ctrl Antibody (400108, BioLegend, London, UK), APC Mouse IgG1, κ isotype Ctrl (FC) Antibody (400122, BioLegend, London, UK), Mouse IgG1 κ isotype Control PE (555749, BD Biosciences, Oxford, UK), PE/Cy7 Mouse IgG1, κ isotype Ctrl Antibody (400126, BioLegend, London, UK) and Rat IgG2b κ isotype Control PerCP-eFluor® 710 (46-4031-82, eBioscience, Thermo Fisher Scientific, Manchester, UK). In addition to isotype controls, staining using a fluorescence minus one (FMO) strategy was employed for all subjects at baseline before each clamp visit. In an FMO control strategy, cells are stained with all but one fluorochrome in a systematic fashion (Roederer, 2001). This allows one to take into account spread of fluorochromes in unlabelled channels thus allowing accurate

gate placement. Table 2.3 represents the FMO staining strategy employed. At time points other than baseline FMO, three Eppendorf tubes (Eppendorf, Stevenage, UK) of whole blood were prepared, tube 1 was an unstained sample and two Eppendorf tubes (tube 2 and tube 3) were stained in duplicate with all antibodies (CD14/CD16/CD66c/CD11b/CX₃CR1). Staining for isotype controls was done at baseline before clamp and repeated before the endotoxin visit for each subject.

Table 2.3 FMO strategy used at baseline for each subject on the day of clamp visit (visit 2). Seven separate 1.5 ml Eppendorf tubes were prepared. One tube was designated unstained and acted as a negative control. An additional tube of whole blood was stained with all antibodies in the panel (CD14/CD16/CD66c/CD11b/CX₃CR1). The remaining five tubes were stained with all conjugated-antibodies minus one and were labelled: - CD16 FITC, - CD66c PE, - CD14 APC, - CD11b PE-Cy7 and - CX₃CR1 PErCP 710. The volume of antibody used was 2.5 µL. Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; PECy7, phycoerythrin-Cy7; (PerCP)-eFluor®710, peridinin chlorophyll protein.

Staining	FITC	PE	APC	PE-Cy7	PErCP 710
Unstained	-	-	-	-	-
CD 16 FITC	-	+2.5 µL	+2.5 µL	+2.5 µL	+2.5 µL
CD 66c PE	+2.5 µL	-	+2.5 µL	+2.5 µL	+2.5 µL
CD14 APC	+2.5 µL	+2.5 µL	-	+2.5 µL	+2.5 µL
CD 11b PE-Cy7	+2.5 µL	+2.5 µL	+2.5 µL	-	+2.5 µL
CX ₃ CR1 PErCP 710	+2.5 µL	+2.5 µL	+2.5 µL	+2.5 µL	-

Whole blood (5 ml) was anticoagulated in EDTA (BD Vacutainer™ K3E) and gently mixed to avoid activation. One hundred microliters of whole blood was transferred to 1.5 ml Eppendorf tubes and stained using 2.5 µl of antibodies and corresponding isotype controls (1 in 2 dilution from neat). The tube was gently tapped to encourage mixing. The concentration of antibodies and isotype controls was matched. Samples were incubated in the dark for 15 minutes at room temperature

following staining with antibodies and isotype controls. Following staining, 1.4 ml of FACS lyse solution (BD, Oxford, UK) was added to Eppendorf tubes and the tubes were briefly vortexed to lyse erythrocytes. FACS lysed samples were centrifuged using a micro-centrifuge at 2000 rpm for 4 minutes. The resulting supernatant was carefully removed in 500 μ l aliquots in order not to disturb the white blood cell pellet and the latter was resuspended in 450 μ l of PBS. 100 μ l of 1% w/v formaldehyde was added to each sample. To allow enumeration of the absolute number of monocyte subsets per μ l of blood, CountBright™ beads (15 μ l, Life Technologies, USA) were added. Samples were immediately processed using the LSRII flow cytometer. Flow cytometry was performed for all subjects on the day of sampling. Compensation was performed using positive and negative anti-mouse and anti-rat compensation beads (Molecular Probes, Life Technologies, USA). Monocytes were gated using morphology on forward and side scatter and through exclusion of neutrophils using CD66c. Flow cytometry data were analysed using Flow Jo (FlowJo, version 10, Ashland, Oregon, USA). Figure 2.2 illustrates the gating strategy employed.

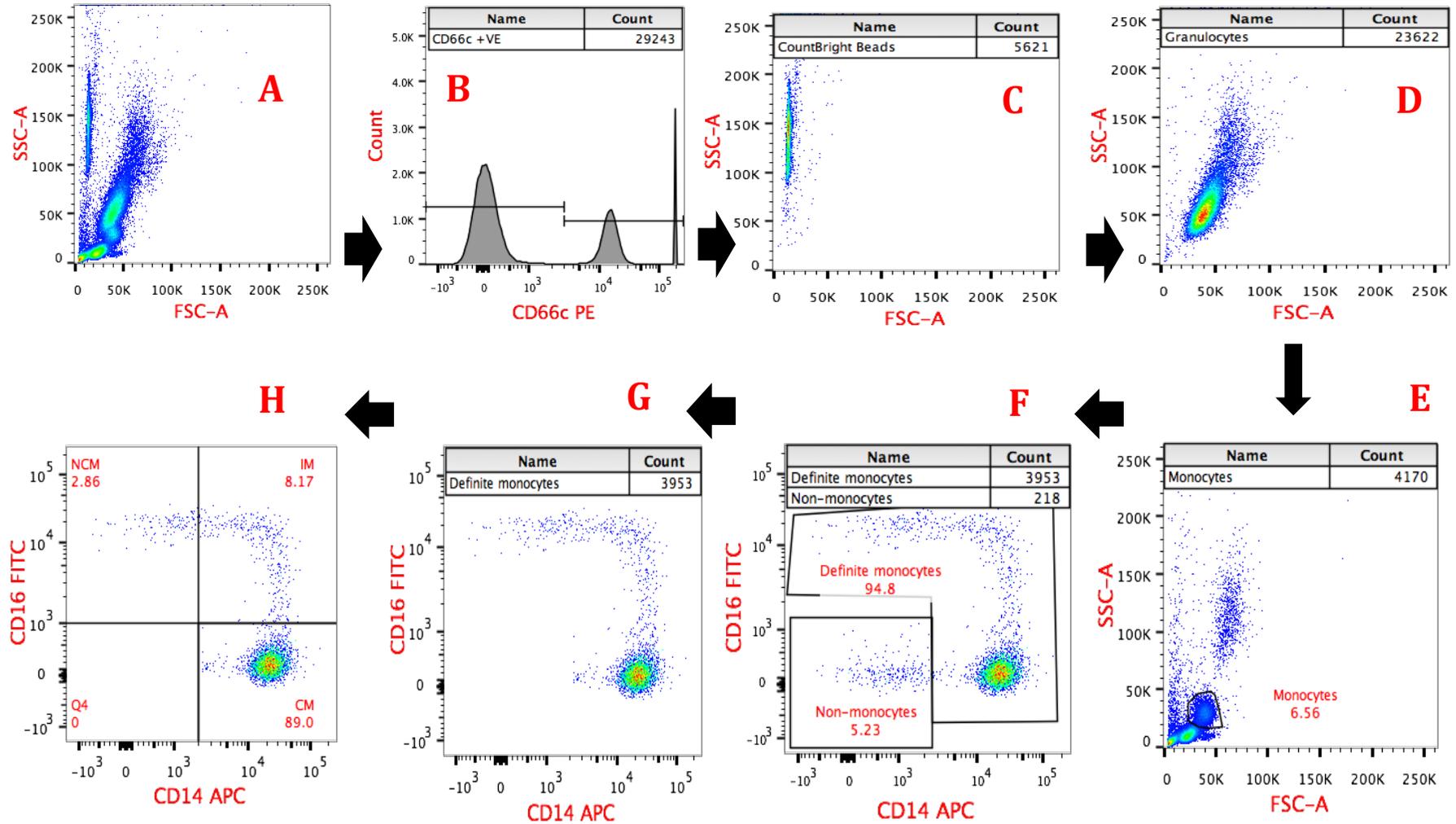


Figure 2-2 Monocyte gating strategy. A) All events acquired are shown on the FSC and SSC axes. B) CountBright™ are dissected out based on intense fluorescence and granulocytes are separated based on positivity for CD66c. Beads (C) and granulocytes (D) plotted on FSC and SSC respectively. E) All CD66c negative events are plotted on FSC and SSC and monocytes are gated around based on their FSC and SSC profile. F) All cells in the monocyte gate are plotted on CD14 APC (x-axis) vs. CD16 FITC (y-axis). Cells that are dual negative for CD14 and CD16 are designated non-monocytes and gated out. G) Monocyte subsets are identified based on their CD14 (x-axis) and CD16 (y-axis) profiles. H) Monocytes are dissected out into classical monocytes CD14⁺⁺CD16⁻, intermediate monocytes CD14⁺⁺ CD16⁺ and non-classical monocytes CD14⁺ CD16⁺⁺. Abbreviations: FSC, forward scatter; SSC, side scatter; FITC, fluorescein isothiocyanate; APC, allophycocyanin; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes.

2.1.7 Platelet aggregation

Venous blood (2.7 ml) was collected into a 3 ml hirudin tube (Multiplate®, Verum Diagnostica GmbH, Munich, Germany) and precautions taken to avoid agitation. Platelet aggregation was measured using impedance aggregometry (Multiplate®, Verum Diagnostica GmbH, Munich, Germany). Aliquots of 300 µl saline and 300µl of hirudin-anticoagulated blood were added to the cuvette immediately after venepuncture and incubated at 37 °C for 3 minutes. Agonist (20 µl ADP 1 at a fixed concentration of 6.45 µM) was added and the assay commenced. The area under the curve (AUC) was measured which represents the level of platelet aggregation.

2.1.8 Statistical analysis

My pilot data indicated that a sample size of 7 participants per group would have 90% power to a significance of 5% to detect a 50% relative difference in mobilisation of monocytes between hypoglycaemia and controls. Eight subjects were recruited per group to allow for a 13% drop out rate. Mean baseline measurements of glucose were compared between groups using analysis of

variance (ANOVA). Mean measurements of glucose, insulin, and adrenaline at 60 minutes post clamp were compared adjusting for clamp baseline measurement, using analysis of covariance (ANCOVA). In the event of unequal variance between groups, a log transform was applied and the analysis repeated. Longitudinal and between-group comparisons were made for post-endotoxin measurements using mixed effects linear models. For models examining between-group differences, the baseline endotoxin measurement was included as a covariate. For all mixed-effects linear models, an autoregressive correlation structure (AR1) was used to allow for the correlation between multiple measurements on the same person. Planned contrasts were made versus baseline and between groups at equivalent time points with Sidak's correction for multiple comparisons. All data are expressed as mean \pm SEM, unless otherwise specified, and a p value of < 0.05 was deemed statistically significant. Analysis was performed using SPSS (version 22.0, IBM, Chicago, Illinois, USA).

2.2 Animal studies: effects of experimental hypoglycaemia on plaque biology in a novel murine model of combined atherosclerosis and hypoglycaemia

2.2.1 Overview of study

There was scant data exploring the relationship between hypoglycaemia and CVD in a robust animal model. The aim of these experiments was therefore to firstly, establish a robust murine model of experimental hypoglycaemia in pre-established atherosclerosis and secondly employ this model to study the consequences of hypoglycaemia on atherosclerotic lesion burden and markers of vulnerability.

2.2.2 Regulatory approvals

Animal experiments were carried out under the project licence PPL 70/7992 (previously 40/3307) held by Professor Sheila Francis, University of Sheffield. A personal licence numbered PIL IC801F8F8 was obtained by Dr Ahmed Iqbal to carry out all work in compliance with UK Animals (Scientific Procedures) Act 1986.

2.2.3 Animals, husbandry and diet

Male ApoE^{-/-} mice on a C57BL/6J background (JAX B6.129P2-ApoE^{tm1Unc/l}) were purchased from Charles River laboratories (Wilmington, Massachusetts, USA) and identified by ear clips and a corresponding unique identification number. All animals were housed at the University of Sheffield animal research facility based at the Royal Hallamshire Hospital in a controlled environment at 22°C with constant air pressure and 12 hour cycle of alternating light/darkness. Mice were specific pathogen free and housed in open top cages. The maximum number of mice housed in a cage was 4 with littermates being housed together when possible. Mice had free access to water and were fed *ad libitum* a standard chow diet (Teklad Global 18% protein rodent diet; Harlan, Idaho, USA which contained 6% w/w fat, 18.8% protein, 45% starch and 5% sugar) unless otherwise stated.

Atherogenic Western diet [Diet W, no 4021.06; 20 % casein, 16 % fat (15 % cacao butter, 1 % corn oil), 0.25 % cholesterol] was externally sourced from ABdiets (Woerden, The Netherlands) and used to expedite atherosclerotic plaque formation in ApoE^{-/-} mice. All mice were 8-13 weeks of age when feeding with Western diet was commenced. Western diet was stored in a refrigerator at 5-7°C.

2.2.4 Food consumption and body weight monitoring

Western diet was fed to ApoE^{-/-} mice such that each animal received 5 g of diet/day for a period of 7 weeks. Diet was weighed up and added to individual cages every week based on 5 g of diet/day/mouse. Diet remaining in each cage at the beginning of every feeding week was weighed up and not removed, additional fresh diet was simply added to meet the weekly requirements. Mice were weighed before the start of diet designated week 0 and weekly thereafter with measurements rounded to the nearest 0.1 g.

2.2.5 Experimental hypoglycaemia

A rodent model of recurrent insulin induced hypoglycaemia had been developed by our collaborators (McNeilly *et al.*, 2017). However, a model of combined experimental hypoglycaemia and atherosclerosis in ApoE^{-/-} mice fed Western diet was hitherto untested. Thus, in order to achieve experimental hypoglycaemia, optimisation of experimental conditions was undertaken in both fed and non-fed animals with an insulin dose finding exercise and variation of fasting conditions. Experimental conditions employed in the definitive experiment are described below. Methodological details of optimisation experiments that differ are included in chapter 4: Development of a murine model of experimental hypoglycaemia in pre-established atherosclerosis.

In order to control for stress hormones that are counter-regulatory to the action of insulin, ApoE^{-/-} mice were familiarised with the experimental laboratory by exposure to light levels, room temperature and noise levels on a weekly basis 7 weeks prior to induction of hypoglycaemia. Mice were placed on a heated platform

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of the Bp-2000 blood pressure analysis system (Visitech Systems Inc, Apex, NC) during hypoglycaemia or sham saline injection. In order to acclimatise mice to these conditions they were trained on this platform at the same time of day as planned experiments on a weekly basis (60 minutes/week) for 7 weeks during the feeding period and efforts were made to ensure that animal handling was performed by the same operators.

After an overnight fast, ApoE^{-/-} mice were placed in a warmed incubator at 33°C for 10 minutes to allow vasodilation. This facilitated blood sampling via a tail vein snip. Mice were placed on the heated Bp-2000 blood pressure analysis platform, the lateral tail vein was identified and a non-terminal lateral tail vein incision was made using a sterile surgical blade (Swann Morton Ltd, Sheffield, UK). Approximately 5 µL of blood was encouraged to form a micro droplet by application of gentle pressure at both ends of the small cut and blood glucose values were determined using a FreeStyle Optium Neo blood glucose meter (Abbott, Lake Bluff, Illinois, USA). The FreeStyle Optium Neo satisfies the International Organisation for Standardisation (ISO) 2013 blood glucose meter accuracy standards (ISO: 15197:2013) and was calibrated to high and low sugar values using manufacturer provided control solutions at the start of each experiment. A baseline blood glucose was measured and hypoglycaemia induced by an intraperitoneal (i.p.) injection of 2.5-3.7 mU/g Actrapid insulin (Human Actrapid, Novo Nordisk Pharmaceuticals Ltd, Crawley, West Sussex, UK). Actrapid insulin was diluted 1:500; 10 µL of neat insulin diluted in 5 ml of sterile 0.9% sodium chloride.

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Blood glucose values were measured at 15 minute intervals for a total duration of 120 minutes by gently massaging the tail veins snip site. Animals were closely monitored for adverse signs of hypoglycaemia and in this scenario rescued by an i.p. of 1 ml 20% Dextrose (Baxter, Baxter Healthcare Ltd, Norfolk UK) and placed in an incubator at 33°C. In the event that hypoglycaemia was irrecoverable, mice were euthanised using a Schedule 1 method. At the end of the experiment, mice were returned to their cages and fed a mash chow diet.

Control animals were handled identically but instead of insulin received a fixed volume (200 µl) injection of 0.9% sodium chloride.

2.2.6 Experimental design of recurrent hypoglycaemia

Having established a protocol for experimental hypoglycaemia in Western diet fed ApoE ^{-/-} mice, a definitive experiment was designed to recurrently induce hypoglycaemia. Mice were randomly allocated at the start of the feeding period to one of two groups:

- Group 1: experimental hypoglycaemia n=10
- Group 2: sham saline controls n=10

Following feeding, mice in each group underwent either hypoglycaemia or sham-saline injection on an alternate day basis for 4 consecutive weeks. Thus, each animal underwent 8 episodes of insulin induced hypoglycaemia or sham-saline

injection over the experimental period prior to sacrifice. Figure 2.3 is a schematic representation of the experimental design.

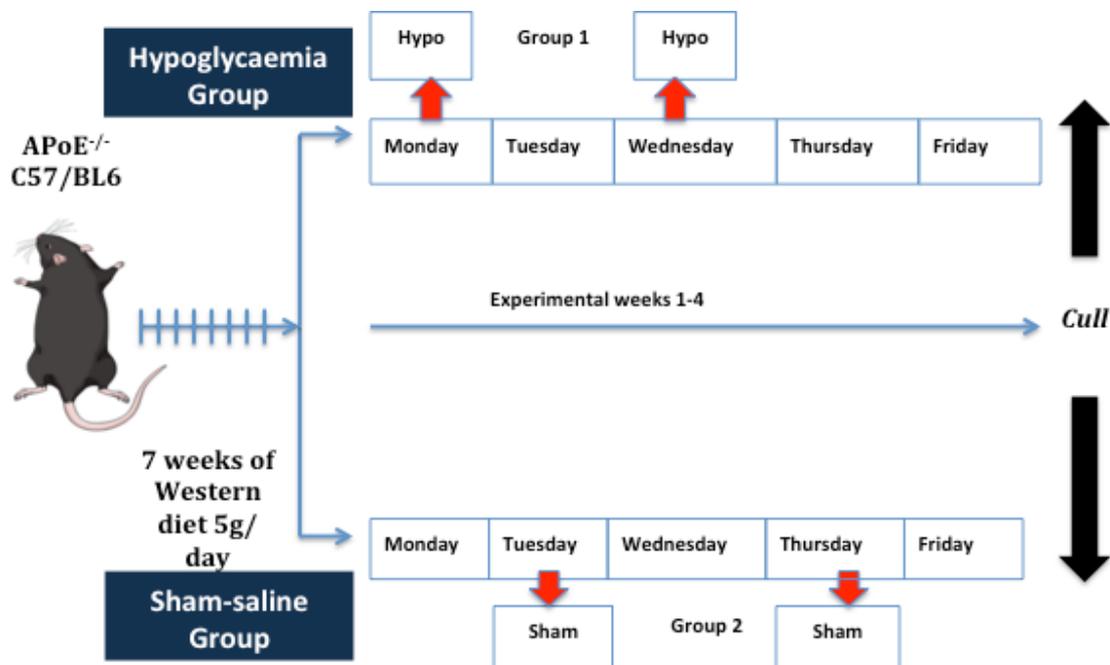


Figure 2-3 Schematic representation of the experimental protocol used to induce recurrent hypoglycaemia vs. sham-saline injection. ApoE^{-/-} mice fed Western diet for 7 weeks were allocated to either receive hypoglycaemia (group 1) or a sham-saline (group 2). Group 1 mice underwent hypoglycaemia on Mondays and Wednesdays. Group 2 mice received a saline control injection on Tuesdays and Thursdays. The experiment was conducted over 4 consecutive weeks. Mice from each group were rested on respective non-experimental days.

2.2.7 Harvesting of tissue

Animal sacrifice

At the end of the study, mice were weighed and sacrificed by an overdose of sodium pentobarbital (J M Loveridge Ltd, Southampton, UK) at a dose of 0.2 ml (200 mg/ml) given via an i.p. injection.

Cardiac puncture and perfusion fixation

Blood was collected via cardiac puncture once the pedal reflex was lost. A 1 ml heparinised syringe (1:100 in blood) was inserted using a left lateral approach through the intercostal spaces in the upper part of the thoracic cavity aiming for the left ventricle in order to obtain a single, good quality sample of mouse blood. Care was taken to obtain the maximum volume of blood at first attempt so as to minimise potential damage and contusions to cardiovascular tissue that was to be excised. In order to isolate plasma, whole blood was centrifuged at 3000g for 5 minutes in a microcentrifuge (Beckman Coulter, High Wycombe, UK). Isolated plasma was stored at -80°C in miniature cryovials for future analysis where performed.

Once respiration had completely ceased, the abdominal cavity was exposed and a small incision made in the abdominal aorta to allow extravasation of blood. The thoracic cavity was then exposed by performing a median sternotomy. The left ventricle was identified and injected with 1 ml of PBS followed by 1 ml of 10% v/v buffered formalin (33.3 mmol/l NaH₂PO₄, 50 mmol/l Na₂HPO₄, (Sigma-Aldrich, UK), 10% v/v formaldehyde (VWR International Ltd, BDH Chemicals, UK).

Dissection of cardiovascular tissue

Following perfusion fixation, CV tissue was excised. Firstly, the thoracic cage was removed to allow direct visualisation of the heart, great vessels and lungs. Next, using careful blunt dissection, the thymus, thyroid, trachea and oesophagus were identified and dissected. Both lungs were then dissected at the hilum. The diaphragm was preserved to allow the thoracic aorta to be distinguished from the

abdominal aorta. Starting at just above the diaphragm, the thoracic aorta was identified and carefully separated from the inferior vena cava and connective tissue. Working in a cephalic direction, fat was dissected from the aorta and small branching vessels removed using blunt dissection only. Upon arriving at the aortic arch, the superior vena cava was identified and detached from the heart thereby exposing the aortic arch fully. Careful dissection of fat then allowed the aortic arch and its 3 main vessels, the brachiocephalic, left common carotid and left subclavian arteries to be dissected free of surrounding tissue. The origin of the brachiocephalic artery was identified at the aortic arch and the vessel was excised from here to its bifurcation point into the right common carotid and right subclavian arteries and placed in 10% v/v buffered formalin.

The left common carotid and left subclavian arteries were then cut and the aorta mobilised by excising it at its origin at the heart. The descending aorta was then excised at just above the diaphragm. The whole aorta was transferred to a Petri dish and kept moist with PBS. Under microscopic guidance, blunt forceps were used to separate the aorta from closely adhering adventitial fat with utmost care to avoid damaging the vessel. Aortae were then cut using sprung vannas (curved pointed tip) scissors. The inside curvature was cut along its entire length and on the outside curvature the cutting edge ended at the top of the descending aorta. This allowed the entire inside surface of the vessels to be exposed. The vessels were stored in 4% w/v paraformaldehyde (PFA; VWR International Ltd, BDH Chemicals, UK) in PBS. Figure 2.4 is a diagrammatic representation of brachiocephalic artery excision and aortic dissection.

All tissue was stored at 4°C in either 10% v/v buffered formalin or % w/v paraformaldehyde for 24 hours prior to being transferred to PBS and stored 4°C until analysis.

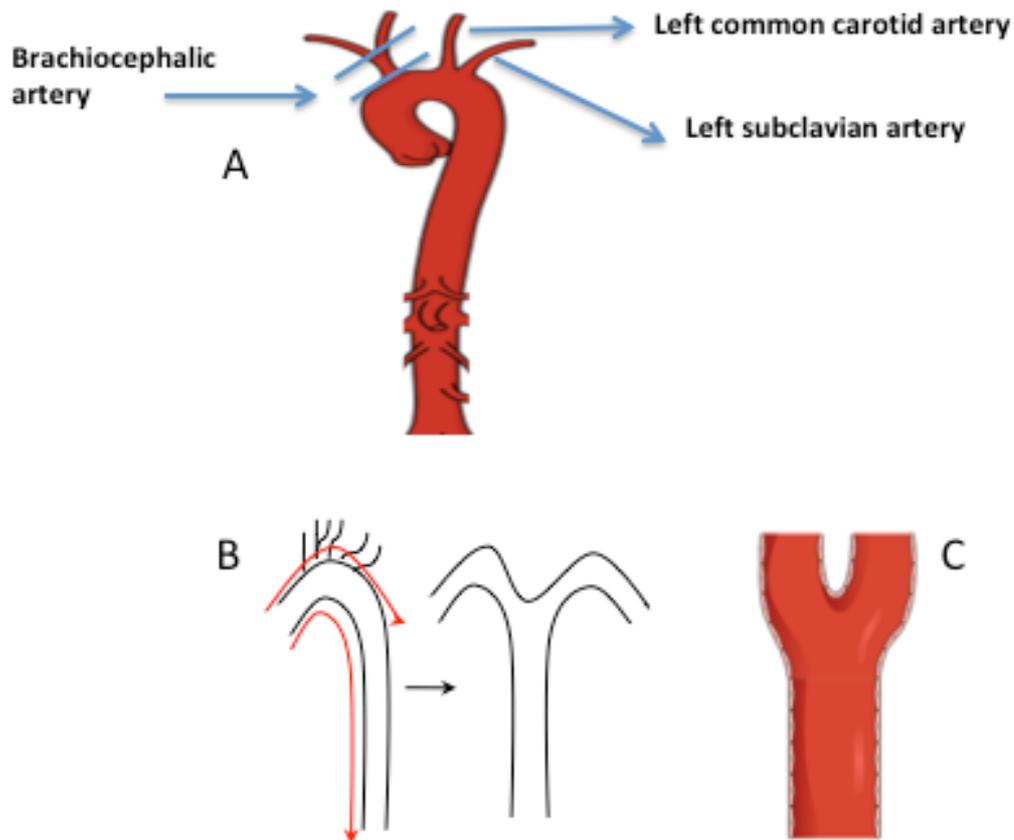


Figure 2-4 Excision of brachiocephalic arteries and aortic dissection. A) The brachiocephalic arteries were cut at their point of origin at the aortic arch to their bifurcation as indicated by the blue horizontal lines. In order to prepare aortae for *en face* staining, vessels were cut along both the inside and outside curvatures (B) to open up the vessels as represented (B&C).

2.2.8 Histological studies

Tissue preparation

Brachiocephalic arteries were removed from PBS, straightened and gently sandwiched between two sheets of Whatman grade 1 filter paper (Whatman International, UK). The samples with the filter papers were placed inside a plastic histological cassette (Thermo Fisher Scientific, UK) to undergo dehydration through graded alcohols and xylene prior to wax embedding and sectioning.

Tissue dehydration

Brachiocephalic arteries in histological cassettes were submerged in progressively higher concentrations of alcohol (50%, 70%, 90% and 100% v/v ethanol) for 1 hour per concentration of alcohol. There was also a change of ethanol after submersion in 100% ethanol. Tissue was then placed in a 1:1 mixture of 100% ethanol and xylene (Thermo Fisher Scientific, UK) for 1 hour. Following this, tissue was placed in xylene over two x 1 hour steps with a change of liquid after the first xylene step. Tissue was then stored in molten wax in 60°C overnight. This temperature was chosen to ensure infiltration of wax into tissues. Briefly, molten wax should be held at 1-2°C above its melting point and as the melting point was determined to be 58°C, the oven was set at 60°C. Storing at higher temperatures may reduce the ability of wax to successfully set into tissue.

Wax embedding of brachiocephalic arteries

A two-step embedding process was used to correctly orientate the brachiocephalic artery for sectioning. Following the dehydration protocol, histological cassettes were removed from the oven and brachiocephalic arteries retrieved and each

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placed horizontally at the base of a shallow plastic mould. Firstly, molten paraffin wax was poured around the vessel and allowed to cool and set by being left at room temperature for 3 hours. Once the wax had set, the wax block was trimmed to a small cuboidal shape containing the artery. Secondly, this piece of wax was rotated 90 degrees and placed at the bottom of another mould with the vessel perpendicular to the base. Molten wax was then poured around this mould which was later covered with a microtome block. The wax was then left to set overnight at room temperature.

Sectioning

Wax blocks were pressed out of mould and placed on ice for at least 60 minutes prior to sectioning using a Leica RM2135 microtome (Leica Microsystems, Wetzlar, Germany). Cut vessel sections were floated out on a water bath at 34-35°C as this aided in flattening the extremely thin slices of tissue. Polysine glass slides (VWR International Ltd, UK) were gently submerged just below floating sections of tissue in the water bath and then pulled out to seamlessly collect sections of tissue. Tissue on slides was firstly air dried in racks prior to being placed at 40°C in an oven overnight to facilitate complete adherence of tissue onto the slide. Slides were then stored at room temperature until staining.

Each brachiocephalic artery was sectioned from its origin at the arch of aorta to its point of bifurcation at 5 µm intervals. Four 5 µm sections were then mounted on each slide covering 20 µm vessel length. These samples were all individually inspected under the microscope and a 200 µm section of each artery containing the

largest neointima was identified. Eight sections were taken from this part of the artery, at approximately 40 μm intervals for analysis of atherosclerotic lesion area.

Oil red O staining of whole aortae

En face oil red O staining was used to quantify atherosclerotic lesion area in whole mounted aortae as previously described (Nunnari *et al.*, 1989). Oil red O stain was prepared by adding 1 ml of distilled water to 99 ml of isopropanol (Propran-2-ol, Sigma-Aldrich, UK). Oil red O powder (Sigma-Aldrich, UK) was then added to the solution until saturation. The saturated solution was filtered using Whatman grade 1 filter paper (Whatman International, UK) and the final stain diluted to 60% v/v using distilled water. Individual vessels were then stained in 1.5 ml Eppendorf tubes by initially rinsing aortae in distilled water followed by 60% v/v isopropanol for 2 minutes. Following this step, vessels were immersed in oil red O stain for 15 minutes prior to being rinsed with 60% v/v isopropanol for 2 minutes. Finally, aortae were immersed in distilled water prior to being mounted *en face*. In order to perform this, molten wax was poured onto 15 cm Petri dishes and allowed to set at room temperature reasonably firm yet malleable consistency. Individual vessels were then placed on the wax and opened gently with blunt forceps to reveal the inner surface with the lumen facing up. Once in position, 0.2 mm diameter micro-needles (Fine Science Tools, Heidelberg, Germany) were used to pin the aortae aided by a magnifying lamp. When adequately positioned, the vessels were submerged in PBS prior to image capture and analysis.

Elastin Van Gieson staining of brachiocephalic artery sections

Brachiocephalic artery cross sections identified for atherosclerotic lesion analysis were dyed with the Miller's Elastin/Modified Van Gieson (MVG) protocol. Sections on slides were de-waxed in xylene for 10 minutes and rehydrated through graded alcohol for 2 minutes as follows: 100%, 90%, 70% and 50% v/v ethanol and then rinsed in tap water. Tissue sections then underwent oxidation with 0.25% w/v aqueous potassium permanganate for 3 minutes. Following this, sections were bleached with 1% w/v oxalic acid for 3 minutes prior to being rinsed in water. Nuclei were then stained with Carazzi's haematoxylin for 2 minutes and differentiated with 1% v/v hydrochloric acid in 70% v/v ethanol for 5 seconds. Tissue sections were then exposed to hot running tap water for 5 minutes.

Slides with sections were then stained with alcian blue (1% w/v in 3% aqueous acetic acid, pH 2.5) for 5 minutes and the stain rinsed in water and then 95% v/v ethanol before staining with Miller's elastin for 30 minutes. Following this, tissue was differentiated in 95% v/v ethanol and rinsed in water prior to being stained with Curtis' modified Van Gieson reagent (10 ml 1% w/v Ponceau S in 90 ml saturated aqueous picric acid, 1 ml glacial acetic acid) for 6 minutes. Slides were then rinsed in water and dehydrated through graded alcohols ending in xylene as a reverse of the first step in the protocol. Finally, tissue sections were mounted under cover slips using DPX resin (VWR International Ltd, UK).

2.2.9 Immunohistochemistry

Brachiocephalic artery sections were stained for MAC-3 which is an established macrophage marker. Slides containing sections were de-waxed in xylene prior to rehydration in graded alcohols to water. Treatment with hydrogen peroxide (30% v/v; Sigma-Aldrich, UK) blocked endogenous peroxidases and incubation in 1% Marvel buffer (commercially available 0.1% w/v milk buffer) in PBS for 30 minutes blocked non-specific antibody binding. Antigen retrieval was performed if necessary prior to blocking with milk buffer. Slides were incubated with rat anti-mouse CD107b (MAC-3, 550292, BD Biosciences, Oxford, UK, diluted 1 in 100 in PBS) antibody at 4°C overnight prior to washing thrice with PBS. Primary antibody binding to target antigen was determined by incubating sections with biotinylated rabbit anti-rat secondary antibody (BA4000, Vector Laboratories, UK, diluted 1 in 200 in PBS) for 30 minutes at room temperature. In order to amplify positive staining, this step was followed by incubation of sections with avidin-biotinylated enzyme complexes with horseradish peroxidase (Vectastain ABC-HRP kit, Vector Laboratories, UK) at room temperature for 30 minutes prior to washing thrice with PBS. In order to detect staining with light microscopy, sections were then incubated with enzyme substrate SigmaFAS 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, UK) until colour development was witnessed when the reaction was stopped by rinsing slides first in distilled water and then PBS. Lastly, slides were counterstained with Carazzi's haematoxylin for 1 minute, dehydrated and mounted using cover slips with DPX.

2.2.10 Morphometry

Stained sections images were acquired by using a camera connected to a light microscope for brachiocephalic arteries. A camera connected to a dissecting microscope was used for *en face aortae*. Atherosclerotic lesion area was calculated by analysis of captured images using the NIS-elements image analysis software (Nikon, Kingston-upon-Thames, UK). To eliminate any potential bias, atherosclerotic lesion analyses were performed by myself and confirmed by Dr Jessica Johnston who was blinded to study group allocation.

Atherosclerotic lesion analysis in whole mounted aortae

A 10 cm ruler was first captured using the camera at x15 magnification in order to calibrate the software. Calibration ensured subsequent image capture of aortae was accurate. Individual vessels were captured at x15 magnification. The total surface area of each aorta was manually traced by closely following the vessel outline. Using the software threshold function that incorporated hue, saturation and intensity filters, areas positively staining for oil red O were identified. Total atherosclerotic lesion area was calculated and expressed as a percentage of the total surface area of each vessel. Lesion area was assessed in whole aortae, and regional analysis performed in the aortic arch and descending parts. Figure 2.5 demonstrates quantification of atherosclerosis in whole aortae.

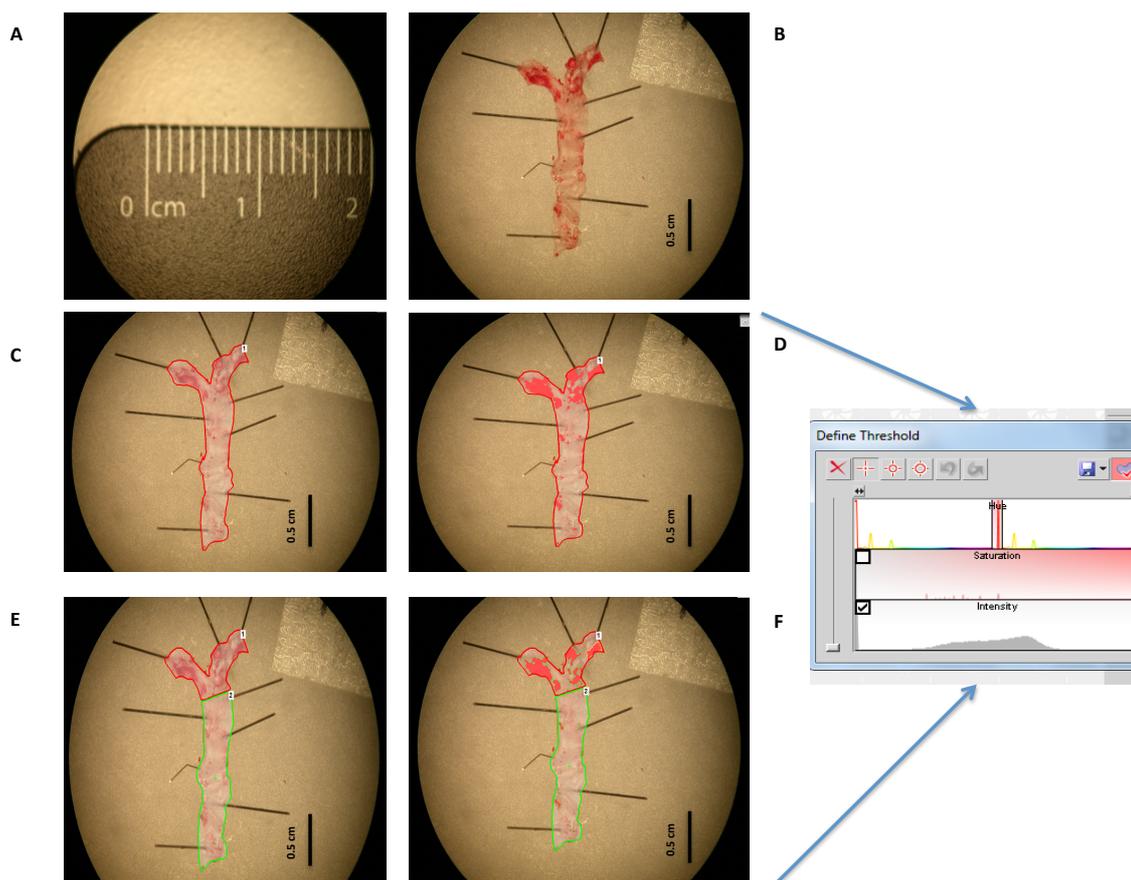


Figure 2-5 Quantification of atherosclerotic lesion burden in *en face* oil red O stained whole aortae. A) Original images were captured at x15 magnification and an image of a ruler was used to calibrate the software. B) Image of an aorta with positive areas stained in red. C) The total surface area of each vessel was calculated by manually tracing the vessel as shown in red. D) The NIS-elements software was used to select areas of positive staining highlighted in red by using the threshold function and applying hue, saturation and intensity filters. E) Regional analysis of atherosclerosis was performed within the aortic arch (outlined in red) and the descending aorta (outlined in green). F) The threshold function of the software was then used to separately identify areas of positive staining within the two aortic regions. Scale bars = 0.5 cm.

Atherosclerotic lesion analysis in brachiocephalic arteries

Brachiocephalic artery sections were captured at x100 magnification. Eight sections taken from a 200 μm section of the brachiocephalic artery containing the largest neointima were analysed from each vessel to generate a mean total atherosclerotic lesion area measurement for each mouse. The cross sectional area

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(CSA) for each vessel was defined as the external elastic lamina (EEL) and this was quantified by manually tracing the EEL for each vessel in the NIS-elements software. Atherosclerotic lesion area was quantified by manually tracing around the lesion. Total atherosclerotic lesion area was expressed as a percentage of the total CSA. As vessels are prone to distortion during processing, a calculation (see below) was applied to adjust measured EEL cross-sectional area working under the assumption that the brachiocephalic artery was originally a perfect circle.

Calculation for corrected cross-sectional area of brachiocephalic arteries where the measured circumference corresponds to the EEL:

$$\text{Circumference (C)} = 2\pi r, \text{ so therefore with rearrangement } r = C/2\pi$$

$$\text{Area of a circle} = \pi r^2$$

$$\text{Corrected area} = \pi(C/2\pi)^2$$

Figure 2.6 demonstrates quantification of atherosclerotic plaque lesion area in a brachiocephalic artery.

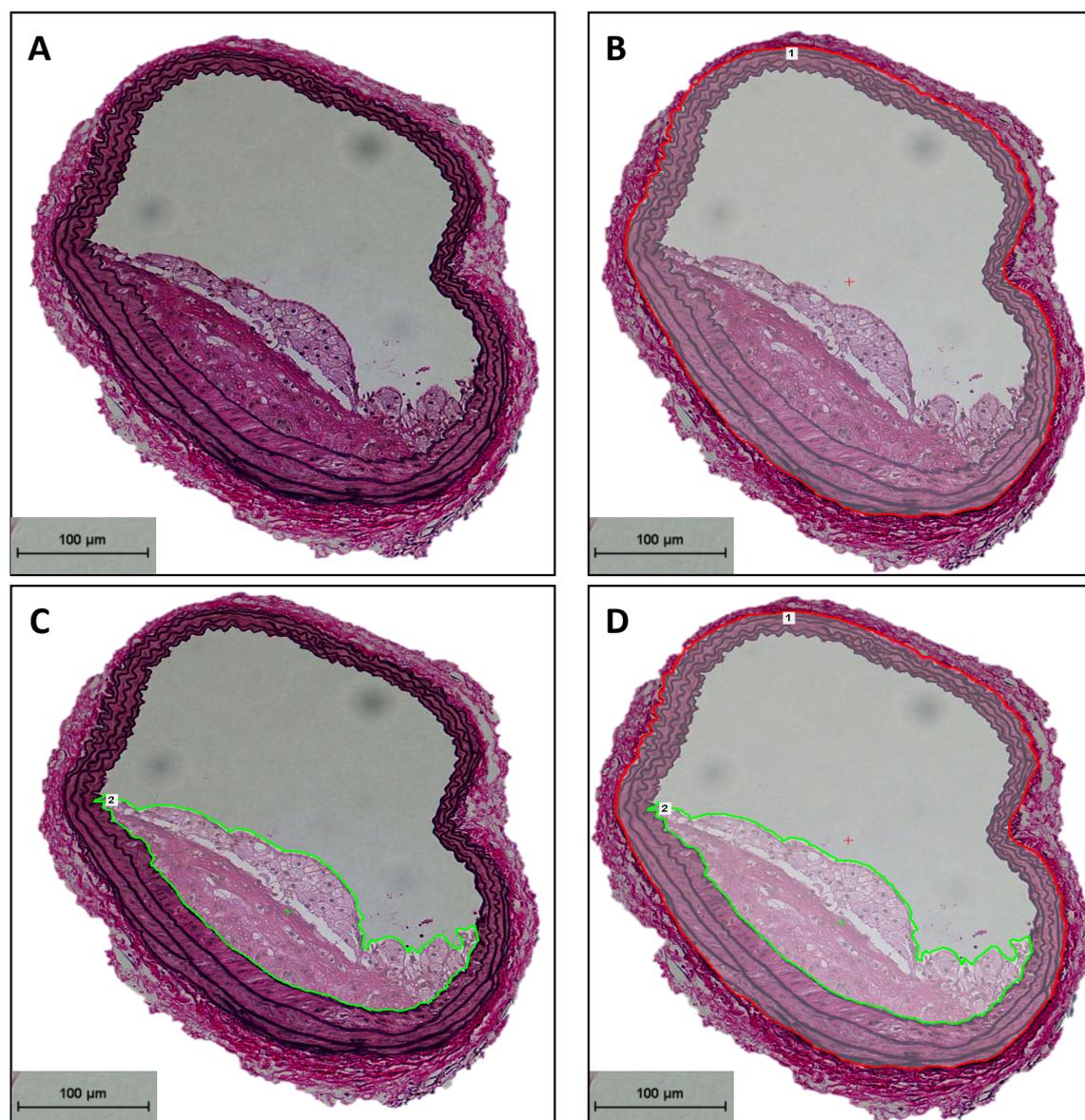


Figure 2-6 Measurement of atherosclerotic plaque lesion area in a brachiocephalic artery. A) MVG stained brachiocephalic artery, scale bar = 100 µm. B) The EEL was used to measured the vessel CSA and manually traced as shown in red using the NIS-elements software. C) The atherosclerotic plaque was manually traced in green using the software. D) Total plaque area (green) was calculated by the software and expressed as a percentage of the calculated and equation corrected total vessel CSA (red). Abbreviations: MVG, Miller's Elastin/Modified Van Gieson; EEL, external elastic lamina; CSA, cross sectional area.

Quantification of positive immunohistochemistry staining

Positive immunohistochemistry staining for MAC-3 was identified on account of the brown precipitate that forms with use of DAB substrate. The NIS-elements

software threshold function which incorporates hue, saturation and intensity filters was used to identify and then quantify areas within lesions corresponding with positive staining. Figure 2.7 illustrates positive staining for MAC-3.

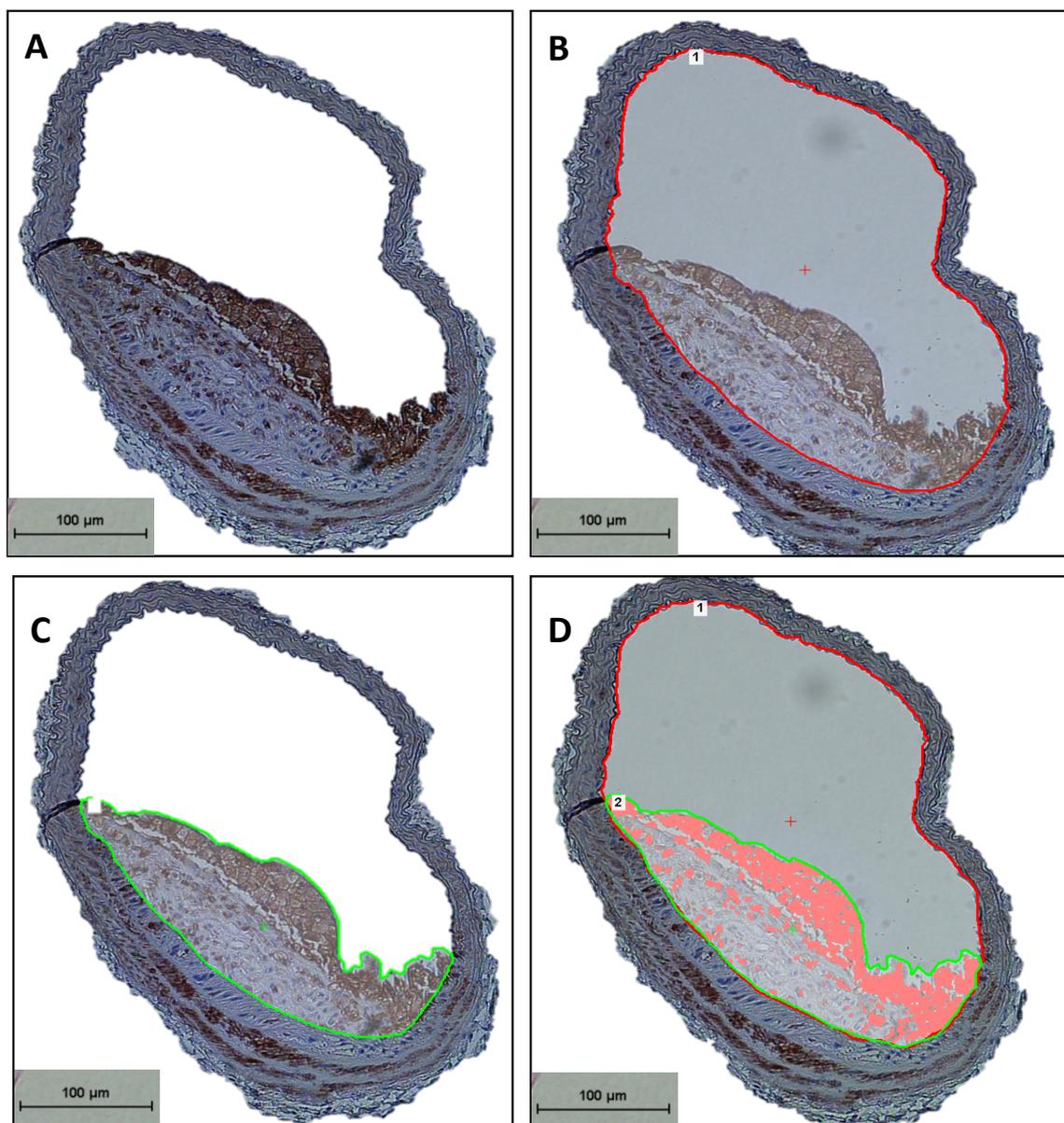


Figure 2-7 Quantification of positive immunohistochemical staining for macrophages. A) MAC-3 staining in a brachiocephalic artery, positive areas are brown, scale bar = 100 µm. B) The internal elastic lamina (red) was manually traced to define the lumen of the vessel as a region of interest in the NIS-elements software. C) The atherosclerotic plaque (green) was manually traced to define a secondary region of interest within the lumen. D) Using the threshold function and hue, saturation and intensity filters areas of positive staining for MAC-3 were quantified in the software. Abbreviations: MAC-3, CD107b.

2.2.11 Biochemical analyses

Catecholamines

Adrenaline was measured at 0 minutes and 60 minutes following i.p. insulin injection on the 7th episode of hypoglycaemia or sham-saline injection (week 4). In unrestrained mice, whole blood (40 µl) obtained from the tail vein cut was collected into EDTA coated Microvette capillary tubes (Sarstedt, Nümbrecht, Germany) and microcentrifuged (Beckman Coulter, High Wycombe, UK) at 3000g for 5 minutes to obtain plasma that was stored at -80°C until analysis. Adrenaline concentrations in plasma were measured using a commercially available kit (LDN, Germany) by Dr Alison McNeilly at the University of Dundee. During this assay, adrenaline was extracted from the plasma (10µl per sample) using a cis-diol-specific affinity gel, acylated and converted enzymatically. In this competitive enzyme-linked immunosorbent assay (ELISA), the antigen was bound to the solid phase of the microtiter plate. The derivatised standards, controls and samples and the solid phase bound analytes compete for a fixed number of antiserum binding sites. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG-peroxidase conjugate using 3,3',5,5' tetramethylbenzidine (TMB) as a substrate. The reaction is monitored at 450 nm. Quantification of unknown samples is achieved by reference to a standard curve generated using a 4-Parameter Logistic (4-PL) curve method.

Serum lipids

Whole blood (200 µl) obtained during cardiac puncture at sacrifice was aliquoted in a 1.1 ml neonatal SST tube (Sarstedt, Nümbrecht, Germany) and centrifuged to

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obtain serum at STH laboratories. Total cholesterol, HDL and triglycerides were measured using a commercial assay (Cobas system, Roche Diagnostics, Burgess Hill, West Sussex, UK). The LDL values were collected using the following equation:

$$\text{LDL-C} = \text{Total cholesterol} - \text{HDL} - (\text{triglycerides}/2.2)$$

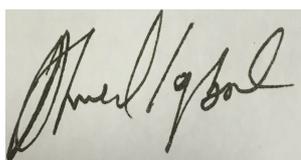
2.2.12 Statistical analysis

All data are expressed as mean \pm SEM, unless otherwise specified, and a p value of < 0.05 was deemed statistically significant. Analyses were performed using Graph Pad Prism version 7 (GraphPad, San Diego, California, USA). In order to compare two experimental groups, data were analysed using a two-tailed unpaired Student's t test for normally distributed data. For multiple comparisons, I employed analysis ANOVA. Based on data from Prof. Francis' group, power calculations suggested that to detect a 5-10% difference in total plaque macrophages or lesion area using histology, with a power of 80% and significance of 5%, a sample size of 10 mice per group would be needed.

Chapter 3- Effects of hypoglycaemia and low dose endotoxaemia on inflammatory responses in humans

This chapter takes the form of a published paper upon which I am first author. I was the primary contributor to this manuscript and my contribution included: experimental design (with guidance from supervisory team), performing all experimental work (with some technical assistance) and data analysis, preparation of figures and writing of the manuscript (which was edited by the co-authors where necessary). The format of this chapter was determined by the publisher's requirements. Additional explanatory text pertaining to methodology is found in chapter 2.

I can confirm that I obtained permission from The University of Sheffield and the Journal of Clinical Endocrinology and Metabolism to include this published material in this thesis.



Ahmed Iqbal



Prof Simon Heller



Prof Ian Sabroe

3.1 Introduction

Hypoglycaemia is associated with a greater propensity to adverse CV outcomes in diabetes (Hsu *et al.*, 2013; Khunti *et al.*, 2015; Lu *et al.*, 2016). To determine if such outcomes were dependent upon changes in innate immune responses, I devised a novel model whereby subjects were challenged with a hypoglycaemic clamp, and then the durable effects on the innate immune system probed by an *in vivo* endotoxin challenge 48 hours later.

Iatrogenic hypoglycaemia remains a major barrier to effective treatment of insulin-treated diabetes (Cryer, 2008). The ACCORD trial showed that intensive glucose control, during which patients were exposed to significantly more hypoglycaemia (Gerstein *et al.*, 2008), was associated with excess CV mortality. Despite the evidence confirming an association between hypoglycaemia and mortality, cause and effect has not been established. Trial evidence suggests that the relationship is, at least in part, explained by 'confounding', i.e. that hypoglycaemia identifies individuals with comorbidities who are both vulnerable to hypoglycaemia and more likely to die for other reasons (Zoungas *et al.*, 2010). Nevertheless, a recent large meta-analysis (Goto *et al.*, 2013) has suggested that comorbidities alone are unlikely to explain this relationship. Furthermore, there is a growing body of evidence highlighting a number of mechanisms whereby hypoglycaemia may lead to CV events (Chow *et al.*, 2014, 2017).

Hypoglycaemia has pro-inflammatory consequences, including increases in levels of factor VIII and von Willebrand factor and impaired fibrinolysis

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(Dalsgaard-Nielsen, Madsbad and Hilsted, 1982; Fisher *et al.*, 1991; Gogitidze Joy *et al.*, 2010). In addition, hypoglycaemia has been shown to increase pro-inflammatory cytokines (Dotson *et al.*, 2008; Razavi Nematollahi *et al.*, 2009; Gogitidze Joy *et al.*, 2010) and promote rises in the levels of pro-atherogenic cell adhesion molecules (Gogitidze Joy *et al.*, 2010). Repeated episodes of hypoglycaemia have also been reported to impair nitric oxide-mediated vasodilation (Joy *et al.*, 2015).

Monocytes are phagocytes that are central to the aetiology of atherosclerosis (Oude Nijhuis *et al.* 2007) and play a role in precipitating acute CV events by promoting plaque destabilisation and rupture (Newby *et al.*, 2009). The extent to which monocytosis and monocyte activation is modified by hypoglycaemia remains uncertain. Recent studies have also determined that monocytes can be classified into 3 distinct subsets, termed classical monocytes (CM: CD14⁺⁺ CD16⁻; 'Mon1'), intermediate monocytes (IM: CD14⁺⁺ CD16⁺, 'Mon2'), and non-classical monocytes (NCM: CD14⁺ CD16⁺⁺, 'Mon3') (Ziegler-Heitbrock *et al.*, 2010; Weber *et al.*, 2016). A number of observational studies indicate that IMs may be particularly pro-atherogenic. Elevated levels of IMs are associated with adverse CV outcomes (Rogacev *et al.*, 2011, 2012; Tapp *et al.*, 2012; Zhou *et al.*, 2016), independently predict future CV events (Rogacev *et al.*, 2012), and have been associated with coronary plaque vulnerability in patients with angina (Kashiwagi *et al.*, 2010). Elevated levels of CMs may also independently predict CV events (Berg *et al.*, 2012).

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AMI results in monocytosis, mediated by sympathetic nervous system activation (Dutta *et al.*, 2012). In humans, CD16⁺ monocytes selectively mobilise, in a catecholamine-dependent fashion, following exercise (Birgit Steppich *et al.*, 2000). As epinephrine (adrenaline) is the key counter-regulatory hormone produced in response to hypoglycaemia, I hypothesised that hypoglycaemia would also exert significant effects on monocytes. I further hypothesised that we would see additional synergistic changes in monocyte and platelet activation, as revealed by formation of monocyte-platelet aggregates, which are increased after AMI (Furman *et al.*, 2001; Tapp *et al.*, 2012) To probe how hypoglycaemia may modulate monocyte function in the human *in vivo*, I chose to combine a classical hypoglycaemic stimulus with a subsequent *in vivo* systemic stimulus of the innate immune system. To achieve this, I combined hyperinsulinaemic hypoglycaemic-euglycaemic and sham-saline clamps with low-dose intravenous endotoxin challenge 48 hours later in healthy participants.

3.2 Methods

A detailed description of the study design, clamp and endotoxin methods, biochemical, flow cytometry and other assays are described in chapter 2.

3.3 Results

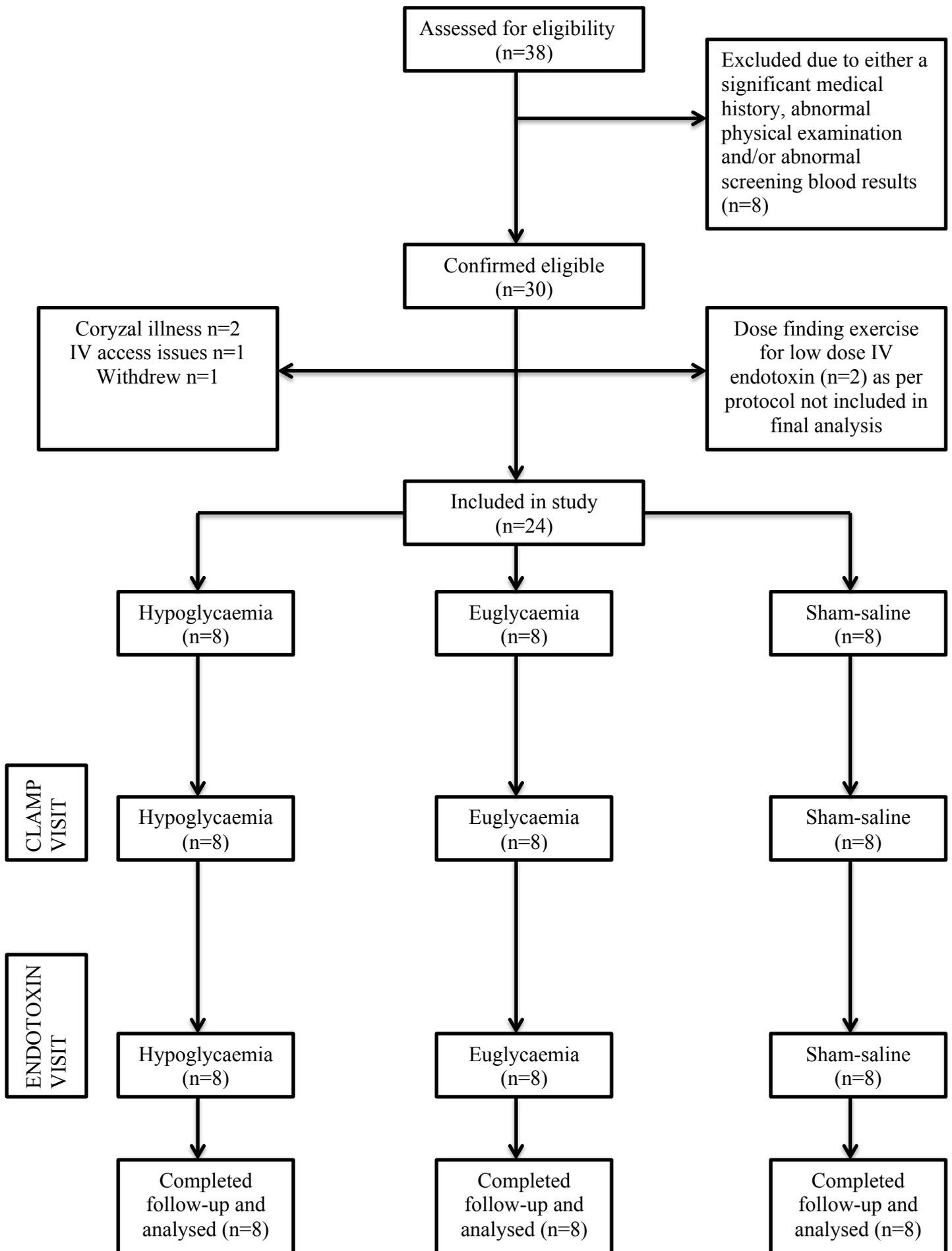
3.3.1 Participants

Study participants across the three groups were well matched for age, sex, BMI, HbA1c and total WBC count with no significant differences at screening (Table 3.1). Participant numbers at each stage of study is illustrated in a flow diagram

on page 130. Data shown in Figures: 3.2, 3.3 and 3.4 A, 3.4 C, 3.4 D, 3.4 E and 3.4 F are Sysmex data from n = 6 in each study group.

Table 3.1 Comparison of participant characteristics at baseline. Data are mean \pm SD or median (interquartile range). p values indicate comparisons between study groups via parametric or nonparametric testing. Abbreviations: BMI, body mass index; WBC, white blood cells.

Parameter	Hypoglycaemia	Euglycaemia	Sham-saline	p
Total n=24	8	8	8	N/A
Sex (M/F)	4/4	4/4	4/4	N/A
Age (years)	21 (19-22)	21 (20-23)	21.5 (21-26)	0.299
BMI (kg/m²)	24 \pm 2	23 \pm 2	24 \pm 4	0.638
HbA1c				
%	5.2 \pm 0.31	5.2 \pm 0.26	5.1 \pm 0.14	0.792
mmol/mol	34 \pm 3.6	33.5 \pm 2.8	32.6 \pm 1.4	0.616
Total WBC (X10⁹/l)	6.26 \pm 1.42	4.83 \pm 0.91	4.50 \pm 1.69	0.102



3.3.2 Clamp studies

Glucose, insulin and other counter-regulatory hormones

Arterialised blood glucose values are shown in Figure 3.1 A. The glucose values were 2.51 ± 0.11 mmol/l and 6.04 ± 0.16 mmol/l at the end of the hypoglycaemia and euglycaemia clamps, respectively. Glucose values at the end of the sham-saline clamp were 4.64 ± 0.09 mmol/l. A counter-regulatory response to hypoglycaemia was evident with epinephrine (adrenaline) levels during hypoglycaemia (1.87 ± 0.25 nmol/l) being significantly higher ($p < 0.001$) compared to euglycaemia (0.07 ± 0.01 nmol/l) and sham-saline (0.10 ± 0.04 nmol/l) (Figure 3.1 B). Free insulin levels at the end of clamp were similar between hypoglycaemia (968.5 ± 149.1 pmol/l) and euglycaemia groups (1025.4 ± 81.4 pmol/l, $p = 0.996$) but significantly higher ($p < 0.001$) than those in the sham-saline (31.3 ± 6.3 pmol/l) group (Figure 3.1 C). Serum cortisol and growth hormone were significantly higher in the hypoglycaemia group compared to euglycaemia and sham-saline controls (Figure 3.1 D&E).

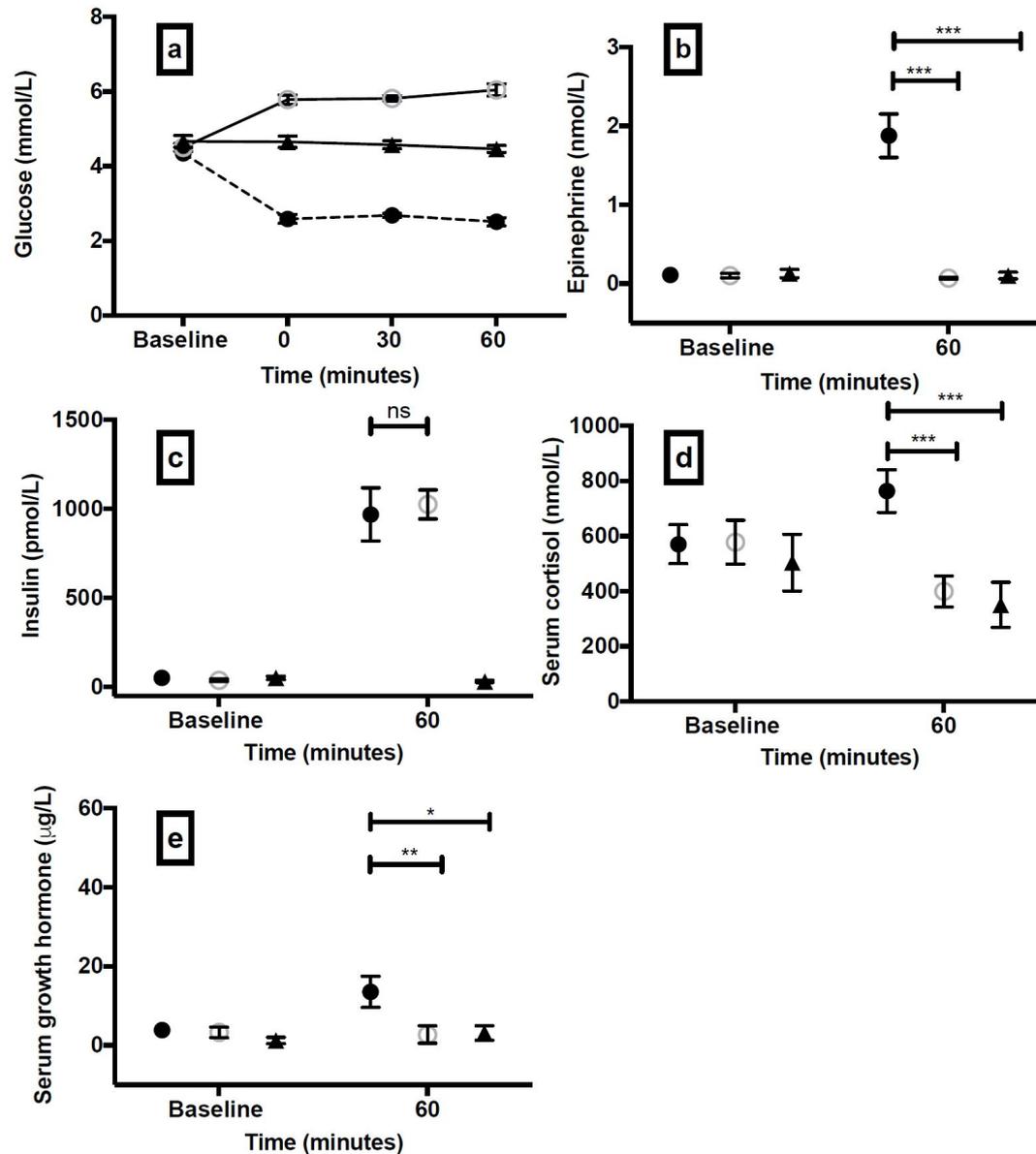


Figure 3-1 Glucose, insulin and counter-regulatory hormones in clamp studies. Arterialised whole blood glucose values during hyperinsulinaemic hypoglycaemic, euglycaemic and sham-saline clamps (a), epinephrine (adrenaline) (b), free insulin (c), cortisol (d) and growth hormone values (e) after 60 minutes of hypoglycaemia, euglycaemia or sham-saline injection. Data are mean (SEM), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns-non-significant, p-values are provided for comparison between study groups. Black circles (and dashed line 3.1 A)-hypoglycaemia group; open circles-euglycaemia group; black triangles (and solid line 3.1 A)-sham-saline group.

Total and differential leukocyte count

I determined if hypoglycaemia results in changes in circulating leukocytes. Hypoglycaemia significantly increased the total number of WBCs compared to controls (Figure 3.2 A). There was an increase across all classes of leukocytes studied, including neutrophils (Figure 3.2 B), lymphocytes (Fig 3.2 C) and total monocytes (Fig. 3.3 A).

Monocyte subsets

I sought to determine if hypoglycaemia exerted specific effects on monocyte subsets associated with cardiac pathology. Hypoglycaemia increased the absolute number of all three circulating monocyte subsets compared to euglycaemia and sham-saline (Fig. 3.3 B-D). The number of circulating NCMs after 60 minutes of hypoglycaemia compared to baseline (17.6 ± 2.9 cells/ μ l) increased twofold. IM numbers after 60 minutes of hypoglycaemia compared to baseline (23.2 ± 4.5 cells/ μ l) increased by a factor of 1.81 and CMs after 60 minutes of hypoglycaemia compared to baseline (442.4 ± 55.3 cells/ μ l) increased by a factor of 1.29. There were no significant differences in the baseline values of all three monocyte subsets between the study groups.

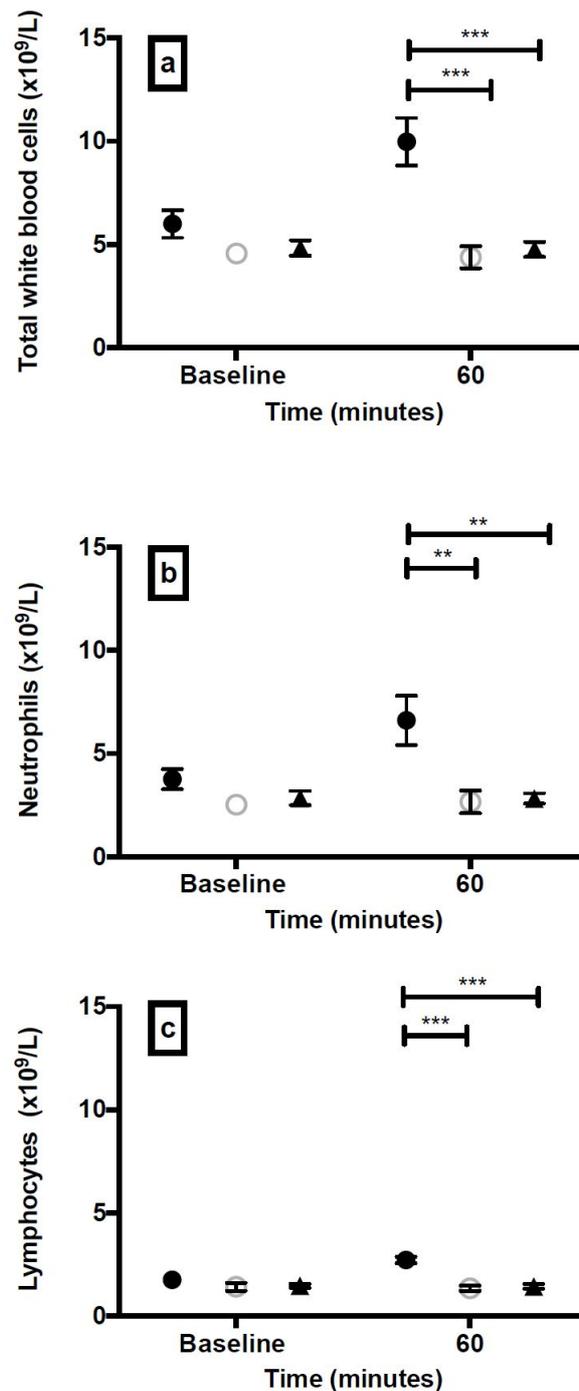


Figure 3-2 Peripheral total white blood cell, neutrophil and lymphocyte kinetics in experimental hypoglycaemia and controls. Number of circulating total WBCs (a), neutrophils (b) and lymphocytes (c) after 60 minutes of hypoglycaemia, euglycaemia or sham-saline injection. Data are mean (SEM), **p<0.01, *p<0.001, p-values are provided for comparison between study groups. Black circles-hypoglycaemia group; open circles-euglycaemia group; black triangles-sham-saline group. WBC, white blood cells.**

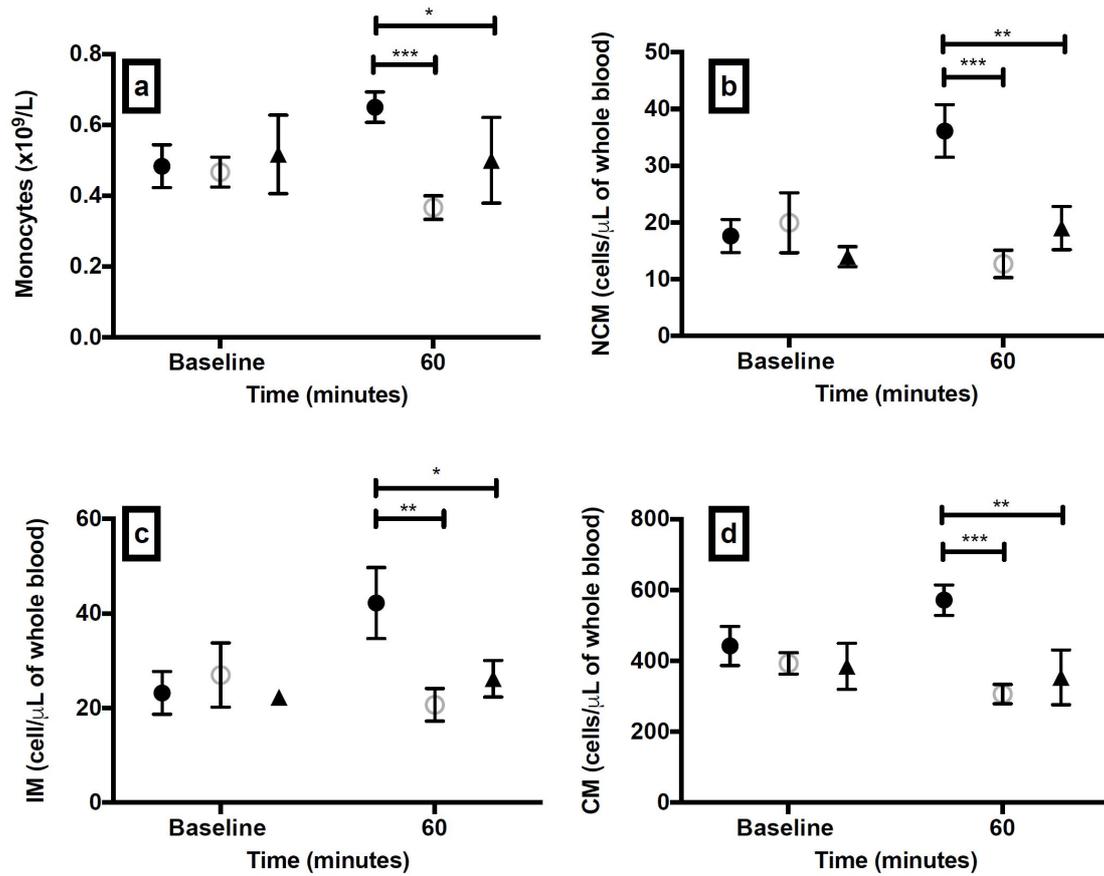


Figure 3-3 Total monocyte count and monocyte subset kinetics in experimental hypoglycaemia and controls. Absolute circulating numbers of total monocytes (a) and monocyte subsets comprising of NCMs (b), IMs (c) and CMs (d) after 60 minutes of hypoglycaemia, euglycaemia or sham-saline injection. Data are mean (SEM), * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, p-values are provided for comparison between study groups. Black circles-hypoglycaemia group; open circles-euglycaemia group; black triangles-sham-saline group. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical monocytes.**

Platelet count, aggregation and monocyte-platelet aggregates

Activation of platelets and generation of platelet-leukocyte aggregates contribute to leukocyte mobilisation and inflammation in the vasculature (Totani and Evangelista, 2010). I therefore studied platelet number and function and their interaction with leukocytes. Total platelet count increased in hypoglycaemia compared to euglycaemia and sham-saline controls (Figure 3.4 A). ADP-induced platelet aggregation increased following 60 minutes of hypoglycaemia versus euglycaemia ($p = 0.014$) and there was numerically, but not statistically significantly, higher platelet aggregation detected in the hypoglycaemia group compared to sham-saline group ($p = 0.064$) (Figure 3.4 B). The total number of MPAs increased following 60 minutes of hypoglycaemia compared to euglycaemia (Figure 3.4 C). Whilst total MPAs were not significantly higher in hypoglycaemia compared to sham-saline controls at 60 minutes (Figure 3.4 C), I observed specific increases in non-classical monocyte (NCM) and intermediate monocyte (IM)-platelet aggregates (Figure 3.4 D&E). Classical monocyte (CM)-platelet aggregates appeared to increase following 60 minutes of hypoglycaemia versus euglycaemia and sham-saline but this was not statistically significant ($p = 0.054$) (Figure 3.4 F).

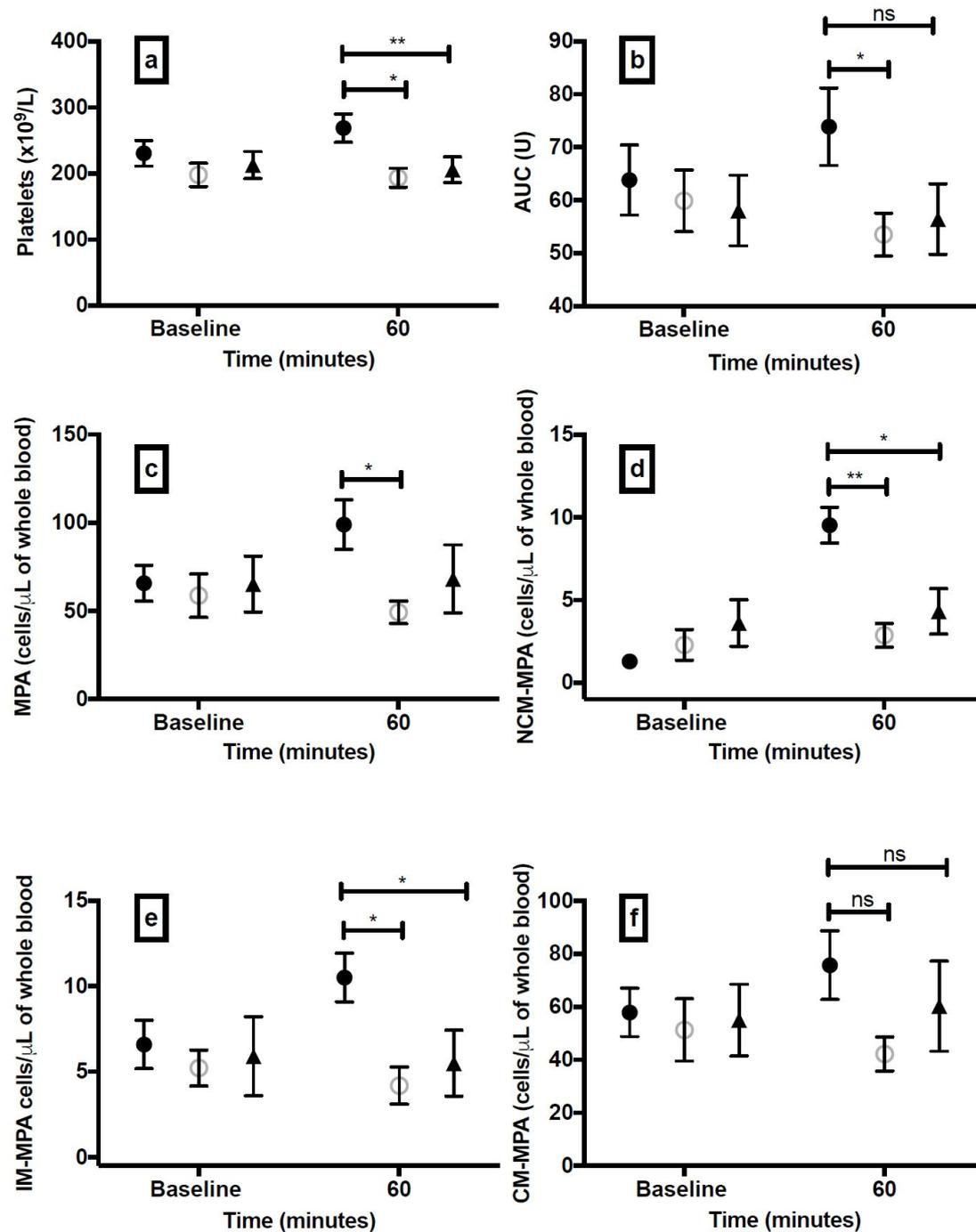


Figure 3-4 Platelet reactivity and monocyte-platelet aggregate formation in experimental hypoglycaemia and controls. Total platelet count (a), platelet aggregation to ADP 6.45 μ M (b), total MPA formation (c) and MPA formation within monocyte subsets; NCM-MPAs (d), IM-MPAs (e) and CM-MPAs (f) after 60 minutes of hypoglycaemia, euglycaemia or sham-saline injection. Data are mean (SEM), *p<0.05, **p<0.01, ns-non-significant, p-values are provided for comparison between study groups. Black circles-hypoglycaemia group; open circles-euglycaemia group; black triangles-sham-saline group. MPA, monocyte-platelet aggregates; NCM-MPA, non-classical monocyte-platelet aggregates; IM-MPA, intermediate monocyte-platelet aggregates; CM-MPA, classical monocyte-platelet aggregates.

Cell surface markers

To further explore the activation state of monocytes after hypoglycaemia, I studied expression levels of chemokine receptor CX₃C chemokine receptor 1 (CX₃CR1) and integrin CD11b. Hypoglycaemia did not alter the expression of CX₃CR1 or CD11b (Figure 3.5 A&B).

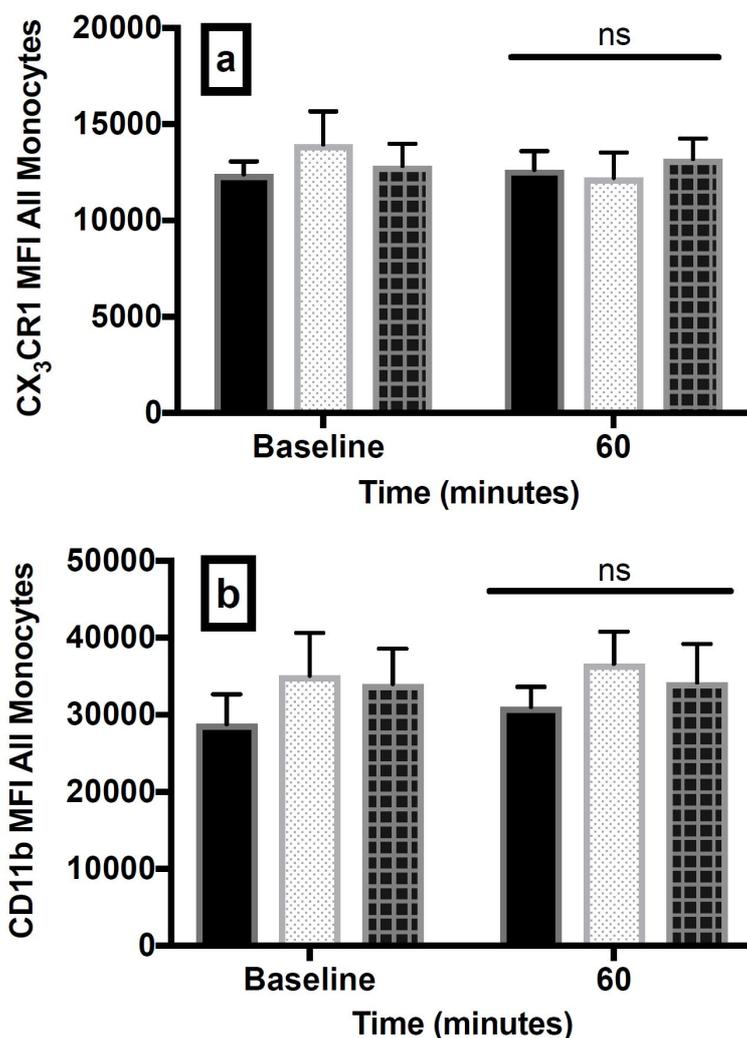


Figure 3-5 Expression of CX₃CR1 and CD11b on monocytes in clamp studies. Expression of CX₃CR1 (a) and CD11b (b) on all monocytes after 60 minutes of hypoglycaemia, euglycaemia or sham-saline injection. Data are geometric mean (SEM), ns-non-significant, solid horizontal lines represent significance for comparison between study groups. Black bars-hypoglycaemia group (n=8); white bars-euglycaemia group (n=8); striped black bars-sham-saline group (n=8). MFI, mean fluorescence intensity.

3.3.3 Endotoxin challenge

To determine if prior hypoglycaemia affected the subsequent response to a classical immune activator, and thus to reveal if hypoglycaemia had any longer-lasting effects on the innate immune system, I next proceeded to a low-dose (0.3 ng/kg) intravenous endotoxin challenge 48 hours after the hypoglycaemic challenge in all subjects. Consistent with the low-dose model employed, no fever or significant change in mean arterial blood pressure was recorded following endotoxin challenge across the study groups.

Epinephrine, cortisol and growth hormone

In contrast to the stress response induced by hypoglycaemia, epinephrine levels were not significantly different between study groups 6 hours following endotoxin administration (Figure 3.6 A). In the hypoglycaemia group, epinephrine levels were 0.15 ± 0.04 nmol/l versus 0.06 ± 0.01 nmol/l in euglycaemia group and 0.09 ± 0.01 nmol/l in sham-saline group. There were also no differences detected between groups in serum cortisol and growth hormone levels following endotoxin administration (Figure 3.6 B&C). However, a rise versus baseline in the stress hormone cortisol was evident whereby serum cortisol levels peaked at 4 hours following endotoxin challenge in all study groups ($p = 0.005$) (Figure 3.6 B).

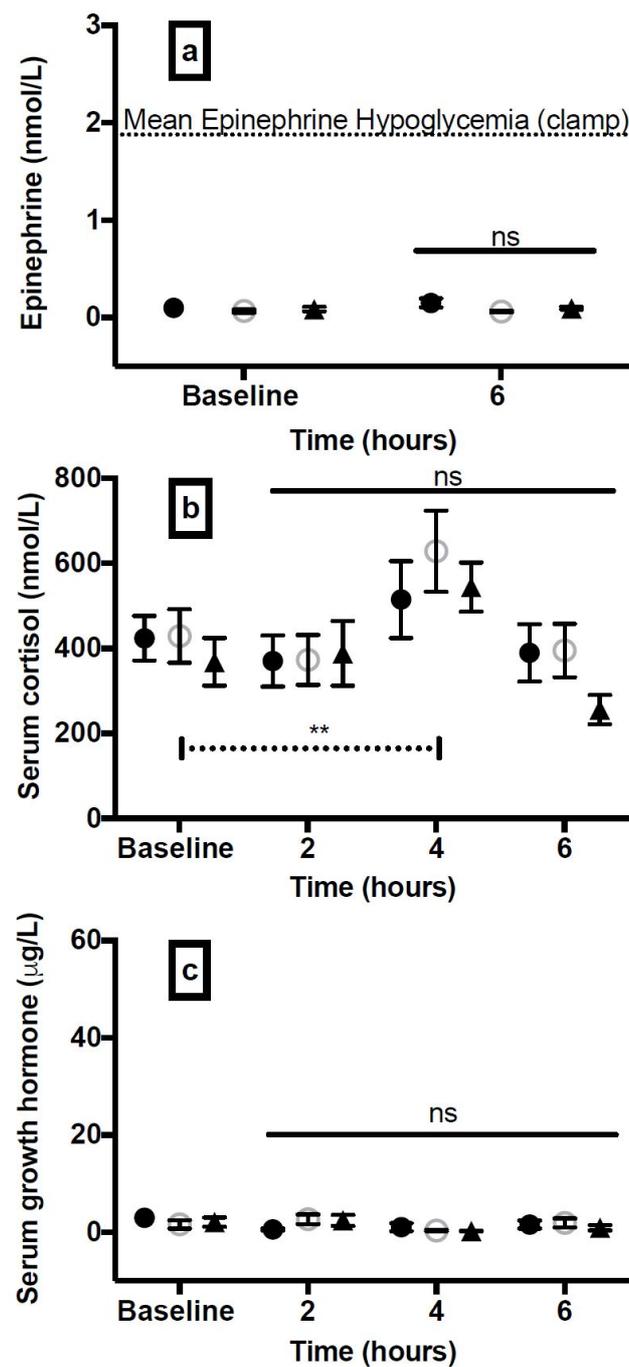


Figure 3-6 Changes in epinephrine (adrenaline), cortisol and growth hormone response post endotoxin challenge. Epinephrine (a), cortisol (b) and growth hormone (c) responses 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycaemia, euglycaemia or a sham-saline clamp 48 hours earlier. Data are mean (SEM), ** $p < 0.01$, ns-non-significant, p-value on dashed line in (b) represents change in cortisol at 4 hours compared to baseline in all groups, solid horizontal lines represent significance for comparison between study groups. Dashed line in (a) illustrates the mean epinephrine response in hypoglycaemia clamp subjects. Black circles-hypoglycaemia group; open circles-euglycaemia group; black triangles-sham-saline group.

Total and differential leukocyte count

I observed that antecedent hypoglycaemia modulated the subsequent WBC response to endotoxin. Total number of WBCs increased significantly following endotoxin in all the study groups (Figure 3.7 A). The peak WBC response occurred at 4 hours post endotoxin and this was significantly higher in the hypoglycaemia group at $10.96 \pm 0.97 \times 10^9/l$ vs. $8.21 \pm 0.85 \times 10^9/l$ in the euglycaemia group ($p = 0.012$) (Figure 3.7 A). Total WBC count 4 hours post endotoxin in the sham-saline group was $10.65 \pm 0.64 \times 10^9/l$ and this was significantly higher compared to euglycaemia ($p = 0.033$), but not hypoglycaemia ($p = 0.974$). The rise in WBCs was mainly a consequence of an increase in neutrophil count (Figure 3.7 B). The lymphocyte count decreased following endotoxin (Figure 3.7 C) and the monocyte count initially decreased prior to recovery 6 hours post endotoxin (Figure 3.8 A). There was a trend towards a higher total monocyte count in the hypoglycaemia group 4 hours post endotoxin compared to euglycaemia but this comparison did not reach statistical significance ($p = 0.085$). The absolute number of circulating monocyte subsets did not differ significantly between study groups (Fig. 3.8 C-D). NCM and IM numbers decreased significantly following endotoxin compared to baseline values in all groups ($p < 0.001$) (Figure 3.8 B&C). Compared to baseline, CM numbers significantly declined at 2 hours ($p < 0.001$), prior to rising and reaching a peak at 6 hours ($p < 0.001$) (Figure 3.8 D).

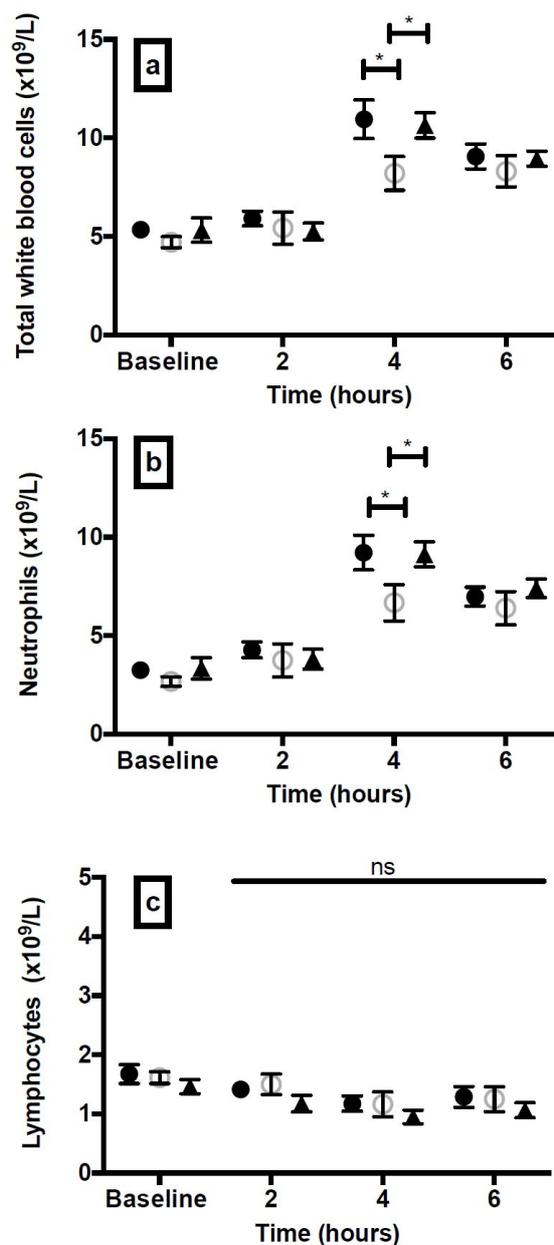


Figure 3-7 Peripheral total white blood cell, neutrophil and lymphocyte kinetics post endotoxin challenge. Number of circulating total WBCs (a), neutrophils (b) and lymphocytes (c) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycaemia, euglycaemia or a sham-saline clamp 48 hours earlier. Data are mean (SEM), *p<0.05, ns-non-significant, p-values are provided for comparison between study groups, solid horizontal line in (c) represents significance for comparison between study groups. Black circles-hypoglycaemia group; open circles-euglycaemia group; black triangles-sham-saline group.

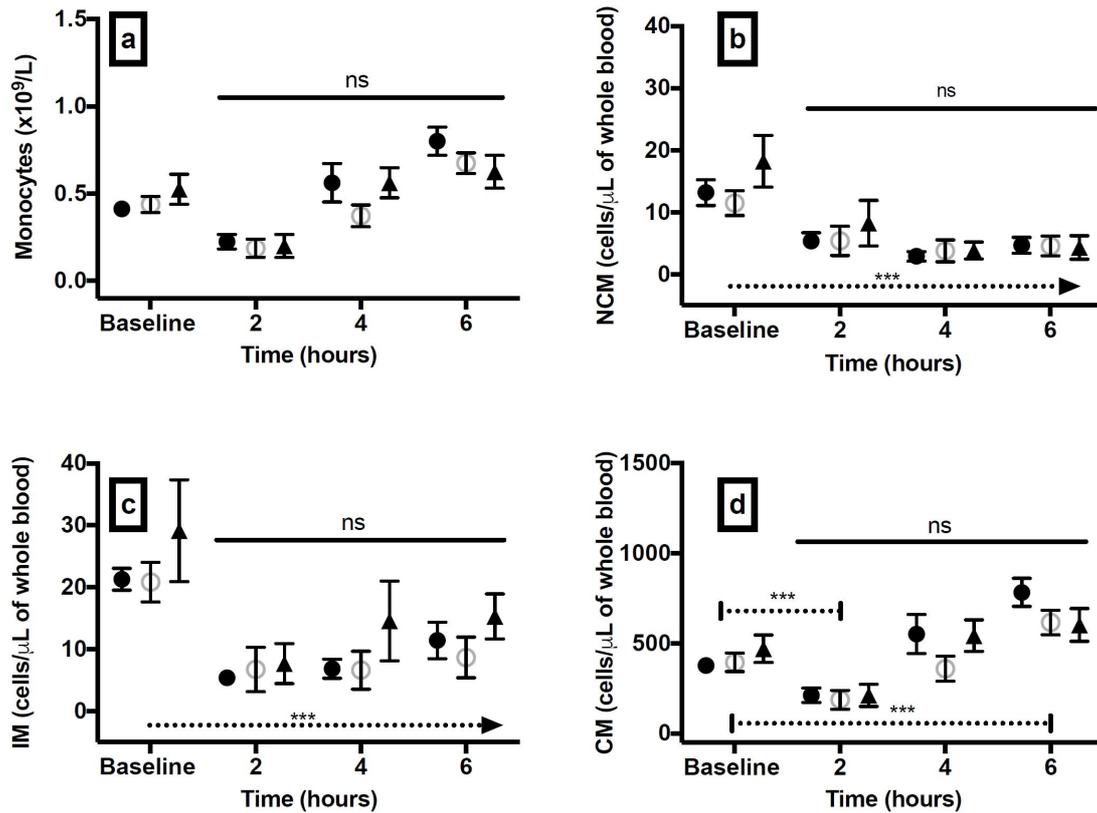


Figure 3-8 Total monocyte count and monocyte subset kinetics post endotoxin challenge. Absolute circulating numbers of total monocytes (a) and monocyte subsets comprising of NCMs (b), IMs (c) and CMs (d) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycaemia, euglycaemia or a sham-saline clamp 48 hours earlier. Data are mean (SEM), * $p < 0.001$, ns-non-significant, p-value on dashed line in (b) represents change in number of NCMs at 2, 4 and 6 hours compared to baseline in all study groups. p-value on dashed line in (c) represents change in number of IM at 2, 4 and 6 hours compared to baseline in all study groups. P-value on dashed lines in (d) represent change in number of CMs at 2 and 6 hours compared to baseline in all study groups. Solid horizontal lines represent significance for comparison between study groups. Black circles-hypoglycaemia group; open circles-euglycaemia group; black triangles-sham-saline group. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical monocytes.**

Cell surface markers

I examined monocyte activation following endotoxin exposure by measurement of cell surface marker CX₃CR1 expression. Endotoxin administration caused a significant decline in expression of this marker across all study groups compared to baseline ($p < 0.001$) (Figure 3.9 A). This was accompanied by an increase in the concentration of CX₃C chemokine ligand 1 (CX₃CL1) in plasma at 4 and 6 hours compared to baseline in all groups ($p < 0.001$) (Figure 3.9 E). Activation of monocytes was also revealed by increased expression of CD11b expression at 4 and 6 hours following endotoxin compared to baseline in all groups ($p < 0.001$) (Figure 3.10 A). In addition, the percentage of total monocytes that were positive for CD11b expression was higher in hypoglycaemia group versus euglycaemia group at 2 hours post endotoxin ($p = 0.007$) (Figure 3.10 B).

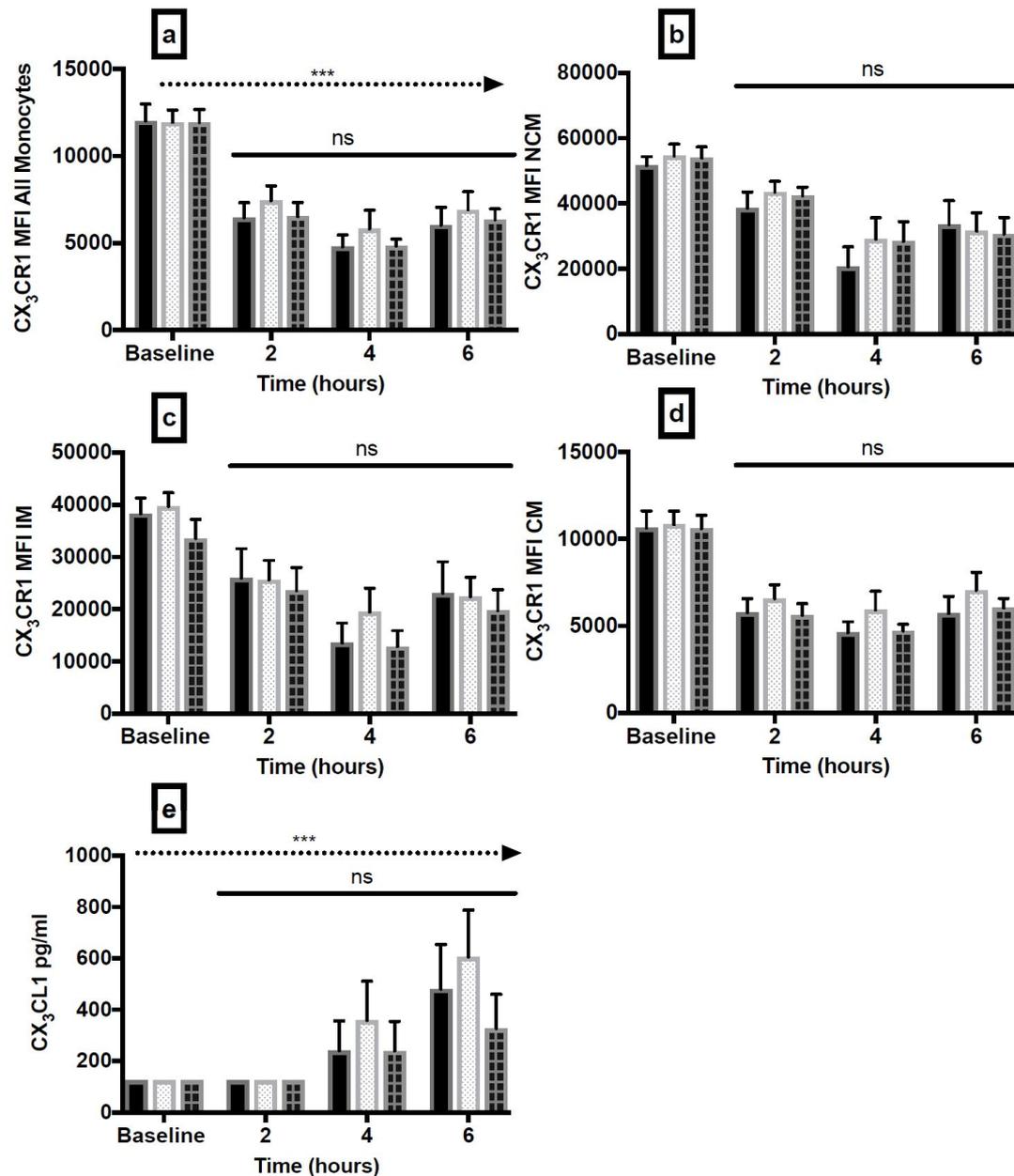


Figure 3-9 Expression of CX₃CR1 on monocytes and plasma levels of CX₃CL1 post endotoxin challenge. CX₃CR1 expression on total monocytes (a) and monocyte subsets comprising of NCMs (b), IMs (c) and CMs (d) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycaemia, euglycaemia or a sham-saline clamp 48 hours earlier. CX₃CL1 values determined in plasma are shown in (e). Data are geometric mean (SEM), *p<0.001, ns-non-significant, p-values on dashed line in (a) and (e) represent changes in CX₃CR1 on all monocytes and plasma CX₃CL1 expression respectively at 2, 4 and 6 hours compared to baseline in all study groups. Solid horizontal lines represent significance for comparison between study groups. Black bars-hypoglycaemia group (n=8); white bars-euglycaemia group (n=8); striped black bars-sham-saline group (n=8). NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical monocytes; MFI, mean fluorescence intensity.**

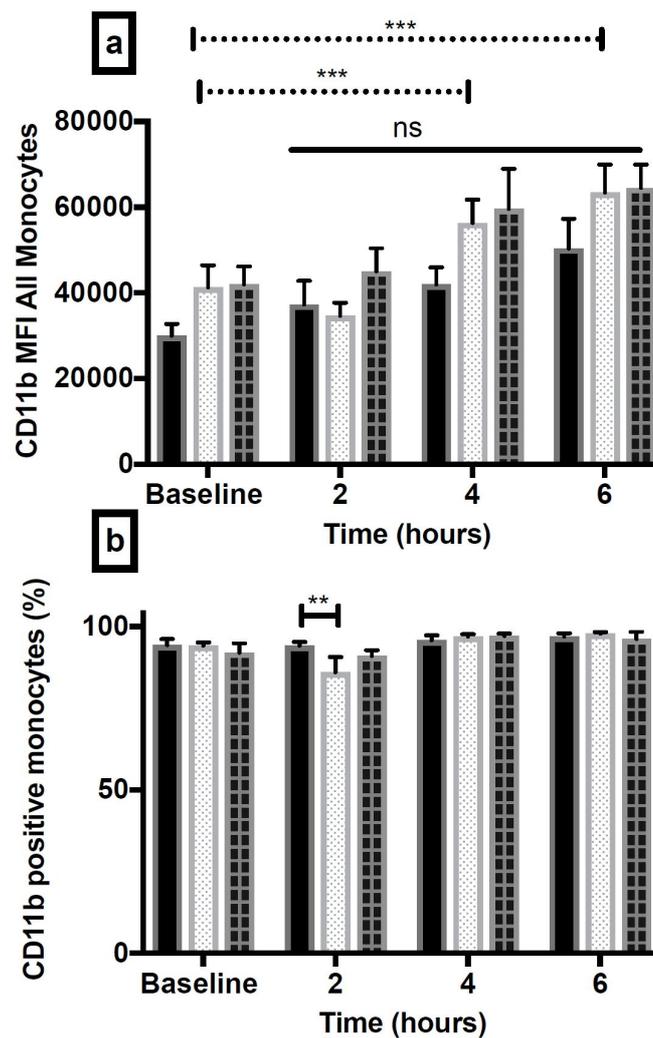


Figure 3-10 Expression of CD11b on monocytes and percentage of CD11b positive monocytes post endotoxin challenge. CD11b expression on total monocytes (a) and percentage (%) of monocytes that positively express CD11b (b) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycaemia, euglycaemia or a sham-saline clamp 48 hours earlier. Data are geometric mean (SEM) in (a) and mean (SEM) in (b), ** $p < 0.01$, *** $p < 0.001$, ns-non-significant, dashed lines in (a) represents change in CD11b expression on all monocytes at 4 and 6 hours compared to baseline in all study groups. P-value in (b) is for comparison of CD11b percentage expression between hypoglycaemia and euglycaemia. Solid horizontal line in (a) represents significance for comparison between study groups. Black bars-hypoglycaemia group (n=8); white bars-euglycaemia group (n=8); striped black bars-sham-saline group (n=8). MFI, mean fluorescence intensity.

3.4 Discussion

Episodes of hypoglycaemia may increase both the rate of progression of atherosclerosis and increase the risk of thrombosis of the unstable plaque. I aimed to investigate the effect of acute experimental hypoglycaemia and subsequent low-dose endotoxaemia on aspects of the innate immune response (total leukocytes, leukocyte subsets and specifically monocyte subsets), thrombosis (platelet aggregation) and cross-talk between inflammation and thrombosis (monocyte-platelet aggregates). My main findings were: (1) hypoglycaemia increased the number of all three circulating monocyte subsets, in association with a stress response characterised by increased plasma epinephrine levels; (2) hypoglycaemia increased platelet reactivity, promoted formation of MPAs and promoted aggregate formation between pro-inflammatory monocytes and platelets; (3) leukocyte mobilisation to the stress response of low-dose endotoxin was independent of epinephrine, and antecedent hypoglycaemia resulted in a significantly higher inflammatory leukocyte response to low-dose endotoxin administered 48 hours later.

As shown previously (Razavi Nematollahi *et al.*, 2009; Ratter *et al.*, 2017), I confirm that hypoglycaemia results in leukocytosis. In addition, I present, for the first time, the effect of hypoglycaemia on monocyte subset kinetics and demonstrate an increase in the absolute number of all three circulating monocyte subsets. The largest increase was observed in numbers of circulating NCMs (2-fold) and IMs (1.8-fold) with a modest increase in the number of CMs (1.3-fold). These data are in keeping with an observed selective mobilisation of CD16⁺ monocytes in response to exercise (B Steppich *et al.*, 2000; Hong and

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Mills, 2008) and epinephrine infusion (Dimitrov, Lange and Born, 2010). Ratter *et al.* also recently determined that hypoglycaemia might modify selective monocyte mobilisation (Ratter *et al.*, 2017). However, they did not phenotype monocyte subsets but rather measured total levels of CD16 on peripheral blood mononuclear cells isolated from both healthy participants and those with T1D in experimental hypoglycaemia settings. Our data identify specific changes in monocyte subsets that have been previously linked to monocyte activation and atherogenesis. As observational data support the notion of CD16⁺ monocytes being pro-atherogenic (Kashiwagi *et al.*, 2010; Rogacev *et al.*, 2012; Tapp *et al.*, 2012; Zhou *et al.*, 2016), and adrenergic modulation of monocytes induces pro-inflammatory changes (Sarigianni *et al.*, 2011), an increase in the circulating number of these cells following hypoglycaemia may enhance CV risk in diabetes.

Previous studies investigating effects of hypoglycaemia on platelet biology have suggested an increase in platelet reactivity; however, this was in the context of significant hypoglycaemic stimulus as part of an insulin stress test (Hutton *et al.*, 1979). An older investigation into the effect of hypoglycaemia on monocyte-platelet interactions in T1D and healthy controls has also suggested a trend towards increased MPA formation, but these data were not conclusive with little difference between euglycaemic and hypoglycaemic conditions (Wright *et al.*, 2010). My study also recapitulates and extends previous findings that hypoglycaemia is prothrombotic, as evidenced by an increased platelet count and increased platelet reactivity to ADP (Trovati *et al.*, 1986). I have now conclusively demonstrated an overall increase in formation of MPAs in hypoglycaemia in comparison to euglycaemia. Furthermore, I provide novel data

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demonstrating MPA formation within monocyte subsets in experimental hypoglycaemia. MPA formation is a highly sensitive marker of both monocyte and platelet activation (Michelson *et al.*, 2001; Wrigley *et al.*, 2013). MPA formation promotes monocyte release of the pro-inflammatory cytokines; TNF- α , C-X-C motif chemokine ligand 8 (CXCL8) and C-C motif chemokine ligand 2 (CCL2) (Neumann *et al.*, 1997; Bournazos *et al.*, 2008) and increases adhesive properties of monocytes (Martins *et al.*, 2006), thereby representing a bridge between inflammation and thrombosis, that may serve to increase CV risk. In acute coronary syndromes, MPA formation correlates with troponin elevation, risk of in-hospital cardiac events including death and risk of future cardiac events (Zhang *et al.*, 2007; Tapp *et al.*, 2012). I have also shown that NCMs and IMs aggregate more readily with platelets in response to hypoglycaemia compared to CMs. A similar observation of proportionally higher IM-MPA and NCM-MPA formation has been reported in patients following an ST-elevation myocardial infarction (STEMI) with higher IM-MPAs in particular being a poor prognostic indicator at 6 weeks following STEMI (Tapp *et al.*, 2012). Thus, my data suggest that hypoglycaemia not only increased circulating numbers of CD16⁺ monocytes, but also promoted increased interaction between these pro-inflammatory monocyte subsets and platelets.

In a first model of its type, I wished to determine whether antecedent hypoglycaemia modulated responses to low-dose endotoxin. I chose a low-dose endotoxin model firstly because I felt it the safest way to combine the clamp and endotoxin human models, secondly because future extension to the study of people with diabetes would be more feasible with this model, and finally because

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people with diabetes are often exposed to chronic low-grade infections through foot ulceration and periodontitis which might further increase the risk of CV mortality (Brownrigg *et al.*, 2012; P. M. Preshaw *et al.*, 2012). In my model, I observed in all groups that monocytes were activated even in response to low-dose endotoxin, as indicated by upregulation of systemic levels of the CX₃CR1 ligand CX₃CL1, and the upregulation of the adhesion molecule CD11b on the monocytes themselves. Interestingly, compared to euglycaemia, hypoglycaemia resulted in greater leukocyte mobilisation in response to low-dose intravenous endotoxaemia 48 hours later. Furthermore, I noted a non-significant trend towards a higher total monocyte count in the hypoglycaemia group 4 hours post endotoxin compared to euglycaemia. The percentage of monocytes that were CD11b positive was also higher in hypoglycaemia group compared to euglycaemia group at 2 hours post endotoxin. Levels of leukocyte mobilisation were similar between groups who received prior sham-saline or hypoglycaemia. These data suggest that euglycaemia with insulin suppressed leukocyte mobilisation in response to endotoxin 48 hours later, consistent with the known anti-inflammatory actions of insulin (Aljada and Dandona, 2000; Dandona *et al.*, 2010) and that the physiological stress of hypoglycaemia overcame this insulin-mediated suppression of inflammatory responses. My data show that drivers for differential leukocyte mobilisation to endotoxin are unlikely to be due to differences between groups in epinephrine, cortisol and growth hormone levels post endotoxin, as these were not significantly different. My observation that a single episode of hypoglycaemia compared to euglycaemia invokes a stronger pro-inflammatory response to endotoxin up to 2 days later is of potential clinical

relevance given that trial data suggest downstream mortality following hypoglycaemia (Duckworth *et al.*, 2009).

The strengths of my study include use of a novel human experimental model and detailed flow cytometric analysis that allowed me to comprehensively describe immune cell kinetics and activation status in response to experimental hypoglycaemia and endotoxin challenge *in vivo*. The separation of clamp and endotoxin studies by 48 hours allowed me to probe the longitudinal effects of hypoglycaemia on innate immunity. Moreover, by using a sham-saline group, I specifically controlled for the immunological effects of insulin, thereby robustly investigating pro-inflammatory changes in response to hypoglycaemia.

One limitation was my decision to study a relatively small number of young healthy participants. This limits the applicability of my findings to older patients with diabetes, established CV risk factors and atherosclerosis. For ethical and safety reasons, I decided to examine my novel experimental model initially in healthy participants. I also specifically adopted a low-dose endotoxin model with future translatability in older, higher-risk participants in mind. Future studies should therefore confirm my findings in those with diabetes. In addition, it is worth noting that I studied cell numbers, phenotypic changes and activation status in circulating immune cells and this may not necessarily reflect the functional capacity of these cells in an atherosclerotic plaque. An animal model of combined experimental hypoglycaemia and atherosclerosis would help to resolve these questions, which I subsequently developed and tested as described in chapters 4 and 5.

In conclusion, hypoglycaemia mobilised pro-atherogenic monocyte subsets and induced prothrombotic changes by increasing platelet reactivity. In addition, hypoglycaemia amplified interactions between platelets and monocytes by promoting MPA formation with enhanced aggregation of pro-inflammatory monocytes with platelets. Hypoglycaemia may also prime the innate immune system to respond more robustly to stimuli such as endotoxin. This implies pro-inflammatory consequences of hypoglycaemia beyond the acute episode. These data provide novel mechanistic insights into how hypoglycaemia could increase CV risk through upregulation of inflammatory responses.

Chapter 4- Development of a murine model of experimental hypoglycaemia in pre-established atherosclerosis

4.1 Introduction

Evidence from clinical trial data in T2D suggest that aiming for tighter glycaemic control and reducing HbA1c does not reduce CVD (Gerstein *et al.*, 2008; Patel *et al.*, 2008; Duckworth *et al.*, 2009). Further, there is accumulating evidence associating hypoglycaemia with all-cause mortality and an increased incidence of CV events (Khunti *et al.*, 2015) which cannot be explained by confounding factors alone (Goto *et al.*, 2013). Moreover, analyses from clinical trials show that potentially deleterious effects of hypoglycaemia may persist for weeks downstream from the episodes (Duckworth *et al.*, 2009; Pieber *et al.*, 2018).

In exploring a possible relationship between hypoglycaemia and CV events, retrospective analyses of clinical trials is problematic, as these studies were not designed to test this relationship a priori. However, in a number of observational human experimental studies, hypoglycaemia has been shown to exert effects on inflammation and thrombosis that could serve to increase CV risk through pro-thrombotic (Hutton *et al.*, 1979; Wright *et al.*, 2010; Chow *et al.*, 2018) and pro-inflammatory effects consequent upon acute leucocytosis (Razavi Nematollahi *et al.*, 2009; Ratter *et al.*, 2017) in addition to increased levels of pro-inflammatory cytokines and cell adhesion molecules (Gogitidze Joy *et al.*, 2010). These studies, whilst lending biological plausibility to relationship between hypoglycaemia and CV events are limited as they studied relatively small number of young participants with and without diabetes. Furthermore, whilst informative, these

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studies measured circulating mediators of inflammation and changes in immune cell function that are not necessarily indicative of pathology within CV tissue.

In order to examine cause and effect, therefore, the direct consequences of hypoglycaemia on the atherosclerotic plaque need to be studied over a number of weeks. This is, however, challenging and ethically questionable in humans in light of clinical trial data, particularly as intensive glycaemic control appears to confer a greater risk in those with a pre-existing high CV burden (Reaven *et al.*, 2009). Thus, an animal model was needed to robustly test this relationship and to explore causality. Animal experimentation in non-obese diabetic Goto-Kakizaki rats had suggested that repetitive hypoglycaemia resulted in increased sympathetic activity with resulting induction of monocyte adhesion to endothelial cells in the aorta (Jin *et al.*, 2011) and augmented intimal thickening and smooth muscle proliferation following vascular injury in the carotid artery (Yasunari *et al.*, 2014). At the time of my study, however, no robust animal model of recurrent hypoglycaemia and atherosclerosis existed.

A murine model of ApoE ^{-/-} mice was chosen as a model of atherosclerosis. ApoE ^{-/-} mice have several practical advantages over other species including: relative ease of breeding, low cost of maintenance and reliable development of atherosclerosis over short periods of time (Getz and Reardon, 2012). ApoE is essential for catabolism of triglyceride rich lipoproteins (Schaefer *et al.*, 1986). In humans, a familial form of ApoE deficiency results in defective binding to the LDL receptor and results in the clinical phenotype of type III hyperlipoproteinaemia which is characterised by severe hypercholesterolaemia,

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premature atherosclerosis and CV events (Schaefer *et al.*, 1986). ApoE^{-/-} mice develop plaques which are morphologically similar to those found in humans and thus have a fibrous cap containing smooth muscle cells supported by a connective tissue matrix that encapsulates a necrotic core containing foamy macrophages (Piedrahita *et al.*, 1992; Nakashima *et al.*, 1994). ApoE^{-/-} mice spontaneously develop advanced atherosclerotic lesions by 20 weeks of age even when fed a chow diet (Nakashima *et al.*, 1994). Feeding these pre-disposed animals a highly atherogenic, high fat content Western diet accelerates the process through rapid induction of severe hypercholesterolaemia with advanced lesions forming within 6-8 weeks of feeding (Nakashima *et al.*, 1994).

In this chapter I describe the development of a novel murine model of hypoglycaemia on the background of pre-established atherosclerosis. Data from clinical studies suggest that an episode of severe hypoglycaemia confers durable effects. Furthermore, patients rarely suffer just one episode of severe hypoglycaemia in clinical practice. The aim of these experiments, therefore, was to develop a robust model of recurrent hypoglycaemia of significant depth to invoke physiological counter-regulatory responses yet high to ensure survivability to allow experimentation over an extended period.

4.2 Methods

Standard methodological details for animal experimentation have been provided in chapter 2. Here, I will detail specific methods employed that differed whilst establishing the model.

4.2.1 Experimental hypoglycaemia in chow fed male and female ApoE^{-/-} mice

ApoE^{-/-} mice: JAX B6.129P2-ApoE^{tm1Unc/J} were purchased from Charles River laboratories (Wilmington, Massachusetts, USA) aged approximately at 12-13 weeks. Mice were left undisturbed in the animal facility for a period of at least 1 week prior to experimentation and were fed a standard chow diet. Collaborators (Prof Rory McCrimmon, University of Dundee) suggested an initial dose of 0.75 mU/g of Actrapid insulin (Human Actrapid, Novo Nordisk Pharmaceuticals Ltd, Crawley, West Sussex, UK) would be a safe starting point for mice with a C57BL/6J background. Animals were fasted for 3 hours prior to i.p. injection of insulin and tail vein blood glucose was obtained at baseline and regularly thereafter for at least 90 minutes, later extended to 135 minutes and then 150 minutes. Following initial experiments in a male mouse, the dose of insulin was sequentially increased in subsequent animals giving a dose range of 0.75 mU/g, 0.825 mU/g (10% increase from base dose) and 1.00 mU/g (33.5% increase from base dose).

4.2.2 Insulin dose finding in Western diet fed male ApoE^{-/-} mice

Male ApoE^{-/-} mice were purchased from Charles River laboratories (Wilmington, Massachusetts, USA) aged approximately at 6-7 weeks. Following 1 week of acclimatisation, mice were fed 5g/day/animal of Western diet (ABdiets, Woerden, The Netherlands) for 6 weeks and hence were aged between 12-14 weeks when hypoglycaemia was induced. Anticipating higher insulin resistance given a higher total body weight and based on experience from non-fed animals, an initial insulin dose of 1.5 mU/g was selected. Mice were fasted for 3 hours prior to i.p. insulin. To promote animal welfare, the dose of insulin was increased or decreased following initial results from the first mouse undergoing the protocol.

4.2.3 Development of a model of recurrent hypoglycaemia in Western diet fed ApoE^{-/-} mice

Male ApoE^{-/-} mice were purchased from Charles River laboratories (Wilmington, Massachusetts, USA) aged 9-10 weeks and following 1 week of acclimatisation fed 5g/day/animal Western diet (ABdiets, Woerden, The Netherlands) for 7 weeks. Mice were randomly allocated at the start of the feeding period to one of two groups:

- Group 1: experimental hypoglycaemia n=8
- Group 2: sham saline controls n=8

The experiment lasted 3 consecutive weeks. Two experimental weeks (weeks 1 and 3) were separated by a rest week (week 2). Mice in each group underwent

either hypoglycaemia or sham-saline injection on an alternate day basis. Thus, each animal underwent 4 episodes of insulin induced hypoglycaemia or sham-saline injection over the experimental period prior to sacrifice. Figure 4.1 is a schematic representation of experimental design.

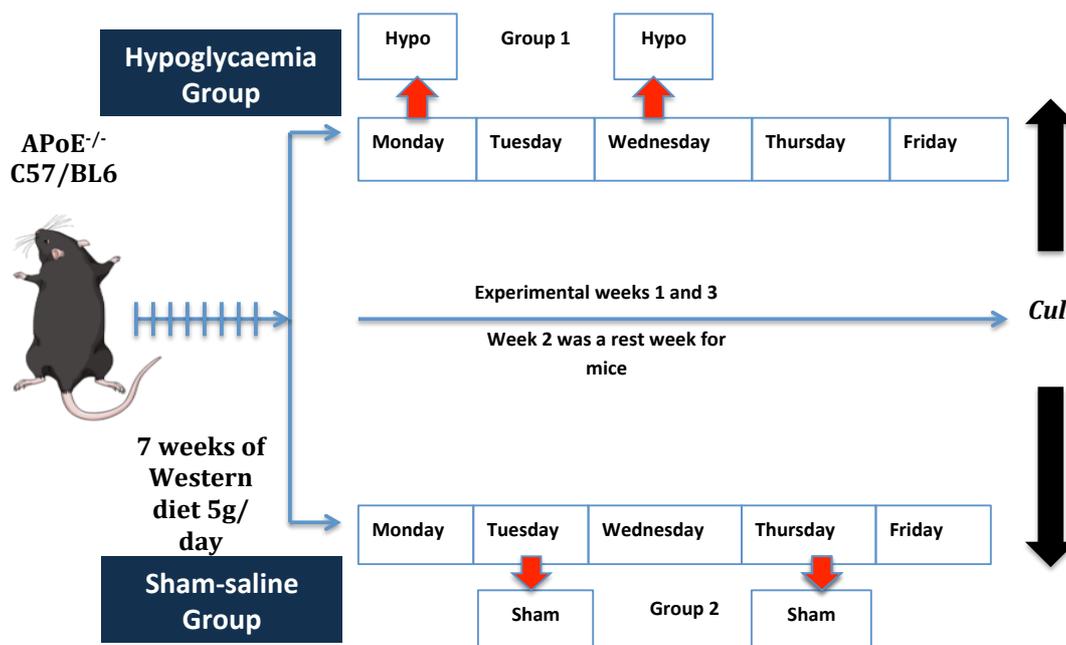


Figure 4-1 Experimental design used to develop a protocol for recurrent hypoglycaemia vs. sham-saline injection. ApoE^{-/-} mice fed Western diet for 7 weeks were allocated to either a receive hypoglycaemia (group 1) or a sham-saline (group 2). Group 1 mice underwent hypoglycaemia on Mondays and Wednesdays. Group 2 mice received a saline control injection on Tuesdays and Thursdays. The experiment was conducted over 3 consecutive weeks. Mice from each group were rested on respective non-experimental days and week 2 which was designated a rest week.

All animals were fasted for 3 hours before i.p. insulin. Due to heterogeneity between mice in the dose-response relationship of i.p. insulin injection and tail vein blood glucose values, an insulin dose of 1.3 mU/g was selected as a starting point for safe induction of hypoglycaemia in Western diet fed mice. A final insulin dose range of 1.3 mU/g to 2.2 mU/g was used. Control animals were

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handled identically but instead of insulin received a fixed volume (200 μ l) injection of 0.9% v/v sodium chloride.

Adrenaline was measured at 0 minutes and 60 minutes following i.p. insulin injection on the 3rd episode of hypoglycaemia or sham-saline injection (week 3). Serum lipids were measured by the Cobas analyser at sacrifice and whole aortae were harvested and stained with oil red O as described in chapter 2.

4.3 Results

4.3.1 Experimental hypoglycaemia in chow fed male and female ApoE^{-/-} mice

Baseline data from this first experiment indicated that chow fed ApoE^{-/-} mice (JAX B6.129P2-ApoE^{tm1Unc/J}) had a mean body weight at age 12-13 weeks of age of 21.4 grams (Table 4.1).

Table 4.1 Weight of chow diet fed ApoE^{-/-} male and female mice at 12-13 weeks of age.

Mouse number	Sex	Body weight (grams)
1	Male	25.0
2	Male	20.8
3	Female	19.5
4	Female	20.3

Mouse 1 underwent induction of hypoglycaemia first with an i.p. injection of 0.75 mU/g (absolute insulin dose = 18.75 mU). The baseline blood glucose was 13.1 mmol/l and a glucose nadir of 4.7 mmol/l was reached at 90 minutes (Figure 4.2 A). Mouse 1 died within 24 hours of the protocol. This prompted a reduction of the fasting period to 2 hours, an extension of blood glucose monitoring to 135 minutes and no dose escalation of insulin for mouse 2. Mouse 2 had a fasting

blood glucose of 14.4 mmol/l, achieved a blood glucose nadir of 5.1 mmol/L 60 minutes following i.p. insulin and survived the protocol (Figure 4.2 B). Mice 3 and 4 were females and received 0.85mU/g and 1mU/g i.p. insulin respectively. The blood glucose response to insulin was, however, not consistent in female mice (Figure 4.2 C&D).

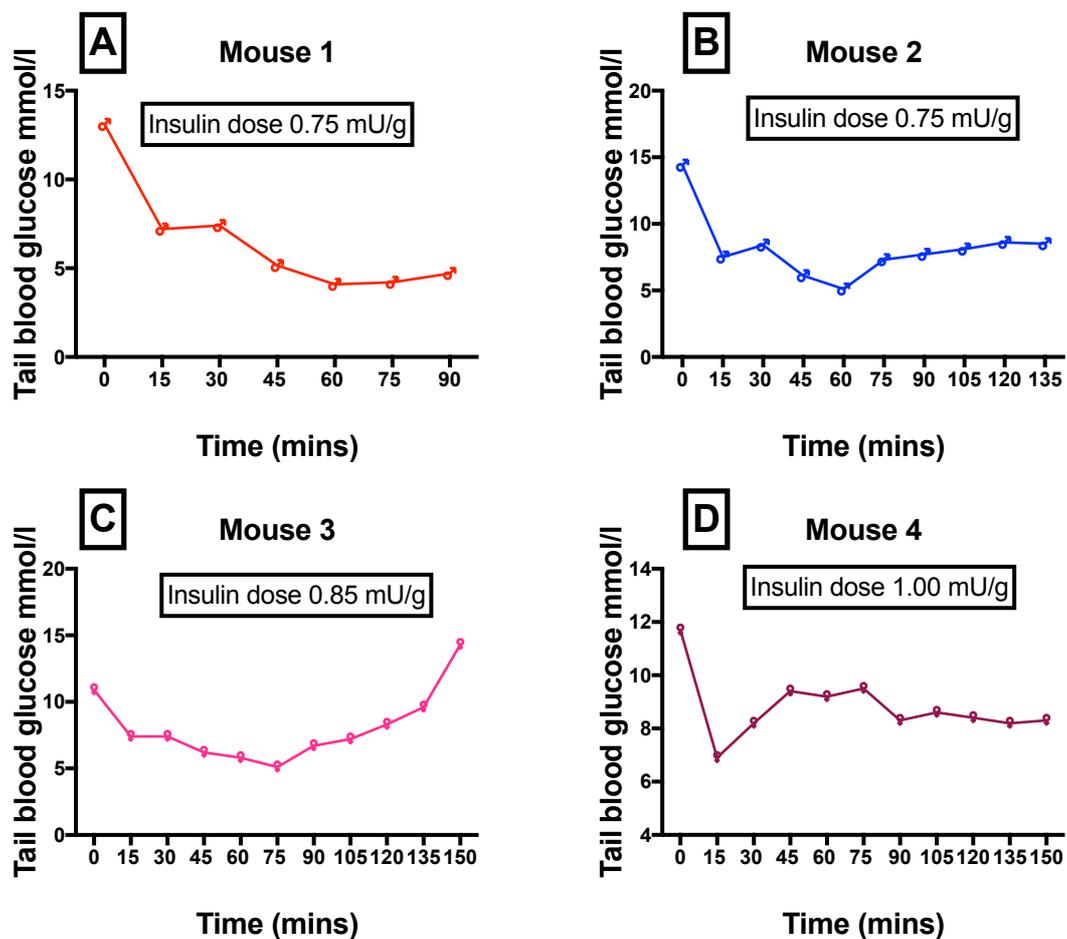


Figure 4-2 Blood glucose profile following i.p. insulin injection in male and female ApoE^{-/-} mice on a chow diet (n=4). A&B) Glucose profile in male mice. Mouse 1 did not survive the protocol. C) Glucose profile in a female mouse with a dose escalation to 0.85 mU/g. D) Failure to achieve a glucose nadir at between 60-90 minutes despite insulin dose escalation to 1.00 mU/g in a female mouse.

4.3.2 Insulin dose finding in Western diet fed male ApoE^{-/-} mice

Having established a local protocol for insulin induced hypoglycaemia in ApoE^{-/-} mice in 4.3.1, I next conducted experiments on Western diet fed mice to: a) establish an insulin dose range in fed mice and b) test the severity and survivability of hypoglycaemia in fed animals. Given the variability observed in glucose response to insulin in female mice, I decided to take experiments forward in male mice.

Western diet fed ApoE^{-/-} mice had a mean body weight of 27.9 ± 2.3 grams following 6 weeks of feeding with a mean fasting glucose of 11.6 ± 0.7 mmol/l.

The first mouse received 1.5 mU/g of i.p. insulin (absolute insulin dose = 44.55 mU) displayed in red in Figure 4.3. The baseline blood glucose was 12.8 mmol/l and a glucose nadir of 2.6 mmol/l was reached at 60 minutes. Tail blood glucose, however, continued to fall and the animal developed severe hypoglycaemia with a complete lack of response and activity. Rescue i.p. injections of 20% Dextrose were administered but the mouse remained unresponsive and the last measured blood glucose was 1.2 mmol/l at 120 minutes (Figure 4.3). This mouse was sacrificed using an overdose of sodium pentobarbital as per schedule 1. In light of this, the remaining three mice had a reduced dose of insulin at 1.3 mU/g and had mash chow diet made available in the cage once the hypoglycaemia nadir had been reached. The three remaining animals survived the experiment. Two out of three mice were administered 150 μ l i.p. injection of 20% dextrose to aid recovery from hypoglycaemia, upon reaching blood glucose values below 2.0 mmol/l as past experience in the first animal suggested vulnerability below this

threshold. The mean blood glucose nadir was 1.7 mmol/l between 45-60 minutes following i.p. insulin (Figure 4.3).

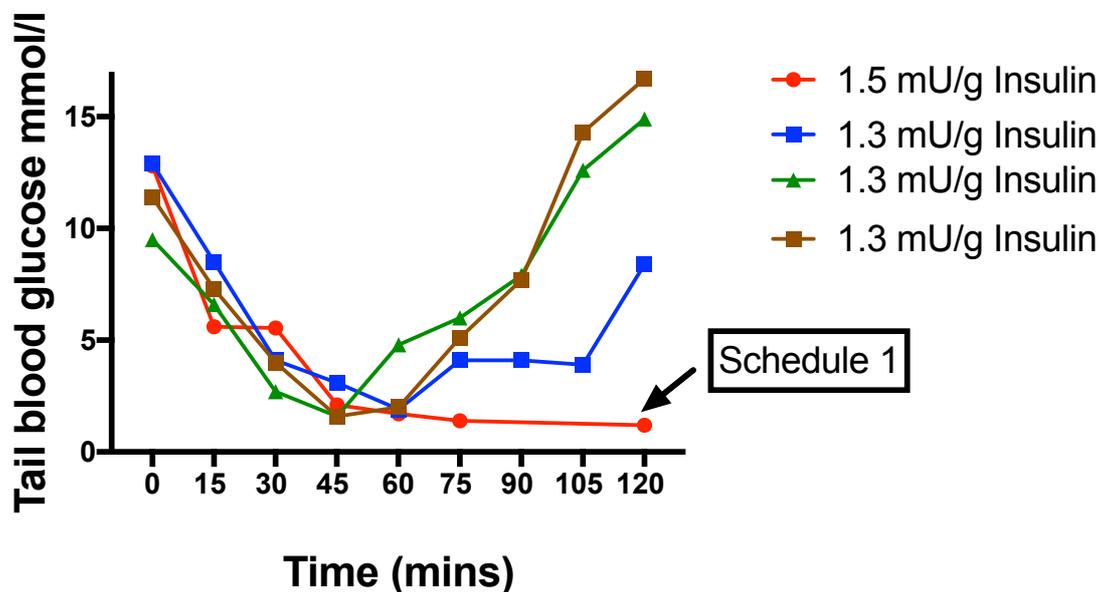


Figure 4-3 Blood glucose profile following i.p. insulin injection in Western diet fed male ApoE^{-/-} mice (n=4). A dose of 1.5 mU/g resulted in the loss of a mouse to irrecoverable hypoglycaemia (red dots). Subsequently, hypoglycaemia was successfully induced in three mice at a reduced insulin dose of 1.3 mU/g (blue squares, green triangles and brown squares). Two of these mice (green triangles and brown squares) were administered 150 μ l of 20% Dextrose i.p. to aid recovery from hypoglycaemia at 60 minutes. One of the mice (blue squares) was fed mash chow diet only at 60 minutes.

4.3.3 Development of a model of recurrent hypoglycaemia in Western diet fed ApoE^{-/-} mice

Following insulin dose finding experiments in fed mice, I then developed a model of recurrent hypoglycaemia in ApoE^{-/-} mice fed a Western diet. The aims of this experiment were to: a) establish a protocol for recurrent hypoglycaemia over an extended period, b) demonstrate biochemical validity of the model by measurement of plasma adrenaline and serum lipids c), explore atherosclerotic disease burden at one anatomical site in fed mice in order to compare

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differences between hypoglycaemia and control conditions. The experiment was also designed to incorporate sequential dose increments or decrements of insulin as part of a dose range based on glucose data obtained following the first episode of hypoglycaemia thus allowing titration of insulin doses on subsequent experimental hypoglycaemia days. Also, as rodents have been shown to develop defective counter-regulatory responses to recurrent insulin induced hypoglycaemia (McNeilly *et al.*, 2017) an insulin dose range provided the flexibility, if required, to down-titrate insulin on subsequent experimental days to ensure maximal survivability. No mouse was lost to hypoglycaemia during this experiment.

Body weight

Baseline body weight prior to feeding was 24.0 ± 0.6 grams in mice allocated to the hypoglycaemia group vs. 25.2 ± 0.3 grams in mice allocated in to the sham-saline group ($p = 0.750$). Body weight at the end of the feeding period was 31.5 ± 0.6 grams in the hypoglycaemia group vs. 32.1 ± 0.6 grams in the sham-saline group ($p = 0.997$). Mice in both groups displayed a positive weight trajectory during the feeding period (Figure 4.4 A). There was no between group difference in body weight during the experimental days and immediately pre-sacrifice ($p = 0.999$) (Figure 4.4 B).

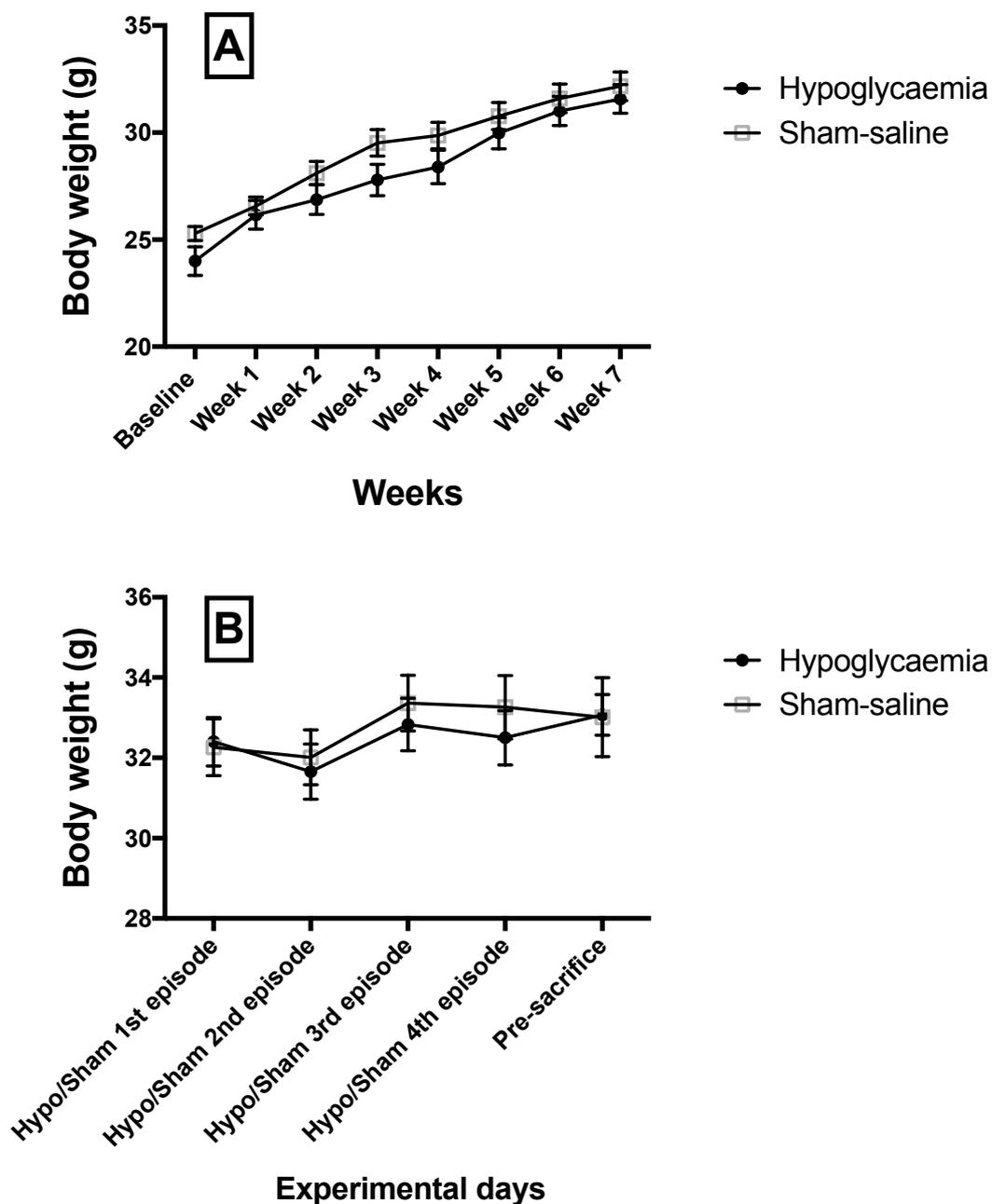


Figure 4-4 Body weight in Western diet fed male ApoE^{-/-} mice. **A)** Mice in the hypoglycaemia (n=8) and sham-saline group (n=8) steadily gained weight through the feeding period with no significant difference at the end of feeding. **B)** During recurrent hypoglycaemia or sham-saline injections, there was fluctuation in weight but no between group differences. Data are mean \pm SEM. Hypo = hypoglycaemia, sham = sham-saline group. Two-way ANOVA.

Serum lipids

Serum lipids were biochemically analysed for mice in the hypoglycaemia and sham-saline group at sacrifice. All lipid parameters were elevated in mice on Western diet for 7 weeks. There were, however, no significant differences between experimental groups (Table 4.2).

Table 4.2 Serum lipids in ApoE^{-/-} mice fed a Western diet for 7 weeks prior to exposure to recurrent hypoglycaemia or sham-saline injection. Data are mean \pm SEM. p values are for between group comparisons.

Serum lipids (mmol/l)	ApoE ^{-/-} Hypoglycaemia n=8	ApoE ^{-/-} Sham-saline n=8	p values
Total cholesterol	29.8 \pm 1.3	27.3 \pm 3.2	0.534
HDL cholesterol	7.9 \pm 1.1	5.9 \pm 0.8	0.178
LDL cholesterol	21.2 \pm 1.5	20.5 \pm 2.6	0.854
Triglycerides	1.5 \pm 0.2	1.7 \pm 0.3	0.637
Total cholesterol/HDL	4.2 \pm 0.8	5.0 \pm 0.8	0.533

Blood glucose

Baseline fasting blood glucose values were 10.4 \pm 0.3 mmol/l in the hypoglycaemia group vs. 10.3 \pm 0.3 mmol/l in sham-saline group (p = 0.981). Blood glucose values fell steadily following i.p. insulin in the hypoglycaemia group to reach a nadir of relative hypoglycaemia at 6.6 \pm 0.4 mmol/l compared to 11.8 \pm 0.5 mmol/l in sham-saline group at 60 minutes (p < 0.001) (Figure 4.5). Following recovery from relative hypoglycaemia, blood glucose values steadily rose to 14.7 \pm 0.5 mmol/l vs. 12.5 \pm 0.4 at 120 minutes (hypoglycaemia vs. sham-saline: p < 0.05) (Figure 4.5). A change of behaviour was also

consistently noted in mice in the hypoglycaemia group between 40-60 minutes corresponding with the relative hypoglycaemia nadir. Mice displayed reduced levels of activity and demonstrated diminished interest in their surroundings with a return to normal behaviour following resolution of hypoglycaemia thus suggesting observed behaviour may have been indicative of neuroglycopenia (Figure 4.6).

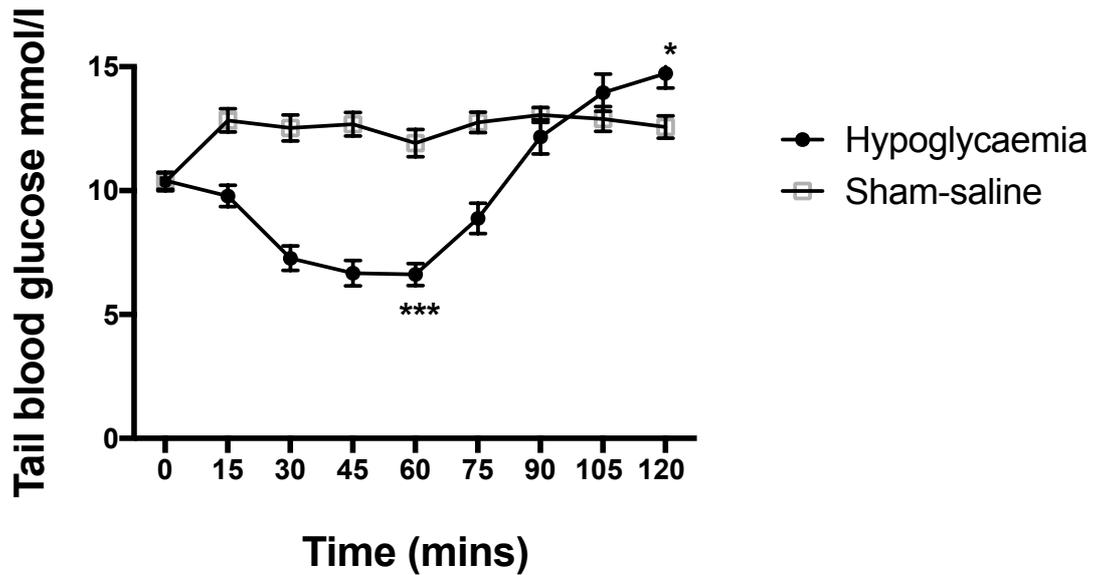


Figure 4-5 Cumulative blood glucose profiles following i.p. insulin (dose range 1.3-2.2 mU/g) (n=8) vs. control mice (n=8) that received a sham-saline injection (200 μ l) in a model of recurrent relative hypoglycaemia lasting 3 weeks in Western diet fed ApoE $^{-/-}$ mice. The glucose nadir was consistently reached at 60 minutes in the hypoglycaemia group. Blood glucose values were higher at 120 minutes in the hypoglycaemia group. Data are mean \pm SEM, *p<0.001, *p<0.05. Two-way ANOVA with Sidak's multiple comparison test.**

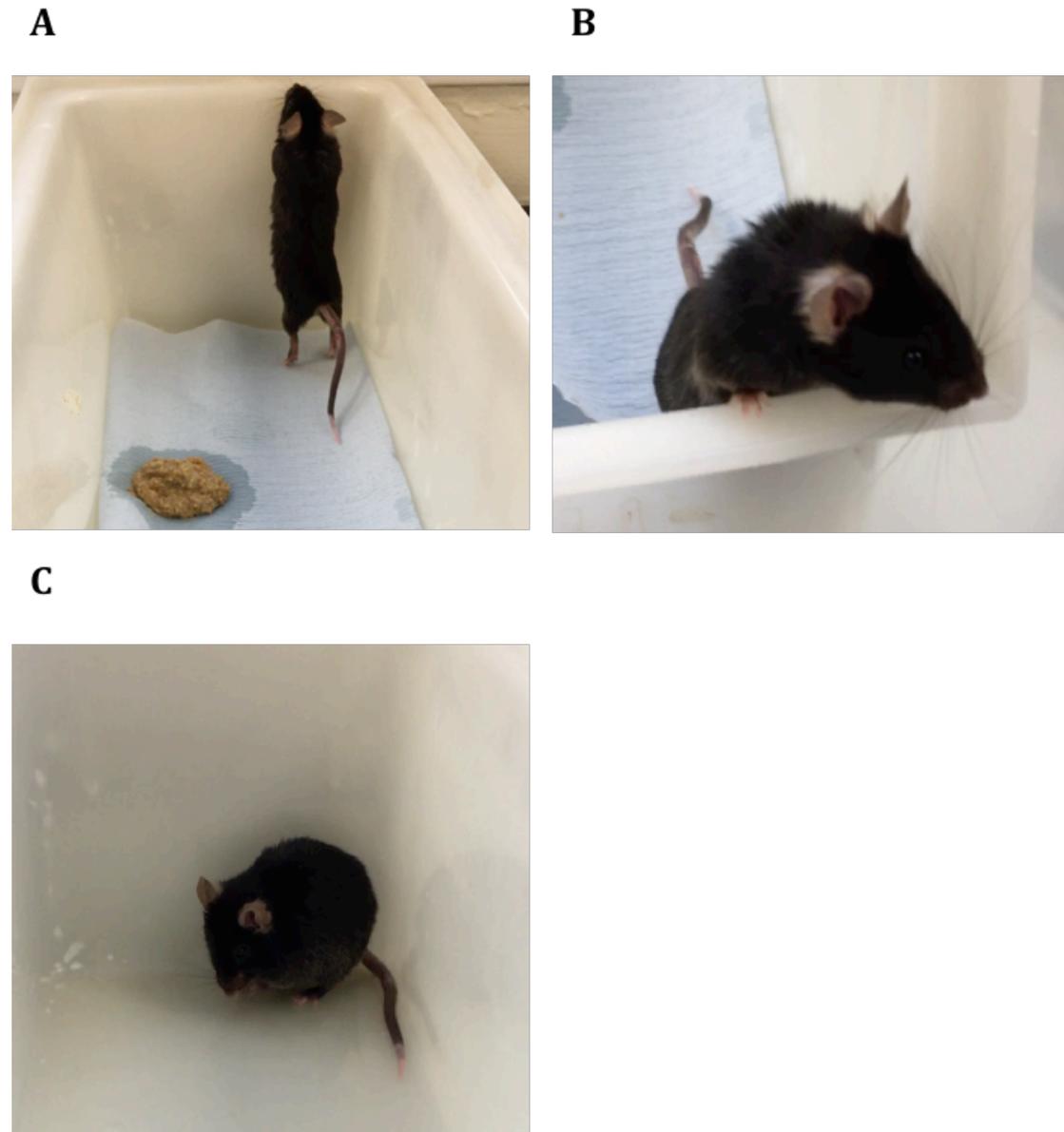


Figure 4-6: Visual signs of hypoglycaemia in an ApoE^{-/-} mouse. A&B) Mouse activity during euglycaemia and in C) the same mouse displaying a change in activity once relative hypoglycaemia was induced with 1.80 mU/g of i.p. insulin. The tail vein blood glucose value was 4.0 mmol/l at 50 minutes in this mouse when the photograph was taken.

Plasma adrenaline

Baseline plasma adrenaline in the hypoglycaemia group was 0.61 ± 0.17 nmol/l vs. 0.69 nmol/l ± 0.11 nmol/l in the sham-saline group ($p = 0.916$). As expected, plasma adrenaline levels rose to 4.92 ± 1.22 nmol/l following 60 minutes of relative hypoglycaemia compared to 1.16 nmol/l ± 0.16 nmol/l 60 minutes following sham-saline injection ($p < 0.001$) (Figure 4.7).

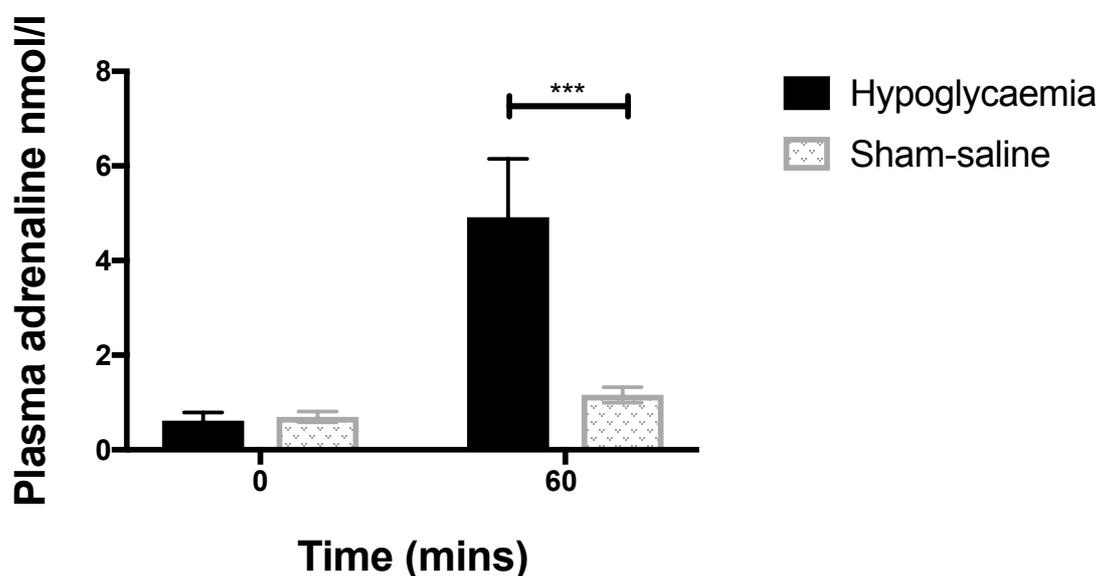


Figure 4-7 Plasma adrenaline levels at baseline (0 minutes) and following 60 minutes of relative hypoglycaemia (n=8) or sham-saline injection (n=8) in Western diet fed ApoE^{-/-} mice. Data are mean \pm SEM, * $p < 0.001$, 60 minutes data in the hypoglycaemia group are for n=6 mice due to haemolysis in the remaining two samples. Two-way ANOVA with Sidak's multiple comparison test.**

Oil red O staining

Analysis of oil red O stained *en face* aortae demonstrated no difference in plaque lesion area. In the hypoglycaemia group, total lesion area was $7.51\% \pm 1.85$ vs. $11.08\% \pm 2.88\%$ in the sham-saline group ($p = 0.331$)(Figure 4.8).

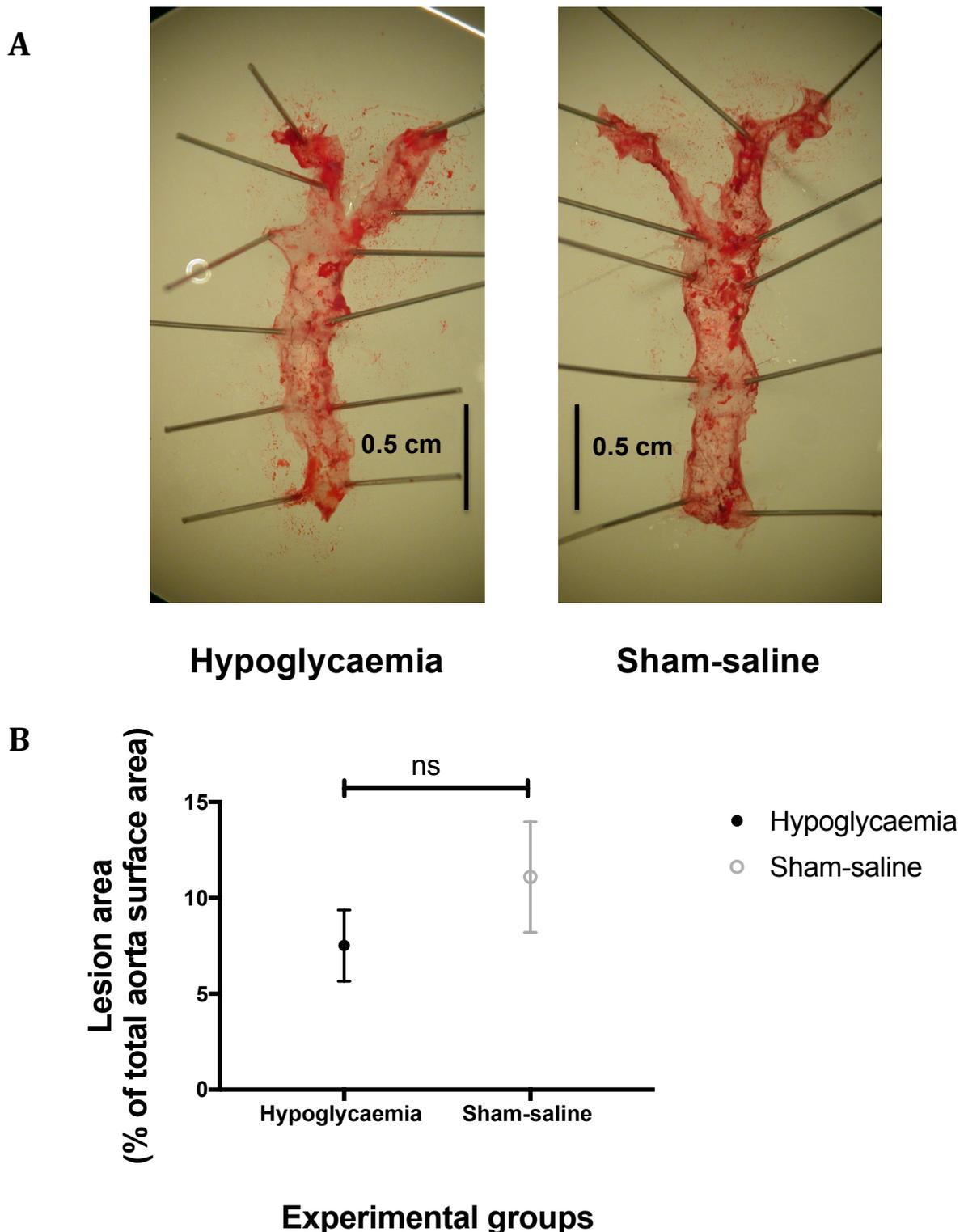


Figure 4-8 Atherosclerotic lesion burden in *en face* oil red O stained whole aortae following 4 episodes of insulin induced relative hypoglycaemia (n=7) compared to sham-saline injection (n=8) in Western diet fed ApoE ^{-/-} mice. A) Representative images demonstrate atherosclerotic burden from a mouse in the hypoglycaemia group vs. sham-saline. B) No significant difference in lesion area between hypoglycaemia and sham-saline groups was noted (p=0.331). Data are mean ± SEM, ns = non-significant. Scale bars = 0.5 cm. Unpaired two-tailed Student's t test.

4.4 Discussion

Data obtained from study of humans suggest that hypoglycaemia is associated with increased CV risk (Khunti *et al.*, 2015). In this chapter, I have described the development of a novel murine model of combined recurrent relative hypoglycaemia (relative to high tail blood glucose values at baseline) and pre-established atherosclerosis to enable studies into the effects of hypoglycaemia on the atherosclerotic plaque.

Preliminary experiments were conducted in chow fed mice to establish a protocol for insulin induced hypoglycaemia and to explore differences between male and female C57BL/6J ApoE^{-/-} mice in their response to hypoglycaemia. One interesting finding from these experiments was that whilst the two male mice had a similar blood glucose profile in response to i.p. insulin, the response between the two female mice was inconsistent. Despite similar body weights, handling and a dose escalation of insulin, there was an initial sharp decline in the blood glucose profile for female mouse 4 at 15 minutes post i.p. insulin to 6.9 mmol/l prior to a sharp rise in blood glucose to 9.4 mmol/l at 45 minutes implying stronger counter-regulation compared to other animals. This experiment was limited, however, as counter-regulatory hormones were not measured in these mice.

It was important to study female mice as previous work has shown that atherosclerotic lesions are larger and more advanced in young female ApoE^{-/-} mice compared to male mice (Caligiuri *et al.*, 1999). This makes female ApoE^{-/-} mice appealing to study when setting up a model of atherosclerosis. This

important sex difference in atherosclerotic lesion development has also previously been noted in other C57BL/6 mouse models of atherosclerosis (Paigen *et al.*, 1987) and is thought to be mediated, at least in part, by oestrogen induced proliferative T-cell responses to oxidised LDL which were not seen in male mice (Caligiuri *et al.*, 1999). Further, several studies in healthy humans and those with diabetes have revealed an intriguing gender difference in the counter-regulatory response to insulin induced hypoglycaemia (Amiel *et al.*, 1993; Davis *et al.*, 1993; Fanelli *et al.*, 1994). Collectively, all three studies demonstrated that the sympathetic response to insulin-induced hypoglycaemia was lower in human females compared to males. A diminished glucagon response to hypoglycaemia in human females was also demonstrated in two studies (Davis *et al.*, 1993; Fanelli *et al.*, 1994). It was therefore, of additional relevance to study female mice in order to develop a murine model of hypoglycaemia that mirrors the human condition as closely as possible.

My preliminary data demonstrating a heterogeneity between male and female mice in their response to insulin induced hypoglycaemia are in keeping with previous data that showed a gender difference in the response to insulin in mice (Karlsson, Scheurink and Ahrén, 2002). It is known that female mice have a larger α cell mass and stronger glucagon response to hypoglycaemia, whereas the sympathetic response to hypoglycaemia is stronger in male mice (Karlsson, Scheurink and Ahrén, 2002). Thus, taken together, initial experiments highlighted potential inter-species differences between man and mouse in gender responses to hypoglycaemia. On balance, and in light of preliminary findings, female mice were not used in the final optimised model in order to

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minimise the introduction of confounding factors that would potentially preclude reliable induction of recurrent hypoglycaemia on a consistent atherosclerotic plaque burden.

Male ApoE ^{-/-} mice fed high fat Western diet had, as expected, a higher body weight compared to chow fed mice. As Western diet feeding is known to induce insulin resistance in male ApoE ^{-/-} mice (Schierwagen *et al.*, 2015; Han *et al.*, 2017), a dose increment of i.p. insulin was performed compared to chow fed mice. One mouse was lost to an apparent hypoglycaemia-related death with a suggestion of pre-terminal hypoglycaemic seizure. The loss of this animal highlighted ApoE ^{-/-} mice vulnerability to hypoglycaemia prompting a dose reduction of insulin. This finding is consistent with previous work demonstrating Western diet fed ApoE ^{-/-} mice of a C57BL/6J strain are particularly susceptible to insulin induced hypoglycaemia displaying a deeper and longer lasting fall in blood glucose when compared to a strain of ApoE ^{-/-} BALB/cJ mice (Li *et al.*, 2011).

The insulin dose-finding exercise yielded a consistent blood glucose response to i.p. insulin and a dose range of insulin (1.3-2.2 mU/g) derived from this experiment that was comparable to insulin doses (0.75-2 mU/g) used in ApoE ^{-/-} and other murine models of hypoglycaemia (Havel *et al.*, 1993; Karlsson, Scheurink and Ahrén, 2002; Li *et al.*, 2011).

Data generated during the development of a model of recurrent relative hypoglycaemia in male ApoE ^{-/-} mice demonstrated a final body weight

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consistent with published data from ApoE ^{-/-} mice fed Western diet for an equivalent period (Schierwagen *et al.*, 2015). My finding of non-significant difference in the body weights between hypoglycaemia and control mice at the end of the feeding period and during experimental weeks was notable for two reasons. Firstly, it demonstrated comparable diet consumption between the experimental and controls groups and secondly, it highlighted that recurrent relative hypoglycaemia did not induce significant weight loss in mice which is a marker of vulnerability and reduced survivability in murine models (Burkholder *et al.*, 2012). Non-significant body weight differences pre-sacrifice between the hypoglycaemia and control group were additionally important as adipose tissue drives production of pro-inflammatory cytokines and chemokines (Weisberg *et al.*, 2003; Sell and Eckel, 2010; Osborn and Olefsky, 2012) and low-grade inflammation is critical to the aetiology of atherosclerosis (Ross, 1999).

In line with previous observations (Nakashima *et al.*, 1994), Western diet induced hyperlipidaemia in ApoE ^{-/-} mice. Biochemical analyses of serum lipids, however, revealed no significant difference between groups at sacrifice. Thus, the effects of dyslipidaemia on potential between group differences in atherosclerosis were controlled for in this model.

In my study, blood glucose profile data revealed lower mean fasting blood glucose values than obtained in an older study (Li *et al.*, 2011). The latter investigated hyperglycaemia in female ApoE ^{-/-} C57BL/6J mice fed a Western diet for 12 weeks. The authors performed insulin tolerance tests thereby yielding comparative data on an identical strain of mice. Lower fasting glucose in my

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model may be explained by differences in gender and a shorter feeding duration of 7 weeks. In my study, recurrent application of i.p. insulin reliably induced relative hypoglycaemia with a nadir at 60 minutes post-injection. Blood glucose kinetics resembled previous data from Li *et al.*, although the mean glucose nadir (6.61 mmol/l) in my model was higher than previously reported (3.1 mmol/l) (Li *et al.*, 2011). The final body weight of mice in my study was also higher compared to mice studied by Li *et al.* and thus a lower glucose nadir may reflect a higher total insulin resistance. Indeed, whilst optimising my model, I was able to achieve deeper hypoglycaemia with a mean glucose of 1.7 mmol/l (Figure 4.3) in mice fed Western diet for 6 weeks and a lower body weight. However, one animal had irrecoverable hypoglycaemia and two out of three animals needed i.p. dextrose for glycaemic recovery suggesting that at this depth of hypoglycaemia, there would be reduced animal survivability raising ethical and feasibility concerns.

Plasma adrenaline levels demonstrated unequivocally, however, that at a relative hypoglycaemic nadir of 6.61 mmol/l, mice in my model were exhibiting strong counter-regulatory responses compared to controls. This provided biochemical proof of hypoglycaemia and augmented observed physical findings of altered mouse behaviour suggestive of neuroglycopenia in hypoglycaemic mice. Further, as adrenaline levels were measured after the third episode of hypoglycaemia, elevated levels suggested that the counter-regulatory response was still preserved despite recurrent relative hypoglycaemia in my model. Data in humans (Heller and Cryer, 1991) and rodents (McNeilly *et al.*, 2017) demonstrates that recurrent hypoglycaemia impairs the hormonal counter-

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regulatory response to future episodes of hypoglycaemia. As I hypothesised that hypoglycaemia may aggravate atherosclerosis at least in part through sympathetic mediated activation of inflammatory pathways (Dutta *et al.*, 2012), I wanted to avoid significant diminution of the sympathetic response to hypoglycaemia in my model. Further, clinical trials of intensive glycaemic control demonstrating an association between hypoglycaemia and CV risk were conducted in patients with T2D in whom the hypoglycaemia burden is significantly lower than in those with T1D (Donnelly *et al.*, 2005) and defective counter-regulatory responses are therefore less prevalent (Schopman, Geddes and Frier, 2010). Thus, by preserving counter-regulatory responses in my mice, I also aimed to make my study more relevant to the human condition.

Rebound hyperglycaemia as a consequence of hypoglycaemia recovery was noted at 120 minutes in the hypoglycaemia group of mice with blood glucose values being higher than fasting levels and significantly higher compared to controls. This limited my model as it was designed to study the effects of hypoglycaemia on atherosclerosis. Evidence from healthy humans and those with T1D suggests that hyperglycaemia after recovery from hypoglycaemia can worsen endothelial dysfunction, increase oxidative stress and promote inflammation (Ceriello *et al.*, 2012). Further, data from ApoE^{-/-} mice fed maltose twice weekly with resultant repetitive postprandial hyperglycaemia demonstrated an increased adherence of macrophages to the vascular endothelium and an increase in atherosclerotic lesion size compared to controls (Mita *et al.*, 2007). Collectively, therefore, fluctuations in blood glucose can influence inflammation and progression of atherosclerosis. Thus, as part of

further model refinement, care was taken to avoid correctional rebound hyperglycaemia so as not to confound the potential effects of hypoglycaemia on the vasculature. It is important to note that high baseline fasting glucose values in both experimental groups were potentially related to the stress of the animals at the starting point. In particular, as observed in the sham-saline control group, an initial blood glucose rise at 15 minutes implied adrenergic stress. In order to minimise confounding from this, mice were carefully familiarised to experimental conditions at multiple times during the feeding period as described in chapter 2.

Exploratory analyses of atherosclerotic lesion burden in *en face* whole aortae did not reveal a significant difference between hypoglycaemia and sham-saline. Total atherosclerotic lesion burden was, however, in line with published data from studies conducted locally using an identical strain of mice (West *et al.*, 2014). A trend towards a lower total atherosclerotic burden in the hypoglycaemia group of mice that received i.p. insulin compared to the sham-saline group is of interest and could have been further explored by in-depth analysis of more robust histological endpoints as well as measurement of differences in plasma cytokines between the two groups. Insulin has been shown to have a number of anti-inflammatory effects. Insulin via activation of phosphatidylinositol kinase (PI3K) and Akt kinase, induces NO synthase which in turn generates NO (Aljada and Dandona, 2000). Studies from humans show that insulin through effects that are NO dependent, inhibits platelet aggregation (Trovati *et al.*, 1997) and attenuates vasoconstriction induced by catecholamines (Grover *et al.*, 1995). Further, in those with T2D, a low dose intravenous insulin

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infusion has been shown to significantly suppress TLRs 1,2,4,7 and 9 mRNA expression in mononuclear cells (Ghanim *et al.*, 2008). TLRs are important in the pathophysiology of atherosclerosis as they induce inflammation through activation of a number of pro-inflammatory transcription factors. In addition, to dampening inflammatory indices, insulin has been shown to exert anti-atherosclerotic effects. In the DCCT study, intensive insulin therapy demonstrated a trend towards reduced CV events in those with T1D (Diabetes Control and Complications Trial Research Group *et al.*, 1993). The Epidemiology of Diabetes Interventions and Complications (EDIC) more latterly demonstrated that carotid intima-media thickness as a marker of atherosclerosis was reduced in the DCCT group that had received intensive insulin therapy (Nathan *et al.*, 2003). Also, in experimental animals, ApoE^{-/-} mice that received oral insulin had significantly attenuated atherosclerotic plaque progression through reduction in serum and macrophage oxidative stress (Shamir *et al.*, 2003). Conversely, in a mouse model where insulin signalling was disrupted through lack of insulin receptor substrate 2, the vasculature was more susceptible to atherosclerosis (Kubota *et al.*, 2003). In light of these data, a trend towards a lower atherosclerotic burden in mice subjected to recurrent insulin induced hypoglycaemia maybe reflective of the anti-inflammatory and anti-atherogenic effects of insulin overcoming pro-inflammatory and pro-atherosclerotic effects of hypoglycaemia. This would be in keeping with data from my human experiments (chapter 3), where prior hyperinsulinaemic-euglycaemia had an inhibitory effect on endotoxin mediated inflammation. However, in humans a sufficient hypoglycaemic stimulus with an equivalent hyperinsulinaemia abrogated the anti-inflammatory effects of insulin. Thus, in refining my animal model further, I

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decided to extend the experiment duration to double the hypoglycaemic stimulus to a total of 8 episodes. To facilitate more readily a lower hypoglycaemic stimulus, I also elected to fast animals overnight to deplete glycogen stores. These changes are reflected in the final model employed as described in chapters 2 and 5.

Strengths of my novel model include a reliable induction of atherosclerosis and moderate recurrent relative hypoglycaemia over a number of weeks with good mouse survivability.

One limitation is that I did not specifically induce diabetes in my mice through the use of streptozotocin for example and then insulin treat them to emulate more closely the T2D population studied in various clinical trials. It is conceivable that in my model, in atherosusceptible animals, high fat Western diet for 7 weeks induced abnormal glucose tolerance, if not frank T2D, which has been observed in ApoE^{-/-} mice fed Western diet for 12 weeks (Li *et al.*, 2011). However, ethical constraints meant that I could not take more than 10% of overall blood volume (approximately 100 μ l) on any experimental day and therefore could not perform oral glucose tolerance tests or measure HbA1c to confirm this. Furthermore, in aiming to test the effects of hypoglycaemia on the vasculature, induction of diabetes and then insulin treatment in mice prior to superimposed recurrent relative hypoglycaemia would have added potential confounding of glucose variability between mice and groups which would have been difficult to control. Experiments would also be strengthened by measurement of insulin in all mice in addition to inflammatory cytokines,

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additional counter-regulatory hormones including glucagon and circulating leukocytes including monocytes. Limits to blood letting in mice, however, precluded this.

In conclusion, over a number of optimisation steps, I successfully developed a novel murine model of recurrent relative hypoglycaemia and pre-established atherosclerosis. I then employed this model to test the effects of recurrent hypoglycaemia on atherosclerosis as described in chapter 5.

Chapter 5- Consequences of recurrent hypoglycaemia on atherosclerotic lesion burden and markers of plaque vulnerability

5.1 Introduction

There is a growing body of evidence linking hypoglycaemia with increased CV risk. There remains debate, however, as to whether the relationship between hypoglycaemia and CV risk is causal or is present because of confounding. In the ADVANCE trial, an association between severe hypoglycaemia and mortality was thought to be due to frail and vulnerable participants with comorbidities being more prone to adverse outcomes and thus also at a higher risk of encountering severe hypoglycaemia (Zoungas *et al.*, 2010). Other clinical trials of CV outcomes have also suggested that confounding due to comorbidities may explain the relationship between hypoglycaemia and mortality (Heller *et al.*, 2017; Pieber *et al.*, 2018). Evidence for a causal relationship between hypoglycaemia and CV events is supported by systematic reviews that have included a large number of participants from observational studies and performed statistical analyses to account for confounding from comorbidities (Goto *et al.*, 2013; Yeh *et al.*, 2016). Further, recent human experimental medicine studies in both healthy volunteers and those with diabetes have offered mechanisms that explain a relationship between hypoglycaemia and CV events (Ratter *et al.*, 2017; Chow *et al.*, 2018; Iqbal *et al.*, 2019).

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To date, few studies either in humans or animal models have directly explored a causal relationship between hypoglycaemia and CV events. Atherosclerotic lesion burden and plaque rupture is responsible for acute CV events and I therefore hypothesised that recurrent hypoglycaemia increases total plaque burden and causes inflammatory changes within the plaque that increase the risk of plaque rupture. In order to test this hypothesis, I first developed a mouse model of recurrent relative hypoglycaemia and atherosclerosis as described in chapter 4. In this chapter, I describe experiments using my model to explore causality between recurrent relative hypoglycaemia and aggravation of pre-established atherosclerosis.

5.2 Methods

Male ApoE^{-/-} mice on a C57BL/6J background (JAX B6.129P2-ApoE^{tm1Unc/J}) aged 8-13 weeks were fed a Western diet for 7 weeks. Mice were randomly allocated at the start of the feeding period to either experimental hypoglycaemia (n=10) or sham-saline injection (n=10). Each animal underwent 8 episodes of insulin induced hypoglycaemia or sham-saline injection over 4 consecutive weeks. Atherosclerotic lesion analysis was performed in brachiocephalic arteries and whole aortae. Brachiocephalic artery sections were stained for the macrophage marker MAC-3. A detailed description of these methods is in chapter 2.

5.3 Results

One mouse was lost to irrecoverable hypoglycaemia on day 1 and week 1 of experiments. Another mouse from the sham-saline group was lost to a laceration sustained from tail vein injury within the first week of experiments. Data was therefore not available for analysis from these animals and thus data shown below is for n=9 in each group unless otherwise stated.

5.3.1 Body weight

Body weight was not significantly different between mice in each group at baseline, end of feeding and immediately prior to sacrifice. Baseline body weight in mice allocated to the hypoglycaemia group was 25.0 ± 0.3 grams vs. 26.6 ± 0.7 grams for mice allocated to the sham-saline group ($p = 0.477$). Weight at the end of the feeding period was 28.9 ± 0.6 grams for mice in the hypoglycaemia group vs. 31.0 ± 1.0 grams for mice in the sham-saline group ($p = 0.169$). Mice in both groups displayed a positive weight trajectory during feeding. The pre-sacrifice weight at end of the experiment was 29.9 ± 0.8 grams in the hypoglycaemia group vs. 31.8 ± 0.8 grams in the sham-saline group ($p = 0.617$).

5.3.2 Serum lipids

There were no significant differences in serum lipid parameters between the experimental hypoglycaemia and control sham-saline groups at sacrifice (Table 5.1).

Table 5.1 Serum lipids in ApoE^{-/-} mice fed a Western diet for 7 weeks prior to exposure to recurrent hypoglycaemia or sham-saline injection. Data are mean \pm SEM. p values are for between group comparisons.

Serum lipids (mmol/l)	ApoE ^{-/-} Hypoglycaemia n=9	ApoE ^{-/-} Sham-saline n=9	p values
Total cholesterol	28.8 \pm 2.3	28.5 \pm 1.7	0.928
HDL cholesterol	8.0 \pm 0.5	7.7 \pm 0.2	0.560
LDL cholesterol	19.9 \pm 2.0	19.9 \pm 1.5	0.988
Triglycerides	1.7 \pm 0.3	2.0 \pm 0.3	0.529

5.3.3 Blood glucose

Fasting blood glucose values were 12.0 ± 0.18 mmol/l in the hypoglycaemia group vs. 12.0 ± 0.2 mmol/l in the sham-saline group ($p = 0.985$). Following i.p. insulin, blood glucose values fell to a relative hypoglycaemia nadir of 6.1 ± 0.3 mmol/l compared to 13.4 ± 0.3 mmol/l in the sham-saline group ($p < 0.001$) (Figure 5.1). At recovery (120 minutes), blood glucose values were 13.2 ± 0.4 mmol/l in the hypoglycaemia vs. 13.6 ± 0.2 mmol/l in the sham-saline group ($p = 0.992$).

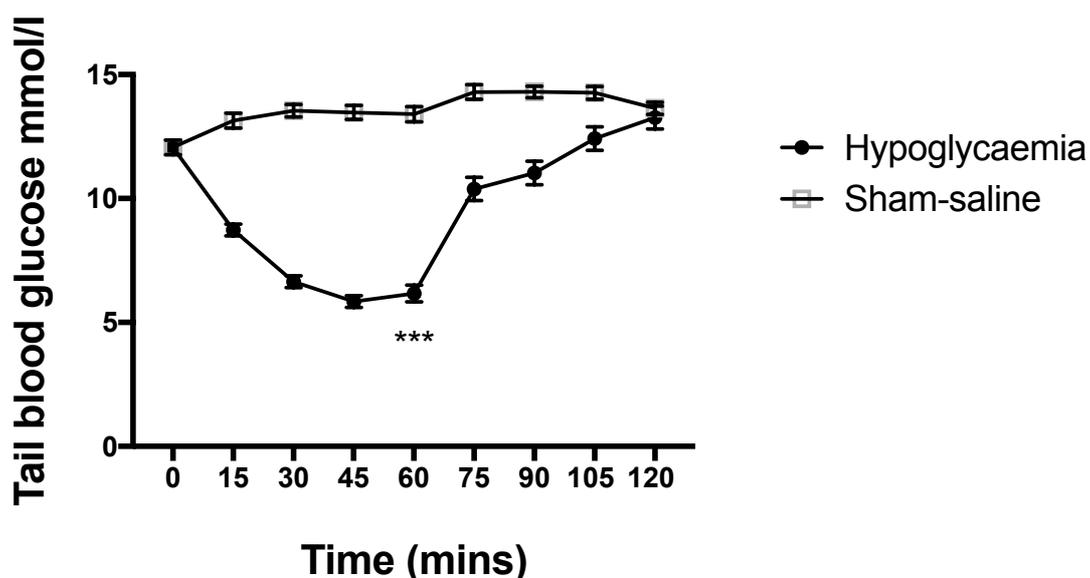


Figure 5-1 Blood glucose kinetics following i.p. insulin (dose range 2.5-3.7 mU/g) in the hypoglycaemia group (n=9) vs. control sham-saline injection (n=9) over a 4 weeks experiment in Western diet fed ApoE $^{-/-}$ mice. Data are mean \pm SEM, * $P < 0.001$. Two-way ANOVA with Sidak's multiple comparison test.**

5.3.4 Plasma adrenaline

Baseline plasma adrenaline in the hypoglycaemia group was 0.42 ± 0.13 nmol/l vs. 0.61 ± 0.09 nmol/l ($p = 0.958$). Plasma adrenaline levels rose to 3.87 ± 1.01 nmol/l following 60 minutes of hypoglycaemia group compared to 2.11 ± 0.26 nmol/l 60 minutes following sham-saline injection ($p = 0.050$) (Figure 5.2).

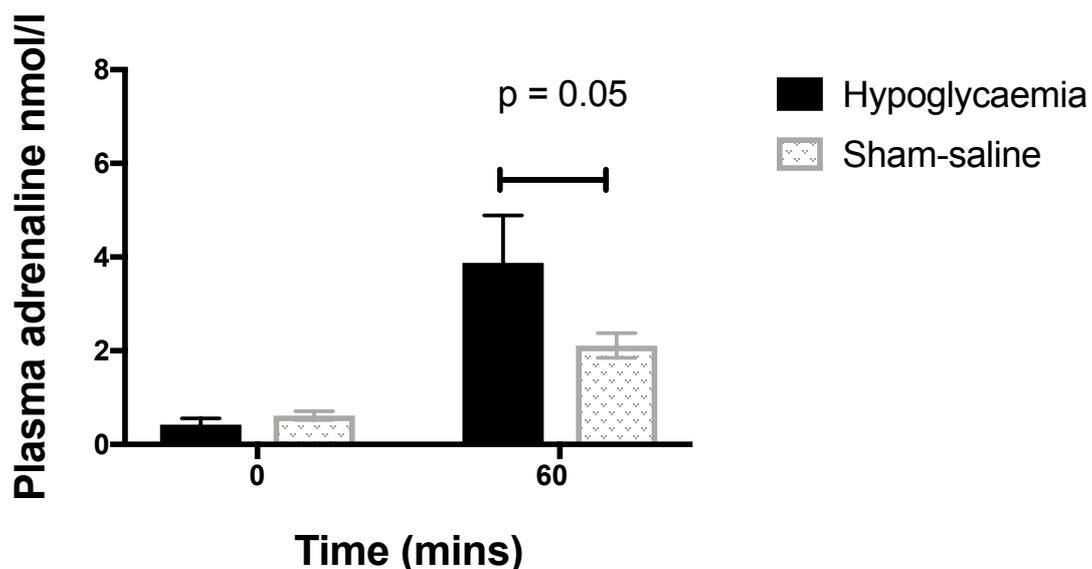


Figure 5-2 Plasma adrenaline levels at baseline (0 minutes) and following 60 minutes of relative hypoglycaemia (n=9) or sham-saline injection (n=9) in ApoE^{-/-} mice. Plasma adrenaline levels were obtained following the 7th episode of hypoglycaemia or sham-saline injection in the final (week 4) of the experiment. Data are mean \pm SEM, 60 minutes data in both groups are for n=8 mice due to haemolysis in the remaining samples. Two-way ANOVA with Sidak's multiple comparison test.

5.3.5 Atherosclerosis in whole aortae

Oil red O staining of aortae did not demonstrate a significant difference in total plaque area between hypoglycaemia and sham-saline groups. Total lesion area presented as a percentage of the total aortic surface area in the hypoglycaemia group was $10.8 \pm 0.9\%$ vs. $8.2 \pm 0.8\%$ in the sham-saline group ($p = 0.062$) (Figure 5.3 A). In order to assess regional differences in atheroma formation, lesion analysis was performed in aortic arch and descending aorta. There were no significant differences between groups at these sites. Aortic arch lesion area in the hypoglycaemia group was $22.4 \pm 2.8\%$ vs. $19.3 \pm 1.8\%$ in sham-saline controls ($p = 0.380$)(Figure 5.3 B). Descending aortic analyses showed a lesion area of $4.5 \pm 1.0\%$ in the hypoglycaemia group compared to $3.1 \pm 0.8\%$ in the sham-saline mice ($p = 0.280$)(Figure 5.3 C).

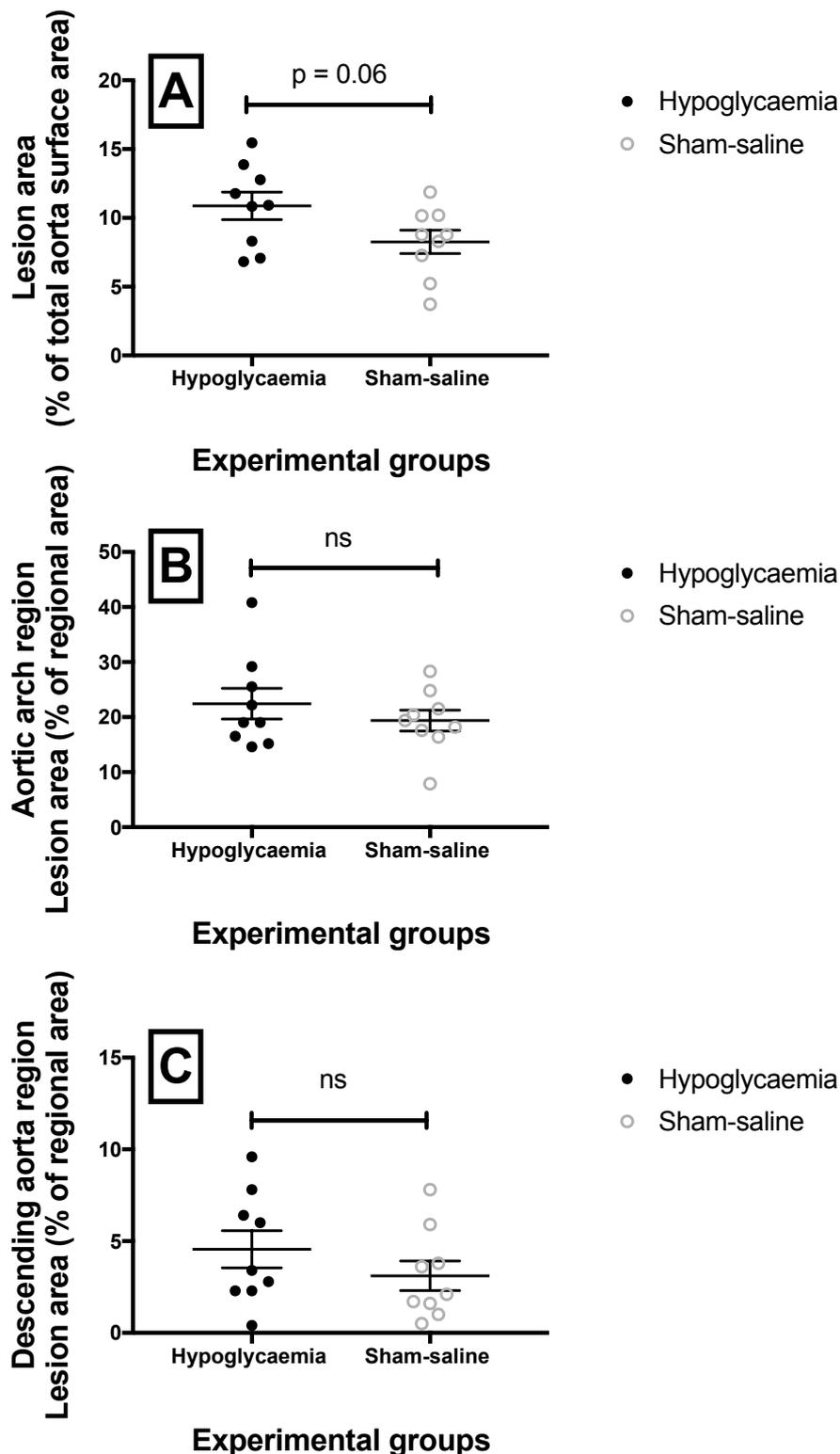


Figure 5-3 Atherosclerotic lesion burden in *en face* oil red O stained whole aortae following 8 episodes of relative hypoglycaemia (n=9) or sham-saline injection (n=9) in Western diet fed ApoE^{-/-} mice. A) Lesion area calculated as a percentage of total surface area for the whole aorta. B) Lesion area in the aortic arch and C) descending aorta. Data are mean \pm SEM, ns = non-significant. Unpaired two-tailed Student's t test.

5.3.6 Atherosclerosis in brachiocephalic arteries

Atherosclerotic lesion area in brachiocephalic arteries expressed as a percentage of the total vessel CSA was not significantly different between groups. Plaque area in the hypoglycaemia group was $17.8 \pm 1.9\%$ vs. $18.2 \pm 3.2\%$ in the sham-saline group ($p = 0.919$)(Figure 5.4 A&B).

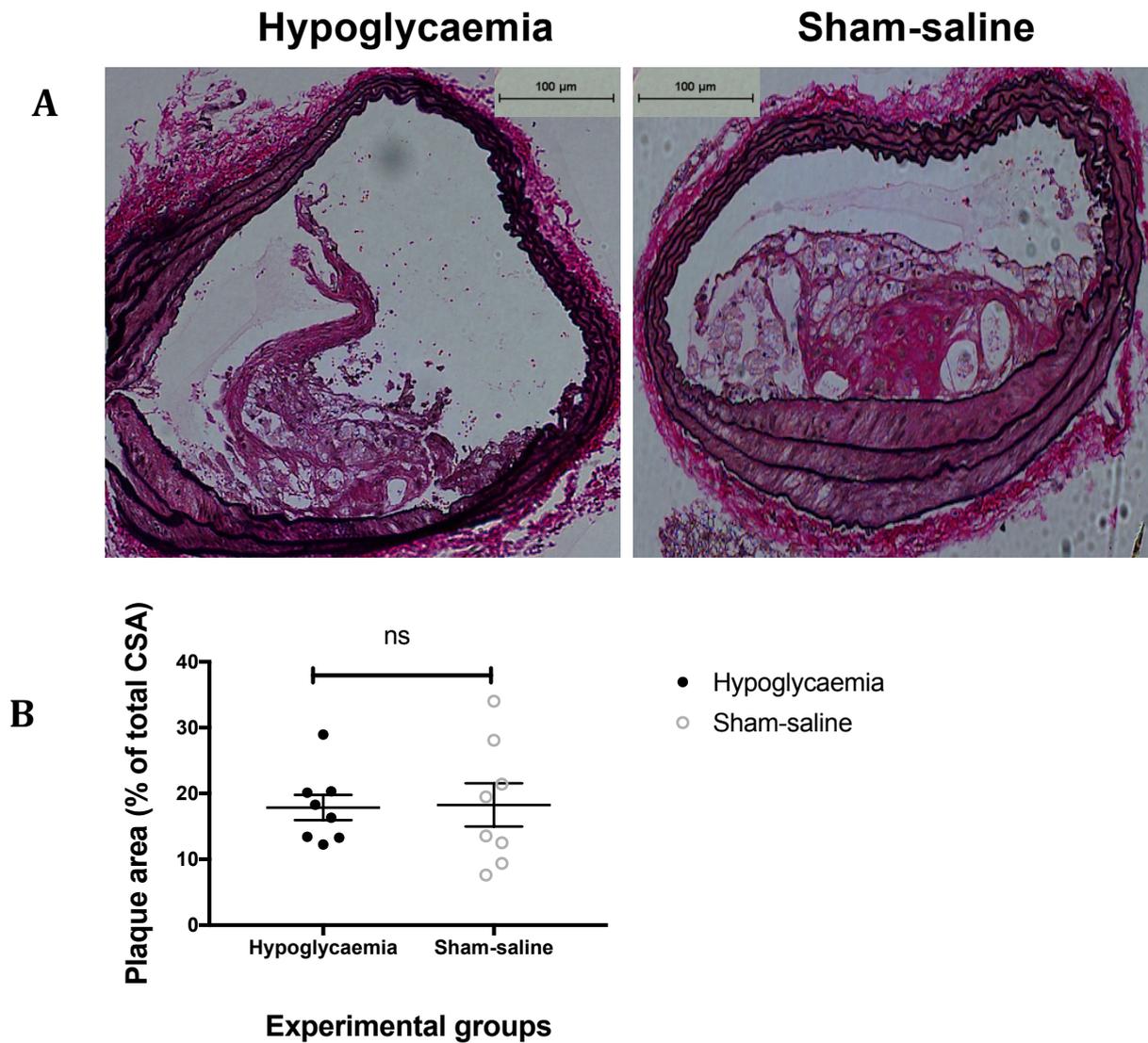


Figure 5-4 Atherosclerotic lesion area in cross sections of brachiocephalic arteries. A) Representative images of MVG stained sections demonstrating plaque formation in hypoglycaemia and sham-saline groups. B) Plaque area expressed as a percentage of the total vessel CSA in hypoglycaemia (n=8) and sham-saline mice (n=8). Data are mean \pm SEM, ns = non-significant. Scale bars = 100 μ m. Unpaired two-tailed Student's t test.

5.3.7 Macrophage staining in brachiocephalic arteries

Immunohistochemical staining for the macrophage marker MAC-3 expressed as a percentage of positive staining over total brachiocephalic lesion area did not significantly differ between groups. Positive staining for MAC-3 in the hypoglycaemia group was $29.1 \pm 1.9\%$ vs. $33.3 \pm 4.0\%$ in sham-saline mice ($p = 0.383$)(Figure 5.5 A&B).

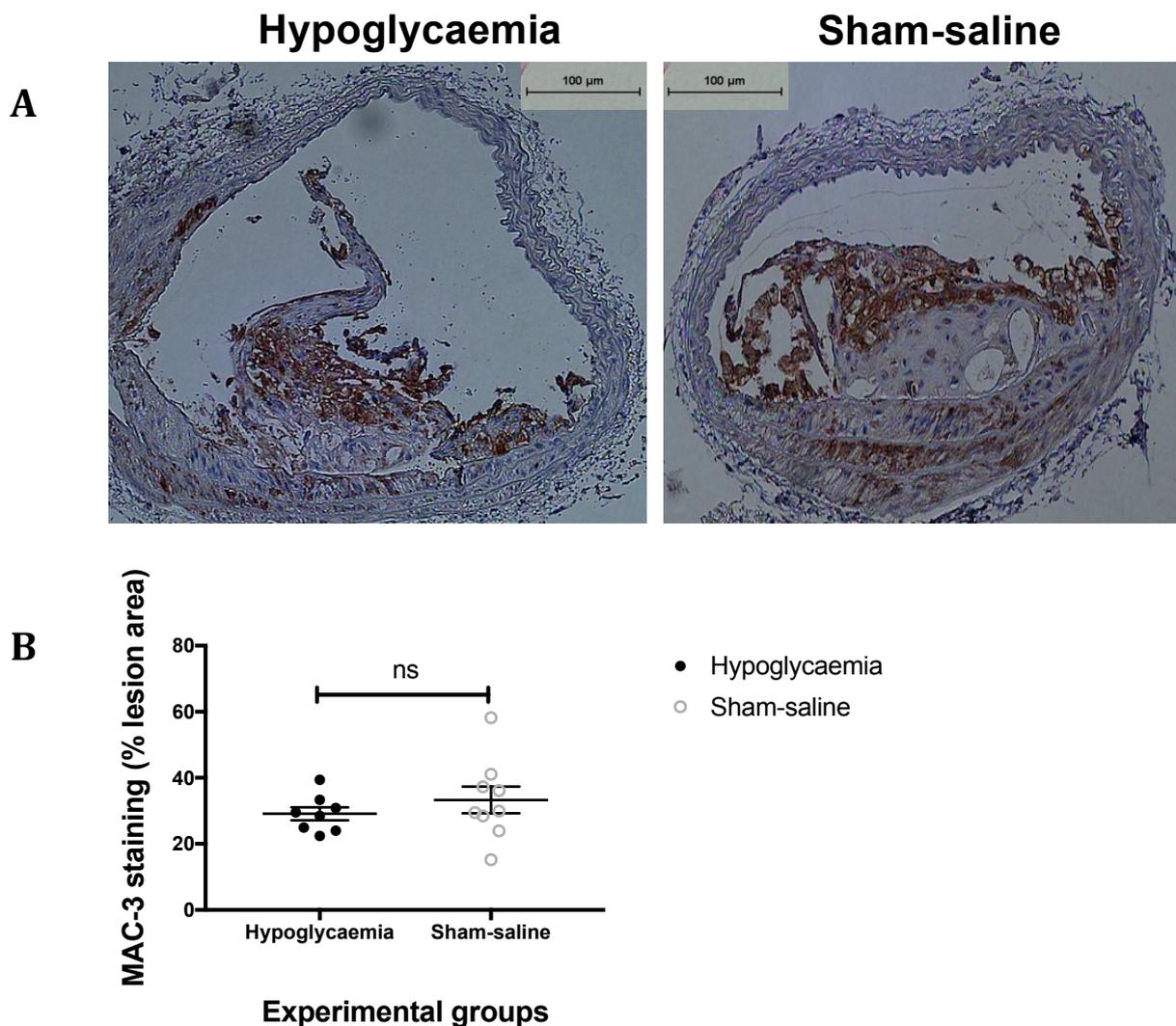


Figure 5-5 Macrophage staining positive for MAC-3 in cross sections of brachiocephalic arteries. A) Representative images demonstrating positive immunohistochemical staining in hypoglycaemia and sham-saline groups. B) MAC-3 staining expressed as a percentage of total lesion area in hypoglycaemia (n=8) and sham-saline mice (n=9). Data are mean \pm SEM, ns = non-significant. Scale bars = 100 μ m. Unpaired two-tailed Student's t test.

5.4 Discussion

Hypoglycaemia is associated with CV risk, but there remains controversy as to if this relationship is causal. Here, I aimed to explore causality between recurrent moderate relative hypoglycaemia and aggravation of pre-established atherosclerosis in a combined murine model. My main findings are that compared to sham-saline controls: (1) hypoglycaemia resulted in a trend towards increased atherosclerotic lesion burden in oil red O stained whole aortae; (2) hypoglycaemia did not increase total lesion burden in the brachiocephalic artery; (3) hypoglycaemia did not increase plaque macrophage content in the brachiocephalic artery.

Whilst completing my studies, Nakajima et al. published data using their own combined model of recurrent hypoglycaemia in ApoE ^{-/-} mice (Nakajima *et al.*, 2015). The latter studied the effects of repetitive hyperglycaemia, hypoglycaemia and control conditions on the progression of atherosclerosis in multiple groups of ApoE ^{-/-} mice on a C57BL/6J background. The authors found that recurrent hypoglycaemia, hyperglycaemia or their combination did not increase total atherosclerotic lesion burden quantified by oil red O staining at the aortic sinus and whole aortae (Nakajima *et al.*, 2015). These findings are broadly in keeping with my own data that overall, repetitive relative hypoglycaemia had no statistically significant impact on atherosclerosis. Methodologically, my model differed from the Nakajima study. Nakajima and colleagues studied a larger number of mice (n=22 vs. n=10) over a longer period (15 weeks vs. 4 weeks), used far higher doses of insulin (8 IU/kg vs. 2.5-3.7 IU/kg) and fed animals a standard rodent as opposed to a high fat Western diet. Thus, whilst a lower

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hypoglycaemia nadir was achieved (3.2 mmol/l vs. 6.1 mmol/l) there was poor mouse survivability in their model with a loss of 20% of animals in the hypoglycaemia group. The authors do not mention rescue glucose therapy and infrequently measured blood glucose suggesting that a significant number of animals were lost to severe hypoglycaemia. Further, as a high dose of i.p. insulin was given once a week over 15 weeks, it is plausible that mice became increasingly vulnerable on account of developing defective counter-regulation. Surprisingly, however, the authors did not measure counter-regulatory hormones to assess this. Overall, it is likely that in the Nakajima model, chronic hyperinsulinaemia resulted in reduced atherogenesis, thus opposing any pro-atherosclerotic effects of recurrent hypoglycaemia in addition to animal loss during the experiment confounding results.

It is also possible that in my experiments the hypoglycaemia stimulus employed was not sufficient to overcome known anti-inflammatory and anti-atherogenic effects of insulin as previously described. This is supported by an increase in total lesion formation at the aorta following a total of 8 episodes of relative hypoglycaemia as seen in this chapter compared to mice described in chapter 4 that were exposed to a total of 4 episodes of recurrent relative hypoglycaemia. In addition to an increase in total atheroma, doubling the number of hypoglycaemic episodes appears to have also reversed the trend where I previously observed higher atheroma formation at the aorta in the sham-saline groups vs. hypoglycaemia (Figure 4.8, chapter 4). In both experiments, identical ApoE ^{-/-} mice were fed Western diet for 7 weeks and had comparable body weights, lipid profile and hypoglycaemic nadirs. Thus, it may be that to assess more

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definitively the effects of hypoglycaemia on lesion formation, the experiment needs to be extended to incorporate more hypoglycaemic episodes. This would, however, potentially reduce mouse survivability due to progressive blunting of counter-regulatory responses and as a result demand a larger number of mice. This is supported by my observation that doubling the number of hypoglycaemic episodes of the same severity reduced the peak adrenaline response to 3.87 ± 1.01 nmol/l as described in this chapter compared to 4.92 ± 1.22 nmol/l in mice described in chapter 4. Overall, the magnitude of effect of recurrent relative hypoglycaemia vs. sham-saline injection on atherosclerotic lesion burden at the aorta suggests that the study was underpowered which limits the conclusions that can be drawn. Further work with an adequate sample size will allow a more robust assessment of atheroma formation at the aorta.

Whilst total lesion area appeared to increase at the aorta in the hypoglycaemia group, there was no difference or suggestion of a trend in atherosclerotic lesion burden between groups in MVG stained cross sections of brachiocephalic arteries. Whilst little is known about the effects of hypoglycaemia on atherosclerosis at regional sites in the vasculature, there are data to suggest that the rat thoracic aorta is prone to monocyte attachment following recurrent hypoglycaemia (Jin *et al.*, 2011). This makes it tempting to reason that the aorta is an early site of atherosclerosis progression following recurrent hypoglycaemia and shear stress and flow patterns at regional sites may potentially exacerbate the effect of hypoglycaemia on atheroma formation. Initially, however, future work needs to investigate the effects of recurrent hypoglycaemia on lesion

burden at a third anatomical site of known atherosclerosis predilection for example the aortic sinus.

Acute repetitive relative hypoglycaemia over 4 weeks did not modulate plaque macrophage numbers compared to sham-saline controls. These data are in a way consistent with clinical experience, trial data and human experimental studies that have studied the relationship between hypoglycaemia and CV risk. In clinical practice, hypoglycaemia is relatively commonly encountered, yet CV mortality following a few episodes is, thankfully, rare. Further, clinical studies suggest that hypoglycaemia may serve to increase CV risk longitudinally over many months (Duckworth *et al.*, 2009; Pieber *et al.*, 2018). Also, as I show in chapter 3 (Iqbal *et al.*, 2019), hypoglycaemia induces relatively transient pro-inflammatory and pro-atherothrombotic changes that are likely consequent upon sympatho-adrenal activation, whilst the innate immune system may be primed to respond more robustly in a durable fashion. It then follows that the catecholamine response to episodes of acute relative hypoglycaemia in ApoE ^{-/-} mice may have mobilised monocytes and thus transiently increased monocyte/macrophage traffic to the plaque with a decline back to baseline levels in between repeated episodes. Thus, this relatively brief experiment may not have captured an increase in plaque macrophage numbers that may occur insidiously over several weeks to months and cumulatively increase the risk of plaque instability and AMI as clinical data suggests. This hypothesis could be tested in further work by prolonging the duration of experiments or looking at immediate acute monocyte recruitment after episodes of hypoglycaemia. Since it has also been shown that in ApoE ^{-/-} mice extramedullary haematopoiesis plays a role in chronic

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monocyte/macrophage accumulation in plaques weeks after events associated with significant systemic stress and sympatho-adrenal activation (Dutta *et al.*, 2012), current experiments would have been strengthened by harvesting mouse spleens for flow cytometric analyses of progenitor cells. In addition, as extramedullary haematopoiesis generates pro-inflammatory Ly-6c^{high} monocytes that infiltrate atherosclerotic lesions (Robbins *et al.*, 2012), flow cytometric analyses in whole blood to enumerate and phenotype mouse HSCs and monocyte subsets during repeated hypoglycaemia would have been of interest. Isolation of plaque monocytes/macrophages in mice exposed to recurrent hypoglycaemia to assess for molecular signals of activation for example through quantification of mRNA encoding pro-inflammatory genes including IL-1 β would also be warranted in future studies.

In summary, as a first step towards elucidating potential relationships between hypoglycaemia and aggravation of atherosclerosis, I was able to quantify the extent and inflammatory burden of the atherosclerotic plaque in my murine model. Whilst there was a suggestion of accelerated atherosclerosis at the aorta in the hypoglycaemia group, one limitation was the lack of statistical power. In addition, due to time constraints, lesion burden was only assessed at two sites and the aortic sinus not studied. Also, smooth muscle and collagen content and markers of plaque vulnerability including neovascularisation, buried cap formation and fibrous cap thickness were not studied further limiting a definitive conclusion. Further work needs to address these limitations to better characterise the effects of hypoglycaemia on atherosclerosis.

Chapter 6- Final Discussion

The early termination of the ACCORD trial (Gerstein *et al.*, 2008) on account of increased mortality in the intensive glycaemic treatment arm provoked interest and fuelled debate in understanding a potential relationship between hypoglycaemia and adverse CV events. There was existing data from human studies that demonstrated hypoglycaemia exerted pro-inflammatory, pro-atherothrombotic and arrhythmogenic effects. These studies, however, ignored the effects of hypoglycaemia on monocyte mobilisation, activation and monocyte-platelet interactions all of which are important in the pathophysiology of acute CV events. Further, longer lasting effects of hypoglycaemia on the innate immune system that could serve to increase CV risk had not been performed. Another issue was that cause and effect had not been demonstrated between hypoglycaemia and CV risk with ethical limitations preventing experimentation in humans that would yield these data. This has led some to argue that hypoglycaemia is simply a risk marker for susceptibility to adverse outcomes, including CV events, as it is more frequently encountered in those with underlying severe comorbid illnesses.

With existing deficiencies in the literature in mind, in this thesis I set out to investigate mechanisms through which hypoglycaemia could increase CV risk. I developed a novel human experimental model of combined hyperinsulinaemic hypoglycaemic, euglycaemic and sham-saline clamps with low dose intravenous endotoxin challenge 48 hours later to study innate immune cell function and platelet biology. In ApoE ^{-/-} mice fed a high fat Western diet, I first developed a

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novel model of recurrent hypoglycaemia on pre-established atherosclerosis. I then used my model to study the effects of recurrent hypoglycaemia on plaque biology in comparison to controls.

In this chapter I will describe the key findings from both human and murine experiments, explore limitations and suggest future work prior to drawing relevant conclusions.

6.1 Summary of human studies

The main findings from human work (chapter 3) were that an episode of acute moderate hypoglycaemia lasting 60 minutes in healthy volunteers: mobilised monocytes with preferential mobilisation of CD16⁺ pro-inflammatory subsets, increased platelet reactivity, encouraged formation of MPAs between pro-inflammatory monocytes and platelets and resulted in a significantly higher leukocyte response to low dose endotoxin challenge 48 hours later.

I showed for the first time that hypoglycaemia mobilises pro-inflammatory subsets of monocytes. This adds to the existing paradigm that an acute leukocytosis ensues following hypoglycaemia and is likely consequent upon sympatho-adrenal activation. I postulate that the catecholamine response to hypoglycaemia mobilises CD16⁺ monocytes from the marginal pool and these cells were preferentially mobilised on account of either richer β 2 adrenoreceptor expression or by being particularly sensitive to modulation through catecholamine signalling via β 2 adrenoreceptors. This hypothesis is supported

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by recent work that demonstrates that beta blockade inhibits NCM mobilisation in response to the adrenergic stress of acute exercise but fails to inhibit CM mobilisation (Graff *et al.*, 2018). Also, quantifying expression of $\beta 2$ adrenoreceptors on the three different monocyte subsets under steady state conditions and following adrenergic stress would provide data to test my hypothesis. Whilst this has been studied in other leukocyte subpopulations (Landmann, 1992), monocytes have received surprisingly little attention. Ratter *et al.* also recently studied leukocyte mobilisation in hypoglycaemia compared to euglycaemia in healthy volunteers and those with T1D with intact and impaired awareness of hypoglycaemia (Ratter *et al.*, 2017). The latter showed an increase in CD16 expression on peripheral blood mononuclear cells following hypoglycaemia which is complementary with my own findings, but they did not phenotype and enumerate distinct monocyte subsets. The authors also demonstrated that pro-inflammatory leukocyte mobilisation was diminished in those with T1D and impaired awareness of hypoglycaemia highlighting that counter-regulatory hormones modulate these changes. The same group have also in a subsequent publication demonstrated that hypoglycaemia mobilises NCM in those with T1D (Ratter *et al.*, 2018). These data, have therefore, reproduced and confirmed my original finding in healthy volunteers in those with T1D.

A consistent picture is now therefore emerging that hypoglycaemia mobilises pro-inflammatory monocyte subsets and that this may increase CV risk. However, it is well known that the adrenergic stress of exercise also preferentially mobilises CD16⁺ monocytes (B Steppich *et al.*, 2000; Graff *et al.*,

2018). Furthermore, indices of inflammation that have previously been shown to increase following hypoglycaemia including: platelet activation, endothelial dysfunction, pro-inflammatory cytokines and selectin release are also elevated following acute strenuous exercise in those without diabetes (Cwikiel *et al.*, 2018). The beneficial cardioprotective and anti-atherogenic effects of exercise are however, well established. In understanding this paradox it is important to revisit clinical trial data that suggest that in those with low atherosclerotic burden, intensive glycaemic control had potentially favourable effects in comparison to those that had a high CV disease burden where intensive glycaemic control was deleterious (Reaven *et al.*, 2009). It is therefore plausible that pro-inflammatory monocyte mobilisation and activation in those with little or no CAD serves as a normal physiological response to adrenergic stress in preparing the body for anticipated insult or injury. In those with diabetes and pre-established atherosclerosis, this inflammatory response in the context of the adrenergic stress of hypoglycaemia is, however, maladaptive as it may increase CV risk through plaque progression and/or instability.

I found that platelets are activated following hypoglycaemia and that they interact more readily with monocytes to form MPAs. Further, I showed that within monocyte subsets, IMs and NCMs aggregate with platelets more readily than CMs. As described in chapter 1, platelet activation following hypoglycaemia is catecholamine driven and MPA formation is a sensitive marker of both platelet and monocyte activation. Increased MPA formation following hypoglycaemia can therefore promote a pro-inflammatory milieu specifically by encouraging production of pro-inflammatory cytokines including TNF- α and CCL2 which can

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encourage monocyte recruitment to the atherosclerotic plaque (Neumann *et al.*, 1997; Martins *et al.*, 2006). Preferential formation of CD16⁺ monocyte specific MPA following hypoglycaemia is a novel and important finding given these have shown to be elevated post STEMI (Zhou *et al.*, 2016). My observation is at least in part due to a higher NCM and IM counts that were observed following hypoglycaemia, but also likely highlights the higher inflammatory potential of these subsets expressed as increased interaction with platelets under hypoglycaemic conditions. MPA formation following hypoglycaemia also demonstrates that both inflammatory and thrombotic pathways are activated creating a pro-atherogenic ensemble. Given that P2Y₁₂ antagonism using clopidogrel and ticagrelor significantly reduces MPA formation (M. R. Thomas *et al.*, 2015) these complexes maybe an important future target in mitigating hypoglycaemia associated CV risk. As a first step however, future studies of experimental hypoglycaemia in those with and without diabetes are needed to confirm or refute my observations of MPA formation and kinetics.

A key finding from my work was that hypoglycaemia compared to euglycaemia resulted in greater leukocyte mobilisation in response to low dose intravenous endotoxin challenge 2 days later. Hypoglycaemia thus appeared to prime the innate immune system and exerted durable effects. This finding is clinically important as it suggests that the pro-inflammatory effects of hypoglycaemia extend beyond the acute episode making it more likely to increase CV risk. These data are also in keeping with recent work from my group which showed that in those with T2D, hypoglycaemia exerted pro-thrombotic effects for up to 7 days (Chow *et al.*, 2018).

Interestingly, in my study, levels of leukocyte mobilisation post endotoxin were in fact very similar between sham-saline controls that received no insulin and the hyperinsulinaemic-hypoglycaemia group. Importantly, insulin exposure was matched between hypoglycaemia and euglycaemia groups. There are two important conclusions that can be drawn from this finding. First, consistent with known anti-inflammatory effects of insulin that I have previously described (chapters 1, 2, 3 and discussion chapter 4) hyperinsulinaemic-euglycaemia suppressed leukocyte mobilisation upon classical stimulation of the innate immune system with endotoxin challenge 48 hours later. Hypoglycaemia was, however, able to abolish these anti-inflammatory effects and resulted in a leukocyte response on endotoxin challenge that was comparable to those that had no prior insulin exposure (sham-saline group). Secondly, counter-regulatory hormones did not differ significantly between groups following endotoxin challenge and thus differences in leukocyte mobilisation were independent of this. This raises important questions as to how a single episode of moderate hypoglycaemia modulates the immune system to respond more robustly 2 days later. It is known that tissue exposure to chronic hyperglycaemia can result in epigenetic modifications which result in persistent vascular complications even if current glucose control is optimised (Keating *et al.*, 2018). Thus, hypoglycaemia may exert a variety of short-term effects on bone marrow cell production rates, intracellular signalling and sensitivity to or resistance to endotoxin signalling, and potential long-term changes in cellular function through epigenetic pathways, but which of these is operating here is not clear. This important issue needs to be disentangled in future studies.

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One limitation of my model is that it studied a relatively small number of healthy people that had no CV risk factors and did not have diabetes. This meant that my cohort was not representative of those studied in trials of intensive glycaemic control and also limits the applicability of my findings to older people with T1D or T2D and atherosclerosis. I was cognisant of this when designing my experiments, however, I wanted to safely establish my hitherto untested model of combined hypoglycaemia and endotoxin challenge in healthy volunteers first. I also chose the lowest possible dose of intravenous endotoxin that would yield sufficient stimulation of the innate immune system. This allows me to seamlessly test my model in older and more vulnerable people with diabetes in the future. Arguably, having three independent parallel groups is also a limitation in my study design as considerable heterogeneity can exist between individuals. A more robust model would involve each subject undergoing three separate clamps studies (hypoglycaemia, euglycaemia and sham-saline) followed by an endotoxin challenge 2 days later on each occasion with in-built washout periods of at least 2 weeks. Using this approach, each subject would thus act as their own control minimising the effects of inter-individual variability. I deemed this design, however, to be neither practical given time constraints nor ethically acceptable given the high experimental burden for each subject.

In addition to signalling via β 2 adrenoreceptors, adrenaline also promotes monocyte demargination from the vascular endothelium, mostly likely through modulating CD11a and CX₃CR1 mediated adhesion to the vessel wall (Dimitrov, Lange and Born, 2010). I quantified CX₃CR1 and CD11b expressions on monocytes, however, my data would have been strengthened further by

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quantifying CD11a expression on monocyte subsets following hypoglycaemia vs. controls. Also, in addition to focusing on monocyte subsets, in an ideal scenario, I would have liked to have studied the effects of hypoglycaemia on other cytotoxic leukocytes. Specifically, CD8⁺ T cells, NK and NKT cell kinetics would have been insightful given these cells have been implicated pathologically in atherosclerosis (Selathurai *et al.*, 2014; Cochain and Zerneck, 2016; van Puijvelde and Kuiper, 2017). Further, I would have wished to probe deeper the effects of hypoglycaemia on the inflammatory potential of monocytes. This could have been explored by monocyte purification following clamp and endotoxin challenge and study of MMP, IL-1 β , and IL-1ra protein expression and IL-1 β processing using qPCR and Western blotting. Finally, fate-mapping of human monocyte subsets in my model by using *in vivo* deuterium labelling for example would have allowed me to trace elegantly immune cell kinetics following hypoglycaemia and endotoxaemia.

6.2 Summary of animal studies

In the first phase of murine experimentation (chapter 4), I set out to develop and refine a novel combined model of recurrent hypoglycaemia and atherosclerosis in ApoE^{-/-} mice fed a high fat Western diet. Whilst developing my model, I elected to study male mice given observed sex differences in the glucose response to insulin. I then established a safe insulin dose range in Western diet fed mice. Next, I proceeded to induce recurrent hypoglycaemia vs. sham-saline injection in Western diet fed mice over a 3 week experiment. Here, I was able to demonstrate that I had successfully controlled for differences in body weight and

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serum lipids between groups. Importantly, I also proved biochemically that my hypoglycaemic stimulus was sufficient to induce a catecholamine response in my chosen strain. I noted rebound hyperglycaemia following recovery from hypoglycaemia in mice. I recognised this as a potential confounder given glycaemic flux from hypoglycaemia to hyperglycaemia in humans has been shown to worsen endothelial dysfunction and activate thrombosis (Ceriello *et al.*, 2012). In a further refinement, I avoided this when definitively testing my model (chapter 5). Exploratory assessment of atherosclerotic burden at the aorta during model development demonstrated no difference in atherosclerosis between groups and a higher lesion burden was noted in the control group. I reasoned that observed results may have been a consequence of the burden of hypoglycaemia not being sufficient to counter anti-atherogenic effects of hyperinsulinaemia. In further development, I therefore elected to double the hypoglycaemic exposure from 4 to 8 episodes as described in chapter 5. To my knowledge, I was able to develop for the first time a model of recurrent hypoglycaemia with established atherosclerotic plaque formation and a high level of animal survivability. One strength of my model is that it is amenable to modifications including increasing or decreasing the burden and severity of hypoglycaemia and atherosclerotic lesion burden. This model can be developed further and serve as a foundation for work exploring causality between hypoglycaemia and atherosclerosis.

In the second phase of animal experiments (chapter 5), I employed my mouse model to test the hypothesis that repetitive hypoglycaemia would increase atherosclerotic plaque burden and modulate the inflammatory phenotype of the

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plaque. My main findings were: compared to controls, recurrent hypoglycaemia resulted in a trend towards higher atherosclerotic burden at the aorta, but did not increase lesion burden or macrophages in brachiocephalic arteries, a common site of predilection for experimental atherosclerosis.

A trend towards higher atheroma formation following recurrent hypoglycaemia vs. sham-saline injection is an interesting finding. I was however short of power to enable me to draw any definitive conclusions. A published study has also reported no effect of recurrent hypoglycaemia on plaque burden in ApoE^{-/-} mice (Nakajima *et al.*, 2015). As discussed in chapter 5, however, the high insulin dose, infrequent glucose monitoring, high animal mortality and lack of high fat diet makes me sceptical of the methodology employed by Nakajima and colleagues.

In contrast to the aorta, MVG stained cross sections of the brachiocephalic artery demonstrated similar atherosclerotic lesion burden following hypoglycaemia vs. controls. It is tempting to hypothesise that hypoglycaemia may preferentially promote atheroma formation at some sites over others on account of local factors consequent upon haemodynamic and flow patterns in vascular beds. However, prior to exploring potential regional variation, it is imperative to first explore the effects of hypoglycaemia on lesion burden at the aortic sinus which I could not perform due to a lack of time. The aortic sinus is a known site of predilection for atheroma formation in murine models of atherosclerosis and has been extensively studied in this context (VanderLaan, Reardon and Getz, 2004).

There was no difference in plaque macrophage content between hypoglycaemia and control conditions at sacrifice. As I discuss in chapter 5, this finding may not be out of keeping with clinical data in humans. I postulate that the effects of hypoglycaemia in modulating the inflammatory phenotype of the plaque may not have been captured over the relatively short course of my experiments and that cumulative exposure to repeated episodes of hypoglycaemia over a longer period would definitively test this. Overall, whilst suggesting emerging trends, my experiments using this model did not definitively confirm nor refute my hypothesis that hypoglycaemia worsens atherosclerosis. Thus, the question of a direct causal relationship between hypoglycaemia and CV events at least in the context of experimental animal models still remains largely unanswered.

One major limitation of my animal model was the lack of sufficient power to enable me to test my hypothesis more robustly. Whilst statistical power appeared to just fall short of significance for some data, having had more animals in each group would have certainly strengthened the study. The primary reason for this was difficulty in accurately estimating power prospectively whilst designing my experiments in a first of its kind model. Further, I was conservative in my use of animals in line with the 3R's principles. Also, it can be argued that selecting the total experiment duration over 4 weeks was a somewhat arbitrary time point. I wanted to ensure mice were exposed to repeated episodes of hypoglycaemia over a number of weeks but also had to balance this with the severity of protocol allowed on the licence and ethical limits to the frequency and volume of blood sampling. This alongside side practical, monetary and time considerations meant I could not perform additional assays to study: insulin,

glucagon, platelets, monocytes and progenitor cells. These data would have been of value.

A more fundamental limitation of animal experimentation is its applicability to humans given known differences in biology between man and mouse. Even with best efforts to mimic human disease, data yielded from animal experimentation is open to question in its applicability to humans. There are also inherent differences in atherosclerosis and CV physiology between these species. ApoE ^{-/-} mice unlike humans for example rarely develop plaques within the coronary arteries (Nakashima *et al.*, 1994). In addition, elevated heart rate and diurnal heart rate variation are both associated with atherosclerotic plaque formation (Bassiouny *et al.*, 2002) and in a C57BL/6 mouse the average heart rate is 550 beats per minute compared to 70 in an average human (Tiemann *et al.*, 2003). Despite the challenges of recapitulating human disease in animal models and then translating findings back, causality between hypoglycaemia and atherosclerosis cannot be proved ethically in human studies. In lieu of this, use of animal models and particularly ApoE ^{-/-} mice can offer valuable insights. This is because whilst complex atherosclerotic plaques can be established in a number of animal models, plaque rupture and thrombosis are critical to acute CV events. ApoE ^{-/-} mice have been shown to display features of plaque rupture in their brachiocephalic arteries that are consistent with human pathology (Johnson and Jackson, 2001).

I studied atheroma formation by examining *en face* oil red O stained aortae. This is a recognised technique in assessing lesion burden. Interpretation of data

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derived from this method is, however, caveated. Whilst I meticulously dissected fat from the outer vessel wall of all specimens as described in chapter 2, invariably some tissue may have adhered to the vessels. As I quantified atheroma in all vessels relative to the total vessel area, residual fat on certain vessels could have influenced results. Thus, this method of lesion assessment is somewhat crude. Being aware of this, and also of the potential for introduction of bias, I had my histological data for oil red O stained aortae analysed by a colleague blinded to mouse group allocation which yielded results that were comparable to my analysis.

6.3 Future directions

The findings presented in my thesis present ample opportunities for advancing the field through parallel human and animal experimentation which I will discuss below. Some of the described work is along themes that are going to be explored in the Innovate Medicine Initiative (IMI) funded 'Hypoglycaemia redefining solutions for better lives' (Hypo-RESOLVE) project (<https://hypo-resolve.eu/project>). I am, as part of my research group in Sheffield, a future collaborator in these studies.

Human studies

I and other have demonstrated that acute hypoglycaemia can mobilise pro-inflammatory leukocyte subsets in healthy volunteers and those with T1D (Ratter *et al.*, 2017, 2018; Iqbal *et al.*, 2019). I also showed that hypoglycaemia activates the immune system for at least 2 days where the acute adrenergic

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stress from the initial episode is not implicated (chapter 3)(Iqbal *et al.*, 2019).

Whilst circulating cell kinetics have been studied, deeper effects of hypoglycaemia on the pro-inflammatory and thus pro-atherosclerotic properties of monocytes and other leukocytes remains unresolved. Also, the mechanisms for the longer lasting pro-inflammatory effects of hypoglycaemia are unknown.

A logical extension of this work is to study subjects with insulin treated T2D, but in the absence of established CVD or significant CV risk factors. By employing hyperinsulinaemic hypoglycaemic (2.5 mmol/l) and euglycaemic clamps (6.0 mmol/l), one could first enumerate and phenotype monocytes, T cells, NK, NKT and monocyte subset specific MPAs in this cohort to confirm or refute findings existing findings. To explore the impact of hypoglycaemia on inflammatory potential, it will then be important to isolate monocytes immediately following clamp and 7 and 30 days later to probe longitudinal effects. One could then examine the impact of hypoglycaemia on monocytes by assessing *ex vivo* the pro-inflammatory cytokine response (TNF- α , IL-1 β , IL-1ra, sCX3CL1 and CCL2) to LPS stimulation. Further, it will be important to prepare isolated monocytes for RNA sequencing of pro-inflammatory genes (IL-1 β , MMP) and epigenetic analysis (DNA methylation and histone acetylation). In addition to focus on monocytes, another interesting angle would be to examine both the short and medium term effects of hypoglycaemia on circulating progenitor cells that contribute to plaque structure. Circulating haematopoietic stem cells for example have been shown to differentiate into VSMCs that are known to play a part in arterial remodelling and atherosclerosis (Sata *et al.*, 2002).

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If one of the mechanisms through which hypoglycaemia does indeed promote atherosclerosis is the cumulative and chronic traffic of pro-inflammatory monocytes to the atherosclerotic plaque, then what potential therapeutic strategies can be employed to mitigate this? In translating this body of work for future patient benefit, two potential therapeutic strategies could be explored. First, pro-inflammatory NCMs and IMs have a unique CX₃CR1^{high} CCR2^{low} chemokine receptor profile. CCR2 plays an integral role in monocyte egress from the bone marrow, recruitment to atherosclerotic lesions (Zernecke, Shagdarsuren and Weber, 2008) and CCR2 gene deletion in a double knock-out mouse model has delayed atherosclerotic lesion progression (Boring *et al.*, 1998). Thus, modulating the CCL2-CCR2 axis maybe an appealing target in potentially reducing hypoglycaemia associated CV risk. An oral antagonist to CCR2 has been tested in human patients with rheumatoid arthritis (Vergunst *et al.*, 2008). Whilst the results were disappointing in rheumatoid disease, there maybe merit in exploring this avenue in those with diabetes, CVD and a propensity to recurrent hypoglycaemia. As diabetes is a chronic condition and hypoglycaemia commonly encountered over many years, any agent would conceivably need to be taken on a prophylactic basis to reduce CV risk. This of course raises issues around drug tolerability, potential immunosuppression and off target effects.

As pro-inflammatory leukocyte subsets are likely mobilised via the adrenergic stress associated with hypoglycaemia, beta blockade could inhibit this effect. As demargination of CD16⁺ cells is primarily mediated through β₂ adrenoreceptors (Graff *et al.*, 2018) a non-specific beta blocker such as nadolol or propranolol

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would need to be used. Also as an additional potential mechanism linking hypoglycaemia and CV risk is related to haemodynamic changes and tachyarrhythmias as described in chapter 1, combined β_1 and β_2 blockade would potentially target two distinct risk factors. Indeed, combined β_1 and β_2 blockade has been shown to reduce lethal cardiac arrhythmias in rats (Reno *et al.*, 2013), although the hypoglycaemic stimulus in this study was extreme (< 1.0 mmol/l) and not commonly encountered in humans. It seems somewhat counterintuitive, however, to consider at least combined beta blockers in those already at risk of hypoglycaemia as these agents are known to impair glycogenesis, gluconeogenesis and lipolysis thus prolong and enhance hypoglycaemia (Mills and Horn, 1985). The answer could lie in the use of selective blockade of β_1 adrenoreceptors by using agents such as bisoprolol for example, however, this would not inhibit CD16⁺ monocyte mobilisation. Worryingly, a group that recently studied the ACCORD population reported that beta blocker use *per se* was associated with a high risk of severe hypoglycaemia and an increased risk of CV events (Tsujiimoto *et al.*, 2017).

Currently, glycaemic targets need to be individualised and relaxed in those with diabetes that have a history of hypoglycaemia and CV risk as recently recommended by the ADA (American Diabetes Association, 2019).

Animal studies

In order to examine the relationship between hypoglycaemia and CV events a more complete and robust animal model is required. Building on my model, future studies could study ApoE ^{-/-} mice fed Western diet to develop

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atherosclerosis and then treatment with low dose streptozotocin to induce T2D, thus yielding a combined polygenic model of atherosclerosis and diabetes. Then by using standard insulin release implant (Linplant) mice could be insulin treated to achieve moderate glucose control thus more closely mimicking the ACCORD population. On this background of chronic hyperglycaemia, episodes of recurrent hypoglycaemia or control saline injection would then be superimposed over several weeks. In this model, *in vivo* assessment would involve assessment of CV function including telemetry and endothelial function assessed by flow mediated dilatation as previously described (Wang *et al.*, 2015). Flow cytometric assays to phenotype murine monocyte subsets and circulating progenitor cells during hypoglycaemia and over longitudinal time points would compliment future human experiments as described. In order to assess inflammation further, mice would be splenectomised at sacrifice to study immune cells focussing on HSCs (Dutta *et al.*, 2012; Robbins *et al.*, 2012). Assessment of atherosclerotic lesion burden would also demand assessment at three anatomical sites including the aorta, brachiocephalic artery and aortic sinus. To further characterise the inflammatory signal of the plaque following hypoglycaemia, one option could be to isolate plaque macrophages using laser capture micro-dissection and extracting RNA (Feig and Fisher, 2013) for micro-array analysis to assess expression of genes that maybe differentially expressed following recurrent hypoglycaemia compared to controls. Additionally, epigenetic analysis of CV tissue at sacrifice would be important to assess the chronic impact of hypoglycaemia.

6.4 Conclusions

Hypoglycaemia remains a major barrier to intensive treatment with insulin in those with diabetes. A significant body of data now exists to support a relationship between hypoglycaemia and CV events or death. In this thesis, I present novel findings from human mechanistic studies to support my original hypothesis that hypoglycaemia induces durable activation of the innate immune system which can accelerate atherosclerosis. Furthermore, I developed for the first time a murine model of recurrent hypoglycaemia and combined atherosclerosis to explore in more detail this relationship. My animal model can be developed further to test more definitively the consequences of hypoglycaemia on the plaque. Also, further investigation in humans with diabetes is warranted to first dissect further, and then potentially therapeutically target the inflammatory pathways I have identified. This could ultimately impact positively on the lives of millions of people with diabetes who encounter hypoglycaemia and its deleterious consequences.

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Appendix

Effect of Hypoglycemia on Inflammatory Responses and the Response to Low-Dose Endotoxemia in Humans

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Context: Hypoglycemia is emerging as a risk for cardiovascular events in diabetes. We hypothesized that hypoglycemia activates the innate immune system, which is known to increase cardiovascular risk.

Objective: To determine whether hypoglycemia modifies subsequent innate immune system responses.

Design and Setting: Single-blinded, prospective study of three independent parallel groups.

Participants and Interventions: Twenty-four healthy participants underwent either a hyperinsulinemic-hypoglycemic (2.5 mmol/L), euglycemic (6.0 mmol/L), or sham-saline clamp (n = 8 for each group). After 48 hours, all participants received low-dose (0.3 ng/kg) intravenous endotoxin.

Main Outcome Measures: We studied *in-vivo* monocyte mobilization and monocyte-platelet interactions.

Results: Hypoglycemia increased total leukocytes ($9.98 \pm 1.14 \times 10^9/L$ vs euglycemia $4.38 \pm 0.53 \times 10^9/L$, $P < 0.001$; vs sham-saline $4.76 \pm 0.36 \times 10^9/L$, $P < 0.001$) (mean \pm SEM), mobilized proinflammatory intermediate monocytes ($42.20 \pm 7.52/\mu L$ vs euglycemia $20.66 \pm 3.43/\mu L$, $P < 0.01$; vs sham-saline $26.20 \pm 3.86/\mu L$, $P < 0.05$), and nonclassic monocytes ($36.16 \pm 4.66/\mu L$ vs euglycemia $12.72 \pm 2.42/\mu L$, $P < 0.001$; vs sham-saline $19.05 \pm 3.81/\mu L$, $P < 0.001$). Following hypoglycemia vs euglycemia, platelet aggregation to agonist (area under the curve) increased (73.87 ± 7.30 vs 52.50 ± 4.04 , $P < 0.05$) and formation of monocyte-platelet aggregates increased ($96.05 \pm 14.51/\mu L$ vs $49.32 \pm 6.41/\mu L$, $P < 0.05$). Within monocyte subsets, hypoglycemia increased aggregation of intermediate monocytes ($10.51 \pm 1.42/\mu L$ vs euglycemia $4.19 \pm 1.08/\mu L$, $P < 0.05$; vs sham-saline $3.81 \pm 1.42/\mu L$, $P < 0.05$) and nonclassic monocytes ($9.53 \pm 1.08/\mu L$ vs euglycemia $2.86 \pm 0.72/\mu L$, $P < 0.01$; vs sham-saline $3.08 \pm 1.01/\mu L$, $P < 0.05$), with platelets compared

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Abbreviations: CM, classic monocyte; CV, cardiovascular; CX₃CR1, CX₃C chemokine receptor 1; FACS, fluorescence-activated cell sorting; IM, intermediate monocyte; MPA, monocyte-platelet aggregate; NCM, nonclassic monocyte; PE, phycoerythrin; WBC, white blood cell.

with controls. Hypoglycemia led to greater leukocyte mobilization in response to subsequent low-dose endotoxin challenge (10.96 ± 0.97 vs euglycemia $8.21 \pm 0.85 \times 10^9/L$, $P < 0.05$).

Conclusions: Hypoglycemia mobilizes monocytes, increases platelet reactivity, promotes interaction between platelets and proinflammatory monocytes, and potentiates the subsequent immune response to endotoxin. These changes may contribute to increased cardiovascular risk observed in people with diabetes. (*J Clin Endocrinol Metab* 104: 1187–1199, 2019)

Hypoglycemia is associated with a greater propensity to adverse cardiovascular (CV) outcomes in diabetes (1–3). To determine whether such outcomes were dependent on changes in innate immune responses, we devised a model whereby subjects were challenged with a hypoglycemic clamp, and then the durable effects on the innate immune system were probed by an *in vivo* endotoxin challenge 48 hours later.

Iatrogenic hypoglycemia remains a major barrier to effective treatment of insulin-treated diabetes (4). The Action to Control Cardiovascular Risk in Diabetes trial showed that intensive glucose control, during which patients were exposed to significantly more hypoglycemia (5), was associated with excess CV mortality. Despite the evidence confirming an association between hypoglycemia and mortality, cause and effect has not been established. Trial evidence suggests that the relationship is, at least in part, explained by confounding, that is, that hypoglycemia identifies patients with comorbidities who are both vulnerable to hypoglycemia and more likely to die for other reasons (6). Nevertheless, a recent large meta-analysis (7) suggested that comorbidities alone are unlikely to explain this relationship. Furthermore, there is a growing body of evidence highlighting a number of mechanisms whereby hypoglycemia may lead to CV events (8, 9).

Hypoglycemia has proinflammatory consequences, including increases in levels of factor VIII and von Willebrand factor and impaired fibrinolysis (10–12). In addition, hypoglycemia has been shown to increase proinflammatory cytokines (12–14) and promote rises in the levels of proatherogenic cell adhesion molecules (12). Repeated episodes of hypoglycemia have also been reported to impair nitric oxide–mediated vasodilation (15).

Monocytes are phagocytes that are central to the etiology of atherosclerosis (16) and play a role in precipitating acute CV events by promoting plaque destabilization and rupture (17). The extent to which monocytosis and monocyte activation are modified by hypoglycemia remains uncertain. Recent studies have also determined that monocytes can be classified into three distinct subsets, called classic monocytes (CMs: CD14⁺⁺ CD16[–], and Mon1), intermediate monocytes (IMs: CD14⁺⁺ CD16⁺, Mon2), and nonclassic monocytes (NCMs: CD14⁺ CD16⁺⁺, Mon3) (18, 19). A

number of observational studies indicate that IMs may be particularly proatherogenic. Elevated levels of IMs are associated with adverse CV outcomes (20–23), independently predict future CV events (22), and have been associated with coronary plaque vulnerability in patients with angina (24). Elevated levels of CMs may also independently predict CV events (25).

Acute myocardial infarction results in monocytosis, mediated by sympathetic nervous system activation (26). In humans, CD16⁺ monocytes selectively mobilize, in a catecholamine-dependent fashion, after exercise (27). Because epinephrine is the key counterregulatory hormone produced in response to hypoglycemia, we hypothesized that hypoglycemia would also exert significant effects on monocytes. We further hypothesized that we would see additional synergistic changes in monocyte and platelet activation, as revealed by formation of monocyte-platelet aggregates (MPAs), which are increased after acute myocardial infarction (20, 28). In large prospective studies, CV events did not appear to occur during the hypoglycemic episode *per se*, but there was an increased risk of events in the weeks and months after the episode (29–31). Therefore, we hypothesized that acute hypoglycemia may prime the innate immune system, leading to a more pronounced inflammatory response to a subsequent inflammatory stimulus downstream from the initial episode of hypoglycemia. It is also relevant to note that people with diabetes experience increased incidences of acute and chronic infections that will further activate innate immunity. To reveal whether hypoglycemia modulated monocyte function in the human *in vivo*, we chose to combine a classic hypoglycemic stimulus with a subsequent *in vivo* systemic stimulus of the innate immune system. To do so, we combined hyperinsulinemic-hypoglycemic, euglycemic, and sham-saline clamps with low-dose intravenous endotoxin challenge 48 hours later in healthy participants. Endotoxin, otherwise known as gram-negative bacterial lipopolysaccharide, was used because it induces a short-lived, sterile inflammation that is both safe and reproducible (32).

Materials and Methods

Study design and participants

This was a single-blinded, prospective study of three independent parallel groups (hyperinsulinemic-hypoglycemia, euglycemia, and sham-saline controls) conducted in a random group order at the Clinical Research Facility, Northern General Hospital, Sheffield, United Kingdom between January 2015 and April 2016. We therefore had three groups that had euglycemia with insulin, hypoglycemia with insulin, or saline. Each then received endotoxin. Baseline values at the start of endotoxin administration were studied in all groups, providing a set of data obtained before endotoxin. A total of 24 healthy participants without diabetes were recruited from the University of Sheffield and Sheffield Teaching Hospitals, with written informed consent in accordance with a protocol approved by Yorkshire and the Humber-Sheffield Research Ethics Committee (REC 14/YH/1264). All participants had a HbA1c <6.5% (<48 mmol/mol), measured with ion exchange high-performance liquid chromatography, and none had impaired glucose tolerance based on HbA1c as judged by the American Diabetes Association criteria (33, 34). Participants were in good health, as determined by a medical history, physical examination, vital signs, and clinical laboratory test results including full blood count and renal and liver function. Those with an intercurrent illness in the previous 4 weeks were excluded. Participants taking beta-blockers, QT interval-prolonging agents, and anticoagulant, antiplatelet, or anti-inflammatory medications were also excluded. Female participants were on secure contraception and also had negative urinary pregnancy tests on the morning of the clamp and endotoxin studies.

Clamp studies

All participants attended at 0800 hours after an overnight fast and were blinded to their group allocation. Participants were instructed to avoid caffeine, alcohol, and vigorous exercise 24 hours before the study visit. An intravenous cannula was inserted into the antecubital fossa of the nondominant arm for insulin and dextrose infusion. A second intravenous cannula was inserted into the antecubital fossa of the dominant arm for all blood measurements except glucose. After application of a local anesthetic cream (lidocaine/prilocaine; Astra-Zeneca, Macclesfield, UK) to the dorsal hand or wrist of the nondominant arm, a retrograde cannula was inserted and the hand placed in a warming chamber (The Sheffield Hand Warmer, Sheffield, UK) at 55°C to allow arterialization of venous blood for glucose measurement. In the hypoglycemia and euglycemia study groups, a primed continuous insulin (Human Actrapid; Novo Nordisk Pharmaceuticals LT, Crawley, UK) infusion was administered at a rate of 90 mU/m²/min, with total insulin exposure matched between groups. A 20% dextrose (Baxter Healthcare Ltd., Thetford, UK) variable-rate infusion was administered simultaneously and the rate adjusted according to arterialized whole blood glucose concentrations measured every 5 minutes with a glucose oxidase method (Yellow Springs Instrument 2300 STAT, Yellow Springs, Ohio). After a brief (30-minute) euglycemic phase in both groups, blood glucose was lowered to 2.5 mmol/L in the hypoglycemia group and maintained for 60 minutes at this level. In the euglycemia group, blood glucose was maintained at 6 mmol/L for 60 minutes. Participants in the sham-saline group were investigated identically but did not receive insulin/dextrose infusions and instead

received a slow intravenous infusion of 0.9% NaCl (Baxter, Baxter Healthcare Ltd.) at a predetermined fixed rate. Thus, participants in the sham-saline group were under normoglycemic conditions, allowing us to control for the effects of insulin and dextrose. Blood was sampled at baseline and at 60 minutes. Members of staff processing assays were blinded to glucose group allocation.

Endotoxin challenge

Endotoxin challenge is a safe and well-studied model of innate immune activation *in vivo* (35). Forty-eight hours after the clamp, participants reattended at 0800 hours, having fasted overnight and refrained from caffeine, alcohol, and vigorous exercise since the clamp visit. An intravenous cannula was inserted into the antecubital fossa of the nondominant arm for administration of endotoxin and a second cannula inserted into the contralateral antecubital fossa for blood sampling. All participants received 0.3 ng/kg *Escherichia coli* O:113 lipopolysaccharide (Clinical Centre Reference Endotoxin; National Institutes of Health, Bethesda, MD). Endotoxin powder was reconstituted in 1 mL sterile 0.9% NaCl to form a solution at a concentration of 1000 ng/mL, which was vortexed for 60 minutes. The weight-adjusted dosage of endotoxin was obtained from this solution, added to 5 mL of 0.9% NaCl, and administered as a slow bolus injection over 1 minute. An intravenous infusion of 500 mL of 0.9% NaCl (Baxter Healthcare Ltd.) then continued for 4 hours after endotoxin to avoid hypotension. Venous blood was sampled at baseline and 2, 4, and 6 hours after endotoxin. All laboratory measurements were performed by staff blinded to glucose group allocation.

Biochemical analysis

To measure epinephrine, venous forearm blood was collected into chilled lithium heparin tubes and centrifuged at 4°C, 1000g for 10 minutes. The resulting supernatant was stored at -80°C until assayed by high-performance liquid chromatography. To determine insulin levels, EDTA-anticoagulated blood was centrifuged at 3000g for 10 minutes, and free insulin levels were measured in the resulting plasma with an immunoassay (Roche Cobas; Roche Diagnostics, Burgess Hill, West Sussex, UK). Venous blood was centrifuged at 3000g for 10 minutes, and the resulting serum was used to measure cortisol and GH with an immunoradiometric assay (Roche Cobas). Sample collection for cortisol and GH was controlled for time of day across the three study groups.

Cell counts and flow cytometry

Total and differential white blood cell (WBC) and platelet counts in EDTA-anticoagulated blood were determined with an automated clinical grade Sysmex cell counter (XN-9000; Sysmex, Milton Keynes, UK). For the clamp visit, alternative WBC counting methods were piloted for the first two subjects in each group, but these were later deemed less accurate than the Sysmex cell counter. Data shown in Figs. 2, 3, and 4a, 4c, 4d, 4e, and 4f are Sysmex data from n = 6 in each study group. Flow cytometry was used to determine MPAs: blood was collected into tubes containing trisodium citrate dihydrate (3.13% w/v) and incubated in a heat block at 37°C for 10 minutes; erythrocytes were lysed with fluorescence-activated cell sorting (FACS) lyse solution (BD, Oxford, UK) and stained with fluorescein isothiocyanate-conjugated CD16 (BioLegend, London,

UK), allophycocyanin-conjugated CD14 (BioLegend), and phycoerythrin (PE)-conjugated CD42a (BD) in addition to matched isotype controls. Cells were fixed with FACS Fix (BD) and analyzed with flow cytometry (Accuri C6 multicolor flow cytometer; BD) within a consistent time frame for all subjects. Monocytes were gated based on morphology and CD14 expression. Neutrophils were gated on morphology and through exclusion of monocytes. Monocyte-platelet aggregation was determined by measuring monocyte mean fluorescence of the platelet-specific marker CD42a. To phenotype and enumerate monocyte subsets, anticoagulated blood was stained with fluorescein isothiocyanate-conjugated CD16 (BioLegend), allophycocyanin-conjugated CD14 (BioLegend), PE-conjugated CD66c (BD), PE-Cy7-conjugated CD11b (BioLegend), and (PerCP)-eFluor® 710-conjugated CX₃C chemokine receptor 1 (CX3CR1; eBioscience, Altrincham, UK). Matched isotype controls and a “fluorescence minus one” strategy optimized compensation. Stained whole blood was lysed with FACS lyse as above and the pellet resuspended in PBS before fixation with 1% w/v formaldehyde. Samples were immediately processed for analysis with flow cytometry (LSRII; BD). Monocytes were gated based on morphological characteristics and through the exclusion of neutrophils with CD66c. Monocyte subsets were identified based on relative expression of CD14 and CD16. Flow cytometry data were analyzed with FlowJo version 10 (FlowJo LLC, Ashland, OR).

Platelet aggregation

Platelet aggregation was measured with impedance aggregometry (Multiplate®; Verum Diagnostica GmbH, Munich, Germany). Aliquots of 300 μ L saline and 300 μ L hirudin-anticoagulated blood were added to the cuvette and incubated at 37°C for 3 minutes. Then 20 μ L ADP (at a final concentration of 6.45 μ M) was added as agonist, and the assay commenced. The area under the curve was measured, which represents the level of platelet aggregation.

Statistical analysis

Our pilot data indicated that a sample size of seven participants per group would have 90% power to detect a 50% relative difference in mobilization of monocytes between hypoglycemia and controls. Eight subjects were recruited per group to allow for a 13% dropout rate. Mean baseline measurements of glucose were compared between groups with ANOVA. Mean measurements of glucose, insulin, and epinephrine at 60 minutes after clamp were compared, adjusting for clamp baseline measurement, with analysis of covariance. In

the event of unequal variance between groups, a log transform was applied and the analysis repeated. Longitudinal and between-group comparisons were made for postendotoxin measurements with mixed effects linear models. For models examining between-group differences, the baseline endotoxin measurement was included as a covariate. For all mixed-effects linear models, an autoregressive correlation structure was used to allow for the correlation between multiple measurements on the same person. Planned contrasts were made with baseline and between groups at equivalent time points with Sidak correction for multiple comparisons. All data are expressed as mean \pm SEM, unless otherwise specified, and a $P < 0.05$ was deemed statistically significant. Analysis was performed in SPSS version 22.0 (IBM, Chicago, IL).

Results

Participants

Study participants across the three groups were well matched for age, sex, body mass index, HbA1c, and total WBC count, with no significant differences at screening (Table 1). Participant numbers at each stage of study are illustrated in a flow diagram (Supplemental Flow Diagram).

Clamp studies

Glucose, insulin, and counterregulatory hormones

Arterialized blood glucose values are shown in Fig. 1a. The glucose values were 2.51 ± 0.11 mmol/L and 6.04 ± 0.16 mmol/L at the end of the hypoglycemia and euglycemia clamps, respectively. Glucose values at the end of the sham-saline clamp were 4.64 ± 0.09 mmol/L. A counterregulatory response to hypoglycemia was evident, with epinephrine levels during hypoglycemia (1.87 ± 0.25 nmol/L) being significantly higher ($P < 0.001$) than those during euglycemia (0.07 ± 0.01 nmol/L) and sham-saline (0.10 ± 0.04 nmol/L) (Fig. 1b). Free insulin levels at the end of clamp were similar between the hypoglycemia (968.5 ± 149.1 pmol/L) and euglycemia groups (1025.4 ± 81.4 pmol/L, $P = 0.996$) but significantly higher ($P < 0.001$) than those in the sham-saline (31.3 ± 6.3 pmol/L) group (Fig. 1c). Serum cortisol and

Table 1. Comparison of Participant Characteristics at Baseline

Parameter	Hypoglycemia	Euglycemia	Sham-Saline	<i>P</i>
Total (n = 24)	8	8	8	N/A
Sex, M/F	4/4	4/4	4/4	N/A
Age, y	21 (19–22)	21 (20–23)	21.5 (21–26)	0.299
Body mass index, kg/m ²	24 \pm 2	23 \pm 2	24 \pm 4	0.638
HbA1c				
%	5.2 \pm 0.31	5.2 \pm 0.26	5.1 \pm 0.14	0.792
mmol/mol	34 \pm 3.6	33.5 \pm 2.8	32.6 \pm 1.4	0.616
Total WBCs, $\times 10^9$ /L	6.26 \pm 1.42	4.83 \pm 0.91	4.50 \pm 1.69	0.102

Data are mean \pm SD or median (interquartile range). *P* values indicate comparisons between study groups via parametric or nonparametric testing.

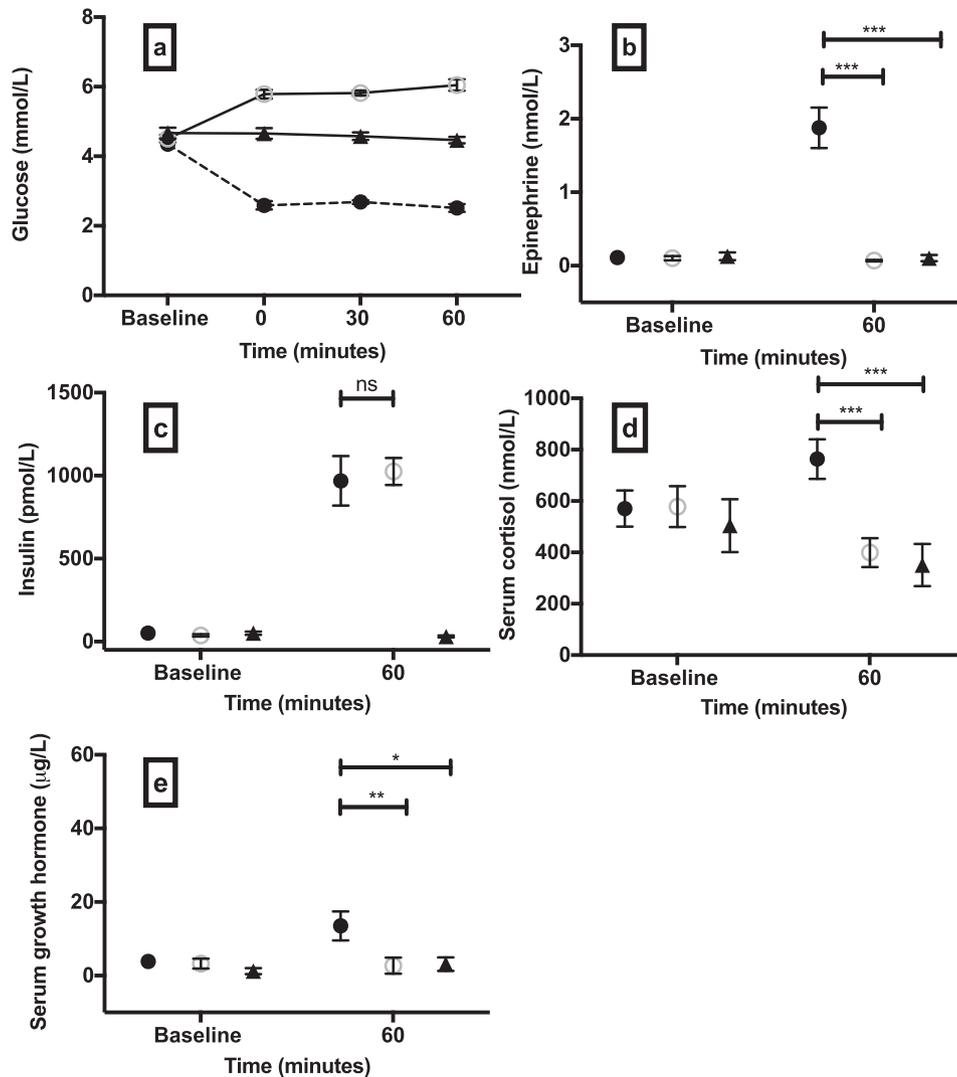


Figure 1. Glucose, insulin, and counterregulatory hormones in clamp studies. (a) Arterialized whole blood glucose values during hyperinsulinemic hypoglycemic, euglycemic, and sham-saline clamps, (b) epinephrine, (c) free insulin, (d) cortisol, and (e) GH values after 60 min of hypoglycemia, euglycemia, or sham-saline injection. Data are mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns-nonsignificant; P values are provided for comparison between study groups. Black circles (and dashed line 1a), hypoglycemia group; open circles, euglycemia group; black triangles (and solid line 1a), sham-saline group.

GH were significantly higher in the hypoglycemia group than in the euglycemia and sham-saline groups (Fig. 1d and 1e).

Total and differential leukocyte count

We determined whether hypoglycemia results in changes in circulating leukocytes. Hypoglycemia significantly increased the total number of WBCs compared with controls (Fig. 2a). There was an increase across all classes of leukocytes studied, including neutrophils (Fig. 2b), lymphocytes (Fig. 2c), and total monocytes (Fig. 3a).

Monocyte subsets

We sought to determine whether hypoglycemia exerted specific effects on monocyte subsets associated with cardiac pathology. Hypoglycemia increased the absolute

number of all three circulating monocyte subsets compared with euglycemia and sham-saline (Fig. 3b–3d). The number of circulating NCMs after 60 minutes of hypoglycemia compared with baseline (17.6 ± 2.9 cells/ μ L) increased twofold. IM numbers after 60 minutes of hypoglycemia compared with baseline (23.2 ± 4.5 cells/ μ L) increased by a factor of 1.81, and CMs after 60 minutes of hypoglycemia compared with baseline (442.4 ± 55.3 cells/ μ L) increased by a factor of 1.29. There were no significant differences in the baseline values of all three monocyte subsets between the study groups.

Platelet count, aggregation, and MPAs

Activation of platelets and generation of platelet-leukocyte aggregates contribute to leukocyte mobilization and inflammation in the vasculature (36). We therefore studied platelet number and function and their

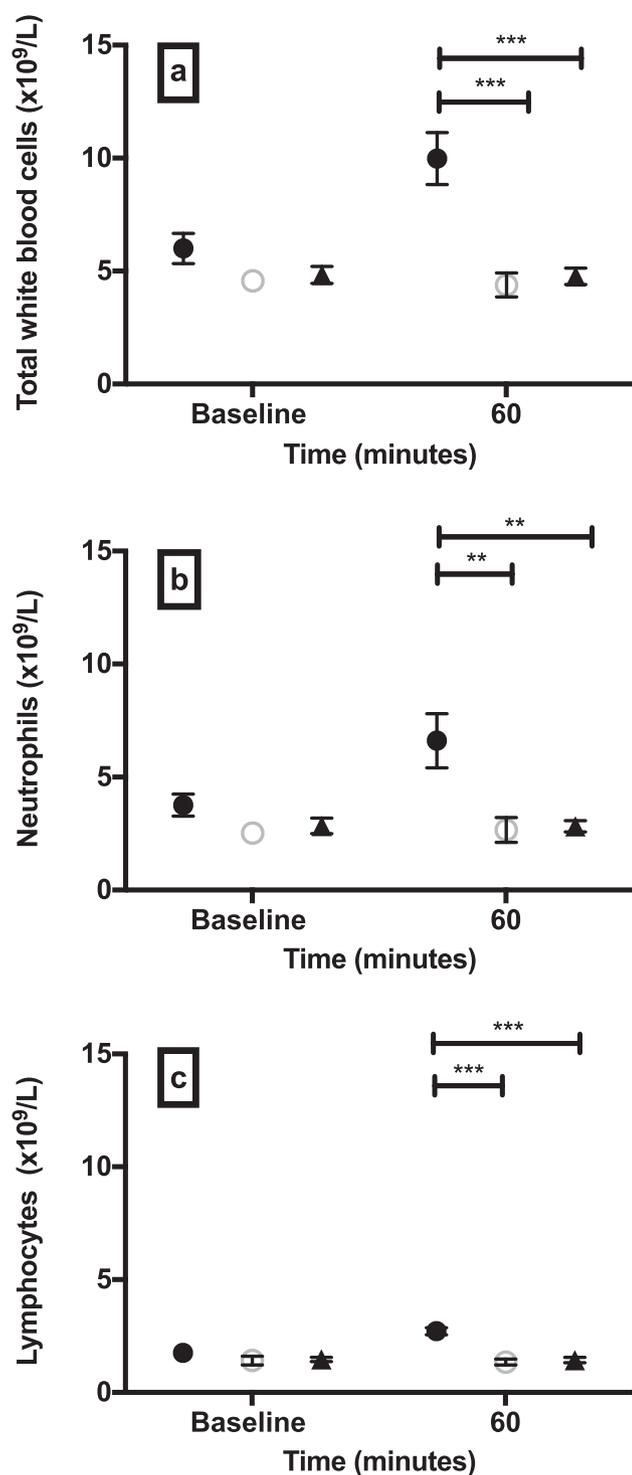


Figure 2. Peripheral total WBC, neutrophil, and lymphocyte kinetics in experimental hypoglycemia and controls. Number of circulating (a) total WBCs, (b) neutrophils, and (c) lymphocytes after 60 min of hypoglycemia, euglycemia, or sham-saline injection. Data are mean (SEM). ** $P < 0.01$; *** $P < 0.001$; P values are provided for comparison between study groups. Black circles, hypoglycemia group; open circles, euglycemia group; black triangles, sham-saline group.

interaction with leukocytes. Total platelet count increased in hypoglycemia compared with euglycemia and sham-saline controls (Fig. 4a). ADP-induced platelet

aggregation increased after 60 minutes of hypoglycemia vs euglycemia ($P = 0.014$), and numerically (but not statistically significantly) higher platelet aggregation was detected in the hypoglycemia group compared with the sham-saline group ($P = 0.064$) (Fig. 4b). The total number of MPAs increased after 60 minutes of hypoglycemia compared with euglycemia (Fig. 4c). Although total MPAs were not significantly higher in the hypoglycemia group compared with sham-saline controls at 60 minutes (Fig. 4c), we observed specific increases in NCM-MPAs and IM-MPAs (Fig. 4d and 4e). CM-MPAs appeared to increase after 60 minutes of hypoglycemia vs euglycemia and sham-saline, but this increase was not statistically significant ($P = 0.054$) (Fig. 4f).

Cell surface markers

To further explore the activation state of monocytes after hypoglycemia, we studied expression levels of chemokine receptor CX₃CR1 and integrin CD11b. Hypoglycemia did not alter the expression of CX₃CR1 or CD11b (Supplemental Fig. 1a and 1b).

Endotoxin challenge

To determine whether previous hypoglycemia affected the subsequent response to a classic immune activator and thus to reveal whether hypoglycemia had any longer-lasting effects on the innate immune system, we performed a low-dose intravenous endotoxin challenge 48 hours after the hypoglycemic challenge in all subjects. Consistent with the low-dose model used, no fever or significant change in mean arterial blood pressure was recorded after endotoxin challenge across the study groups.

Epinephrine, cortisol, and GH

In contrast to the stress response induced by hypoglycemia, epinephrine levels were not significantly different between study groups 6 hours after endotoxin administration (Fig. 5a). In the hypoglycemia group, epinephrine levels were 0.15 ± 0.04 nmol/L vs 0.06 ± 0.01 nmol/L in euglycemia group and 0.09 ± 0.01 nmol/L in sham-saline group. There were also no differences detected between groups in serum cortisol and GH levels after endotoxin administration (Fig. 5b and 5c). However, a rise compared with baseline in the stress hormone cortisol was evident whereby serum cortisol levels peaked at 4 hours after endotoxin challenge in all study groups ($P = 0.005$) (Fig. 5b).

Total and differential leukocyte count

We observed that antecedent hypoglycemia modulated the subsequent WBC response to endotoxin. Total

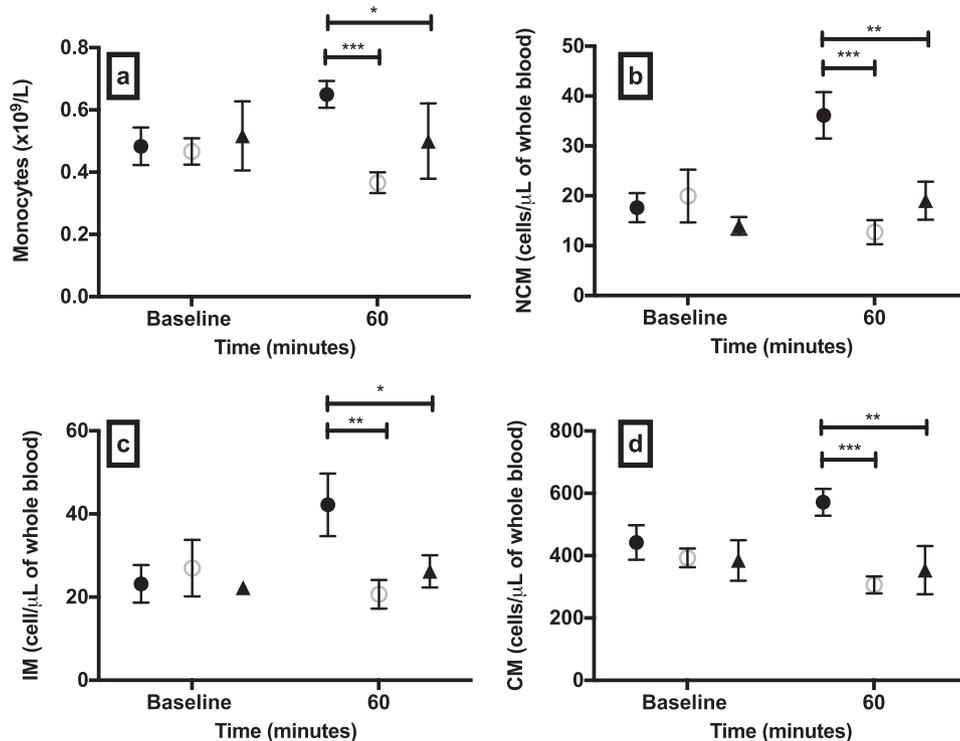


Figure 3. Total monocyte count and monocyte subset kinetics in experimental hypoglycemia groups and controls. Absolute circulating numbers of (a) total monocytes and monocyte subsets (b) NCM, (c) IM, and (d) CM after 60 min of hypoglycemia, euglycemia, or sham-saline injection. Data are mean (SEM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; P values are provided for comparison between study groups. Black circles, hypoglycemia group; open circles, euglycemia group; black triangles, sham-saline group.

number of WBCs increased significantly after endotoxin in all study groups (Fig. 6a). The peak WBC response occurred at 4 hours after endotoxin, and this level was significantly higher in the hypoglycemia group at $10.96 \pm 0.97 \times 10^9/L$ vs $8.21 \pm 0.85 \times 10^9/L$ in the euglycemia group ($P = 0.012$) (Fig. 6a). Total WBC count 4 hours after endotoxin in the sham-saline group was $10.65 \pm 0.64 \times 10^9/L$, and this level was significantly higher than in the euglycemia group ($P = 0.033$) but not the hypoglycemia group ($P = 0.974$). The rise in WBC was mainly a consequence of an increase in neutrophil count (Fig. 6b). The lymphocyte count decreased after endotoxin (Fig. 6c), and the monocyte count initially decreased before recovery 6 hours after endotoxin (Fig. 7a). There was a trend toward a higher total monocyte count in the hypoglycemia group 4 hours after endotoxin compared with euglycemia, but this comparison did not reach statistical significance ($P = 0.085$). The absolute number of circulating monocyte subsets did not differ significantly between study groups (Fig. 7b, 7c, and 7d). NCM and IM numbers decreased significantly after endotoxin compared with baseline values in all groups ($P < 0.001$) (Fig. 7b and 7c). Compared with baseline, CM numbers significantly declined at 2 hours ($P < 0.001$), before rising and reaching a peak at 6 hours ($P < 0.001$) (Fig. 7d).

Cell surface markers

We examined monocyte activation after endotoxin exposure by measuring cell surface marker CX₃CR1 expression. Endotoxin administration caused a significant decline in expression of this marker across all study groups compared with baseline ($P < 0.001$) (Supplemental Fig. 2a). This decline was accompanied by an increase in the concentration of CX₃C chemokine ligand 1 in plasma at 4 and 6 hours compared with baseline in all groups ($P < 0.001$) (Supplemental Fig. 2e). Activation of monocytes was also revealed by increased expression of CD11b expression at 4 and 6 hours after endotoxin compared with baseline in all groups ($P < 0.001$) (Supplemental Fig. 3a). In addition, the percentage of total monocytes that were positive for CD11b expression was higher in hypoglycemia group than in the euglycemia group at 2 hours after endotoxin ($P = 0.007$) (Supplemental Fig. 3b).

Discussion

Hypoglycemia may contribute to exacerbations of ischemic CV disease. We aimed to investigate the effect of acute experimental hypoglycemia and subsequent low-dose endotoxemia on aspects of the innate immune response (total leukocytes, leukocyte subsets, and specifically monocyte subsets), thrombosis (platelet aggregation), and crosstalk

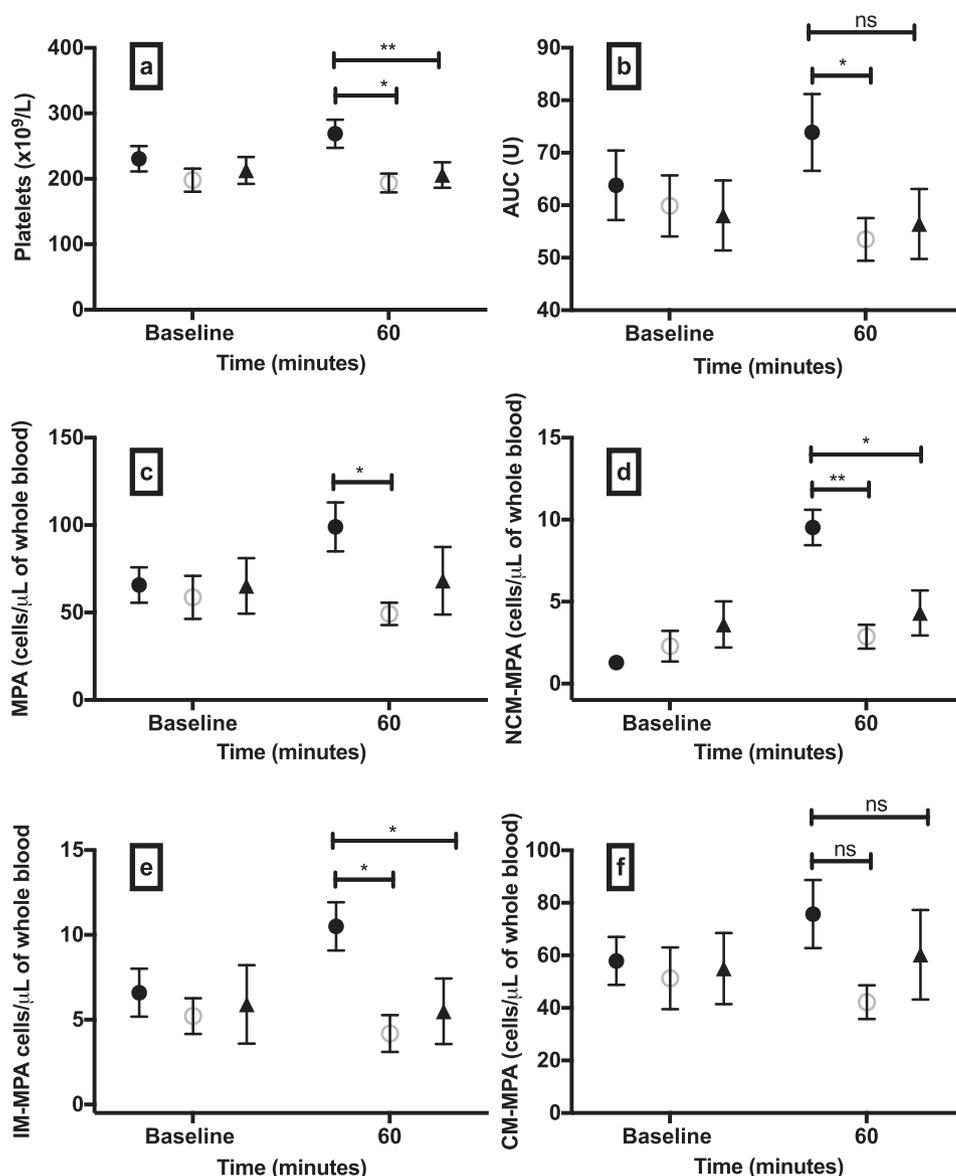


Figure 4. Platelet reactivity and MPA formation in experimental hypoglycemia and controls. (a) Total platelet count, (b) platelet aggregation to ADP 6.45 μM , (c) total MPA formation, and MPA formation within monocyte subsets (d) NCM-MPA, (e) IM-MPA, and (f) CM-MPA after 60 min of hypoglycemia, euglycemia, or sham-saline injection. Data are mean (SEM). * $P < 0.05$; ** $P < 0.01$; ns, nonsignificant; P values are provided for comparison between study groups. Black circles, hypoglycemia group; open circles, euglycemia group; black triangles, sham-saline group.

between inflammation and thrombosis (MPAs). Our main findings were that hypoglycemia increased the number of all three circulating monocyte subsets, in association with a stress response characterized by increased plasma epinephrine levels; hypoglycemia increased platelet reactivity, promoted formation of MPAs, and promoted aggregate formation between proinflammatory monocytes and platelets; leukocyte mobilization to the stress response of low-dose endotoxin was independent of epinephrine; and antecedent hypoglycemia resulted in a significantly higher inflammatory leukocyte response to low-dose endotoxin administered 48 hours later.

As shown previously (13, 37), we confirm that hypoglycemia results in leukocytosis. In addition, we

present the effect of hypoglycemia on monocyte subset kinetics and demonstrate an increase in the absolute number of all three circulating monocyte subsets. The largest increase was observed in numbers of circulating NCMs (twofold) and IMs (1.8-fold), with a modest increase in the number of CMs (1.3-fold). These data are in keeping with an observed selective mobilization of CD16⁺ monocytes in response to exercise (27, 38) and epinephrine infusion (39). Ratter *et al.* (37) also recently determined that hypoglycemia might modify selective monocyte mobilization. However, they did not phenotype monocyte subsets but rather measured total levels of CD16 on peripheral blood mononuclear cells isolated from both healthy participants and those with type 1

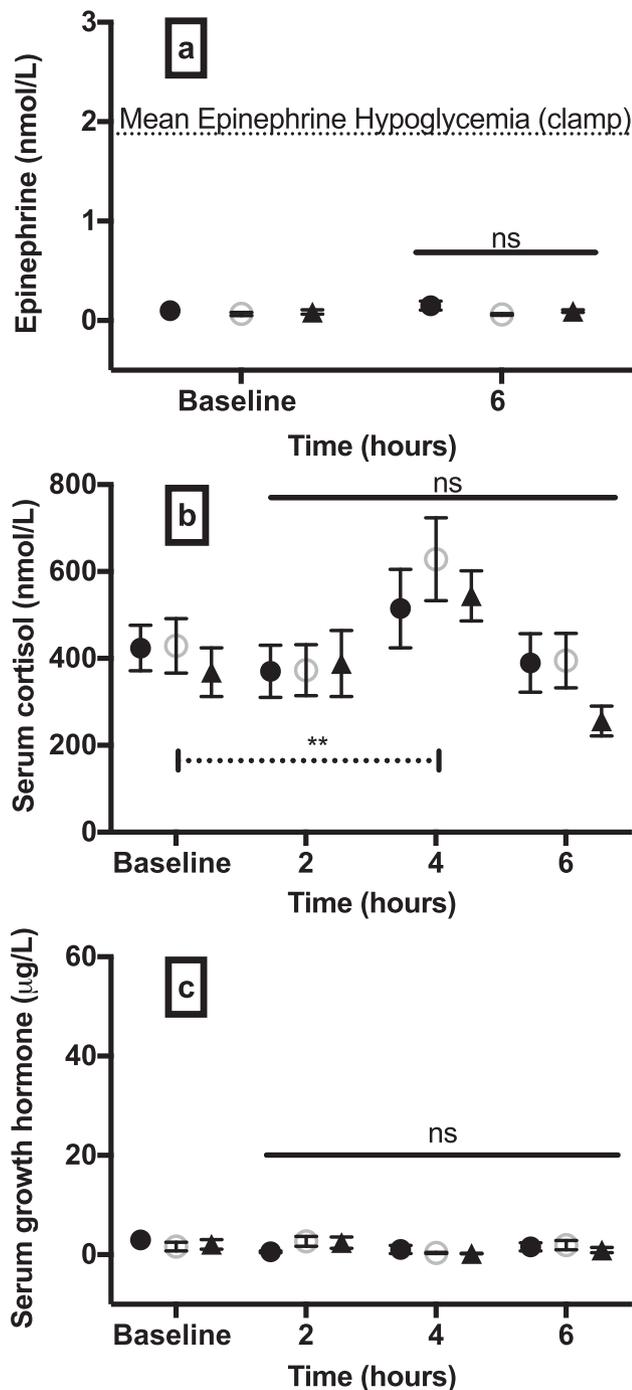


Figure 5. Changes in epinephrine, cortisol, and GH response after endotoxin challenge. (a) Epinephrine, (b) cortisol, and (c) GH responses 2, 4, and 6 h after low-dose (0.3 ng/kg) intravenous endotoxin challenge in participants who underwent hypoglycemia, euglycemia, or a sham-saline clamp 48 h earlier. Data are mean (SEM). ** $P < 0.01$; ns, nonsignificant; P value on dashed line in (b) represents change in cortisol at 4 h compared with baseline in all groups; solid horizontal lines represent significance for comparison between study groups. Dashed line in (a) illustrates the mean epinephrine response in the hypoglycemia clamp group. Black circles, hypoglycemia group; open circles, euglycemia group; black triangles, sham-saline group.

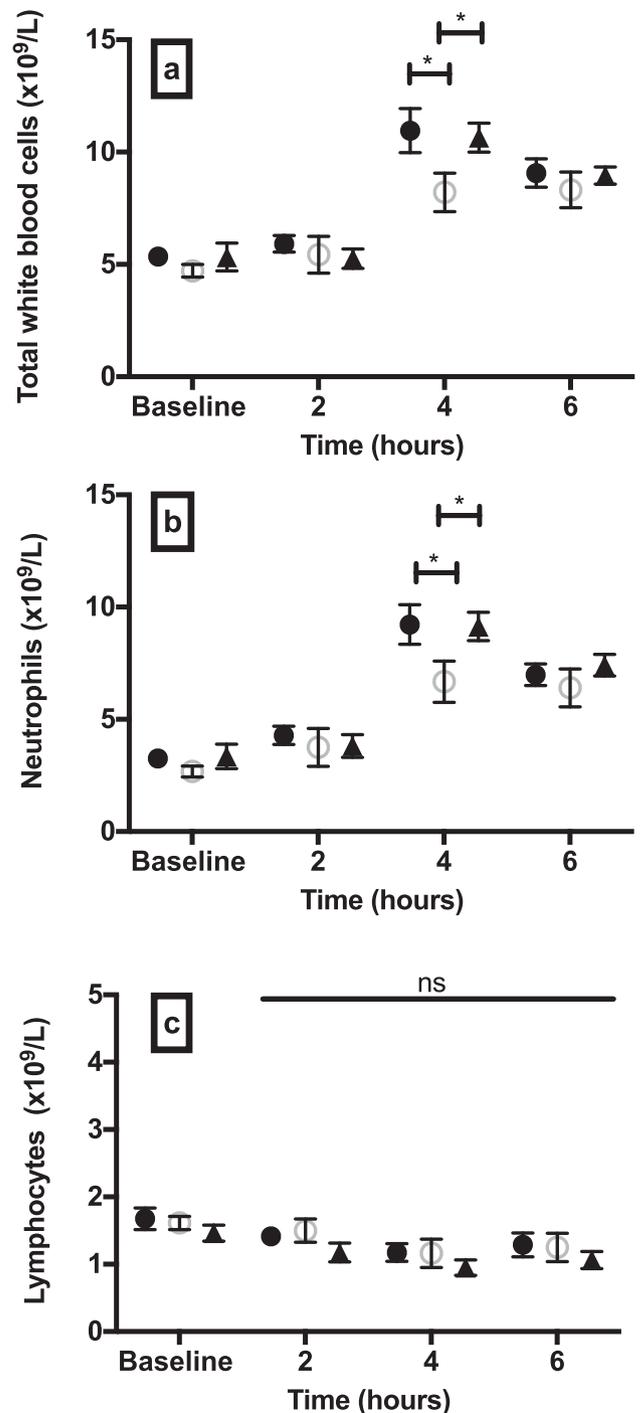


Figure 6. Peripheral total WBC, neutrophil, and lymphocyte kinetics after endotoxin challenge. Number of circulating (a) total WBCs, (b) neutrophils, and (c) lymphocytes 2, 4, and 6 h after low-dose (0.3 ng/kg) intravenous endotoxin challenge in participants who underwent hypoglycemia, euglycemia, or a sham-saline clamp 48 h earlier. Data are mean (SEM). * $P < 0.05$; ns, nonsignificant; P values are provided for comparison between study groups; solid horizontal line in (c) represents significance for comparison between study groups. Black circles, hypoglycemia group; open circles, euglycemia group; black triangles, sham-saline group.

diabetes in experimental hypoglycemia settings. Our data identify specific changes in monocyte subsets that have been previously linked to monocyte activation and

atherogenesis. Because observational data support the notion of CD16⁺ monocytes being proatherogenic (20, 22–24), and adrenergic modulation of monocytes induces

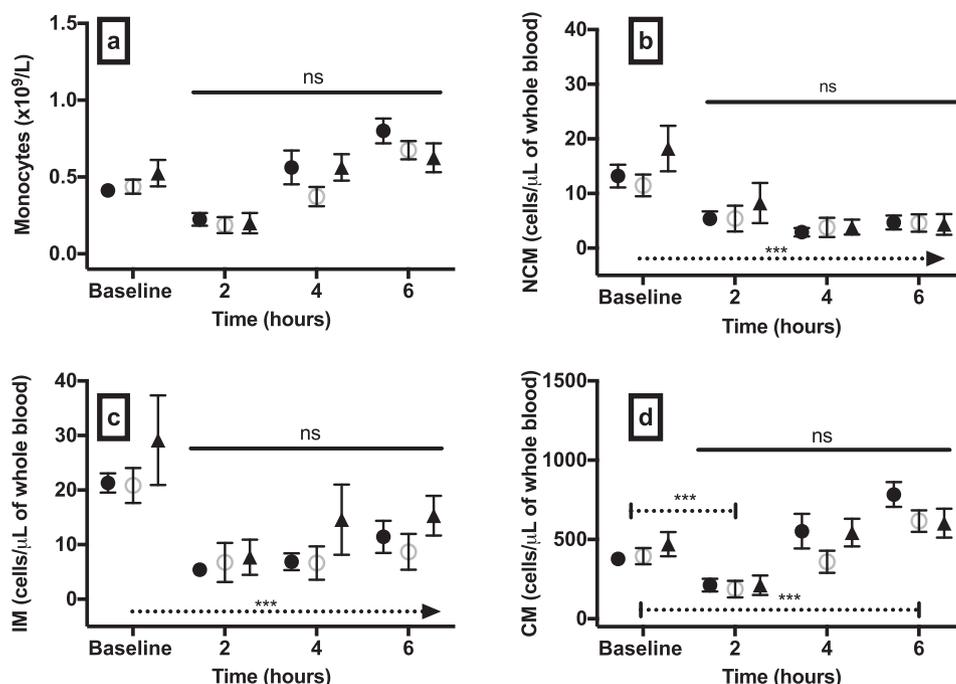


Figure 7. Total monocyte count and monocyte subset kinetics after endotoxin challenge. Absolute circulating numbers of (a) total monocytes and monocyte subsets (b) NCM, (c) IM, and (d) CM 2, 4, and 6 hours after low-dose (0.3 ng/kg) intravenous endotoxin challenge in participants who underwent hypoglycemia, euglycemia, or a sham-saline clamp 48 h earlier. Data are mean (SEM). *** $P < 0.001$; ns, nonsignificant; P value on dashed line in (b) represents change in number of NCMs at 2, 4, and 6 hours compared with baseline in all study groups. P value on dashed line in (c) represents change in number of IMs at 2, 4, and 6 h compared with baseline in all study groups. P values on dashed lines in (d) represent change in number of CMs at 2 and 6 h compared with baseline in all study groups. Solid horizontal lines represent significance for comparison between study groups. Black circles, hypoglycemia group; open circles, euglycemia group; black triangles, sham-saline group.

proinflammatory changes (40), an increase in the circulating number of these cells after hypoglycemia may increase CV risk in diabetes.

Previous studies investigating effects of hypoglycemia on platelet biology have suggested an increase in platelet reactivity; however, this increase was in the context of hypoglycemic stimulus as part of an insulin stress test (41). An older investigation into the effect of hypoglycemia on monocyte-platelet interactions in type 1 diabetes and healthy controls also suggested a trend toward increased MPA formation, but these data were not conclusive, with little difference between euglycemic and hypoglycemic conditions (42). Our study also recapitulates and extends previous findings that hypoglycemia is prothrombotic, as evidenced by an increased platelet count and increased platelet reactivity to ADP (43). We have conclusively demonstrated an overall increase in formation of MPAs in hypoglycemia in comparison with euglycemia. Furthermore, we provide data demonstrating MPA formation within monocyte subsets in experimental hypoglycemia. MPA formation is a highly sensitive marker of both monocyte and platelet activation (44, 45). MPA formation promotes monocyte release of the proinflammatory cytokines $\text{TNF}\alpha$, C-X-C motif chemokine ligand 8, and C-C motif chemokine ligand 2 (46, 47) and increases adhesive properties of monocytes

(48), thereby representing a bridge between inflammation and thrombosis that may increase CV risk. In acute coronary syndromes, MPA formation correlates with troponin elevation, risk of in-hospital cardiac events including death, and risk of future cardiac events (20, 49). We have also shown that NCMs and IMs aggregate more readily with platelets in response to hypoglycemia compared with CMs. A similar observation of proportionally higher IM-MPA and NCM-MPA formation has been reported in patients after an ST elevation myocardial infarction, with higher IM-MPAs in particular being a poor prognostic indicator at 6 weeks after ST-elevation myocardial infarction (20). Thus, our data suggest that hypoglycemia not only increased circulating numbers of CD16^+ monocytes but also promoted increased interaction between these proinflammatory monocyte subsets and platelets.

We wanted to determine whether antecedent hypoglycemia modulated responses to low-dose endotoxin. We chose a low-dose endotoxin model because we thought it was the safest way to combine the clamp and endotoxin human models, because future extension to the study of human diabetes would be more feasible with this model, and because patients with diabetes are often exposed to chronic low-grade infections through foot ulceration and periodontitis, which might further increase

the risk of CV mortality (50, 51). In our model, we observed in all groups that monocytes were activated even in response to low-dose endotoxin, as indicated by upregulation of systemic levels of the CX₃CR1 ligand, CX₃C chemokine ligand 1, and upregulation of the adhesion molecule CD11b on the monocytes themselves. Interestingly, compared with euglycemia, hypoglycemia resulted in greater leukocyte mobilization in response to low-dose intravenous endotoxemia 48 hours later. Furthermore, we noted a nonsignificant trend toward a higher total monocyte count in the hypoglycemia group 4 hours after endotoxin compared with euglycemia. The percentage of monocytes that were CD11b positive was also higher in the hypoglycemia group compared with the euglycemia group at 2 hours after endotoxin. Levels of leukocyte mobilization were similar between groups who received previous sham-saline or hypoglycemia. These data suggest that euglycemia with insulin suppressed leukocyte mobilization in response to endotoxin 48 hours later, consistent with the known anti-inflammatory actions of insulin (52, 53), and that the physiological stress of hypoglycemia overcame this insulin-mediated suppression of inflammatory responses. Our data show that drivers for differential leukocyte mobilization to endotoxin are unlikely to be due to differences between groups in epinephrine, cortisol, and GH levels after endotoxin because these levels were not significantly different. Our observation that a single episode of hypoglycemia compared with euglycemia invokes a stronger proinflammatory response to endotoxin up to 2 days later is of potential clinical relevance given that trial data suggest downstream mortality after hypoglycemia (29–31).

The strengths of our study include use of a human experimental model and detailed flow cytometric analysis that allowed us to comprehensively describe immune cell kinetics and activation status in response to experimental hypoglycemia and endotoxin challenge *in vivo*. The separation of clamp and endotoxin studies by 48 hours allowed us to probe the longitudinal effects of hypoglycemia on innate immunity. Moreover, by using a sham-saline group, we specifically controlled for the immunological effects of insulin, thereby robustly investigating proinflammatory changes in response to hypoglycemia.

One limitation was our decision to study a small number of young, healthy participants, which limits the applicability of our findings to older patients with diabetes, established CV risk factors, and atherosclerosis. For ethical and safety reasons, we decided to examine our experimental model initially in healthy participants. We also specifically adopted a low-dose endotoxin model, with future translatability in older, higher-risk participants in mind. Future studies should therefore confirm our findings in patients with diabetes. In addition, it is

worth noting that we studied cell numbers, phenotypic changes, and activation status in circulating immune cells, and these data may not necessarily reflect the functional capacity of these cells in an atherosclerotic plaque. An animal model of combined experimental hypoglycemia and atherosclerosis may help resolve these questions.

In conclusion, hypoglycemia mobilized proatherogenic monocyte subsets and induced prothrombotic changes by increasing platelet reactivity. In addition, hypoglycemia amplified interactions between platelets and monocytes by promoting MPA formation with increased aggregation of proinflammatory monocytes with platelets. Hypoglycemia may also prime the innate immune system to respond more robustly to stimuli such as endotoxin. This finding implies proinflammatory consequences of hypoglycemia beyond the acute episode. These data provide mechanistic insights into how hypoglycemia could increase CV risk through upregulation of inflammatory responses.

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