UNIVERSITÉ DU LUXEMBOURG

PhD-FSTM-2023-046
The Faculty of Science, Technology and Medicine

DISSERTATION

Defence held on 02/05/2023 in Esch-sur-Alzette
to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

Snehaa SEAL
Born on 28 November 1994 in Kolkata, (India)

CONNECTING THE DOTS IN TYPE-2 DIABETES: EARLY LIFE ADVERSITY, STRESS AND THE IMMUNE SYSTEM

Dissertation defence committee

Dr Jonathan D. Turner, Dissertation Supervisor
Group Leader & Deputy Head (Funding and Finance), Department of Infection and Immunity, Luxembourg Institute of Health

Dr Elif Duman
Assistant Professor, Boğaziçi University

Dr Paul Heuschling, Chairman
Professor, Université du Luxembourg

Dr David Meyre
Associate Professor, McMaster University
Deputy Director, UMRS INSERM 1256, Nutrition-Génétique et Exposition aux Risques Environnementaux, Université de Lorraine

Dr Maria Ruiz-Castell, Vice Chair
Public Health Research, Luxembourg Institute of Health

A dissertation by

Snehaa SEAL

submitted to the University of Luxembourg

in partial fulfilment of the requirements for the degree of

DOCTOR of PHILOSOPHY

Approved by the Dissertation Defence Committee:

Chair of committee: **xxxxxx**

xxxx, Université xxxxxx

Committee members: **Prof. Dr. Hartmut Schächinger**

Head of the Department of Clinical Psychophysiology, Universität Trier

Dr. Elif Duman

Assistant Professor of Biopsychology, Boğaziçi University

Dr. Maria Ruiz-Castell

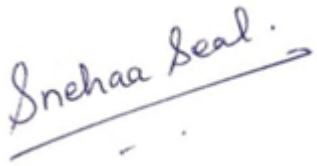
Public Health Research, Luxembourg Institute of Health (LUX)

Supervisor: **Dr. Jonathan D. Turner**

Group Leader & Deputy Head (Funding and Finance), Department of Infection and Immunity, Luxembourg Institute of Health (LUX)

Affidavit

I hereby confirm that the PhD thesis entitled “Connecting the dots in Type-2 diabetes: Early life adversity, stress and the immune system” has been written independently and without any other sources than cited. All necessary ethical approvals have been obtained in accordance with the European Union Council Directive 2010/63/EU (on the use and care of laboratory animals) and with the French ones (Directive 87/148, Ministère de l’Agriculture et de la Pêche – Apafis #16924). The study protocol for the ESCO participants (described in Chapter 2) have been obtained in accordance with the Ethical Committee of the State’s Medical Association (Landesärztekammer Rheinland-Pfalz) in line with the current revision of the Declaration of Helsinki . The EpiPath (described in Chapter 2) human cohort approval was obtained from the NationalResearch Ethics Committee of Luxembourg (no. 201303/10 v1.4) in conjunction with the Ethics Review Panel (University of Luxembourg, no. 13-002).



Luxembourg, 22.02.2023 Snehaa Seal

Funding

Project 'MADAM' was funded by the Fonds National de Recherche Luxembourg (INTER/ANR/16/11568350 'MADAM'). The conducted experiments and the overall progression of the project and thesis were supervised at Luxembourg Institute of Health (LIH) while registered as a student at the University of Luxembourg (uni.lu).

Acknowledgement

I would like to extend my gratitude to the official rat cuddler for my big GTT experiments and the Dr. Father of our team, Jonathan D. Turner, for believing in me, giving me the chance to be a beginner in the laboratory, to make my own mistakes, to learn from them and to ask questions freely. He also taught me to dive and maintain a balance between work and other aspects of my life. This led me to discover my love for travelling to a new country every weekend (in the final year of my PhD and he was very supportive) and I would always come back with newer and better ideas. Talking about travelling, I would like to remind him to not forget his passport again (like how he did in Bordeaux). I would also like to thank everybody who was involved in the writing of MADAM, the partnering laboratories who generated pilot data, the funding bodies who supported this very promising project and the people involved in the recruitment process, who deemed me worthy of being part of it.

As a baby PhD student, I was told on my first day that a PhD is not just a degree, but also a way of life. These 4 years here at LIH, away from home and family, has taught me exactly that. This journey has metamorphosed this 20-something year old, beginner in the lab to a more functional Viv2.0, all thanks to the lovely people around me. During these years, Nathalie Grova, Sophie B. Mériaux and Pauline Guebels have played a gigantic role in the progression of my project, right from the writing of the animal protocol, to animal experimentations and processing of the samples. Nathalie and Sophie have been like family to me away from home.

I feel extremely lucky to have shared my workspace with the nicest set of colleagues ever, namely, Myriam P. Merz (my sweet German sister), Eleftheria G. Charalambous and Cyrielle Holuka, in that specific order. My team mates have always been there for me and have always reminded me that I could do this, especially on days when I needed a little extra motivation or assurance or just a good cry. Over the years, I have also befriended PhD students from other teams who have provided me with fresh perspective and inputs on science and the general workings of the world over lunch and tepid machine coffee. I would like to wish them all success in their respective journeys. The NCP and the IVIP platform deserve special mention, for helping me refine my protocols and overcome occasional hiccups in my experiments and analysis. I also owe a big thanks to Djalil Coowar for allowing me to complete my animal handling training at the university facility next door. Sylvie Delhalle, Malou Fraiture, Carole Weis, Michelle Roderes, Jessica Giro, Magali Guillaume and the LIH helpdesk team deserve a special mention for always having the back of every PhD student, you guys are true super heroes without capes.

I would like to dedicate this thesis to my mum, who was always just a phone call away, on good days and bad ones. My little sister has also been my rock throughout and has never once doubted my abilities, even when I doubted myself at every juncture. Thank you so very much for your constant love and support.

I thank my thesis committee for their valuable insights and guidance to help me better myself as a young scientist. If you're really reading this, I thank you for your time and I swear I will buy you chocolates!

Lastly, I would also like to thank people who I have crossed paths with and who have been a little difficult. It took me a while to understand that this too was a learning on a more personal front, as taught by the wise old tortoise from Kung Fu Panda.

General Information

This thesis has been authored using British English throughout. However the 'Introduction' to this thesis structures around "The 'Jekyll and Hyde' of Gluconeogenesis: Early Life Adversity, Later Life Stress, and Metabolic Disturbances", which was published in the 'International Journal of Molecular Sciences' using American versions of some words and as such the published versions may mave minor differences from the versions included in this thesis.

All publications included in this thesis have been authored as **Snehaa Vivienne Seal** (**SV Seal**).

Table of Contents

List of Figures and Tables

List of Abbreviations

ACTH	Adrenocorticotrophic Hormone
ADC	Apparent Diffusion Coefficient
ANOVA	Analysis of Variance
AUC	Area Under the Curve
ANR	Agence Nationale de la Recherche
CRH	Corticotropin Releasing Hormone
DAMP	Damage-associated molecular patterns
DOHaD	Developmental Origins of Health and Disease
DTI	Diffusion Tensor Imaging
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELA	Early Life Adversity
ELI	Early Life Infection
ELISA	Enzyme-Linked Immunosorbent Assay
EMRA	Effector memory cells re-expressing CD45RA
FA	Fractional Anisotropy
FNR	Fonds National de la Reserche
GC	Glucocorticoids
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GK	Goto-Kakizaki rats
GR	Glucocorticoid Receptor
GRO/KC	Growth-regulated oncogenes/Keratinocyte Chemoattractant
GTT	Glucose Tolerance Test
H1N1	Human Influenza-A-virus H1N1
HPA	Hypothalamus-Pituitary-Adrenal axis
IFN-γ	Interferon Gamma
IgM	Immunoglobulin M
IL	Interleukin
IP-10	Protein 10 From Interferon (Gamma)-Induced Cell Line
MCP1	Monocyte Chemoattractant Protein 1

MD	Maternal Deprivation
MetS	Metabolic Syndrome
MIP	Macrophage Inflammatory Protein
MRI	Magnetic Resonance Imaging
NK cell	Natural Killer cell
PAMP	Pathogen-associated molecular patterns
PND	Post-natal day
PRR	Pattern Recognition Receptor
SECPT	Socially Evaluated Cold-Pressor Test
SEM	Standard Error of Mean
SES	Socio-economic status
Tc	Cytotoxic T cells
T1D	Type-1 diabetes
T2D	Type-2 diabetes
Th	T helper cells
TLR	Toll-like receptors
TSST	Trier Social Stress Test
VEGF	Vascular Endothelial Growth Factor

General Abstract

Psychological stress prompts metabolic reactions aimed at increasing glucose supply to the brain, to initiate fight/flight responses in healthy individuals. The quality of the first 1000 days of life is a critical window which impacts lifelong metabolic trajectories and stress reactions. Exposure to stress during this critical window appears to have an exaggerated long-term effect on glucose availability during stress, leading to an increased risk of cardiometabolic disorders. Early life adversity also appears to have an effect on baseline glucose levels in both pre-clinical models of maternal deprivation and perinatal infection, and in human participants that have experienced early life adversity (institutionalisation-adoption).

In this thesis, we initially critically examined the dogma that the stress induced glucose rise was mediated by glucocorticoids. However, the kinetics of stress-induced glucose release in healthy individuals, peaking 15-28 minutes before cortisol suggests that it is a glucocorticoid-independent process. This was confirmed by the absence of glucocorticoid induced glucose rise after intravenous administration of escalating doses of cortisol in a healthy human cohort. Instead we suggest that this mechanism is most likely orchestrated by a direct innervation in the liver. Results from our initial study indicate that the metabolic status and early life stress are intimately intertwined, and in order to fully understand either profiles, the other should be thoroughly studied.

Type-2 diabetes (T2D) is a complex disorder characterized by insulin resistance, hyperglycaemia, dysregulations in metabolism and is now even considered to have an immune component, with functional impairments in many immune cell types. Through epidemiological data, it is clear that, although the genetic component appears to be weak, the 'pathogenic component' may be transmitted in a pedigree due a common shared environment. This vertical transfer of risk factors through generations may suggest that T2D in itself can also act as a type of early life adversity/stress that further triggers a vicious cycle.

In thesis, we predominantly focus on T2D as a source of early life adversity (nutritional stress) and investigate its effects on stress response and the immune system. Although often accompanied by comorbid obesity, the immune status in Type-2 diabetes independent of obesity is still a knowledge gap. Using Goto-Kakizaki rats as a non-obese Type-2 diabetes model, we demonstrated that these rats have a limited pro-inflammatory baseline immune

profile outside the pancreas. To finally examine when these changes arise in the disease cycle, we conducted a longitudinal study in these rats encompassing the transition period from prediabetes to a fully diabetic phase. Additionally we also studied how the diabetic phenotype impacted lymphocyte redistribution when subjected to a metabolic challenge and restraint stress.

Our study will help understand the immune component of the diabetic aetiology independent of obesity, genetic variations, diet and other confounding parameters. Additionally, our studies pave the way for strategizing effective immunotherapeutic approaches and proposes the nervous system as an effective target in stress biology to neutralise the ELA induced stress-glucose changes.

Chapter 1: General Introduction

Parts of this section have been adapted from the following manuscript:

Seal, S.V.; Turner, J.D. The 'Jekyll and Hyde' of Gluconeogenesis: Early Life Adversity, Later Life Stress, and Metabolic Disturbances. *Int. J. Mol. Sci.* 2021, *22*, 3344. https://doi.org/10.3390/ijms22073344. doi: 10.3390/ijms22073344 . PMID: 33805856; PMCID: PMC8037741.

1.1. T2D: a complex amalgamation of genetic, epigenetic and environmental risks

Type-2 diabetes (T2D) is a complex disorder characterized by insulin resistance, hyperglycemia and inflammation. The global incidence of T2D has increased fourfold in the three decades and this rate is increasing exponentially with the rapid rise in well-known risk factors such as sedentary lifestyle and obesity [1, 2]. However, it is now becoming apparent that T2D is a complex amalgamation of the current as well as the early-life environment along with the genetic component. Thus, the quality of the lifelong exposome, i.e. the accumulation of all physical, social and other forms of environmental exposure throughout an individual's lifetime can dictate one's health status.

The mechanistic link between the environment and the genome is the epigenome, which is passed on mitotically and meiotically to alter succeeding generations at the molecular level [3]. The external environmental cues are annotated either as DNA methylation directly on the genome, or histone post-translational modifications. This intertwining of the environment, genome and epigenome has been summarised in the 'three hit model of vulnerability and resilience' (Figure 1.1). This model suggests that the genetic composition (hit 1) communicates with the early life environment (hit 2), "programming phenotypes with differential susceptibility to later-life challenges (hit 3)" [4]. The "programmed phenotype" during later-life challenges dictates an individual's susceptibility or resilience [4].

The developmental origin of health and disease (DOHAD) theory states that detrimental influences during the critical window of early life development (from conception up to 2 years) can leave an everlasting deleterious effect on an individual's health [5]. The internal (uterine) and external environment of an individual during development has a delicate balance, which when disturbed can result in a plethora of physiological abnormalities which may increase the vulnerability to develop T2D later in life. Gestational diabetes, maternal obesity and maternal age are major prenatal environmental risk factors.

Then, from birth and throughout life, environmental factors such as pollution, ionizing radiations or lifestyle (sedentarily, smoking, alcohol intake, diet, stress) add on to the prenatal risk factors. Finally, at adulthood, age, obesity and depression and other cumulative factors in concertation act as major risk factors for T2D. Early life factors also majorly affect the epigenome and thus may

provide crucial periods for intervention. The following sections bring together the classical parameters such as the genetic component (hit 1) and later life (hit 3) risk factors. Additionally we discuss the early life environment as a hit 2 that acts as a bridge ultimately laying the foundation for T2D.

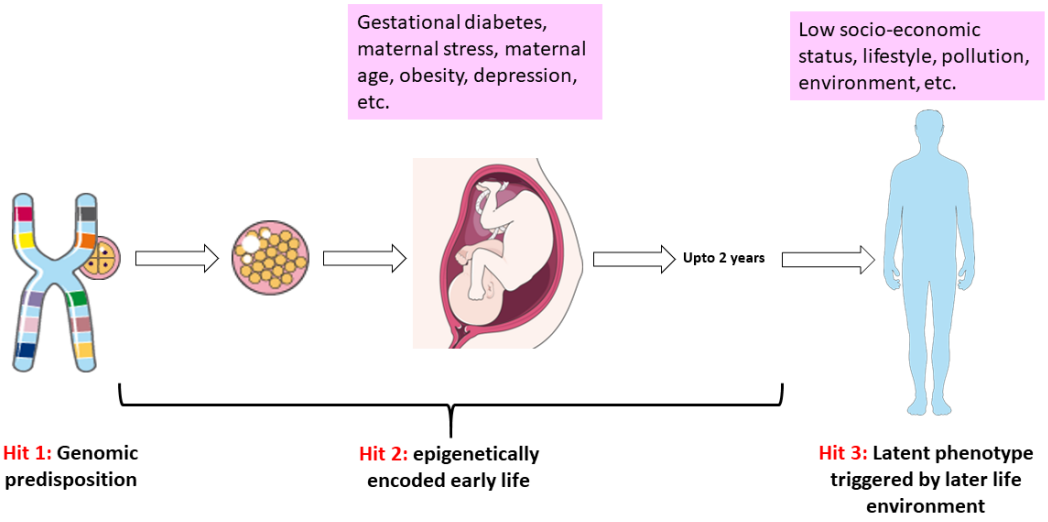


Figure 1.1: The three-hit model of aetiological vulnerability: This concept shows how the three hits-genomic predisposition, epigenetically encoded early life (which is extremely malleable from day 0 up to 2 years of age) and the latent phenotype, which is triggered by the later life exposome leads to diseases like Type-2 diabetes. These hits biologically accumulate the insults inflicted by the risk factors and contribute to the aetiology. (Images adapted from Servier Medical Art under a Creative Commons Attribution 3.0 Unported License.)

1.1.1. Genetic aspect (Hit 1) of T2D

Having obese parents increases the risk of developing obesity more than two fold and furthermore increases the risk of developing T2D [6], although it is now clear that T2D does not follow a strict Mendelian pattern of inheritance. If we look more closely at the epidemiological data, it is clear that there is a family effect. The Framingham offspring study reported a more than double and six times increase in the risk of developing T2D in individuals with one and two diabetic parents respectively [7]. A Swedish study found similar results, which showed that the familial risk was dependent on the number of diabetic relatives [8]. Interestingly, there is evidence for a significant parent-of-origin (maternal) transmission of T2D risk alleles (e.g: *KCNQ1* and *THADA* often reported in GWAS) [9] [10]. Initially it was reported that T2D was seen more frequently in mothers and second-degree maternal relations of diabetic patients than paternal relatives [11]. In an Omani population where 95% of diabetic probands had a familial history of T2D, a similar bias towards

maternal rather than paternal diabetes was seen, with the caveat that there was a high rate of consanguineous marriages among the Omani population [12]. These population studies suggest either a genetic or shared environment component to the pathogenesis of T2D.

Despite many large-scale genome-wide association studies, the most commonly identified genetic variants have not shed light on the exact pathomechanism of T2D. So far, genetic variants explain only 10-15% of T2D inheritance, suggesting only a weak genetic component [13] and only ~5-10% of trait variability and familial aggregation [14, 15]. However, in the GWAS a select series of genes are regularly identified (Reviewed in [16]). Table 1.1 and the references therein summarises these regularly identified genes. Deleterious variants of *PPARG, TCF7L2, KCNQ1, IRS1, PROX1, ADCY5, GIPR, HNF1A* and *SLC30A8* have a direct influence on diabetic mechanisms including, insulin sensitivity, insulin secretion or glucose metabolism and were initially studied using candidate gene approach. These genes also flagged up in GWAS, thereby validating their contribution to the T2D risk. Genes responsible for potassium gated voltage channels like *KCNJ11* and *KCNQ1* in the pancreas have also been implied in directly affecting the insulin secretion. Overexpression of genes associated with the inhibition of insulin secretion such as melatonin encoded by receptor type 1B (*MTNR1B)* have also been associated with T2D risk. [17]. A large number of obesity-related genes like *FTO* and *MC4R* show up in these GWAS [18] [19] [20]. Furthermore, *KLF14* had previously been associated with body fat distribution and function and insulin sensitivity and was suggested to be linked to obesity [21]. Several genes such as *NOTCH2* and *BCL11A* as well as being associated with T2D risk are also involved in diabetic complications like nephropathy and neuropathy respectively [22, 23]. There are however, several genes such as *CDKN2A/2B, CDKAL1, CAPN10,* and *THADA* that confer the T2D risk through mechanisms that remain unclear and needs further investigating [16].

A recent study reported that common variants had a stronger influence (16.3%), while rare variants could successfully explain only 1.1% of the T2D “phenotypic variance” [24]. An East Asian meta-analysis study reported a significant overlap of associated T2D variants with that of European diabetics, and the few unique variants were suspected to be different owing to differential environmental exposures and “larger effect sizes of variants” [25].

Identification of causal variants and “functionally validated genes” is a very challenging task even today after commendable advances in the field of genetics [26]. Causal variants are often named after the closest and most convincing candidate gene, which can be misleading as the T2D phenotype may be crystallised due to the association of multiple genes (candidate/non-candidate) acting in concertation across the genome [26]. In addition, often it is not known if the transcript of

the gene (variant) mediates the T2D risk [27] and analysis of each SNP individually causes geneticists to miss signals due to multiple SNPs with low effects functioning as a unit to amplify the genetic influence [28]. T2D causal variants are often located in non-coding regions of the genome (intronic/intergenic) as shown in Table 1.1, thereby indicating the involvement of (islet specific) regulators (promoters, enhancers, etc.) that probably influences the expression of pancreatic genes [29]. GWAS have clearly shown that beta cell dysfunction is the prime cause of T2D as most of the reported genes have been shown to be active in pancreatic beta cells [18].

Also, the stronger heritability of T2D in monozygotic vs dizygotic twins supports the underlying role of genetics in T2D [30-32], however, this was confounded by the higher (non-twin) sibling disease rates observed in the Botnia family study in previous twin studies [33]. Taken together, this suggests that the genetic basis is not enough to induce T2D, but that something in the environment common to mono- di-zygotic twins, and non-twin siblings is essential in the aetiology of T2D. It is clear that all three types of sibling share a common early-life environment that we propose to be the second hit. We suggest that the large "missing heritability" in T2D and the clear familial clustering and twin concordance depends on this shared, social environment that "primes" the individual, to subsequently develop T2D when, in later life, the classical risk factors are encountered. Indeed, it was suggested in 2013 by Ali that epigenetic mechanisms prompted by the adverse intrauterine environment may be capable, over multiple generations, of producing an "inherited risk" and moreover that the genetic component in T2D was probably overestimated as the "shared environment can be hard to control for in many such studies" [18]. This theory of shared environmental pathogenicity was further supported by an interesting study conducted in pet cats and dogs diagnosed with T2D [34]. It was reported that owners of diabetic pets had a much higher risk of developing T2D, compared to owners of euglycaemic pets.

Initially genomic risk scores were proposed to personalise prediction of T2D and identify high-risk individuals by screening for variants that increase the risk [7]. A polygenic score system for complex diseases like T2D with an increased number of added variants does not necessarily increase the robustness of the risk prediction, but it helps identify individuals who are at higher risk [7]. GWAS often miss rare variants, which might contribute significantly [35]. Thus, it is a good idea to consolidate treatment strategies that take into account the genetic risk, along with the environmental and epigenetic risks to account for the missing heritability of T2D [4].

In conclusion, these genetic and environmental factors function in a concerted manner to determine the transition from a healthy to prediabetic and/or diabetic state (Figure 1.2). Here, we

have proposed a three-hit model leading to the development of T2D. In our three-hit model, the foundation (first-hit) is the invariable genome, while the early life environment and later life risk factors correspond to the second and third hits respectively. Thus, in order to fully understand the phenotypic inheritance, we also need to look at the more malleable Hits 2 and 3 as equally critical driving forces to the T2D aetiology (described in the following sections).

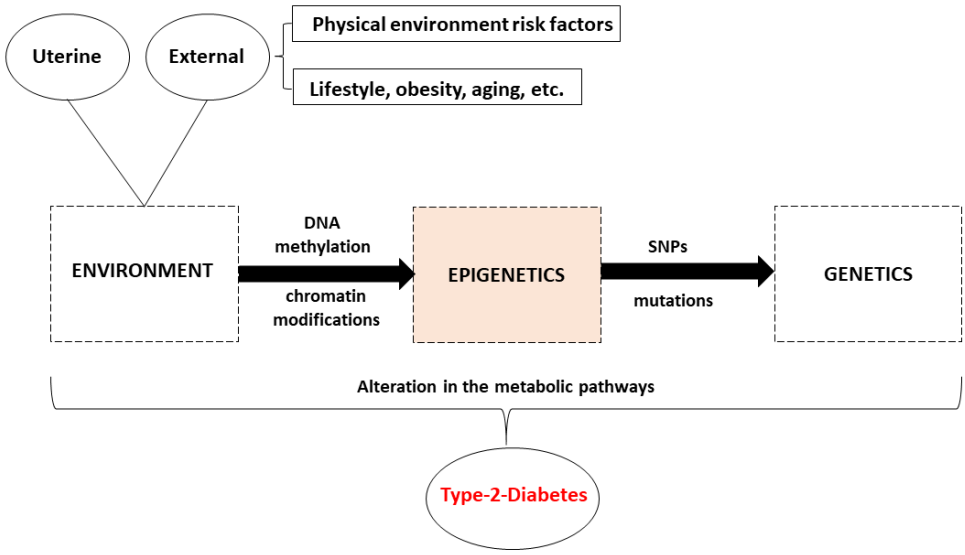


Figure 1.2: Epigenetic factors act as a mediator between the environment and the genetic aspect of type-2-diabetes. The genetic-epigenetic-environmental contribution to Type-2 diabetes is not discrete. The epigenome is altered due to DNA methylation and chromatin modifications, whereas the genome is mutated, concertedly altering key players of metabolic pathways. The uterine and external environment provides risk factors that facilitate these genetic-epigenetic pro-diabetic changes.

Table 1.1: Gene variants identified in GWAS, linkage and candidate studies as Type 2 diabetes risk alleles. * indicates chromosome number.

T2D SNP#	Gene/ nearest gene	Chr *	SNP location	Function	ref
rs1801282	*PPARG*	3	Coding-missense	**Peroxisome Proliferator Activated Receptor Gamma**- codes for glitazone (anti T2D drug that helps increase insulin sensitivity) target and has often been studied using candidate gene approach.	[18, 36, 37]

rs10811661	*CDKN2A/ 2B*	9	intergenic	**Cyclin-dependent kinase inhibitor 2A/B**- both genes have tumor suppressive functions and play a pivotal role in cell cycle regulation. GWAS have shown high association of these SNPs to T2D, but the exact mechanism remains unclear.	[18, 38-40]
rs4506565	*TCF7L2*	10	intron	**Transcription factor 7-like 2**- most T2D GWASes have consistently reported gene variants to be associated with T2D. *TCF7L2 is* active in pancreatic beta cells and liver, and helps regulate glucose balance. Gene variants can impair insulin sensitivity and secretion.	[18, 41]
rs2237895	*KCNQ1*	11	intron	**Potassium Voltage-Gated Channel Subfamily Q Member 1**- codes for a "voltage gated potassium channel" that facilitates "repolarisation of action potential" in the heart muscles. *KCNQ1* expression is also seen in the pancreatic cells and mutations in the gene impair the insulin secretion mechanism. Gene variants have been reported to show parent of origin specific (maternal) effects with regards to T2D.	[9, 42, 43]
rs8050136	*FTO*	16	intron	Plays a prime role in cardiovascular and nervous system. Gene variant has often been implied in obesity (a very important T2D risk factor) and T2D.	[19, 44]
rs7754840	*CDKAL1*	6	intron	**CDK5 Regulatory Subunit Associated Protein 1 Like 1**- function of *CDKAL1* is still not clear but its encoded protein belongs to the methylthiotransferase family. Also the gene variants's contribution to the T2D risk is not clear.	[44, 45]
rs2975760	*CAPN10*	2	intron	**Calcium-Activated Neutral Proteinase 10**- Gene variant was rendered as T2D risk conferring by linkage studies, however the exact mechanism is not clear. It codes for a protein (cysteine) protease that belongs to the family of Calpains and functions as a mediator of	[18, 46]

				cellular signaling and re-modelling. *CAPN10* has not been replicated as consistently as *TCF7L2*.	
rs5219	*KCNJ11*	11	Coding-missense	**Potassium Inwardly Rectifying Channel Subfamily J Member 11**- codes for a protein that is an "inward-rectifier type potassium channel". Deleterious gene variants decrease the efficiency of insulin secretion and have been implicated in neonatal diabetes and T2D. These channels are T2D therapeutic targets using diazoxide to help open them. These ATP potassium channels control insulin secretion.	[16, 18, 47]
rs4675095	*IRS1*	2	intron	**Insulin Receptor Substrate 1**- responsible for "insulin signal transduction" and codes for a protein that undergoes phosphorylation by receptor tyrosine kinase. Gene variants have been strongly associated with dysregulated insulin sensitivity and T2D.	[18, 48]
rs10923931	*NOTCH2*	1	intron	**Neurogenic Locus Notch Homolog Protein 2**- Plays a role in cell-fate determination, differentiation, apoptosis, proliferation and homeostasis. Has been implied in diabetic nephropathy.	[22, 49, 50]
rs340874	*PROX1*	1	intergenic	**Prospero Homeobox Protein 1**-Conserved gene in vertebrates and plays a key role in embryonic development, neurogenesis and pancreatic development and morphogenesis.	[51, 52]
rs7578597	*THADA*	2	Coding-missense	**Thyroid Adenoma Associated**- Function of this gene is not clear yet. Maternal transmission of the risk allele to diabetic offspring has been strongly reported. It has been speculated that *THADA* may be imprinted.	[10]
rs11708067	*ADCY5*	3	intron	**Adenylate Cyclase 5**- responsible for producing cyclic AMP (molecule essential for insulin secretion and signaling in the pancreas). Risk allele reduces the	[53, 54]

				expression of *ADCY5* and disrupt the function of an enhancer region located in the pancreas.	
rs10830963	*MTNR1B*	11	intron	**Melatonin Receptor Type 1B**- codes for a "high affinity melatonin receptor" and is highly expressed in the brain and retina. This particular gene variant results in elevated levels of melatonin receptor 1B in the islets, mediating an inhibitory influence on insulin secretion and thereby contributing to the T2D risk.	[17, 55]
rs12970134	*MC4R*	18	intergenic	**Melanocortin Receptor 4**- mutations in this gene have been implied in obesity and hence potentially also T2D. *MC4R* codes for a "membrane bound receptor" and belongs to the melanocortin receptor family.	[20]
rs8108269	*GIPR*	19	intergenic	**Gastric Inhibitory Polypeptide Receptor**- codes for "G-protein coupled receptor for gastric inhibitory polypeptide (GIP)". Has also been reported to mediate the release of insulin in the presence of high glucose levels.	[56, 57]
rs7305618	*HNF1A*	12	intergenic	**Hepatocyte Nuclear Factor 1-Alpha**- codes for liver and pancreas specific gene transcription factor. Mutation in this gene have been reported to be a causative of maturity onset diabetes of the young type 3 and T2D risk.	[18, 58]
rs243021	*BCL11A*	2	intergenic	**B-Cell Lymphoma/Leukemia 11A**- key player in haemoglobin switching and has been implied in T2D. Has also been reported to cause diabetic neuropathy.	[23, 59]
rs972283	*KLF14*	7	intergenic	**Kruppel Like Factor 14**- an imprinted gene (maternal expression) which also encodes for a "transcriptional co-repressor induced by transforming growth factor-beta (TGF-beta) to repress TGF-beta receptor II gene expression". Mutations (resulting in reduced expression) affect the body fat distribution and function and influence insulin sensitivity.	[21, 60]

rs13266634	*SLC30A8*	8	Coding-missense	**Solute carrier family 30 (zinc transporter), member 8**- Protein expression in pancreatic beta cells responsible for secretion and storage of insulin and mutations can promote the T2D aetiology.	[18, 61]

1.1.2. Early life environment (Hit 2) lays the foundation for T2D

An individual's metabolic health patterns in adulthood are programmed by the quality of life during their formative years [62]. Hales and Barker initially proposed the 'thrifty phenotype hypothesis'. This hypothesis explains how poor maternal gestational diet and the consequent lower availability of nutrients for the foetus reduces foetal growth and concurrently programs the metabolic trajectory of the offspring [63]. Conceptually, this metabolic programming may be advantageous if the post-natal nutritional environment is similarly restricted, however, it will be deleterious if the offspring grows up in an energy-rich environment. Since it was first described, the 'thrifty phenotype hypothesis' has been expanded, now encompassing not only the in-utero period, but throughout infancy and childhood as well.

Environmental factors during early life period (before and after birth) are strongly determined by parental SES. Although low parental SES in the first 1000 days (conception through to 2 years) has significant, negative, long term health consequences [64], low SES in adolescence showed a strong association to the development of T2D up to fifty years later [65]. An independent study reported a similar influence of low early SES on metabolic health but no significant effect of the current SES [66]. Low childhood SES altered stress response associated genes: *AVP*, *FKBP5* and *OXTR* and inflammation associated genes *CCL1* and *CD1D* [67], thus triggering or exacerbating the T2D aetiology. Thus, there are clear mechanistic parallels between SES and T2D.

Other factors such as parental death or separation can lead to trauma, depression and social stress. Similarly, psychosocial stressors such as institutionalization can lead to acute stress, anxiety and depression in children because they do not get used to the different surroundings and treatments in foster homes. Childhood maltreatment or sexual abuse can also induce depression, social stress, anxiety and individuals may fall prey to peer pressure that may lead to alcohol consumption, smoking, drug abuse and other unhealthy lifestyle options. There is now convincing data that poor early-life conditions, are associated with a series of lifelong, pro-inflammatory phenotypes [68-70] and inflammatory diseases such as T2D.

The exact extent of Hit 2 contribution in the aetiology of complex disorders including T2D have been studied sparsely in humans due to obvious ethical codes that prevent experiments involving manipulation of the early life environment. For this reason, scientists have studied 'natural experiments' such as the Canadian 'Project Ice Storm' and the Dutch Hunger Winter in the light of Hit 2 (reviewed in [71]). The severity of Project Ice storm showed a positive correlation with insulin secretion during adolescence which may indicate the incidence of insulin resistance [72]. Similarly, there was a clear evidence that intrauterine exposure to the Dutch Hunger Winter conferred a high risk of developing T2D in adulthood [73]. Therefore it is interesting to posit that the recent COVID-19 pandemic may also act as an early life insult, modulating metabolic pathways that may have similar impacts on coming generations.

Formation of the placenta marks the first stage of embryogenesis and thus is a potential determinant of the quality of the early developmental environment [74, 75]. Pregnant women who have been exposed to the SARS-CoV-2 infection (causative agent of COVID-19 infection) have shown placental injuries resulting in restricted blood flow to the foetus, thus indicating compromised foetal nutrition [74], which may cross over as a future risk of developing metabolic disorders including T2D.

Thus, it is clear that the quality of the early life shapes the metabolic trajectory of an individual's life. Adverse experiences in this critical developmental period leads to higher risks which can crystallise into the T2D phenotype when exposed to 'Hit 3' (detailed in the next section).

1.1.3. Later life T2D risk factors (Hit 3)

While we have only recently started to recognise the importance of early-life environment in the aetiology of T2D, the current environment and risk factors are quite well understood. Obesity and chronic inflammation are two well-established risk factors.

Obesity often results in the deposition of excess fat in tissues and blood vessels. This impedes blood flow and causes the release of pro-inflammatory molecules like TNF-α as well as Interleukins-6 and 1β, which can in turn contribute to the T2D pathogenesis [76] by facilitating apoptosis of islet beta cells [77]. Interestingly, stress has also been shown to mediate Interleukin-6 production from brown fat cells, to induce hyperglycaemia to orchestrate a fight/flight response [78]. Some studies have reported that IL-6 is responsible for insulin resistance [79], which may be exacerbated in T2D patients as they often show a dysregulated HPA axis, resulting in a vicious loop.

In addition to obesity, cigarette smoking, physical inactivity, and low-fibre diets further increase the T2D risk [2]. Conversely, moderate alcohol consumption decreases the risk [2]. Ethnic groups from an underdeveloped region have also shown increased predisposition to T2D on migrating to a Westernised region and on embracing the "westernised lifestyle" and environment [80]. When dietary, lifestyle and behavioural factors are taken together, the concatenation of multiple low-risk behaviours reduced the T2D incidence by 90% [81], suggesting that removing one of the three hits is enough to stop the aetiology.

HPA axis dysfunction has also been implied as a potential mechanism that promotes the T2D pathology [82]. In addition to the well-established classical risk factors for T2D, there is now growing appreciation for non-classical risk factors including low socio-economic status (SES), pollution, and exposure to ionising radiation. Unhealthy living conditions due to low SES, unhygienic surroundings (pollutants, irritants, increased exposure to allergens, antigens and pathogens) and the inability to afford healthy meals, proper sanitation, and lack of vaccination may account for low immunity and stress [83]. Low adult SES has been reported to alter stress associated *AVP* gene and five inflammation associated genes: *CD1D*, *F8*, *KLRG1*, *NLRP12* and *TLR3* [67]. The gene expression patterns of *CD1D*, *F8*, *KLRG1* and *NLRP12* are all altered due to changes in the methylation pattern [67].

Air pollution adversely affects the central nervous system, immune system, liver and adipose tissue and is also a major risk factor for T2D [84]. Chambers et al. conducted an epigenome wide study in Indian Asian and European populations (from blood and liver) and reported a three times higher risk of developing T2D in Indian Asians than Europeans [85]. India has a much higher air quality index i.e. more air pollution than most European countries and it is possible that the quality of the environment contributes to this higher incidence.

Overexposure to ionizing radiations such as ultraviolet rays can also alter the epigenome of metabolically relevant tissues eventually leading to T2D [86]. Ageing has detrimental effects on DNA methylation [86] as it affects the circadian rhythm by epigenetically regulating melatonin receptor genes (*MTNR1a* and *MTNR1b*) to trigger melatonin synthesis, which in turn blocks insulin secretion [87].

Exercise and gastric bypass surgeries (known to control obesity) have shown the potential to alter the methylation pattern in skeletal muscles of T2D patients for the better, strongly indicating that T2D phenotype can be improved by changes in the lifestyle [86]. Increase in physical activities alleviates glucose metabolism by encouraging glucose uptake in the skeletal muscles by

inducing the expression of genes, which have been epigenetically silenced, such as *Glut-4* reviewed in [3]. Ronn et al. showed genome wide alterations in 21 T2D and 18 obesity candidate genes like *FTO*, *TCF7L2*, *THADA*, *CDKN2A*, *CPEB4*, *KCNQ1* etc. after a six-month exercise intervention [88].

Overall, it is clear that non-genetic environmental factors play an important role in T2D incidence. However, intervention studies in individuals predisposed to higher T2D risks have successfully shown that we can delay if not prevent the onset of T2D by resorting to healthier options. Thus, the T2D aetiology like all other complex disorders requires the encounter of all 3 hits of "vulnerability and susceptibility" in order for the disease risk to crystallise. Other than the immutable genetic constitution (which comprises hit 1), hits 2 and 3 have been shown to have roots in physiological and psychosocial stress. The following sections elaborate on the stress response system and ELA as the epicentre of metabolic disturbances.

1.2 Early life stress and metabolic disturbances: 'A tale of two (adver)sities'

The psychophysiological stress reaction is the manner in which the body reacts to an external stressor that requires a fight or flight response disturbing physiological homeostasis. The stress reaction is primarily mediated by catecholamines and glucocorticoids. Initially, they maintain homeostasis and contribute to our overall survival. However, over the long-term, increased exposure to stress (allostatic load) has negative consequences [89]. Activation of stress reaction mobilises stored energy, induces immune cell traficking and biases the immune response as well as increasing heart rate and blood pressure, ensuring that oxygen and energy sources are available where needed.

Carbohydrate metabolism, in particular glucose homeostasis, is a key component of the metabolic reaction to an external stressor. Stress hormones such as the glucocorticoids play an important role in maintaining glucose homeostasis mediated by hepatocytes [90, 91] where glycogenesis (storage of glucose in glycogen chains), glycogenolysis (glucose release from glycogen), gluconeogenesis (de novo glucose production), and glycolysis (ATP release as glucose is converted to pyruvate and ATP) are balanced to maintain plasma glucose levels with tightly controlled parameters.[92, 93]. Under normal physiological conditions insulin, the only known glucose-lowering hormone, is principally counterbalanced by glucagon to control glucose homeostasis [93]. Plasma insulin, glucagon and adrenaline levels are all intimately linked to blood glucose levels [94]. To maintain plasma glucose, insulin activates glucose consuming processes

(glycolysis, glycogenesis) while glucagon and adrenaline increase glucose production (gluconeogenesis, glycogenolysis). During fasting, gluconeogenesis is triggered by glucagon via the cAMP / PKA / CREB / CRTC2 signalling pathway. This terminates at the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1α), which in turn coactivates transcription factors, including hepatocyte nuclear factor 4 α (HNF4α), forkhead box O1 (FOXO1), and GC receptor (GR), to activate hepatic gluconeogenesis [95]. When blood glucose levels rise after a meal, the rise in insulin levels inhibits gluconeogenesis by down-regulating the transcriptional mechanisms (FOXO1, PGC1α, and CRTC2) [95] as well as by activating glucose uptake by peripheral tissues.

The metabolic syndrome (MetS) is the umbrella term that includes impaired glucose metabolism, obesity and hypertension, all of which increases the risk of Type-2 diabetes (T2D) [96]. Over the last decade, MetS has been linked to chronic diseases by altered metabolic and pro-inflammatory pathways, and it has been demonstrated that it originates in early-life, with early-life socioeconomic position being the strongest lifelong driver of MetS [97]. Early-life adversity (ELA) is a broad term that covers all negative experiences affecting an infant's security or safety and inducing a large stress response. It ranges from growing up in a dysfunctional household, abuse or maltreatment to victimisation, bullying or exposure to crime [98, 99] and low socioeconomic status. Parental BMI, acting through a shared cultural environment and learned family eating patterns influences adiposity, BMI, and lipid profiles in their children [97, 100], passing through maternal education [101]. There is a strong epidemiological link between early-life stress or adversity and T2D [66, 102] as well as hypertension and dyslipidaemia [103] that was clear in meta-analyses over the last half-decade [104, 105]. It is probable that the risk of long-term metabolic disturbances passes through "programming" during sensitive developmental windows during early life. Such critical windows of developmental plasticity permit the body to adapt to the environment in which it is developing, and are thought to be mediated by epigenetic changes, potentially in systems such as the HPA axis that are sensitive to the external environment [106] .

Here we review how the early-life period programs the HPA axis, and how it interacts with metabolic pathways at baseline and under acute and chronic stress. We suggest that the link between exposure to chronic stress in early life and changes in the metabolic profile later in life with an the increased risk of metabolic syndrome and type 2 diabetes may occur either through changes in gluconeogenesis in the liver, or through the manner in which HPA axis glucocorticoids regulate gluconeogenesis. "Classic" results have been generated over previous decades, however, only recently has detailed molecular evidence started to become available as to how

glucocorticoids regulate gluconeogenesis [95], and to date, their exact role in the development of MetS after exposure to ELA has been rather under-explored.

1.2.1 The physiological response to external psychosocial stressors

1.2.1.1 The ANS and the HPA axis stress response

Exposure to a stressor activates the paraventricular nucleus of the hypothalamus to secrete corticotropin-releasing hormone (CRH), which in turn activates two synergistic stress response systems, the rapidly responding autonomic nervous system (ANS) [107, 108] with an associated catecholamine release and the second, slower arm of the stress response is the hypothalamus—pituitary—adrenal (HPA) axis [109]. The catecholamines adrenaline and noradrenaline act rapidly and transiently upon stress exposure by increasing the heart rate and raising blood pressure. The sympathetic branch (SNS) of the ANS directly activates preganglionic neurons that project from CRH-containing neurons in the PVN, through noradrenergic centres in the locus coeruleus in the brainstem, then projecting directly through sympathetic preganglionic neurons in the adrenal medulla chromaffin cells from where catecholamines are secreted [110]. This is tempered, controlled, and negated by the parasympathetic branch (PNS) of the SNS, again projecting from the locus coeruleus returning the system to homeostasis. The locus coeruleus integrates signals and balances SNS and PNS activity through activation of the α1- and α2-adrenoceptors on the sympathetic and parasympathetic neurons respectively [111, 112]. Furthermore, SNS activation provides positive feedback, further increasing CRF secretion from the PVN [113]. The hippocampus, now thought to be a key element of the approach-avoidance system which involves weighing probabilistic profits and losses for an experience, thereby playing a prime role in anxiety generation. Hence, it is also part of the conscious stress response [114] and feeds direct inhibitory signals into the PVN, influencing basal GC levels, circadian GC rhythms, as well as inhibiting the HPA axis stress response [115, 116].

The end product of the HPA axis are the glucocorticoids (GC), principally cortisol in humans and corticosterone in rodents that act through their cognate receptor, the glucocorticoid receptor (GR). The presence of 17-α-hydroxylase (CYP17) in the human adrenocortical zona fasciculata means that in addition to cortisol, humans secrete a small amount of corticosterone. The physiological function of corticosterone in man remains to be fully elucidated despite it having analogous metabolism. The two molecules are differentially shuttled across membranes in humans

by ATP binding cassettes (ABC), resulting in differential responses to them in a tissue specific manner [117]. These steroid glucocorticoids subsequently regulate e.g. inflammation, lymphocyte trafficking, metabolic, cardiovascular and behavioural processes amongst others [118] and are regulated by a hormonal cascade which is initiated by the hypothalamic nuclei. Circadian messages from the suprachiasmatic nucleus (SCN) are integrated with physical, emotional and cognitive reactions in the PVN [119, 120]. Activated neurons in the PVN secrete CRH. The cascade is propagated via adreno-corticotropic hormone (ACTH) released from the anterior pituitary gland, which in turn stimulates the adrenal cortex to release glucocorticoids [121, 122].

Cortisol and corticosterone have ultradian and circadian rhythms which are chiefly regulated by the HPA axis (Figure 1.3). Circadian cortisol and corticosterone concentrations peaks around waking; cortisol peaks in the early morning hours while corticosterone levels peak mid-afternoon in nocturnal rodents. This circadian rhythm overlies an ultradian rhythm of the complete HPA axis signalling cascade. The frequency of secretory episodes is relatively stable at one every ~1 h while the amplitude of the secretory episode, and hence the mass secreted, provides variation in the measured concentrations [123-125] as shown in Figure 1.3. The corollary to the pulsatility model is that rapidly rising glucocorticoid levels in the secretory phase induce the necessary glucocorticoid receptor-mediated negative feedback that terminates the hormonal pulse, then, after a constant time interval (the inter-pulse interval) the SCN and the PVN trigger the subsequent pulse [126]. Thus, the HPA axis in conjunction with the ANS provides the molecular weapons needed to elicit a fight/flight reaction in response to stress.

In the early life, the stress hypo-responsive period (SHRP) is a particular period when the HPA axis is poorly responsive. This is between postnatal-days 3—14 (R. norvegicus), and 1—12 (M. musculus) [127]. This period is characterised by extremely low baseline plasma corticosterone levels as well as a reduced ACTH and corticosterone response to stress. This period is complemented by a second critical period. During adolescence, gonadal hormones exert an organizational influence on the HPA axis [128]. As outlined in Section 1.3 stress in these periods, particularly the SHRP, leads to behavioural and neuroendocrine abnormalities, as well as depression and anxiety disorders in a sex-dependent manner.

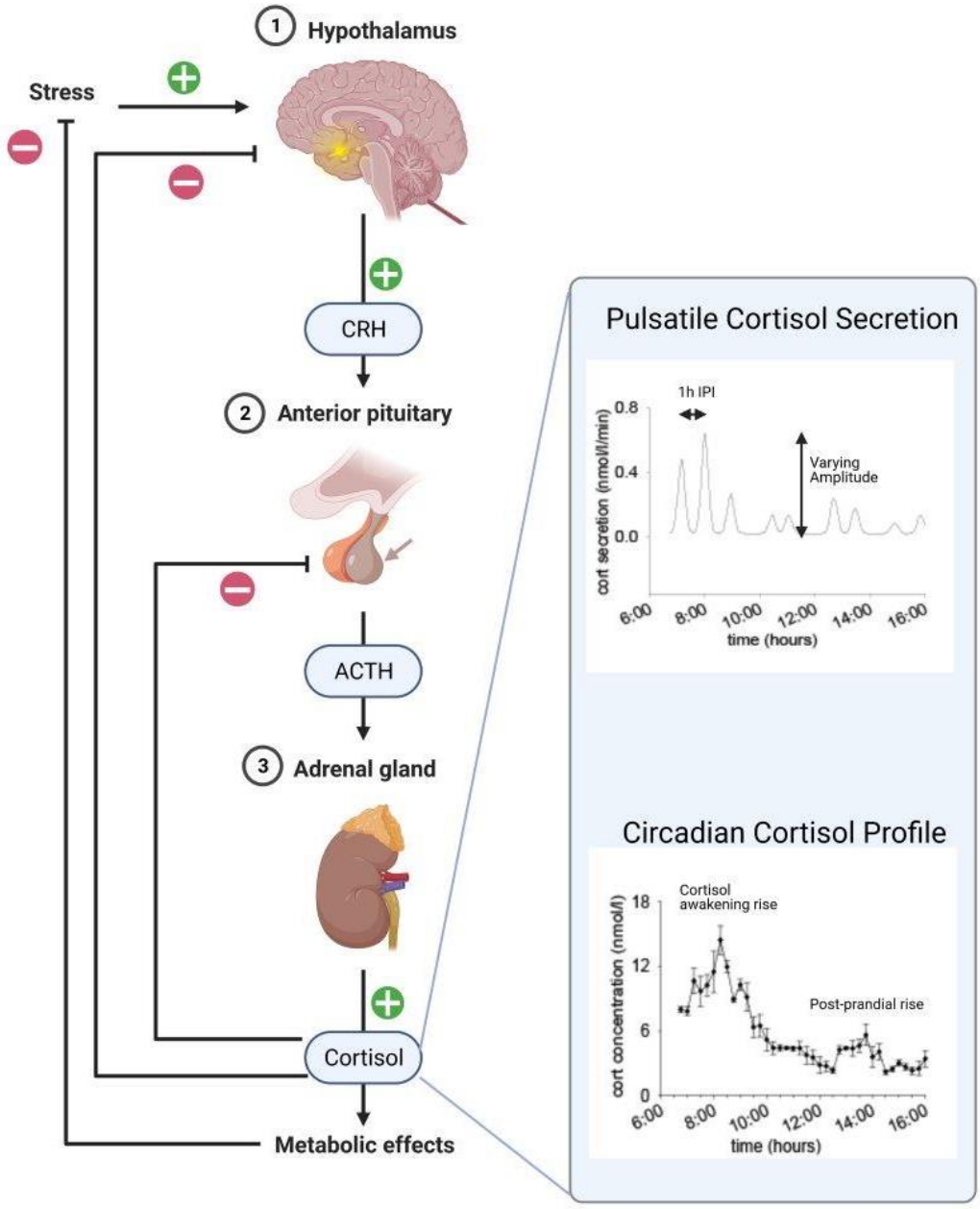


Figure 1.3: The Hypothalamus Pituitary Adrenal axis: This axis controls both ultradian and circadian cortisol rhythms to generate metabolic effects that help the body combat stress and re-establish homeostasis post stress. Pulsatile cortisol profiles adapted from [124].

1.2.2 Metabolic adaption

Metabolic adaptation during stress aims to preserve glucose, providing a short, transient, increase in blood glucose levels temporarily enhancing cognitive processes [129]. In addition to

gluconeogenesis, glucose uptake is reduced in white adipose tissues and skeletal muscles under stress conditions (reviewed in [129]) (Figure 1.4).

Secondary active transport and facilitated diffusion are the two main ways in which glucose is trafficked within the body. Secondary active transport makes use of ATP to distribute glucose in the kidneys. Facilitated diffusion relies on special membrane proteins that allow the ferrying of glucose molecules against a concentration gradient, which may/may not be insulin driven.

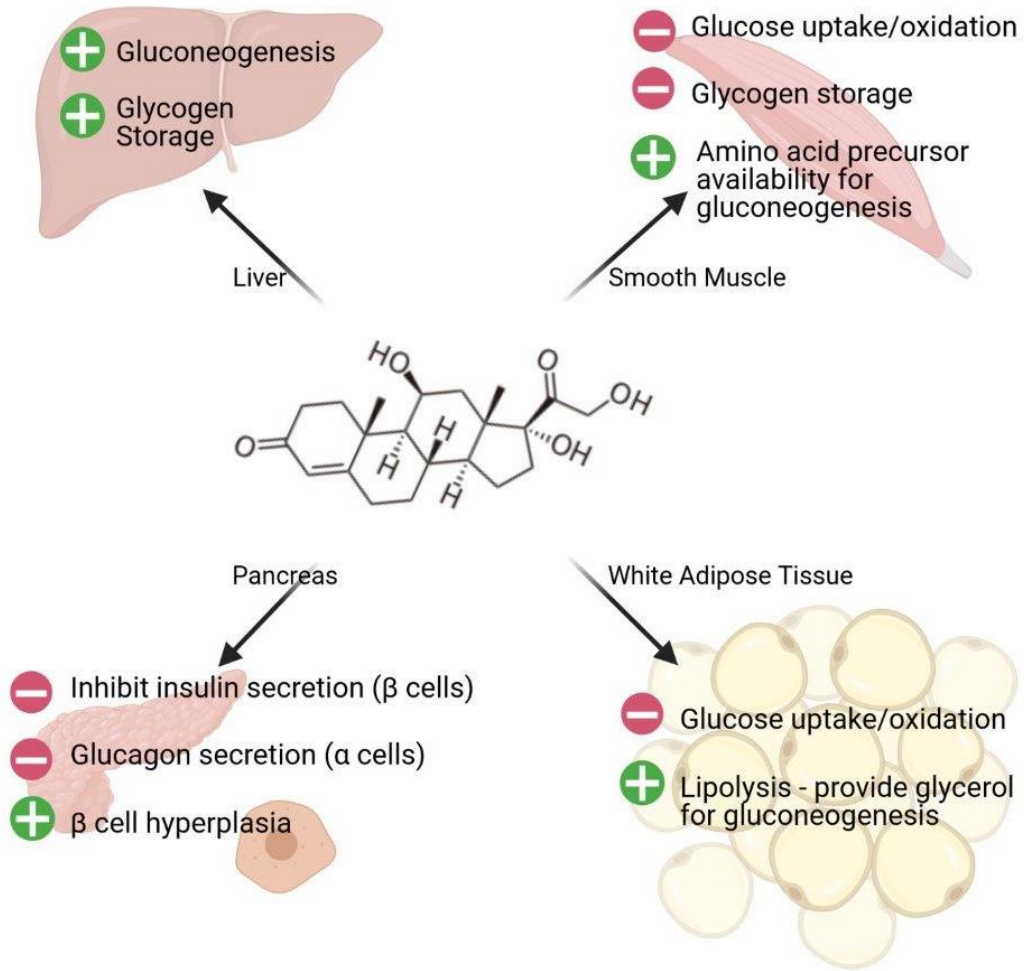


Figure 1.4: Cortisol triggers a cascade of events that affect glucose homeostasis. The liver, skeletal muscles, white adipose tissues and pancreas play a key role in ensuring continuous supply of useable energy for the fight/flight response.

GLUT receptors predominantly govern the uptake of glucose into the cells. There are 14 distinct glucose transporters, of which only GLUT 1-4 are particularly interesting [130]. GLUT 1, a key receptor in the brain and GLUT 3 have been reported to have high preference for glucose. GLUT

2 and GLUT 4 dictate the uptake of glucose in the pancreatic cells and muscles respectively [130, 131].

Absorbed glucose is directed to enter the glycolytic cycle, the final product of which is pyruvate. The synthesised pyruvate is converted into either lactate or carbon dioxide and water depending on the absence or presence of oxygen respectively. Normal physiological conditions strike a balance between the lactate and pyruvate concentrations. Chronic stress increases the net lactate and pyruvate concentrations [132]. This happens so that the produced pyruvate can either enter the glycolytic cycle to produce glucose or be metabolised to generate high concentrations of lactate due to reduced action of pyruvate dehydrogenase (PDH), which normally functions under anaerobic conditions [133]. Chronic stress attenuates the activity of PDH owing to phosphorylation by increasing concentrations of PDH kinase [134]. Stress also causes lipolysis, which provides gluconeogenic substrates [135]. Allostatic adaptations during stress also result in increased activity of the Kreb's cycle due to readily available acetyl coenzyme-A produced by the oxidation of fatty acids. This increased activity of the Kreb's cycle also supplies substrates for gluconeogenesis such as oxaloacetate. Thus, these interconnected metabolic pathways adapt during stress to ensure that the body, in particular the brain, has useable glucose supply.

1.2.3 Metabolism and acute stress interactions

Acute laboratory stressors such as the trier social stress test (TSST) activate the HPA axis inducing cortisol secretion and then the negative feedback loop bringing cortisol levels back to baseline [136]. When the TSST was first developed, the role of energy availability was rapidly investigated. It was initially reported that an 8h fasting period prior to a Trier Social Stress test (TSST) significantly reduced HPA axis reactivity, while glucose supplementation 1h before the paradigm significantly increased cortisol and HPA axis reactivity [137]. Furthermore, when this was extended to food components such as protein or fat, the fasting-induced blunting of the HPA axis response was not restored due to immediate unavailability of useable energy. Moreover, there was a strong link between the increase in cortisol levels during the stress paradigm, and the increase in blood glucose after administration of the glucose bolus [138]. When the mechanisms were further dissected in rats, hypothalamic nuclei such as the ventromedial and paraventricular nuclei (VMN, PVN respectively) stood out. It was proposed that the high insulin and glucose levels observed after glucose loading stimulated VMN, inputting into the PVN, subsequently activating the HPA axis. Importantly, this was pharmacologically validated by colchicine (VMN activity antagonist) disrupting the fasting-induced reduction in HPA axis responsiveness [94, 139] and moreover, low

blood glucose levels significantly inhibit VMN and PVN activity consequently attenuating HPA axis activity and reactivity. The differential response to glucose, fat and protein loading prior to the TSST suggest that, similar to the rat, central mechanisms are also involved in the human situation rather than metabolic pathways such as the Kreb's cycle [138].

At baseline, both hypo- and hyper-glycaemic states alter the HPA axis. In both the fasting and post-prandial state cortisol secretion was increased. Deconvoluting cortisol concentration to access the underlying HPA axis activity, hyper- and hypoglycaemic states had no effect on the number of secretory events, the inter-pulse interval, secretory burst length, or cortisol half-life, however, they modulated the mass secreted during each event [140, 141]. There is however, a gap in the literature on how a stressor such as the TSST activates gluconeogenesis. On the other hand, blood glucose levels are also dependent on catecholamine levels. Release of adrenaline induces a rapid rise in blood glucose levels through a combination of reduced glucose uptake in insulin-dependent tissues as well as a temporary increase in hepatic glucose production from both gluconeogenesis and glycogenolysis. Glycogenolysis wanes rapidly, while gluconeogenesis is mainly responsible for the resultant hyperglycaemia [142]. Therefore, it can be postulated that the status of the HPA axis may be extracted from blood glucose records, and vice versa, and that the nervous system plays a significant role in maintaining homeostasis/allostasis.

1.2.4 Metabolism and chronic stress

Synthesis of glucose using "non-carbohydrate sources" occurs by gluconeogenesis, a central metabolic pathway that also operates during starvation. Stress regulated glucocorticoids transcriptionally trigger key players of the gluconeogenic pathway such as glucose-6-phosphatase (G6Pase), pyruvate carboxylase, pyruvate carboxy kinase (PEPCK) and fructose 1,6 bisphosphatase (FBPase). Interestingly, it has been shown that a doubling in PEPCK expression can lead to insulin resistance and a seven times increase can result in hyperglycaemia [143]. The GC-GR transcriptional dependence is clearly demonstrated in mice lacking hepatic GR that show an exaggerated hypoglycaemia after fasting as neither PEPCK, pyruvate carboxylase, nor G6Pase can be upregulated [144], although all three genes are involved in gluconeogenesis, G6Pase is however, also involved in hepatic glucose release from glycogenolysis [145]. This occurs through direct GR interactions with genomic glucocorticoids response elements (GREs). GR ligation and DNA binding provides a "transcriptional hub" where coactivators such as GR-associated PGC- 1α, FOXO1 and HNF-4 interact to provide the full gluconeogenic response and expression of G6Pase and PEPCK (reviewed in [91]).

The concentration of other gluconeogenic substrates like oxaloacetate and pyruvate also increase during stress owing to increased activity of hormones such as glutamic oxaloacetatic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Thus, chronic stress increases glycogenolysis and gluconeogenesis, while it downweighs glycogenesis and insulin sensitivity, resulting in a diabetogenic physiological state.

1.2.5 Glucocorticoids and diabetes

Type 2 diabetes is largely the result of uncontrolled hepatic gluconeogenesis [146]. When the glucocorticoid actions are impaired by mutations in the GR, mice are protected from experimental models of hyperglycaemia, metabolic syndrome and have reduced gluconeogenic gene expression. While the implication of glucocorticoids in T2D is well established, the underlying mechanisms are still not known, although there are data that suggest changes in expression of the GR itself [147], cofactors such as PGC-1 alpha [148], and inactivation of cortisol by 11-beta HSD, reducing GC signalling [149, 150]. GC effect are also seen in the pancreas.

During metabolic dysfunction, insulin secreting pancreatic beta cells can no longer compensate for hyperglycaemia, and long-term exposure to high levels of exogenous or endogenous GC during therapeutic administration of Cushing's syndrome respectively reduce insulin secretion and induce a diabetes-like phenotype [151]. Overexpression of the GR in pancreatic beta cells leads to reduced insulin secretion associated with an impaired glucose tolerance and eventually hyperglycaemia [152]. Moreover, GC signalling through the GR is a trigger of insulin resistance in muscles, supressing GLUT 4 translocation to the cell surface, as well as downregulating glycogen synthesis [153]. It would appear that the GR interacts directly with both the insulin and insulin-like growth factor 1 (IGF-1) pathways [154], although detailed mechanistic studies of GC effects in muscle carbohydrate metabolism are cruelly lacking [91].

The interaction between the HPA axis and T2D is bidirectional. There is a clear link between T2D, diabetic complications, and HPA axis hyperactivity [155]. Furthermore, hyperglycaemia from T2D can dysregulate the HPA axis, subsequently increasing the risk of major depression [156]. Mechanistically, this passes through altered secretion of ACTH from the pituitary gland, leading to altered GC levels [155, 156]. In a series of particularly well designed experiments, Mosili et al. demonstrated that as rats entered a prolonged pre-diabetic state baseline ACTH levels under non-stressful conditions dropped, but GC levels were significantly elevated [157]. The HPA axis negative feedback loop should, under normal conditions bring down GC levels when ACTH levels are low [158] suggesting that the negative feedback is somehow impaired [155]. Furthermore, when Mosili

et al induced a chronic stress together with pre-diabetes, the rats were unable to mount a normal ACTH or GC response to subsequent acute stressor, further consolidating their observation of either impaired GC signalling or negative feedback [157]. Interestingly, diet would appear to affect the adrenal gland as increased GC secretion, adrenal cortical hyperplasia, and increased steroidogenesis have been reported in T2D- and obesity-inducing high fat diets [159]. Furthermore, certain sugars such as fructose can bind glucose transporter and can pass the blood-brain barrier and be absorbed in both the hippocampus and hypothalamus activating the HPA axis [160, 161]. Thus, poorly controlled T2D can result in an impaired HPA axis, which in turn creates a vicious cycle, where excessive GCs antagonise glucose homeostasis and insulin sensitivity.

1.3 Early life Adversity: the epicentre of metabolic disturbances

1.3.1 Early-life Adversity and changes in the HPA axis

In all societies studied so far, ELA is prevalent, with, for example 59% of the US population reporting at least one adverse event in the BRFSS (Behavioural Risk Factor Surveillance System) study [162]. ELA has broad long-term consequences on the neuroendocrine, immune and metabolic systems (reviewed in [99]) as well as on neuroplasticity and neuronal morphology altering the overall cerebral maturation trajectory (reviewed in [163]). Although the literature is somewhat unclear and contradictory, the most useful classification proposed to date [163] has divided early life stressors into broad categories. “Mild” stress in the neo- and pre-natal period appears to induce HPA-axis hyperactivity such as increase the cortisol responses to a standardized stressor in pre-adolescent children [164, 165]. Slightly higher “moderate” stress levels linked to more clear forms of early life adversity (e.g., frequent emotional maternal withdrawal, corporal punishment, or interparental aggression) increasing both the baseline cortisol level [166], and the HPA axis response to stress [167]. Severe early life stress or adversity e.g., institutionalization, neglect, abuse, or deprivation lowered basal cortisol levels [168, 169] and blunted HPA axis reactivity [170, 171]. Hypocortisolism and reduced HPA axis responsivity was initially proposed to be either due to a reduced pituitary response to hypothalamic CRF [172] or by the hypersensitivity of the final glucocorticoid target tissues and the HPA axis tissues in the negative feedback loop [173]. The latter would appear to be excluded since in our EpiPath institutionalization-adoption “severe” early life stress cohort, peripheral glucocorticoid receptor signalling and functionality was essentially preserved and indistinguishable from non-exposed controls [174]. It should, however, be

remembered that the literature hasn't come to a definitive conclusion as to the exact effects or classification of the different forms of ELA.

The situation may be somewhat more complicated and dependent on the timing of the adversity. Adversity in early childhood, was associated with a decreased hippocampal volume, whilst prefrontal cortex volume was reduced if exposed during adolescence [175, 176]. Psychopathologically, exposure to adversity or trauma before age 12 increased the lifelong risk of developing major depressive disorder, but when occurring between age 12 to 18 the risk of PTSD was increased [177]. It has been suggested that since the human hippocampus is not fully developed before age 2, the frontal cortex primarily matures between age 8 -14 and the amygdala continues developing until early adulthood [178], that the hippocampus is most probably the brain area most affected by early life stress [163]. The sensitivity of the hippocampus to ELA is particularly important, as, outlined above, it plays a key inhibitory role in PVN activation of the HPA axis. Hence, ELA renders the HPA axis impaired, which in turn causes insidious changes to the stress response mechanism along with glucose metabolism, eventually contributing to MetS (Figure 1.5).

There is more to the HPA axis than reactivity to a laboratory stressor. ELA was initially reported to be associated with elements of the cortisol diurnal rhythm such as the cortisol awakening rise (CAR) [179], however, the most recent meta-analysis suggests that this is not the case [180], although the meta-analysis of the stress-induced changes was much stronger [181]. The weakness in the CAR meta-analysis was probably due to large heterogeneity between the techniques employed between the different studies.

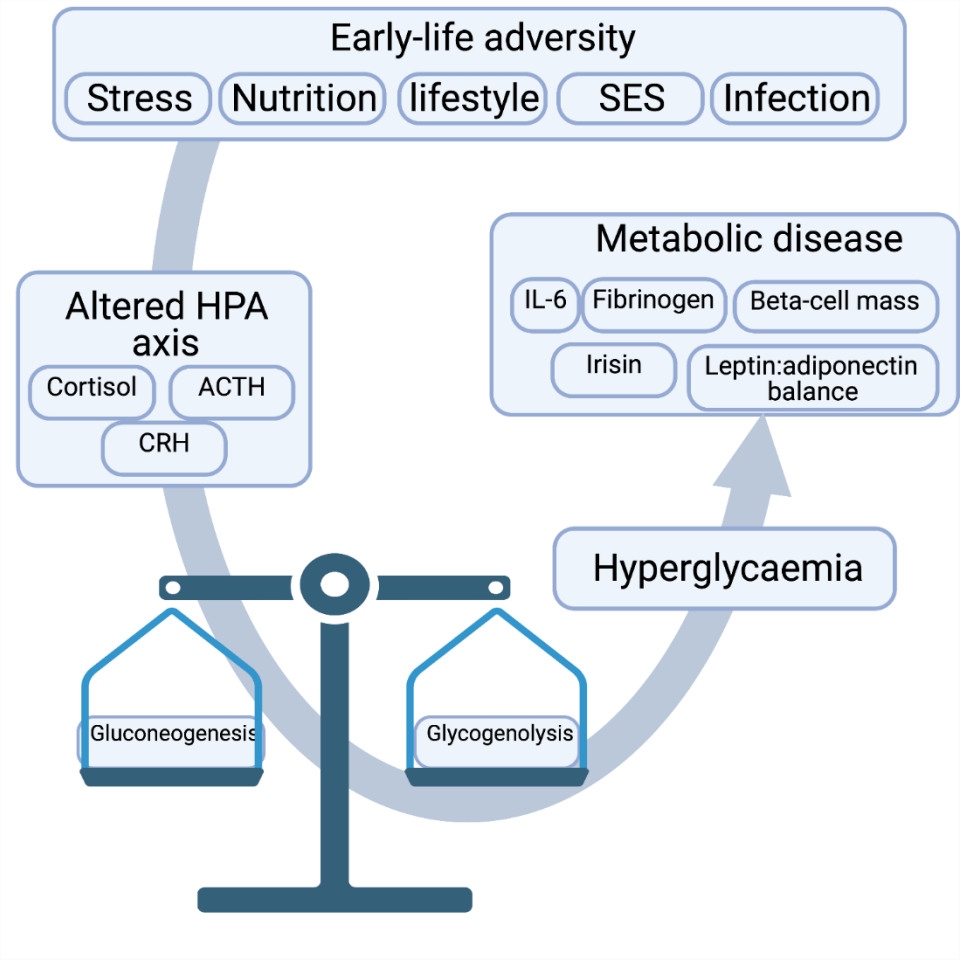


Figure 1.5: Early life adversity dysregulates the HPA axis and its key effector molecules. This dysregulation in turn disrupts glucose homeostatic balance leading to hyperglycaemia and metabolic syndrome if left unchecked.

1.3.2 *Early-life adversity, diabetes and the metabolic syndrome*

MetS has been shown to predict not only cardiovascular mortality, but also the progression to full type 2 diabetes (T2D) [182]. There is growing evidence that early-life nutritional and psychosocial stress or disadvantage determine the trajectory and transition into metabolic dysfunction, MetS, and T2D later in life [97]. There are two principal components of the early-life socioeconomic position that contribute to lifelong MetS and diabetic risk – early-life nutrition and early-life stress. Reports from ourselves and others of early-life adversity have shown effects on either the metabolic profile, obesity, or type 2 diabetes [68, 183, 184]. Socioeconomic status in early life has a similar effect [66], and is associated with T2D 50 years later [65]. ELA also predisposes individuals towards a chronic inflammatory phenotype [68-70, 185]. Furthermore, cardiometabolic disease markers such as fibrinogen, C-reactive protein, and interleukin-6 were elevated [104, 186],

as were endothelial dysfunction markers such as ICAM-1, E-selectin [187], as well as clinical measures such as arterial stiffness [188] or poor blood pressure trajectories with age [189]. Recently, this was replicated by Chandan et al (2020) confirming the role of ELA in determining "a significant proportion of the cardiometabolic and diabetic disease burden may be attributable to maltreatment" [71, 190].

Although there are few mechanistic data available, these metabolic abnormalities may be due to changes in circulating adipokine levels. ELA has been directly associated with an increased leptin:adiponectin ratio [191]. Leptin, secreted by adipocytes, regulates energy balance through decreasing appetite and is associated with the metabolic syndrome [192, 193], whilst adiponectin, has insulin-sensitizing effects. Low adiponectin levels are associated with type 2 diabetes and insulin resistance [193, 194]. Furthermore, Irisin levels were increased by ELA [191]. Irisin, mediates glucose metabolism as well as exercise-related energy expenditure and is a peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α)-dependent myokine [191, 195]. Glucocorticoids may also play a role, as, in utero exposure to maternal under nutritional affects beta-cell number and function lifelong in a manner dependent upon GR and GC since deletion of the GR in foetal pancreatic cell abrogated this effect [91]. These changes may, in part be due to the effects of ELA on the methylation of genes involved in obesity and metabolic pathways [196, 197]. Furthermore, adverse early life conditions induce lifelong changes in gene transcription [67]. Low SES, for example, has been associated with inflammatory and diabetic genes such as TLR3 [198], NLRP12 [199], F8[200], KLRG1 [201], CD1D [202], as well as the stress-associated genes OXTR [203], FKBP5 [204], and AVP [205]. Suggesting, as we have previously proposed, that a negative early life environment acts through inflammatory pathways that are also associated with T2D, targeting pathophysiological factors such as stress and inflammation, participating in the aetiopathology of T2D [71]. Thus, it appears logical to conclude that ELA can effectively alter glucose homeostasis and that the aetiology of MetS and eventual T2D may have strong roots in ELA. Moreover, ELA would appear to be associated with more advanced or complicated diabetic-pathologies requiring more aggressive management [206].

1.3.3 *Glucose metabolism, allostasis and allostatic load*

Ever since Hans Selye described the "general adaptation syndrome" as our response to external stressors [207], there has been a paradox. The ANS and HPA axis protect in the short-term, but over the long-term they may accelerate disease as well as causing lasting damage. Allostatic

Load (AL) is fundamentally a chain of causal events from the primary stress response of SNS and HPA axis activation with adrenaline and cortisol secretion, inflammation [208], leading to secondary markers of stress exposure including hyperglycaemia, hypertension, hyperlipidaemia, and central adiposity. The AL cascade, the overall sequence of responses, as well as their contribution to disease development are not fully understood, and somewhat under-investigated although markers of AL are associated with increased glycaemic measures in women [209]. Similarly, rat chronic stress models, a proxy for AL, consistently report high blood glucose levels up 6 months later [133]. In allostasis these rats also had dysregulated glucose metabolism pathways, in particular increased gluconeogenesis [133]. Furthermore, this stress induced hyperglycaemic state resulted in impaired glucose tolerance and reduced insulin sensitivity [133]. This may be due to metabolic memory, which causes the body to recall the influence of metabolic regulators for a much longer duration. This, in conjunction with persistent stress can lead to maladapted allostasis and eventually AL. Prolonged AL can give rise to metabolic syndrome. There is also recent evidence that diet may act as a stressor. Increased glycaemic load (i.e. dietary sugar intake) is associated with increased markers of AL, particularly in women, suggesting that dietary carbohydrate intake may contribute towards dysregulation of the AL response [209]. It is well established that carbohydrate intake can stimulate the ANS [210], and hypothesised to cascade down increasing AL markers and blood glucose levels [209]. Thus, chronic stress/AL can adversely affect glucose metabolism and mount a faulty bodily adaptation that ultimately leads to MetS and also T2D.

1.3.4 *T2D can itself act as a source of ELA*

Overall, three in-utero conditions, hyper- or hypo-nutrition, and stress have been associated with subsequent T2D in the offspring. Although birthweight is a crude measure of the in-utero environment, they are strongly associated with the nutrients available to the foetus as well as maternal diet and lifestyle factors [211]. Infants born with a low birthweight have a significantly increased risk of metabolic syndrome as well as T2D and cardiovascular disease later in life [212, 213]. Two important natural experiments, the Dutch Hunger Winter of 1944 and the Chinese famine, between 1959 and 1961, clearly demonstrated that exposure in utero to famine induced impaired glucose tolerance, as well as increased the risk of being overweight and having type 2 diabetes, hyperglycaemia and metabolic syndromes many years later [214, 215]. Interestingly, identical twins that are divergent in birthweight have different metabolic trajectories. The twin with

lower birthweight has an increased risk of developing T2D or a metabolic syndrome [216]. Although the literature on restricted uterine nutrition and foetal growth is quite clear, the overall picture is more complicated as a high birthweight, from foetal over nutrition, produces a similar diabetic phenotype, suggesting a biphasic model [217] (Figure 1.6).

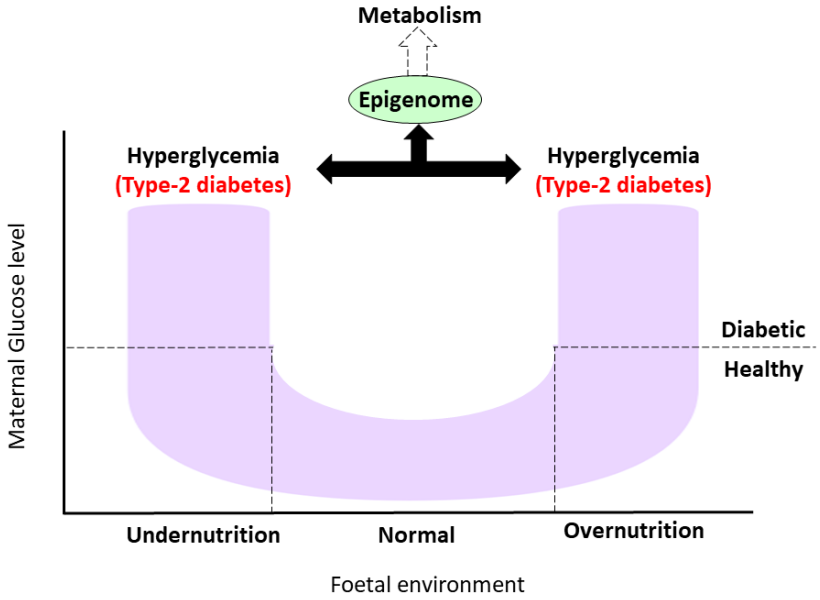


Figure 1.6: Both foetal over and undernutrition due to maternal glucose level triggers the Type 2 diabetes aetiopathology in most of the cases. The nutritional stress reprograms the epigenome, and induces permanent metabolic changes in the foetus as it adapts to the intrauterine environment.

Mechanistically, foetal under nutrition followed by exposure to a normal diet postnatally induces defective insulin signalling not only in peripheral but central tissues as well [218]. The foetal over nutrition hypothesis was translated into murine models successfully [1, 219]. Here, it was possible to see that the phenotype was not only transmitted from one generation to the next, but also survived through to the third generation (only in females). Unlike the global T2D risk passing through the maternal lineage, this phenotype was passed through the paternal lineage [220]. This suggests a broad, stable epigenetic modification of the germ line through imprinted genes potentially providing a mechanism for sex-specificity in the transmission and inheritance of the diabetic phenotype [220]. Foetal over nutrition happens due to hyperglycaemic intrauterine exposure (gestational diabetes mellitus) or obesity [221] and markedly increases the risk of T2D in offspring. [222, 223]. In physiological conditions, there is an increase in maternal insulin secretion during pregnancy that prompts the utilization of fatty acids as a source of energy, while saving glucose as an energy substrate for the foetus [221]. However, when the maternal beta cells fail to

keep up with the increased need to secrete insulin or if the pregnant mother has a pre-existing diabetes condition, this leads to foetal hyperglycaemia. This also induces foetal insulin production and eventually hyperinsulinism and hyperglycemia [224]. This exposure to elevated levels of insulin not only promotes an anabolic reaction (which may lead to foetal overgrowth) but also reprograms the epigenome, and induces permanent metabolic changes in the foetus as it adapts to the hyperglycaemic intrauterine environment (reviewed in [225]).

The effects of maternal stress during gestation on the developing foetus have been studied in several natural experiments. The most convincing was Project Ice Storm, covering pregnant women during the 1998 Quebec Ice Storm [226]. The perceived stress severity (salience) subsequently predicted both the levels of C-peptide ("endogenous measure of insulin secretion") [227] and insulin [72] in the offspring when they reached adolescence. They confirmed a previous report of maternal stress from bereavement during pregnancy that increased the likelihood of the offspring being diagnosed with T2D many years later [72].

An interesting study using "reciprocal embryo transfer" showed that diabetes during pregnancy can cause congenital and fetal growth abnormalities, regardless of the maternal diabetic status during preconception [228]. Thus, in conclusion, the transmittance of risk factors through generations may suggest that T2D in itself acts as a type of ELA or early life stress thereby contributing to T2D related physiological and metabolic anomalies arising due to a vicious cycle.

1.4 Inflammation links the early-life environment to T2D

Children have an immature immune system, which incapacitates their ability to battle early life infections that may alter the gut microbiome, leading to gut dysbiosis which is implicated in T2D, obesity, anxiety and depression [229]. The unbalanced composition of the gut flora has often been linked to inflammation (reviewed in [230]), which may explain the high levels of circulating inflammatory markers and thus a higher T2D predisposition in these individuals. It is interesting to hypothesise that the high sugar availability in diabetics may also disturb the gut flora composition by better nourishing some micro-organisms in favour of the other colonies thereby supporting them to thrive better, which, may lead leading to severe infections [231]. Thus a hyperglycaemic environment may also give rise to gut dysbiosis, thereby disturbing the bidirectional microbiota-immune axis.

While changes in the diabetic innate immune system were thought to cause the persistent, low-grade, inflammation seen in T2D [232], the evidence is now growing for T cell

immunosenescence together with underlying transcriptional changes and the pro-inflammatory "senescence associated secretory phenotype" (SASP) to play a role [70, 233].

The exact extent and mechanism leading to this 'low-grade inflammation' and eventually stubborn chronic diseases is debatable and two hypotheses have been proposed so far; 1. ELA manipulates the stress response system, which then acts as a mediator to epigenetically modify the immune system. 2. ELA independently manipulates the stress response system and the immune system, contributing to disease progression in a concerted fashion [69].

GR expression can be manipulated by early life stressors such as differences in maternal care [234] as well as in adulthood [235] and can thus influence the immune system. These differences are maintained lifelong and have many functional consequences. Rats subjected to maternal deprivation (MD) showed reduced GR expression due to epigenetic alterations (increased GR 1_7 promoter methylation) [236]. Although baseline values were similar, MD dramatically increased NF-kB signalling mediated pro-inflammatory cytokine secretion and inflammatory marker expression after a simple sevoflurane anaesthesia, a phenotype that was reversed by administering Trichostatin A [236], a histone deacytylase inhibitor.

EpiPath, a mechanistic cohort investigated the effects of severe ELA on health in individuals who had been adopted from orphanages. Results from this cohort have shown that despite altered GC secretion to psychosocial stressors [170, 174], the first suggested mechanism i.e. epigenetic programming of the glucocorticoid receptor (GR) is unfortunately incorrect as glucocorticoid (GC) signalling and function remained intact. This cohort provided the first broad description of the immune system (innate, humoral and adaptive arms) in an institutionalisation-adoption model. Overall, these data suggested that ELA programs the immune cells, leading to a long-term shift in T cell differentiation and activation status towards a more pro-inflammatory phenotype. This was accompanied by increased immunosenescence (HLA-DR/CD25 and CD57 expression) and an accumulation of effector memory and terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) [68-70, 237]. In the context of T2D this is important because initial reports suggested that immune senescence was increased in CD4+ [238] and CD8+ cells [239], shown by reduced CD28 levels (helps in T cell proliferation). This was recently confirmed by Lau et al. who conclusively demonstrated that although T2D patients may be lymphopaenic, the T cells present were comprehensively skewed towards the senescent TEMRA CD4 and CD8 subsets [233]. Furthermore, the pro-inflammatory T2D milieu promotes both immunosenescence and diabetic disease progression inducing a vicious cycle.

Repeated chronic stress has been shown to disturb the neuro-endocrine equilibrium and disrupting the ability to maintain physiological balance (allostasis) [240], thereby resulting in an increased risk of developing metabolic syndromes like T2D [97]. Thus, culminating an increased allostatic load ("cumulative physiological wear and tear") due to adversities experienced in early life [241]. This allostatic load prevents the body from adapting to the stress and achieving homeostasis, thereby promoting obesity, inflammation [242] and eventually T2D [243].

'Low-grade inflammation' may also play a significant role in a phenomenon called metabolic memory by means of epigenetic mechanisms [244]. Metabolic memory enables the body to remember the influence of metabolic regulators for a very long time. This can be beneficial for hyperglycemic patients when immediate and aggressive control of hyperglycemia is executed as these patients show a low risk of developing 'hyperglycemia-induced pathologies' regardless of the progression of diabetes [245]. Similarly, hyperglycemia-induced epigenetic changes can remain unaltered for a long time owing to the involvement of inflammation-associated genes, which may induce long-term complications [246]. Joung et al. focused on the effects of early life adversity on adipomyokines like leptin, irisin, adiponectin and CRP (marker of inflammation) from blood serum. Higher levels of leptin leads to leptin resistance where the brain does not receive signals to stop feeding due to the absence of the feedback mechanism leading directly to obesity in the higher ELA group [191]. It is interesting to note that the leptin gene has its promoter region situated within a CpG island indicating its lability to undergo DNA methylation modifications according to the (early) environment, thereby altering its function [3]. Heavy physical activity and exercise is known to release irisin, a hormone that promotes glucose uptake in skeletal muscles and has been strategized as a combating target against obesity related diabetes even though its exact engagement in T2D is unclear [247].

Finally, ELA showed significant shortening of telomere length (a marker of senescence) proportional to the length and type of adversity indicating its contribution to biological ageing [248], another significant T2D risk factor.

It is interesting to note that the type of diet decides the composition and diversity of the gut flora, which in turn dictates the gut barrier integrity [249] and thus the status of the immune system. A compromised gut flora barrier leads to release of inflammatory cytokines in the body, which affects insulin sensitivity [249]. Thus, it is clear that all environmental factors which aided inflammation also promoted inflammation-mediated insulin resistance [246]. This indicates that the immune system is majorly impacted in T2D cases. The following sections of this thesis provide

an outline of the immune system in healthy humans and explains the immune dysregulations observed in diabetics.

1.4.1 Basic concepts of innate and acquired immunity in healthy humans

Innate immunity (first line of defence): The breaching of the epithelial tight junctions and the mucosal barrier by foreign invaders mounts an innate immune response, which is rapid, non-specific and remains phylogenetically unaltered. The components of the innate immune system includes myeloid cells (phagocytes and some antigen presenting cells) such as tissue-resident macrophages, and migratory monocytes, neutrophils, mast cells, NK (Natural Killer) cells, NKT (Natural Killer T) cells, dendritic cells, ILCs (Innate Lymphoid Cells) etc. These innate immune cells use germline encoded pattern recognition receptors (such as TLRs) to observe and identify molecular patterns on the surface of pathogens (pathogen-associated molecular patterns such as LPS) and endogenous damage-associated molecular patterns (DAMPs). Thus, PRR activation prompts signalling mechanisms to combat foreign invaders and induce wound healing. Additionally, these cells also secrete type 1 and type 2 cytokines to activate pro- and anti-inflammatory mechanisms respectively [250], and chemokines to act as chemotactic signalling molecules thereby directing the migration of other immune cells to the site of infection/injury. Both professional phagocytes such as monocytes, macrophages and neutrophils, and non-professional phagocytes such as dendritic cells engulf and digest the cellular contents of the pathogen as reviewed in [251]. This is followed by antigen presentation on the surface of MHC molecules of macrophages and dendritic cells, which then enables activation of the adaptive immune system [252]. NK cells on the other hand possess both “antibody dependent” cytotoxic and cytokine secreting functions and are responsible for eliminating virus infected and tumour cells rapidly reviewed in [231]. NKT cells show dual characteristics of both natural killer cells and T- lymphocytes and can orchestrate a response to an antigen without activation (reviewed in [231]). Similarly, ILCs are the innate equivalents of T cells, although lacking receptors specific for antigens, these cells secrete cytokines and control the functions of other immune cells (reviewed in [253]).

Thus, the innate immune cells work in a concerted manner to mount an initial rapid and non-specific response before the body is ready to launch a more specific immune response aimed at eliminating antigens.

Adaptive immunity: The adaptive immune system, although slower compared to the innate immune system, produces a much more targeted response to a specific pathogen,

orchestrated by B- and T- lymphocytes/cells. It can be broadly classified as humoral immunity, mediated by immunoglobulins secreted by plasma B cells and cell mediated immunity involving cytotoxic- T cells (responsible for eliminating viral infected cells) and T- helper cells that prompt the activation of both B and cytotoxic- T cells [254]. The most unique hallmark of the adaptive immune system is the recombination of VDJ genes, with the numerous "constant region genes" of antibodies to enable "immunoglobulin class switching" [255] and causing rearrangement of T cell receptor genes [256], thereby diversifying both B- and T cell repertoires. Antigen presentation stimulates the migration of adaptive immune cells to the site of infection/inflammation and in the interim, naive T- helper (CD3+, CD4+) and T- cytotoxic (CD3+, CD8+) cells differentiate into T_h1, T_h2, T_h17 and CD4+ Tregs [257], and T_c1, T_c2, T_c17 and CD8+ Tregs respectively. While T_h1 cells primarily secrete pro-inflammatory IFN-γ to promote cell mediated immunity, T_h2 cells produce anti-inflammatory IL-4 and IL-10 cytokines, thereby prompting humoral immunity [258].

T_h17 cells secrete IL-17, thereby inducing a pro-inflammatory phenotype and can also play an insidious role in autoimmune diseases. In order to diminish the action of the pro-inflammatory immune response after the elimination of invading pathogen/infection, CD4+ Tregs produce anti-inflammatory cytokines [259]. Similar to their CD4+ counterparts, T_c1, T_c2, T_c17 and CD8+ Tregs cells show a similar cytokine profile [258, 260, 261].

Subsequently, in order to restore immune homeostasis, most of the newly differentiated subsets of T- and B cells undergo activation induced programmed cell death (AICT). A small fraction of both B and differentiated T- cell subsets are retained to confer "immunological memory". This phenomenon ensures a more rapid and effective immune response, upon re-exposure to the same/similar type of pathogen.

Collectively, a healthy immune response to an invading pathogen starts with the activation of the innate immune system, phagocytosis, antigen presentation to stimulate/activate the adaptive immune system, followed by complete eradication of the pathogen, simultaneous activation induced cell death and finally immunological memory.

1.4.2 *Development and education of the early life immune system*

In the prenatal state, the foetal immune system is uneducated and consequently vulnerable owing to low numbers of functionally reliant immune cells. Thus, the growing foetus is dependent on the maternal immune system for protection. Interestingly, the development of numerous physiological systems including the immune system have been centred around the quality of maternal care [262]. Psychosocial stress in the form of maternal separation, modelled in

non-primates have shown immune anomalies such as a marked decrease in lymphocyte proliferation, circulating T cell numbers and impaired NK cell function in addition to reduced secretion of cytokines [262, 263]. Passive immunity in neonates is conferred in the form of maternal IgG antibodies that cross over through the placenta and secretory IgA antibodies that are transmitted postpartum via breast milk [264, 265]. The placental transfer of antibodies has been reported to significantly increase in the final gestational phase indicating a likely decrease of IgGs in infants born prematurely (reviewed in [262]).

The immune system is intimately connected with the gut microbial composition [266, 267]. The gut microflora in neonates, is largely dependent on the maternal breast milk. A fecal microbiome analysis conducted in baby monkeys born of stressful pregnancies, showed a significant reduction in quantities of Lactobacilli and Bifidobacteria, which constitute two of the most predominant and beneficial microbial gut colonies [268]. Similarly, infections in the early life have been shown to dictate post natal composition of the gut microbiome [269]. Interestingly, the microbial colonies that "seed" the infant's intestinal tract have also been known to depend on the mode of delivery during birth and thus have been thought to determine the long term microflora constitution [270] and also potentially the status of the immune system. Thus, in conclusion changes in the neonatal microbiome signature can alter the overall components of the developing immune system.

The naive immune system in infants is educated based on environmental exposure to antigens [264]. Studies conducted in germ free and gnotobiotic rodent pups born and maintained in sterile environments have been reported to show abnormalities in their immune system [264, 271], most likely due to absence of lymphocyte priming that usually occurs due to "antigenic stimulation". Interestingly, it has been demonstrated that neonatal T cells are epigenetically biased towards producing T_h2 cytokines [272]. This has often been suggested as the lack of education/maturity of the immune system, although recently this phenomenon has been associated with a period of malleabilty to learn about and adapt to the environment [273, 274].

The "hygiene hypothesis" highlights the importance of exposure to a wide spectrum of non-pathogenic microorganisms during early life in order to educate the immature immune system and to build "immune tolerance" [275, 276]. Studies have reported elevated risks of developing allergies and diseases linked to auto-immunity in the absence of immune tolerance [275-277]. Antigenic stimulation and immune tolerance in infants for pathogenic microbes such as *Varicella zostera* virus (causative agent for chicken pox), measles virus, *Corynebacterium diptheriae* (causative agent for diptheria) can be established by vaccination.

Thus, it is clear that the early life is a sensitive developmental window and that disruptions in the early life environment can not only compromise the immediate trajectory of immune system development, but can also have lasting effects. As discussed in section 1.3.4, T2D can act as an ELA and can thus adversely alter the immune system. The next section describes the immune changes associated with glucotoxicity observed in T2D.

1.4.3 Status of the immune system in T2D

It has been established that prolonged stress impairs immune parameters such as inflammation, wound healing, responsiveness to infections and also autoimmunity [278]. However, susceptibility to stress and the magnitude of these deleterious effects on the immune system is somewhat dictated by differences in an individual's coping abilities and perception of stress and adversity [278]. T2D is one of the most commonly diagnosed endocrine disorders and has roots in early life adversity and stress (as discussed in sections 1.2 and 1.3). Since, the endocrine and immune systems are majorly intertwined, it is obvious that T2D also has a profound impact on the immune system as well. T2D is also known to affect cells belonging to both innate and adaptive limbs of the immune system as detailed below:

1.4.3.1 Impact of T2D on the innate immune system

Abnormal activation of immune cells such as myeloid neutrophils and macrophages trigger inflammatory processes that contribute to the vicious circle of disease progression and insulin resistance [279, 280]. T2D patients have been reported to show an increased count of monocytes and neutrophils and increased transcription of inflammatory markers, which were reversed upon observing a strict gylcaemic control [281].

T2D patients show a decline in circulating plasmacytoid and myeloid dendritic cells, thereby weakening their immune system and predisposing them to "opportunistic infections" [282]. Interestingly, Montani et. al reported that serum from diabetics prevented the *in vitro* transition of monocytes into effector dendritic cells and also affected their activation [283]. T2D is known to promote inflammation in diabetic mice by impairing phagocytosis of apoptotic cells by macrophages at sites of injury [284]. T2D animal models show increased activity of NLRP3 inflammasome, which in turn promotes IL-1β and IL-18 secretion in macrophages [285, 286]. Interestingly, peroxisome proliferator-activated receptor (PPAR-γ) expression has been shown to be decreased in T2D macrophages from skin, which results in increased NLRP3 inflammasome

activity and inflammation [286]. Diabetic pancreatic islets also release "damage-associated S100A8", which leads to macrophage infiltration [287]. Neutrophils derived from T2D patients show an activated state and have been known to release ROS [231]. Increased homocysteine levels in diabetic plasma has been indicated in promoting NETs (neutrophil extracellular traps), which also impedes wound healing [288]. Impairment in neutrophil related activities such as migration and phagocytosis also increase susceptibility of T2D patients to infections (reviewed in [231]).

Although T2D patients show an increased frequency of NK cells, most of them are functionally impaired and show decreased secretion of granzymes and perforin (degranulation) [289]. Diabetics have also been observed to show an increase in NKT cells and ILC1s, thereby exacerbating chronic inflammation (reviewed in [231]).

1.4.3.2 Impact of T2D on the adaptive immune system

CD4+ T_h cells have been shown to have a critical role in prompting insulin resistance (reviewed in [290]). The balance between pro- and anti- inflammatory CD4+ effector T cells, i.e. $T_h1$and T_h17, and T_h2 and Treg cells respectively, have often been reported to be disrupted in obese T2D patients (reviewed in [290]). T_h cells in diabetics have been shown to have a tendency of polarising to inflammation promoting T_h1 and T_h17 cells, while anti-inflammatory T_h2 cell numbers have been known to be significantly decreased [291-296]. Additionally, Sireesh et. al have reported a higher T_h1/T_h2 cell ratio in T2D patients [297]. Both T cells and proinflammatory cytokine levels are markedly increased in the T2D pathology and also during obesity induced insulin resistance [298-300]. T_h17 cells possess the ability to suppress the actions of T_h1, T_h2 and T_h17 cells in order to improve insulin resistance, and their percentages have been known to be lowered in T2D patients [301, 302]. Treg/Th17 and Treg/Th1 ratio have also been shown to decreased in diabetics [303].

Accumulation of CD8+ T_c cells have been reported to promote inflammation in addition to insulin resistance [304]. Diabetics and high fat diet fed rodents have been shown to have elevated numbers of T_c cells [304-308], although their percentages were found to reduce 2 hours post glucose administration [309].

B cells play a key role in the progression of insulin resistance [310], via IgG production, and T cell and macrophagic activation [311]. Van Beek et. al have demonstrated an decrease in the percentage of activated B cells in obese diabetics in comparison to eugylcaemic, obese individuals [312]. B cells have been shown to impact T_h17 proliferation along with proinflammatory cytokine

secretion in T2D patients (reviewed in [290]). Furthermore, a decrease in B cell numbers have been shown to bring down T_h17 cell percentages in diabetics but not in healthy controls [313]. Both obese and non obese T2D patients in comparison to control individuals have been reported to show elevated levels of fecal IgG antibodies, secreted by B cells [314, 315].

A hyperglycaemic environment has also been shown to affect the morphology, molecular mass and function of antibodies (reviewed in [231]). Farnsworth et. al have also reported class switch defects in Type-2 diabetics [316]. It is also interesting to note that glycated immunoglobulins in diabetics show defective neutralisation of viruses, thus increasing their susceptibility to viral infections. *Streptococcus pneumoniae* and *Staphylococcus aureus* are the two most prevalent pathogens responsible for causing infections in T2D. Studies in diabetic patients have also reported reduced titers of protective antibodies in addition to compromised phagocytosis, owing to inefficient complement deposition on these pathogens [316, 317].

Thus, in conclusion, we see that T2D has a global impact on the innate and adaptive limbs of the immune system. We have previously discussed (section 1.3.4) how T2D acts as source of ELA and nutritional stress. We also know that uncontrolled gluconeogenesis leads to T2D. The next section explores the role of gluconeogenesis in the context of adversity and metabolism.

1.5 Gluconeogenesis at the heart of adversity and metabolism

It is well established that energy metabolism and psychosocial stress are intimately intertwined. It would also appear that psychosocial adversity in early life sets the individual on a negative trajectory towards either MetS or T2D. The available literature suggests that ELA can effectively alter glucose homeostasis and participate in the aetiology of MetS and eventual T2D. It is possible that MetS and T2D may have very strong roots in the early life environment, with ELA as a strong driver of the eventual diabetic phenotype. This suggests that gluconeogenesis, originally named because of the intimate link between corticoid levels and glucose levels, may be at the heart of the mechanism. We suggest that this may be a double-edged sword. Gluconeogenesis is an integral part of energy homeostasis in response to an external stressor. However, it may show its true “Jekyll and Hyde” nature when the HPA axis is perturbed. Furthermore, the interaction between the HPA axis, glucocorticoids and mechanisms of glucose homeostasis such as gluconeogenesis or insulin resistance may be the link between ELA and lifelong metabolic disturbances. Indeed, one consequence in the neonate of maternal separation is a rapid drop in

blood glucose levels. This, together with increased ghrelin ("hunger hormone") levels may actually trigger HPA axis activation, and glucose supplementation during maternal separation reverses the phenotype confirming the link and providing a potential mechanism to counteract it [318].

Energy homeostasis during psychosocial stress is somewhat underexplored, however, a number of recent studies have started to investigate the full nature of the bi-directional regulation in more detail. There is a wealth of data on how glucose availability modulates the corticosteroid response to psychological and psychosocial stressors, however, the data is sparse or inexistent in the reverse direction. Glucose or levels of gluconeogenesis need to be determined after stress. Furthermore, they need to be recognised as genuine measures e.g. the Trier Social Stress test, providing insight into how the stress axes interact with energy homeostasis. Elucidating these GC-glucose interactions during laboratory stressors is now essential. We need to initially understand the normal blood glucose response to an external stressor. This will subsequently permit investigation of glucose-cortisol coupling in situations such as exposure to ELA where the HPA axis has been significantly programmed, with lifelong changes in reactivity, setpoint, and secreted hormone levels. Recent work on the direct transcriptional control of gluconeogenesis [95], together with recent interest in stress-energy balance [319] open the field for more detailed investigation of the bi-directional regulation of these two essential physiological systems. Thus, it is extremely important to identify if the "Jekyll" or the "Hyde" of gluconeogenesis is at play, and how it balances energy homeostasis under stress, while avoiding gluconeogenesis driven T2D.

In light of the presented narrative, it is clear that there is a direct link between ELA, the HPA axis, glucose metabolism, MetS, and potentially T2D. It is now essential to understand how early life programming of the HPA axis, with lifelong changes in glucocorticoid secretory profiles, influences energy metabolism and processes such as hepatic gluconeogenesis. To do this, and to provide the missing piece of the puzzle we need to consider glucose and energy homeostasis as genuine output measures in standardised laboratory psychological stress tests such as the TSST or the socially evaluated cold pressor test. We need to initially investigate the normal physiological interaction between the HPA axis (or the complete stress system), and energy homeostasis. We hypothesise that lifelong programming of the HPA axis after ELA will fundamentally alter hepatic gluconeogenesis, inducing hyperglycaemia, and the significant lifelong risk or MetS or T2D.

Early life developmental programming of the MetS or T2D risk dovetails nicely into the developmental origins of Health and Disease (DOHaD) model developed by David Barker. The DOHaD model has evolved over the last years into the current 'three hit model' [4, 320]. In the current concept the three 'hits' are defined as (i) invariable genome that fixes genetic risk at

conception, (ii) the early-life environment during which many biological systems are modulated or adapted to the environment the individual is born into and (iii) the later-life environment where a perturbation tips the balance between health and disease. The first and second hits produce a phenotype that remains latent or quiescent until the third hit crystallises the risk and initiates the health-disease transition. In our ELA-metabolic disease paradigm we see the early-life adversity during the SHRP as the second hit and the cluster of metabolic abnormalities as the final disease phenotype. Indeed, there is now evidence that ELA leaves a faint metabolic imprint almost immediately, although the full syndrome doesn't appear until much later in life but usually become manifest not earlier than at adult age [321]. This mirrors the observation from pre-term infants, where increased baseline HPA axis activity, reduced stress reactivity and components of the metabolic syndrome are seen in later life [322]. This may, however, be linked to the use of synthetic glucocorticoids such as dexamethasone in the perinatal period. However, Vargas *et al.*, demonstrated that early life stress could concurrently increase HPA axis activity, and mild metabolic alterations, that were significantly increased with a subsequent environmental challenge akin to the third hit, although their model suggested that it was independent of GC [323].

One potential limitation to our postulate is that chronic stress has been shown to bias feeding choices in both animals and humans to addictive foods such as high sugar and high fat content foods, which can directly contribute to MetS over the years (reviewed in [324]). This could be mediated by alterations in the glucose metabolism, HPA axis related changes, etc. This limitation can be further dissected by studying laboratory models of chronic stress that are only fed standard chow diet to see if gluconeogenesis is indeed altered. It is also interesting to note that chronic stress is frequently accompanied by comorbidities like depression [325], which decrease sucrose preference in depressive animals and is often used as an index of anhedonia [326], providing a conflicting perspective with respect to chronic stress and diet.

Thus, we suggest a "Jekyll and Hyde" role to gluconeogenesis, providing the necessary energy in situations of acute stress, but driving towards pathophysiological consequences when the HPA axis has been altered. Furthermore, if our hypothesised link is correct, we can reflect on whether this output has the predictive power to identify individuals at a higher risk of developing MetS at a later stage in life, before symptom onset.

Thus, it is clear that gluconeogenesis in its "Mr. Hyde" character paves the way for complex metabolic disorders such as T2D. In order to eliminate the diverse genetic constitutions and differing exposomes arising due to different environments in man, we use different models to

dissect the T2D aetiology and disease progression with maturation. The next section gives an account of rodent models used in present day T2D research.

1.6 Rodent models currently used in T2D research

T2D can be modelled in different classifications of animals, ranging from simple non-mammalian models such as roundworms, fruit flies and zebrafish to complex non-human primates such as cats, dogs, pigs and monkeys which are physiologically more similar to humans [327, 328]. However, for the scope of this thesis, we will only discuss different rodent models of T2D in this section. T2D in model rodents can be categorised as monogenic, polygenic or chemically induced pathophysiology [327].

Monogenic models of T2D include Lep $^{ob/ob}$ mice, Lepr $^{db/db}$ mice and inbred Zucker Diabetic Fatty (ZDF) rats (reviewed in [328]). All three models show a dysregulation in Leptin signalling [328]. Leptin is responsible for regulating physiological energy homeostasis by triggering feedback signals to the hypothalamus, thereby establishing satiety (reviewed in [329]). Lep $^{ob/ob}$ mice have been genetically engineered to lack functionally active leptin and developing hyperglycaemia at approximately 4 weeks of age [328], while both Lepr $^{db/db}$ mice and ZDF rats lack leptin receptors, consequently leading to hyperphagia and obesity [330-333]. The ZDF rats however are less obese, although they have been derived by inbreeding obese Zucker Fatty rats, and male rats develop T2D at approximately 8-10 weeks of age [334, 335]. Despite all three monogenic models exhibiting typical symptoms of T2D such as hyperglycaemia, hyperinsulinaemia and insulin resistance, they do not ideally reflect the human diabetic clinical scenario. This is because, as previously mentioned, T2D is a complex, non-Mendelian (polygenic) disorder and thus should be modelled in polygenic conditions similar to human diabetics [327].

Polygenic T2D models include New Zealand Obese (NZO) mice, KK mice, KK-A^{Y} mice, , TallyHo/Jng mice, Otsuka Long-Evans Tokushima Fat (OLETF) rats and NoncNZO10/LtJ mice (reviewed in [328]). NZO [336] and KK [328, 337] mice show common features such as hyperleptinaemia, hyperinsulinism and enlarged pancreatic mass (reviewed in [328]). The KK-A^{Y} mice derived from the KK strain show extreme hyperinsulinism and more severe damage to pancreatic islets [338]. The TallyHo/Jng mice strain also show hyperinsulinaemia and degranulation of pancreatic islets [339]. Interestingly, both OLETF rats (reviewed in [340], [341]) and NoncNZO10/LtJ mice [342, 343] have been reported to show “late onset hyperglycaemia” similar to human diabetics, although they also present the comorbid obese phenotype.

Chemically induced T2D is observed in high fat diet/streptozotocin treated (HFD/STZ) animals [344, 345]. This model comprises an amalgamation of HFD induced obesity resulting in insulin resistance, hyperinsulinaemia and glucose-intolerance followed by administration of Streptozotocin, an antibiotic that destroys healthy pancreatic β cells [344]. T2D is caused by a combination of (early and late) environmental and genetic factors (reviewed in [346]). The main drawback of this model is that it quite likely lacks the early (gestational) environmental and genetic factors responsible for contributing to the diabetic pathophysiology, thereby failing to mimic the human clinical context.

Thus, it is clear that majority of the current day T2D studies fail to differentiate between effects stemming because of T2D in conjunction with obesity, and effects driven by uncomplicated T2D, necessitating uncomplicated (non-obese) T2D models. The "human islet amyloid polypeptide (hIAPP)" mice and Goto-Kakizaki (GK) rats comprise the non-obese category of T2D models (reviewed in [328]).

Islet amyloid polypeptide (IAPP) has often been associated with T2D (reviewed in [347]), which prompts amyloid plaques formation in pancreatic islets, similar to amyloid deposition seen in Alzheimer's disease (also known as Type-3 diabetes) [348]. Although rodent IAPP does not promote amyloid formation, transgenic mice (hIAPP mice) lines have been successfully shown to express human IAPP, thereby leading to the formation of amyloid plaques and subsequent beta cell diminution similar to human diabetics [349].

The GK line was established from normoglycaemic Wistar (W) rats by repeated inbreeding in each successive generation of the siblings with the highest (but still within normal range) blood-glucose levels during an oral glucose tolerance test leading to the development of a non-obese model of uncomplicated diabetes [350]. This means that the T2D causing component in the normoglycaemic control outbred Wistar rats, were condensed through recurrent selective breeding to give rise to the GK diabetic model. Chapters 3 and 4 of this thesis have been modelled on studies conducted using these rats (GK/Par and GK/Mol/Tac colonies). The next section provides a more detailed account of GK rats as a model used to study uncomplicated T2D.

1.6.1 Characteristics of GK rats as a T2D model

T2D is not always associated with obesity as previously discussed [351]. In some populations such as the Japanese, a higher T2D prevalence has been observed in spite of a lower obesity prevalence [352]. The GK rat model has been established as a polygenic non-obese model

that shows a remarkable number of similarities to the human diabetic phenotype (reviewed in [351]).

Adult rats of the GK/Par colony weigh 10-20% less compared to sex and age-matched Wistar rats [351]. The Sendai (GK/Sen) Japanese colony was the first to be established around 1970 [350], and since then more than seven sublines have been in existence (discussed in [351]). Although current day GK colonies show an extent of glucose intolerance, there exists minute differences between sublines in terms of characteristics such as β cell count, islet secretion, constitution and metabolism (reviewed in [353]). These differences in phenotype can arise due to changes in the local and social environment in conjunction with newly arising genetic changes [353]. Thus, comprehension of data from different colonies must take into account the differences in phenotypic properties. Body weight, baseline postabsorptive plasma glycaemia and intra-venous glucose tolerance between the two colonies (GK/Par and GK/Mol/Tac colonies) used to generate data for this thesis have been comparable [351]. However, the GK/Mol/Tac colony shows a subtler impairment of insulin secretion in presence of glucose compared to the GK/Par subline [351].

In addition to fibrosis of islet masses, the Paris colony also shows marked chronic pancreatic inflammation, which now has been established as the pathophysiological driver of the diabetic phenotype [354-356]. It is interesting to note that transcriptome profiling of GK/Par pancreatic islets have shown 34% of the 71 overexpressed genes to be associated with immune response/inflammatory gene class [354]. Macrophage and granulocyte infiltration have also been reported in GK/Par pancreatic tissues [354]. Major histocompatibility complex class II (MHC class II) genes, responsible for driving CD4+ T_h cell development and activation, have been reported to be upregulated in GK/Mol/Tac islets [357], indicating a potential dysregulation/over activation of the GK immune system. The GK/Par subline showed elevated blood flow and a modified pattern of vascularisation in the pancreatic islet possibly due to alterations in vagal nerve regulation [358-360]. A standardised vagotomy in these rats successfully restored normal blood to the islets [359]. Additionally pancreatic islets in these rats showed marks of reactive oxygen species (ROS) [361] possibly indicating cellular damage and oxidative stress, along with a massive decrease in mass [362, 363]. The decreased β cell percentages (almost by 60%) in GK rats have been attributed to reduced cellular replication as opposed to programmed β cell death, implicating a permanent stagnancy of β cell regeneration [363]. GK islets show degranulation as supported by a lower insulin content relative to DNA. Upon further investigation, it was observed that the GK islets majorly consisted of immature granules and contained very few beta granules [364]. The time of onset of β cell destruction and its severity differs in the different colonies, such as in the GK/Par animals,

this phenotype arises at foetal stage [351]. These differences can be a result of diverse nutritional, social and environmental cues [351].

Interestingly, the biosynthesis and downstream processing of proinsulin, the precursor molecule of insulin was found to be normal in diabetic GK rats (reviewed in [351]). This indicates that the defective insulin secretion did not emerge as a result of failure to distinguish glucose as a stimulant for proinsulin synthesis, but rather due to inadequate insulin release (secretory defect) required to meet the hyperglycaemic load in the bloodstream [351]. Although, baseline levels of circulating insulin in GK rats were almost comparable to control Wistar rats, the basal levels were insufficient relative to the glycaemic levels, leading to a dysregulated "glucose-stimulated insulin secretion" [351]. A severe decrease of glucose uptake and insulin resistance have been hallmarks of the GK diabetic model (reviewed in [351]).

GK rats have been known to have an impaired energy metabolism such as an over exaggerated glucose-6-phosphate activity, leading to an escalated "glucose cycling" [365, 366] and stunted pyruvate carboxylate activity [367]. In addition, the proportion of oxidised glucose relative to glycolysed glucose was lower in GK pancreatic islets compared to control rats (reviewed in [351]). GK rats have been reported to show a decrease in mitochondrial DNA [368] and impaired ATP generation [369] in response to high amounts of available glucose. Although GK rats are regarded as a non-obese model, the GK/Par strain has been reported to show excess accumulation of fat reserves in contrast to body weight and an increase in adipocyte size, post hyperglycaemia (reviewed in [351]) .

It is interesting to note that GK/Par neonates show signs of obvious hyperglycaemia as early as 3-4 weeks of age [351]. The fully developed diabetic phase in these rats is thus preceded by a prediabetic phase similar to human diabetics [351]. During this normoglycaemic (still glucose tolerant) prediabetic phase, GK rats still show a massive reduction in pancreatic β cells, which begins during early days of life (foetal day 16) [362]. Just prior to weaning (3-4 weeks of age) GK/Par pups have been shown to develop "insulin hypersensitivity", probably as an adaptive mechanism to compensate for the low levels of circulating insulin [370]. This hypersensitivity is counteracted as the disease progresses leading to insulin resistance in GK rats [370]. Leptin levels in prediabetic animals have been shown to be lower than control Wistar rats, although in the hyperglycaemic state, the situation is reversed [356].

Cross-fostering experiments with GK/Par pups that were placed under the maternal care of control Wistar dams exhibiting normal maternal behaviour and having a normoglycaemic composition of milk, still showed abnormal metabolic parameters [371]. This means that the

metabolic dysregulation emerging at the prediabetic phase has more to do with the genetic predisposition and possible reprogramming due to the (early) prenatal hyperglycaemic intrauterine environment, which cannot be ameliorated with postnatal nurturing [351]. Similarly, it was seen that embryo transfer experiments where GK/Par embryos were implanted into the uterus of Wistar mothers, still developed β cell reduction as adults, again highlighting a key role of genetic factors contributing to the aetiology [372]. However, the same embryo transfer strategy for a Wistar embryo transferred into the uterus of a GK mother conferred a risk of developing T2D later in life [372] implying gestational epigenetic mechanisms to also drive the disease risk. Another seminal study with GK rats concluded that the inheritance of the pathogenic component (genetic and epigenetic) from one diabetic parent is sufficient to prompt a risk of β cell reduction upon adulthood, even if the other parent was non-diabetic [373].

Thus, collectively, we see that GK rats through their genetic predisposition triggering β cell damage, early life epigenetic reprogramming of pancreatic β cells due to gestational diabetes that is intergenerationally passed on, followed by the acquired metabolic and β cell defects due to chronic hyperglycaemia, serve as an excellent model to explore T2D in the human context (reviewed in [351]). The next section outlines the aims and 'Big Picture' overview of the thesis.

1.7 Aims and 'Big Picture' overview of this thesis

The first 1000 days of life coincide with a critical developmental window. The quality of these early days is epigenetically embedded into important interrelated biological systems such as the Hypothalamus Pituitary Adrenal (HPA) axis, metabolism and also the immune system. The introduction to this thesis summarizes how early life adversity (ELA) impairs the HPA axis and (glucose) metabolism, which may trigger a negative trajectory towards the development of complex disorders such as Type-2 diabetes (T2D). The relationship between glucocorticoids (GCs) and psychological stress has been studied thoroughly over decades. However, in this first chapter we highlight the absence of studies exploring energy homeostasis in conjunction with stress response. We think that ELA not only reprograms the HPA axis, but also alters hepatic gluconeogenesis, thereby promoting hyperglycaemia, which when left uncontrolled can snowball into T2D (**working hypothesis 1**).

Following on from this hypothesis, the main objective of **Chapter 2** is to explore basal and stress induced glucose release in different animal models of ELA (perinatal infection and maternal deprivation) in parallel to the human EpiPath (institutionalisation-adoption) cohort. In order to further dissect the stress response, we investigated GC/glucose coupling in normal healthy humans by administering them with escalating doses of GCs and monitoring plasma cortisol and plasma glucose for a series of time points, followed by GR target gene expression analysis. This study has generated compelling evidence to show that rising levels of blood glucose post stress is indeed a hormone (GC) independent process contrary to previous beliefs.

ELA and the stress asscoiated with it can be broadly dissected into different brackets such as maternal separation, early life infection, institutionalisation-adoption, physical abuse, exposure to pollutants, socio-economic status and nutritional stress (T2D and hyperglycaemia, and composition of the gut flora). The 'Big Picture' overview of the following chapters is to focus on T2D as a form of ELA (Working hypotheses 2 and 3).

Although not always associated with obesity, a vast majority of current day preclinical research make use of obese T2D models. In order to understand the deleterious effects of uncomplicated T2D, all studies described in further chapters have been conducted using Goto-Kakizaki rats (a non-obese spontaneous T2D model) with special emphasis on the immune system as T2D has been established to have an underlying inflammatory component. Also very little is known about the immune profile of these Goto-Kakizaki (GK) rats that perfectly mimic the human T2D clinical

scenario. The main objective of **Chapter 3** is to understand the differences arising in the adult immune system of GK rats, the underlying mechanisms and the inflammatory status (Working hypothesis 2).

Finally, in order to get a full understanding of when these immune changes emerge during the diabetes cycle, the objective of **Chapter 4** deals with immune characterisation of GK rats as they mature and also when subjected to stress in the fully diabetic phase (Working hypothesis 3 and 4). The concluding chapter, **Chapter 5** outlines the general discussion, finally connecting the dots between ELA, stress and the immune system in the context of T2D.

Working hypothesis 1: ELA reprograms the HPA axis and alters hepatic gluconeogenesis, which together may prompt hyperglycaemia and eventually T2D.

This hypothesis is tested in **Chapter 2**

Working hypothesis 2: ELA in the form of T2D systematically impacts the immune system (status of adult diabetic GK rats)

This hypothesis is tested in **Chapter 3**

Working hypothesis 3: Immune changes observed in adult GK rats precede the transition into the diabetic phase.

Working hypothesis 4: Metabolic insults and psychosocial stressors induce immune lymphocyte redistribution to the periphery.

These hypotheses are tested in **Chapter 4**

Chapter 2: Stressing glucose: At the crossroads of early life adversity, HPA axis reactivity and carbohydrate metabolism

My contribution to this chapter: Conceptualisation; Literature review; Data curation; Calculation of AUC_g; Data visualisation; Final statistical analysis; Interpretation of results; Making of all figures; and Writing of the article.

Online preprint DOI: 10.20944/preprints202207.0311.v1

2.1. Abstract

External stressors strongly increase cardiovascular activity and induce metabolic and endocrine changes that ensure the availability of glucose and oxygen as part of a co-ordinated stress response. Exposure to stress during early life appears to have an exaggerated long-term effect on this response, leading to an increased risk of cardiometabolic disorders.

In a cross species manner we demonstrate that early life stress (ELS) affects stress-induced glucose release in both pre-clinical models of maternal deprivation and perinatal infection and in human participants that have experienced early life adversity (institutionalisation-adoption). In humans, early life adversity was associated with higher pre-stress glycaemia in women, similar to the mouse perinatal infection and rat maternal deprivation models. We hypothesised that the stress induced glucose rise would be a glucocorticoid (GC) dependent process. However, the kinetics of stress-induced glucose release, peaking 15-28 minutes before cortisol suggest that it is a GC-independent process. This was confirmed by the absence of GC induced glucose rise after intravenous administration of escalating doses of cortisol in healthy humans.

In conclusion, metabolic status and early life stress are intimately intertwined. We demonstrate the importance of collecting early-life stress history in patients suffering from metabolic diseases. Inversely, clinical studies on subjects exposed to early-life stress must systematically explore the metabolic status of these individuals.

Keywords: Glucose; cortisol/corticosterone; stress; early life adversity

2.2. Introduction

External stressors elicit a physiological response that prepares the body for the fight/flight response, disrupting homeostasis. Glucocorticoids (GC) and catecholamines play an important role in driving this stress response by increasing blood pressure and heart rate along with immune and metabolic changes ensuring availability of glucose and oxygen to meet brain and muscle requirements. As a corollary, prolonged exposure to stress impairs the stress response system and this is reflected as changes in metabolic parameters such as glucose [89].

Both the Hypothalamus-pituitary-adrenal (HPA) stress response axis and an individual's metabolic profile adapt to the immediate environment. The Barker theory states that the quality of the first 1000 days of an individual's life (from conception to age 2) predicts their lifelong metabolic status/phenotype [320, 374], and eventual metabolic dysfunction [97]. Chandan et al. (2020) estimated that early life stress (ELS) or adversity (ELA) determines "a significant proportion of the cardiometabolic and diabetic disease burden"[190]. During the first 1000 days of development, individuals may be confronted with numerous adverse and stressful events that can affect life-long health trajectory. Four major sub-categories of ELS have been shown to have major health impact in the literature including: 1) early life infection (ELI) and increased antigenic load, 2) poor perinatal nutrition (quantity and/or quality) , 3) perinatal exposure to toxic environmental agents (e.g. pollutants), and 4) psychological and psychosocial stress, although many commonly investigated measures such as low socioeconomic status (SES) cover multiple sub-categories [320, 375]. Often, multiple stressor come together to make a more severe ELA. The exact contribution of these factors is very difficult to dissect in humans. Several studies have reported a higher risk of metabolic diseases such as type-2 diabetes and obesity in ELA exposed individuals [376-379]. The effects of psychological stress on glucose metabolism are thought to be mediated by insulin, glucagon, GC, and adrenaline [90, 91]. Fasting-induced gluconeogenesis is orchestrated by the cyclic Adenosine Monophosphate/protein kinase A/cAMP response element-binding protein/ CREB-regulated transcription coactivator 2 (cAMP / PKA / CREB / CRTC2) signalling pathway and is mediated by glucagon. Post-prandial increase of blood insulin levels suppress gluconeogenesis by downregulating gluconeogenic transcription factors such as FOXO1 (Forkhead box O1), PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha), and CRTC2 (CREB-regulated transcription coactivator 2) [95], and triggering uptake of glucose via GLUT (glucose transporter) receptors [130]. Gluconeogenesis is also triggered during stress along with glycogenolysis, which rapidly subsides [142]. Thus, gluconeogenic processes are thought to be the

principal mechanism behind the increase in glycaemic measures [142]. However, chronic stress results in consistently high blood glucose levels even up to 6 months after cessation in both rat models of chronic stress [133] and in women where allostatic load positively associates with blood glucose levels [209]. Thus, we have previously suggested that gluconeogenesis may represent a sensitive biological mechanism, which when disturbed can act as a double-edged sword [380]. Recently, Cui et al. showed that synthetic GCs drove the expression of *Klf9* (Kruppel like Factor 9) and *Pgc1α* (PPARG Coactivator 1 Alpha) to promote gluconeogenesis, indicating that gluconeogenesis required gene transcription [95], somewhat at odds with the rapid release of glucose during psychosocial stress [380].

While there are numerous human adversity paradigms, one of the most prevalent is the institutionalisation-adoption model [185, 381-384]. Here, we use the EpiPath cohort of institutionalised-adopted individuals that were investigated some 24 years post adoption. Adopted individuals in the EpiPath cohort had a hyporesponsive HPA axis [385], a clear immune-phenotype reminiscent of accelerated immune ageing [68, 70], although GC signalling and the associated glucocorticoid receptor levels and epigenetic status were unchanged [386]. These two clear physiological sequelae of ELA can be modelled in vivo. The psychosocial aspect of ELA can be studied in laboratory models of chronic maternal separation [387, 388] and the effects of early life infection can also be modelled in young animals by infecting them with a pathogen such as H1N1 [389]. ELA has been known to be a key programmer of the stress response system and can thus potentially dysregulate glucose metabolism and disturb the energy balance, although the exact extent and mechanism behind this is poorly understood.

In this study, using both animal models of early life adversity (maternal separation and perinatal infection) as well as in the EpiPath cohort of individuals that were institutionalised at birth and subsequently adopted, we examine the stress-induced rise in plasma glucose under both normal conditions and after exposure to early life adversity. Additionally, we tested the effects of escalating doses of cortisol boluses on glucose release in a healthy human cohort. Our results reveal a marked effect of ELA on metabolic status in both animal models and humans. We also demonstrated that this effect of stress is not mediated by glucocorticoids contrary to previous beliefs.

2.3. Materials and methods

2.3.1. Human participants and animals

Healthy human (adversity negative) pharmacokinetic cohort: We used an existing cohort (ESCO) of 40 healthy (no history or current record of psychiatric/stress related illnesses) participants (20 females and 20 males, age range from 20-30 years) data from whom have previously been published [390, 391]. Briefly, at study inclusion participants underwent a standard physician administered medical examination and interview procedure. Study exclusion criteria were i) psychiatric, cardiovascular, sleep or stress-related disorders, ii) any somatic illnesses, iii) smoking, excessive caffeine consumption, glaucoma, pregnancy, or recent (6 months) consumption of illicit drugs. Participants were assigned randomly to one of five sex-balanced groups: placebo (NaCl 0.9%, Braun, Melsungen, Germany) or one of five hydrocortisone groups: 3 mg, 6 mg, 12 mg and 24 mg of (Hydrocortison 100 mg, Rotexmedia, Trittau, Germany). Two study participants were included per day, starting with the insertion of a flexible intravenous catheter (Vasofix Braunüle, Braun, Melsungen, Germany) in the right arm between 12:00 and 12:45 (mean time 12:54h ± 00:50 h), when physiological cortisol levels are significantly reduced from the morning peak. After a 45 min rest period the catheter was connected to a syringe pump (Infusomat fm, Braun, Melsungen, Germany). Infusion time was approximately 2 min. EDTA anti-coagulated blood samples were drawn through the iv catheter. The study was approved by the Rheinland-Pfalz state Ethical Committee and performed according to the Declaration of Helsinki. All participants provided written informed consent. Participants received a small monetary reward for participation.

Human early life adversity cohort: We used our previously described EpiPath early-life adversity cohort [68, 70, 385, 386, 392]. Briefly, EpiPath is a cohort of 115 sex matched participants (adoptees and non-adopted controls), median age 24 (IQR 20-25.5) years with or without exposure to ELA that was operationalised as “separation from parents in early life and subsequent adoption”. The vast majority of ELA subjects had institutionalization or unstable foster care. In this cohort, age of adoption has successfully been used as a proxy for the intensity of ELA experienced [68, 70, 385, 386, 392]. The socially evaluated cold pressor test was performed as previously described [385]. Briefly, subjects placed both feet into ice-cooled water (2-3°C) for a period of 3-minutes. Saliva was collected 5 minutes prior to stress onset (-5min), upon stress cessation (+3 min), then at 15, 30, 60, 120 and 180 minutes after stress onset. EpiPath was approved by the Luxembourg National Research Ethics Committee (CNER, No 201303/10 v1.4) as well as the University of Luxembourg

Ethics Review Panel (ERP, No 13-002). All participants provided written informed consent in compliance with the Declaration of Helsinki. All study participants received a small financial compensation for their time and inconvenience.

Rats: Pregnant Wistar dams from Janvier Labs (Le Genest-Saint-Isle, France) were housed in plastic cages (Tecniplast, Varese, Italy) with dimensions 48 x 37.5 x 21 centimetres with access to food and water *ad libitum*, maintained under a 12h light-dark cycle (49-54% humidity and 21° C). Animals were left undisturbed for the entire length of the pregnancy except to conduct routine husbandry. At gestational day 16, nesting material was provided, and cages were not further changed until post-natal day (PND) 2. After natural delivery, litter sizes were adjusted to 12/dam and attributed to one of two treatment groups- control maternal deprivation for 180 min (MD_{180}). Pups belonging to the MD group were deprived of maternal care and contact, daily (MD_{180}: 9 am - 12 am,) from post-natal day 2-14 as previously described [393]. Animals were subjected to a 1-hour restraint stress in a grey plastic tube on PND49 +/- 1 day during the inactive light phase (9 am to 12 pm) and blood was collected from the tail vein after approximately 3 min (pre-stress condition) and 60 min (post-stress condition) of restraint.

Mice: BALB/cJRj mice were originally obtained from Janvier labs (Genest-Saint-Isle, France), and subsequently bred in house. All animals had access to standard rodent chow (#801722; SDS Diets, Witham, UK) and drinking water *ad libitum*. Pups (PND14) were then assigned to one of two groups- the test group that was subjected to H1N1 (25 uL of 1500 focus-forming units of Influenza A virus in PBS) and the controls groups that were administered saline as previously described [389]. On PND42 (±1) a restraint stress test was performed in the inactive phase (between 9 am and 12 pm) by placing the animals in a transparent tube for 1-hour. Blood samples were collected, approximately 3 minutes and 60 minutes after restraint stress to reflect the pre- and post-stress condition. All animal experimental procedures were approved by the institutional animal welfare structure and performed in accordance with the European Union directive 2010/63/EU as well as local guidelines.

2.3.2. *Assessment of cortisol/corticosterone and glucose levels*

Glucose levels were measured using a commercial diagnostic electronic glucometer (Accu-Chek, Roche). Human glucose levels were measured from plasma in the non-fasted condition. Briefly, upon thawing, plasma was vortexed and then placed on a fresh Accu-Chek strip. In both rats

and mice, glucose and corticosterone measurements were performed after approximately 3 min and 60 min of restraint on blood drawn from the tail vein in non-fasted condition. A single blood drop from the site of venipuncture was used to measure glucose levels. All blood samples were centrifuged at 2000 g for 5 minutes and the plasma collected and stored at -80°C, until corticosterone analysis. Plasma corticosterone levels (minimum detection limit = 1.680 nmol/L, analytical sensitivity = 0.589 nmol/L, inter assay variation coefficient = 7.075%, intra assay variation coefficient = 5.15%) were measured by immunoassay (IBL International, Hamburg, Germany), according to the manufacturer's instructions. Human plasma cortisol levels were measured by immunoassay for EpiPath (IBL; analytical sensitivity = 56.72 pg/mL, inter assay variation coefficient = 9.93%, intra assay variation coefficient = 8.13%) and ESCO (Liaison, Diasorin, Saluggia, Italy; analytical sensitivity = 15 ng/mL, inter assay variation coefficient = 5.58%, intra assay variation coefficient = 2.96%) according to the manufacturers' instructions.

2.3.3. *GR target-gene expression*

Total RNA was extracted from whole blood samples using the RiboPure Blood Kit (Ambion, Foster City, Texas, USA) following the manufacturer's instructions. First strand (cDNA) synthesis was performed using 200 U/µl SuperScript III reverse transcriptase, 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl2, 10 mM dithiothreitol, 500 µM dNTPs, and 250 ng/µl dN6 primers (Thermo Fisher, Paisley, UK) in a 60 µl reaction reaction incubated at 55 ˚C for 1h. PCR amplification of cDNA was performed in 25 µl reactions containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 mM dNTPs, 5 U Platinum Taq DNA polymerase (Invitrogen) and 1 x concentrated SYBR Green (Cambrex, Verviers, Belgium). MgCl2 concentration and gene specific primers are shown in Table 2.*1*, Thermal cycling was performed in a CFX 96 (Biorad, NL) starting with one cycle at 95 ˚C, 2 min. This was followed by 40 cycles of denaturation (95 ˚C; 20 s) annealing (20 s), elongation (72 ˚C; 20 s). Annealing temperatures are given in Table 2.1. All primers were synthesised by Eurogentec (Seraing, BE).

Table 2.1: Gene expression PCR conditions

Gene amplified	Primer sequences	Size of amplicon (bp)	Primer concentration (µM)	Mg^{2+} concentration (mM)	Annealing temperature (°C)
GILZ	Fwd : GCACAATTTCTCCATCTCCTTCTT Rev : TCAGATGATTCTTCACCAGATCCA	146	0.5	2	60
FKBP5	Fwd : AAAAGGCCAAGGAGCACAAC Rev : TTGAGGAGGGGCCGAGTTC	236	0.5	4	67
GAPDH	Fwd : GAAGGTGAAGGTCGGAGTC Rev : GAAGATGGTGATGGGATTTC	146	0.5	4	60

Relative gene expression was calculated using the 2-(ΔΔc) relative quantification method [394] using the mean of biological triplicates. Among the three reference genes 18S rRNA, β-actin and GAPDH, the latter was the most stable and was used for normalization, following the procedure of Schote et al. [395]. The relative expression was calculated with reference to the sample immediately preceding the cortisol injection (+57 min).

2.3.4. *Data presentation and statistical analysis*

Data were tested for normality and lognormality using the Kolmogorov Smirnov test. Outliers were identified using the ROUT method (Q = 1-10%) and removed. A paired t-test was conducted to explore blood glucose changes before and after stress, and a 2-way ANOVA was calculated to investigate interactions between different ELA groups and stress induced changes in blood glucose. Additionally, pre-stress blood glucose differences between ELA and control groups separated by sex were investigated using a Mann-Whitney test/unpaired t-test. Area under the curve for both glucose and cortisol were calculated as previously described by Pruessner et al. [396]. A simple linear regression analysis was performed to analyse glucose/cortisol associations. All statistical analyses were performed using GraphPad Prism (version 8.2.0).

2.4. Results

2.4.1. *The effect of ELA on pre-stress and stress-induced rise in blood glucose levels in mice*

The ELA literature (reviewed in [380]) clearly shows that severe adversities such as deprivation, abuse, neglect or institutionalisation result in lower circulating pre-stress cortisol [168, 169] and a stunted HPA axis response [170, 171]. Also, since GCs have been thought to mediate glucose homeostasis, we explored both pre-stress and stress induced glucose release in the ELA models available in our laboratory: murine early life viral infection, rat maternal deprivation and human institutionalisation-adoption (Fig 2.1 A-L; [385, 389, 393]). Perinatal H1N1 exposure significantly increased pre-stress glucose levels in female mice but not in males (Fig. 2.1 A: $p = 0.0051$; Mann-Whitney test, Fig. 2.1 B: $p > 0.05$; Mann-Whitney test). However, in female mice, after stress there was a clear H1N1 and stress effect although the interaction term narrowly missed significance (Fig. 2.1 C: $p < 0.0001$ for all groups, 2-way ANOVA; group effect $p = 0.28$; stress effect $p < 0.0001$; group*stress interaction $p = 0.06$). Similarly, in the male mice there was a clear H1N1 and stress effect although the interaction term was not significant (Fig. 2.1 D: $p < 0.0001$ for all groups, 2-way ANOVA; group effect $p < 0.05$; stress effect $p < 0.0001$; group*stress interaction $p = 0.4$).

2.4.2. *The effect of ELA on pre-stress and stress-induced rise in blood glucose levels in rats*

Interestingly in the rat MD model, although there was no significant MD-induced difference in pre-stress glucose for female rats (Fig 2.1 E, $p > 0.05$, Mann-Whitney test). However, we saw a significant difference between the male control and MD_{180} rats. (Fig. 2.1 F: $p = 0.0039$, Mann-Whitney test). After the 1-hour restraint stress, we observed stress induced glucose release for both the female and male control rats, however the expected rise in glucose levels was completely abrogated in the female MD_{180} (ELA) group, but exaggerated in the male MD_{180} group. For the female rats there was a clear MD and stress effect, although the interaction term was not significant (Fig. 2.1 G: $p = 0.0112$ for controls and $p > 0.05$ for MD_{180} rats; 2-way ANOVA; group effect $p = 0.013$; stress effect $p = 0.0049$; group*stress interaction $p > 0.05$). It is also interesting to note that there was a significant difference in post stress glucose levels between the two female groups ($p < 0.05$). On the contrary, the MD_{180} male rats showed a normal anticipated stress induced rise in blood glucose levels after stress, similar to the controls (Fig. 2.1 H: $p < 0.0001$ for the MD_{180} group and $p < 0.0471$ for controls; 2-way ANOVA; group effect $p > 0.05$; stress effect $p < 0.0001$; group*stress interaction $p = 0.012$).

2.4.3. *The effect of ELA on pre-stress and stress-induced rise in blood glucose levels in humans*

In a manner similar to the H1N1 female mice, female adoptees in the EpiPath cohort had a significantly higher pre-stress blood glucose level (Fig. 2.1 I: $p = 0.0091$; Mann-Whitney test). However, there was no significant difference between the male Epipath adoptees for pre-stress glucose (Fig. 2.1 J: $p > 0.05$; Mann-Whitney test). The female EpiPath participants showed a significant increase of stress induced blood glucose levels in both ELA and control groups (Fig. 2.1 K: $p = 0.0009$ for the control group and $p = 0.017$ for the ELA adoptees; 2-way ANOVA; group effect $p > 0.05$; stress effect $p < 0.0001$; group*stress interaction $p = 0.4$). However, in the male participants, there was no significant change in blood glucose levels before and after the cold pressor test for either group (Fig. 2.1 L: $p > 0.05$ for both groups; 2-way ANOVA; group effect $p > 0.05$; stress effect $p > 0.05$; group*stress interaction $p = 0.28$).

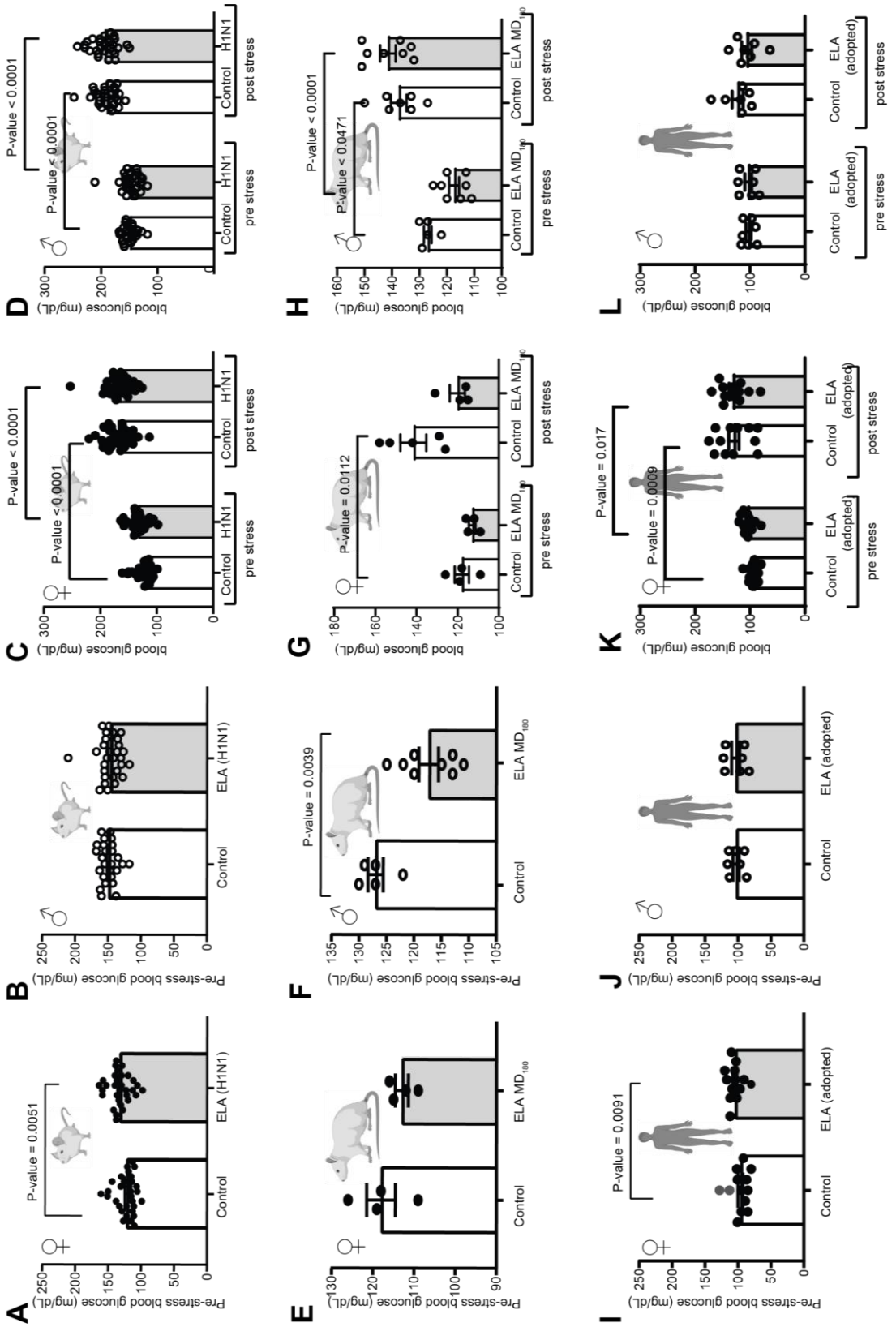


Figure 2.1: The effect of ELA on pre-stress and stress-induced blood glucose levels. Blood glucose levels (mg/dL) A) at pre-stress in female H1N1 and control mice, B) at pre-stress in male H1N1 and control mice, C) after stress in female H1N1 and control mice, and D) after stress in male H1N1 and

control mice. Blood glucose levels (mg/dL) E) at pre-stress in female MD_{180} and control rats, F) at baseline in male MD_{180} and control rats, G) after stress in female maternally separated rats (MD_{180}) and control rats, H) after stress in male maternally separated rats (MD_{180}) and control rats. Blood glucose levels (mg/dL) I) at baseline in female control and institutionalised/adoptees, J) at baseline in male control and institutionalised/adoptees, K) after stress in female control and institutionalised/adoptees, and L) after stress in male control and institutionalised/adoptees. Filled circles: females; Open circles: males. All data are means along with the standard error of mean (SEM). All measurements have been expressed in mg/dL. Each circle represents an individual animal/participant.

2.4.4. *Stress results in elevated blood glucose levels*

Glucose producing processes are activated under stress conditions to ensure a steady supply of energy to the brain. Since we saw an unclear and unexpected pattern of stress induced glucose release in conjunction with ELA and cortisol levels, we decided to further explore this process that has always been assumed to be GC driven in the normal physiological context. In healthy mice and rats, this is reflected by a significant increase in blood glucose level after a 1-hour restraint stress (Fig. 2.2 A, $p < 0.0001$; paired t-test and Fig. 2.2 B, $p = 0.0089$; paired t-test). We had previously shown that the socially evaluated cold pressor test used in the EpiPath study increased blood pressure, heart rate and plasma cortisol levels [170]. In addition to these parameters, in this study we also observe an increase in plasma glucose levels post stress (Fig. 2.2 C). Blood glucose levels rapidly rise after the onset of stress, peaking at 15 minutes with a mean difference of 28 mg/dL (Fig. 2.2 C, $p = 0.0004$, paired t-test compared to t=0), returning to pre-stress levels after approximately 3 hours. The socially evaluated cold pressor test lasted only 3 minutes, explaining the different kinetics from the rodent 1h restraint stress tests. When we extracted the time taken to achieve plasma cortisol peak and plasma glucose peak (Fig. 2.3 A and Fig. 2.3 B), we clearly see that most participants show a cortisol peak 30 minutes after stress onset, while glucose peaks were observed in the 3 or 15 minute post stress samples. Furthermore, this stress induced rise in blood glucose was independent of sex in mice, rats and humans (Fig 2.4 A-F: $p < 0.05$ for all groups (except Fig 2.4 C: female rats, $p = 0.06$; paired t-tests).

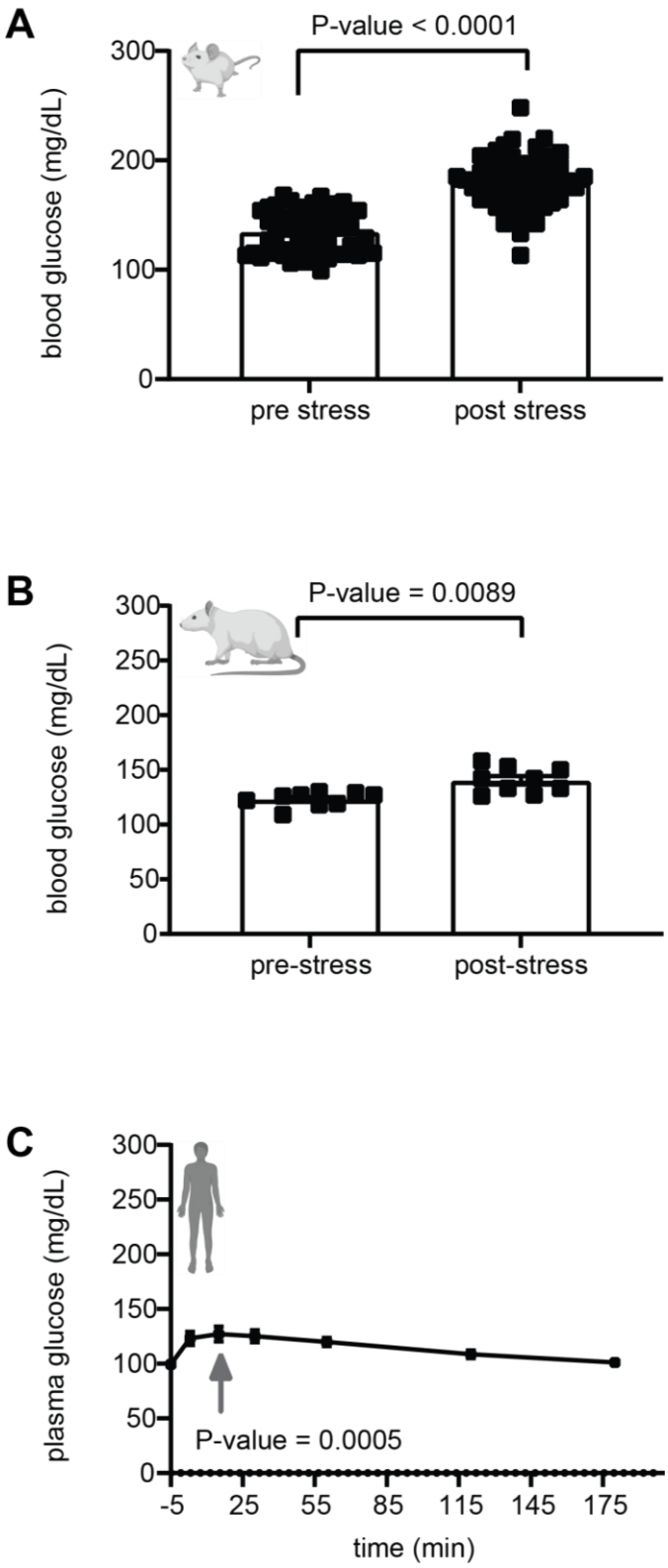


Figure 2.2: Exposure to stress triggers gluconeogenic processes resulting in increased blood glucose levels. A 1-hour restraint stress in both A) mice and B) rats resulted in elevated blood glucose in the post stress condition. C) A socially evaluated cold pressor test (indicated as a grey box) in humans also showed a similar trend of elevated blood glucose (indicated with a grey arrow)

significantly peaking at t = 15 minutes (p = 0.0005, paired t-test), after stress that eventually came back to baseline. All data are means along with the standard error of mean (SEM). All measurements have been expressed in mg/dL. Each square represents an individual animal/participant.

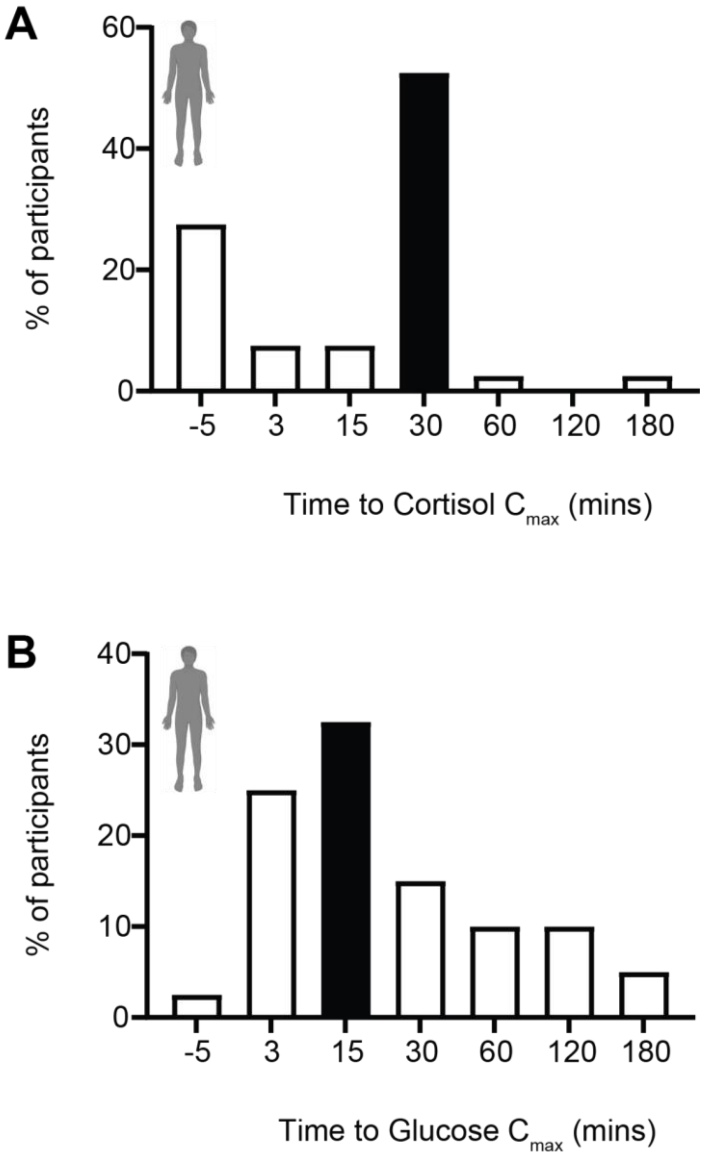


Figure 2.3: Glucose maximum concentration (C_{max}) peak precedes cortisol maximum concentration (C_{max}) peak. Bar graph showing % of participants along with the time (minutes) required for them to achieve A) peak cortisol levels and B) peak plasma glucose levels after the socially evaluated cold pressor test. Black bar represents the time point for which maximum participants achieve peak glucose levels.

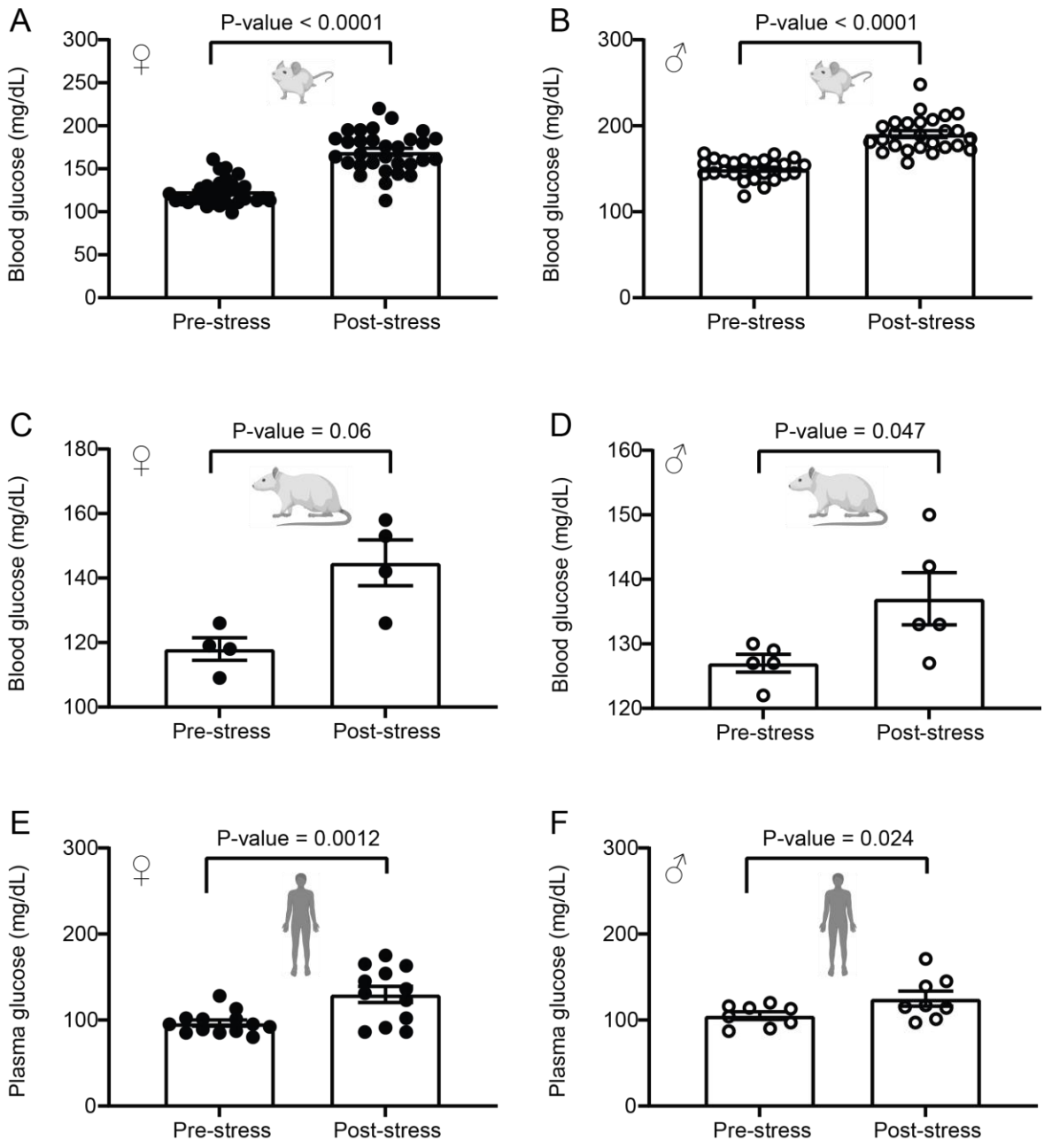


Figure 2.4: Stress induced glucose release is independent of sex. Blood glucose rise post stress was observed in A) female mice, B) male mice, C) female rats, D) male rats, E) human females and F) human males. All data are means along with the standard error of mean (SEM). All measurements have been expressed in mg/dL. Each dot represents individual animals (females = filled circles, males = empty circles).

2.4.5. *I.v. cortisol administration in healthy humans failed to raise blood glucose levels*

In order to better characterize the link between stress and glucose, we further examined blood glucose levels after administration of escalating i.v. doses of cortisol. All groups that received i.v. cortisol showed a peak in plasma cortisol levels approximately 18 minutes after the infusion indicating a successful administration (Fig. 2.5 A). The plasma cortisol levels for the 3 mg and 6 mg groups came back to pre-injection levels after approximately 100 minutes and 160 minutes after iv cortisol administration. The higher dose groups did not return to pre-injection plasma cortisol levels by the end of the experiment. Participants who were administered a higher dose exhibited higher peak plasma cortisol levels at t = 75 mins, meaning that the observed plasma cortisol peak was proportional to the administered dose (Fig. 2.5 B. $p = 0.0027$, $R^2 = 0.97$; linear regression). The plasma cortisol AUC_g showed a high correlation with the administered cortisol (Fig. 2.5 C. $p = 0.0009$, $R^2 = 0.98$; linear regression). However, contrary to our hypothesis, none of the 5 groups showed an increase in the level of plasma glucose up to 2.5 hours post cortisol administration, irrespective of the dose administered (Fig. 2.5 D, $p > 0.05$ for all time points and all doses at time of injection t = 57 mins; Wilcoxon test). We also saw no correlation between the calculated plasma glucose AUC_g and either the dose of cortisol administered (Fig. 2.5 E. $p = 0.85$, $R^2 = 0.015$; linear regression) or the plasma cortisol AUC_g (Fig. 2.5 F. $p = 0.92$, $R^2 = 0.0035$; linear regression).

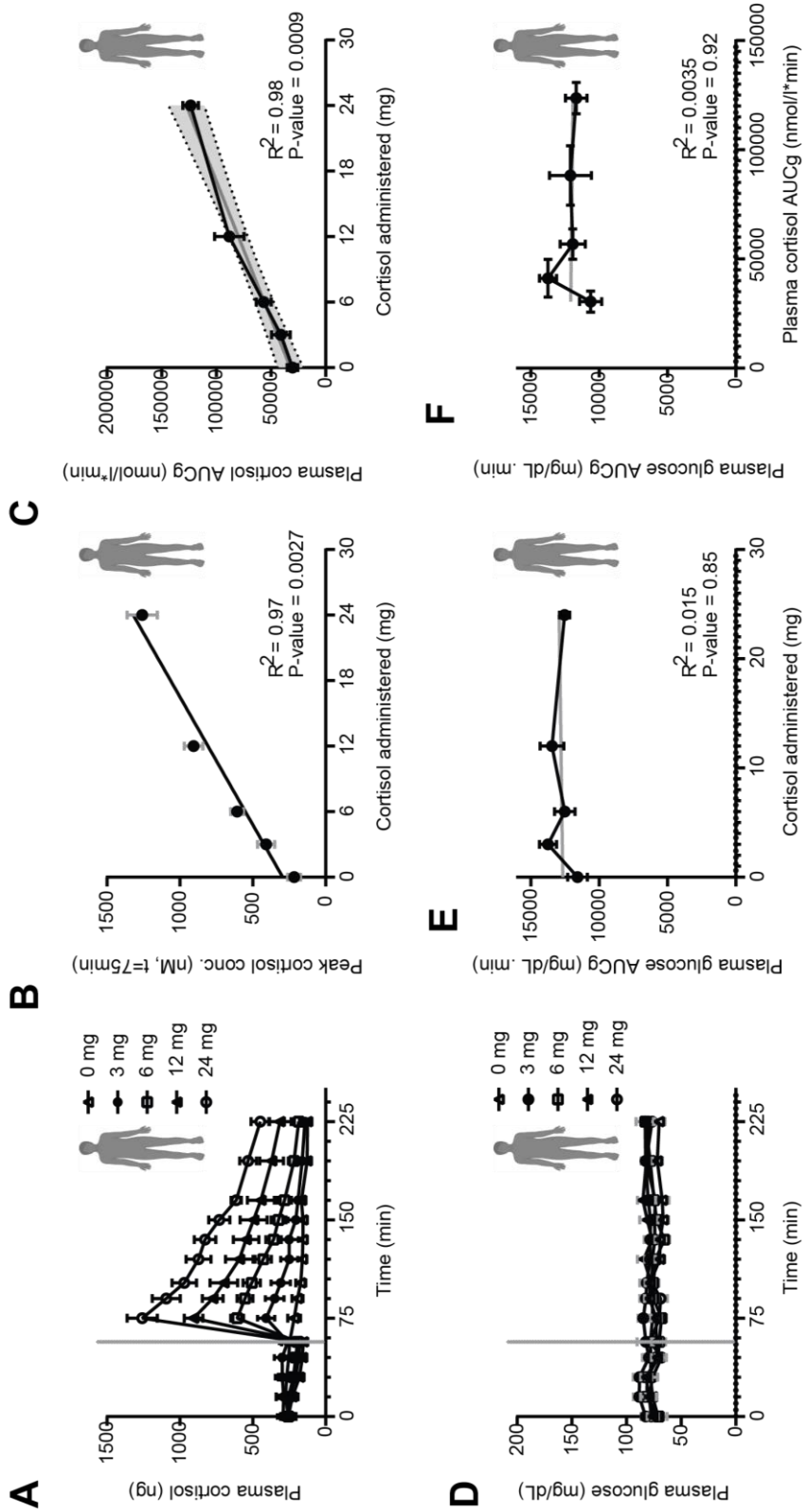


Figure 2.5: Administered cortisol failed to raise blood glucose levels in (ESCO) participants. A) The plasma cortisol readings (in ng) for the control and the 4 test groups (doses in mg) were reflected correctly in the plasma corresponding to the administered dose (mg). The faint dotted line indicates

the time point at which cortisol was infused. The different symbol represents the different doses of administered cortisol. B) The test groups showed a plasma cortisol peak (nmol/l) at approximately 18 minutes after the infusion that was also strongly associated with the administered dose of cortisol (mg). C) There was a clear correlation between calculated plasma cortisol AUC_g (nmol/L.min) and the infused cortisol dose (mg). The grey line represents the line of regression and the light grey area within the dotted lines denotes the area within the error bands. SEM for all panels have been shown as error bars. D) There was no rise in blood glucose level regardless of infused cortisol dose. The faint dotted line indicates the time point (minutes) at which cortisol was infused. The different symbols represent the different doses of administered cortisol. There was no correlation between plasma glucose AUC_g (ng/dL.min) and E) administered cortisol dose (mg) or F) plasma cortisol AUC_g (nmol/L.min). The grey lines for panels C), E) and F) denote the line of regression. SEM for all panels have been shown as error bars.

2.4.6. *Cortisol administration was functionally active: expression of GR target genes GILZ and FKBP51*

As cortisol administration did not induce a glucose rise in the ESCO study participants, we checked the gene expression profile of 2 glucocorticoid receptor target genes known to be induced after GC administration, *GILZ* and *FKPB5* in the plasma of healthy humans administered with escalating doses of cortisol. There was a clear GC-induced gene expression as measured by RT-PCR. For both genes, expression was dose dependent (Fig. 2.6 A. and Fig. 2.6 B), peaking at approximately 195 minutes (135 minutes after injection) although for the highest doses, *FKPB5* expression was still increasing (Fig. 2.6 B). *FKPB5* expression was higher than that of *GILZ* for the same dose of cortisol, however the relative rise was slower than that for GILZ. However, both were significantly dependent on the cortisol dose administered (*GILZ*: Fig. 2.6 C. $p = 0.0011$, $R^2 = 0.98$; linear regression *FKPB5*: Fig. 2.6 D. $p = 0.0014$, $R^2 = 0.98$; linear regression). Interestingly, *GILZ* expression showed a lower percentage of responders to a lower dose of cortisol, in comparison to *FKPB5* expression (Fig. 2.6 E. and Fig. 2.6 F).

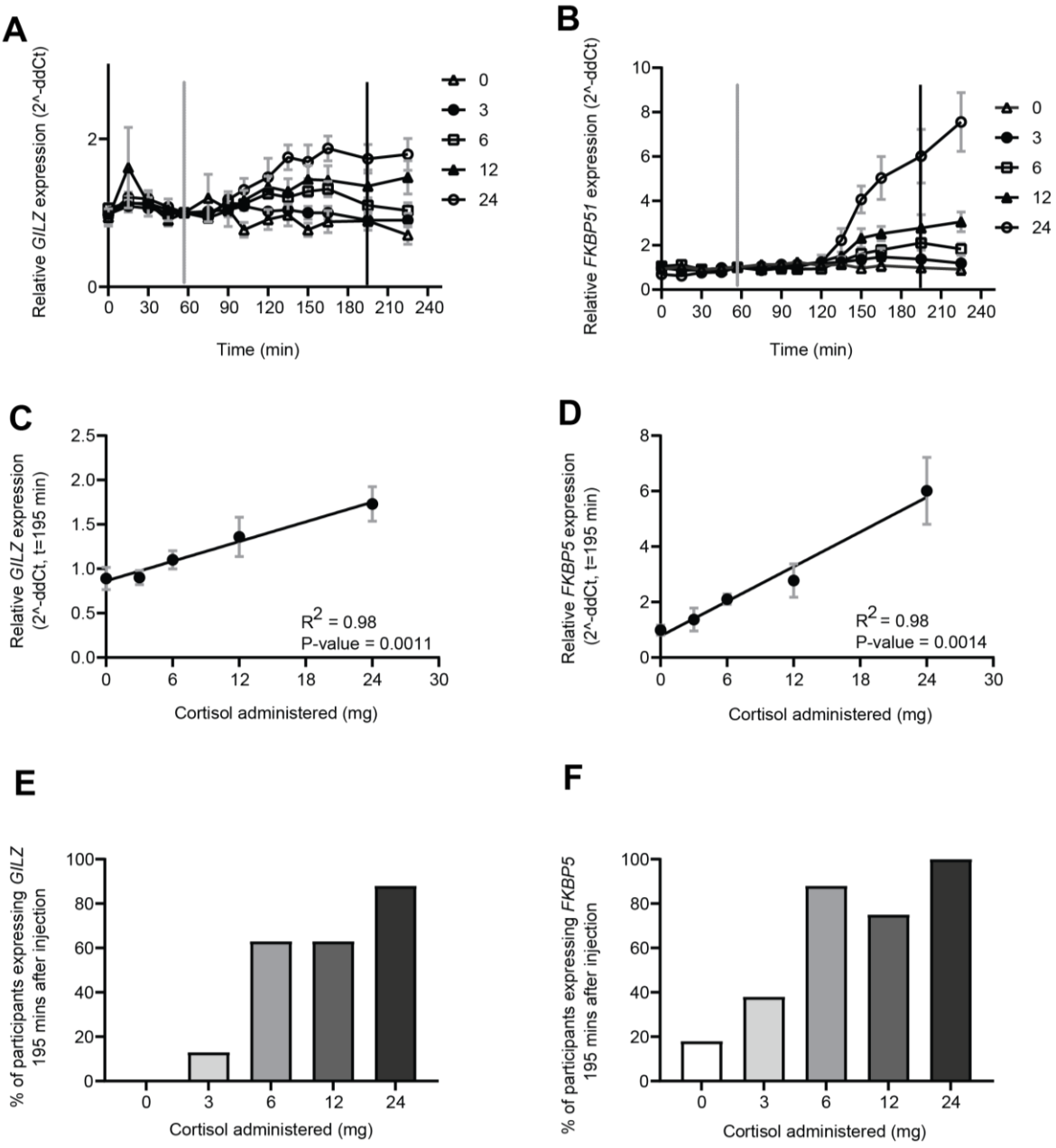


Figure 2.6: Administered cortisol is functionally active and successful in inducing a GC/GR dependent transcriptional response of GR target genes: A) *GILZ* (relative expression) and B) *FKPB5* (relative expression). For both panels, the faint dotted line indicates time point (minutes) at which cortisol was infused. The solid line denotes the best peak of gene expression (195 minutes) and the different symbols represent different doses of administered cortisol. Panels C) and D) show the correlations between the doses of infused cortisol (mg) and C) relative expression of *GILZ* and B) *FKPB5* at 195 minutes. SEM for panels (A-D) have been shown as error bars. Panels E) and F) show the % of responding participants to the infused dose of cortisol (mg), who show gene expression at 195 minutes for E) *GILZ* (relative expression) and F) *FKPB5*. The different colours of the bars indicate the different doses of administered cortisol.

2.5. Discussion

It has previously been reported that ELS may lead to metabolic disturbances later in life in both humans and animal models [104, 397]. Here, we demonstrated that acute stress induces a rapid glucose release that could be impacted by a negative early life environment. Using two animal models of ELS (maternal separation and early infection) we demonstrate that ELS induced pre-stress and acute stress associated changes in blood glucose that were also validated in our institutionalisation-adoption EpiPath cohort. Interestingly, we also showed that the impact of ELS differs according to the type of stressor (psychosocial or immune) and according to the sex. The early life institutionalisation-adoption model showed higher levels of pre-stress glucose in ELA females, similar to the infection rodent model, while the MD males showed a decrease in pre-stress glucose levels compared to the controls. We anticipated that the stress induced glucose rise would be a GC dependent process, however, the kinetics of glucose release and the inability of an i.v. bolus of GC to induce a glucose rise in man suggests that it is a rapid, GC independent, process.

Although there are (limited) data available on how energy availability modulates the stress response [380], knowledge as to how stressors affect energy homeostasis was noticeably absent. It is, however, accepted that the fight/flight response to a stressor requires a rapid mobilisation of resources, mediated through the release of adrenaline and cortisol/corticosterone [380]. At the same time, GC regulates glucose homeostasis, reducing the uptake of glucose by white adipose tissue as well as promoting gluconeogenesis, maintaining glucose availability for central processes [129]. Moreover, multiple studies have demonstrated that chronic stress and/or chronic exposure to glucocorticoids lead to metabolic disturbances [398].

Our data clearly shows the anticipated rapid rise in blood glucose levels after stress. Interestingly, in the EpiPath cohort, this glucose increase slightly preceded peak cortisol levels, while peak cortisol levels were observed 30 minutes after stress onset (Fig. 2.2 A and Fig. 2.2 B), as expected [385, 399], while most participants reached peak glucose levels either 3 or 15 minutes after the onset of stress. This suggests that cortisol does not mediate or initiate glucose release and may not play such an important role in maintaining glucose homeostasis with stress. This was confirmed by our i.v. administration of GC doses up to 24mg. Here, the doses chosen were such that participants receiving 12 or 24 mg cortisol had cortisol levels consistent with a severe psychosocial stressor or excessive physical exercise, while remaining within a range that was physiologically plausible [391, 400]. In the absence of any psychosocial stress signals, the GC bolus did not induce a glucose release. However, the GC bolus was capable of initiating a GC/GR

dependent transcriptional response and was therefore functionally active, confirming the theory that cortisol does not mediate or initiate glucose release as the glucose peak precedes cortisol peak in our experimental paradigm. The kinetics of glucose release, with the most rapidly responding participants reaching peak glucose only after 3 minutes, suggests that this is a direct innervation rather than a hormone-mediated process.

Exposure to stress results in long term metabolic and HPA axis changes, and there is growing evidence that the effects are exaggerated when the exposure period coincides with delicate developmental phases, such as in the case of ELA. These changes, reflect the programming or adapting of physiological systems within the body to cope with the environment in which they are born, and represent the basic principal of DOHaD, or the Barker Theory [374, 401]. DOHaD has now been refined into a 3-hit model [4, 320], with the discordance between the environment to which the individual's body has developmentally adapted to and the actual environment later in life being the tipping point between health and disease [402]. We hypothesised that glucose and cortisol are coupled, and that ELA-induced disturbances of the HPA axis would thus alter glucose homeostasis and vice versa. This was a particularly appealing hypothesis as exposure to adversity in the early-life period in the preclinical and clinical context not only affects the HPA axis, but concurrently induces a long-term metabolic phenotype, and eventual metabolic dysfunction [97, 104, 403, 404]. Furthermore, Chandan et al. (2020) and some other studies estimated that ELA determines "a significant proportion of the cardiometabolic and diabetic disease burden" [71, 99, 190]. In our study, blood glucose was clearly affected by ELS in rodent stress models (MD and ELI), however, there was no clear unique pattern. Although pre-stress and stress-induced glucose release are clearly affected by ELA, there was no association with the HPA axis corticosterone response. This suggests that the HPA axis and metabolic stress responses are independently programmed by the early life environment and that the stress-induced glucose response was not coupled to the GC response.

Acute stress conditions have been known to prompt lipolysis in order to provide energy for fight/flight [405]. Interestingly, Stimson et. al have shown that excess acute cortisol can orchestrate lipid breakdown depending on the availability of high levels of adrenaline and insulin (probable stress induced insulin resistance), with no impact on glucose kinetics [405]. On the contrary, excess chronic exposure to GCs have been reported to induce fat deposition [405]. This contradictory result has been postulated to be a result of differential GC-insulin-adrenaline interaction [405].

It is interesting to note that Cushing syndrome, characterised by excess cortisol in circulation and commonly caused by chronic overadministration of GCs used to treat inflammatory diseases, has been reported to show diabetes as a common comorbidity with a prevalence rate of 20-50% [406, 407]. Chronic inflammation has also been known to promote the T2D aetiology (reviewed in [408]). Thus, it is interesting to hypothesise that the T2D cases observed in conjunction with Cushing syndrome may arise from the underlying inflammation and not due the excess circulating GCs.

This hypothesis is in line with our study, where we conclude that the rapid glucose rise due to stress is not hormone or signalling mediated. Although a completely different experimental paradigm to those used here would be necessary to investigate this, it is interesting to hypothesise that hepatic innervation [409], with counteracting and counterbalancing sympathetic (splanchnic nerves) and parasympathetic (vagus nerve) inputs may be the explanation behind this. Previous findings in mice suggests that exposure to an acute psychological stress induces an acute insulin resistance in the liver and not in either muscles or adipose tissues [410]. The most likely reason that this is restricted to the liver is that there is an established vagal control of hepatic metabolic processes [411-414]. Transcutaneous stimulation of the auricular vagus nerve was also shown to be able to significantly alter 2-hour plasma glucose, fasting plasma glucose, and glycosylated hemoglobin levels over a 12-weeks trial period and improve glucose tolerance [415]. Activation of neurons within the dorsal motor nucleus and solitary tract of the vagus have also been reported to actively decrease hepatic gluconeogenesis, as well as modulate the expression of gluconeogenic enzymes, and glucose production [411]. Cardin et. al reported that vagal blockade decreases glucose production in the liver by diminishing glycogenolysis, while gluconeogenic processes remain unaltered [416]. However, a vagal blockade would most probably block afferent fibers, resulting in a decreased sympathetic input to the liver at the same time [416]. This would decrease the stress-associated signal counterbalancing the vagal parasympathetic input, and potentially controlling gluconeogenic processes.

However, any future investigation of neuronal control of hepatic carbohydrate metabolism should also address neuronal glucose sensors such as Glucokinase and GLUT in the hepatoportal region that also regulate glucose metabolism [409], and may have a direct role in modulating the stress induced glucose release. Additionally, glucagon has been shown to mediate (physical) stress induced hyperglycaemia, but can also be hypothesised to orchestrate psychosocial stress induced blood glucose rise [417].

The differences between the EpiPath and ESCO cohorts are striking. As the experimental paradigm used to administer the GC bolus in the ESCO cohort was designed to blind the participant to the pharmacological manipulation being performed, and to induce a strict minimum of psychological stress, the absence of such visual and psychological stimuli are most probably responsible for the absence of the glucose response. Inversely, in the EpiPath cohort and both rat and mice models had a clear psychological stress component, hence the glucose response. Thus the stress-induced glucose release can be attributed to the psychosocial element of the stressor activating higher functions.

Unfortunately, once our hypothesis was proven to be incorrect, despite having adequate statistical power, investigation of the potential role of the vagal-splanchnic/Glucokinase/glucagon interaction required a completely different experimental setup that was not available to us. Furthermore, we were limited by the lack of data on vagal tone and splanchnic nerve activity from the EpiPath cohort. Importantly, our results clearly disprove cortisol as the key diabetogenic hormone in stress situations and instead suggests rapid inputs from the nervous system as the key gluconeogenic driver. Additionally, in our study we were able to show using cross species models that exposure to adversity in the early-life period plays an important role in determining pre-stress glucose levels, with a clear effect of sex. Moreover, we saw the same effect in the human institutionalisation model as the constituent early life infection and early life psychosocial stressor, maternal deprivation. This was most probably due to an ELA induced epigenetic alteration of the glucose sensors, which in turn impacts the direct neuronal input into the liver.

Another drawback of our study is rooted in the limited evidence from recent ELA literature suggesting that ELA impacts insulin sensitivity, potentially via underlying epigenetic mechanisms thereby affecting neurotransmitters in the brain (reviewed in [225]). We further hypothesise that ELA may also epigenetically program the insulin and glucose receptors. This may contribute to impaired insulin sensitivity and glucose uptake into the cells, consequently disrupting glucose related metabolic pathways in ELA individuals.

Overall, our study provides a novel perspective on the mechanisms linking stress and metabolic changes, and highlights the importance of collecting early life data as a measure to understand an individual's metabolic status in a better light.

Chapter 3: A holistic view of the Goto-Kakizaki rat immune system: Decreased circulating immune markers in non-obese type 2 diabetes.

My contribution to this chapter: Conceptualisation; Literature review; Flow cytometry set up, experimentation and analysis; Final statistical analysis of flow cytometry and cytokine data; Interpretation of results and Writing of the article.

Online DOI: 10.3389/fimmu.2022.896179. eCollection 2022.

Published in: Frontiers in Immunology

3.1 Abstract

Type-2 diabetes is a complex disorder that is now considered to have an immune component, with functional impairments in many immune cell types. Type-2 diabetes is often accompanied by comorbid obesity, which is associated with low grade inflammation. However,the immune status in Type-2 diabetes independent of obesity remains unclear. Goto-Kakizaki rats are a non-obese Type-2 diabetes model. The limited evidence available suggests that Goto-Kakizaki rats have a pro-inflammatory immune profile in pancreatic islets. Here we present a detailed overview of the adult Goto-Kakizaki rat immune system. Three converging lines of evidence: fewer pro-inflammatory cells, lower levels of circulating pro-inflammatory cytokines, and a clear downregulation of pro-inflammatory signalling in liver, muscle and adipose tissues indicate a limited pro-inflammatory baseline immune profile outside the pancreas.

As Type-2 diabetes is frequently associated with obesity and adipocyte-released inflammatory mediators, the pro-inflammatory milieu seems not due to Type-2 diabetes *per se*; although this overall reduction of immune markers suggests marked immune dysfunction in Goto-Kakizaki rats.

Keywords: Diabetes, obesity, cytokines, inflammation, microarrays, Goto-Kakizaki rats.

3.2 Introduction

Type-2 diabetes (T2D) is a complex disorder characterised by hyperglycaemia, insulin resistance (IR) and chronic inflammation of insulin target tissues. T2D is now also considered an inflammatory disease, affecting both innate and acquired immune systems, skewing them towards a pro-inflammatory phenotype (reviewed in [418]). There is also growing evidence for autoimmune involvement in T2D overlapping with the pathophysiology of T1D [419]. Inclusion of cellular autoimmunity with traditional diabetic parameters is now reflected by a number of diabetes sub-types that do not fit into T2D or T1D including type 1.5 diabetes mellitus or latent autoimmune diabetes of the adult (LADA) or the young (LADY) and double diabetes (mixed symptoms of T1D and T2D) [420, 421]. This recalibration of diabetic phenotypes led us to re-examine the use of obese pre-clinical diabetic models, as these no longer adequately reflect the human clinical context [422]. Most of the studies conducted on inflammation in T2D involved overweight or obese subjects and obesity is clearly associated with a low-grade chronic inflammation [423, 424]. Since T2D is not always associated with obesity, it is essential to dissect the exact contributions of obesity and diabetes in the immune phenotype.

Although there are many well-known obese models of T2D, there are few non-obese models [328]. Goto-Kakizaki (GK) rats are a non-obese model that present a pre-diabetic phase before becoming spontaneously diabetic, similar to the human T2D pathophysiology [351]. However, despite the growing immune literature in human T2D, there is little data available on the GK rat immune system. Circulating white blood cell (WBC) levels have been reported to be unchanged in GK rats, although they are known to be biased towards a T_h2 phenotype [425], have fewer B-cells, a higher IgM production and reduced monocyte-phagocytic activity [422, 426]. Surprisingly, this phenotype is somewhat contrary to that observed in the Type 1 model "Diabetes-prone Biobreeding (DP-BB) rats" [422] and high-fat diet induced obesity in mice [427]. In contrast, previous works have demonstrated a marked proinflammatory profile in pancreatic islets in GK animals, with macrophage infiltrations and upregulation of mRNA expression of several proinflammatory cytokines in this tissue [428, 429]. Subsequently fibrosis develops within the islets which further alter their normal secretory function [429].

Given the potential for the GK rat model to represent non-obese and emerging diabetes subtypes, we used a wide-ranging rat immune system profiling panel [393] and multiplex cytokine panel to characterise adult, diabetic GK rats. By investigating levels of principal circulating immune cell subsets and plasma cytokines, we aim to provide a baseline immune profile to further study

the role of immune system in the aetiology of T2D. Furthermore, we reanalysed previous transcriptional datasets [430] for the consequences of exposure to this cytokine milieu in the liver, adipose tissue and muscles.

3.3 Materials and Methods

3.3.1 Animals

GK rats (from B2PE, unit BFA, Diderot and CNRS, Paris colony) and control Wistar rats were bred in the conventional facility of Nutrineuro lab (INRA UMR1286, Bordeaux). Bodyweight and adiposity in animals from the colony have recently been described [431], and are a common finding in GK rats [351, 428, 430] although may depend on colony [432]. Six months old male offspring were used (n=6 Wistar, n=7 GK). All experimental procedures were carried out in accordance with the European Union guidelines for the use of animals for experimental purposes (Council Directive 2010/63/EU) and the French guidelines (Directive 87/148, Ministère de l'Agriculture et de la Pêche – Apafis #16924). Animals were grouped-housed (n=3 per cage) in a 12 h/12 h light:dark cycle (lights on at 06:00 a.m.) at 22 ± 2 °C with access to food (SAFE D113, Augy, France) and water ad libitum.

3.3.2 Blood sampling

Blood samples were collected by tail nick into EDTA-coated tubes. Samples were centrifuged (1800g, 4 °C) for 10 minutes. Plasma was collected and stored at −80 °C for cytokine or insulin assays. For flow cytometry, blood was mixed with Streck cell preservative (1:1 volume) (Biomedical Diagnostics, Antwerp, Belgium), stored and transported at 4°C.

3.3.3 Flow Cytometry

Staining was performed on Streck-preserved samples as described in Supplementary Materials. Briefly, 200,000 events were recorded on an LSRFortessa (BD BioSciences, NJ, USA) and analysed using FlowJo (version 10.6.1, BD BioSciences, NJ, USA). A Streck-preservative optimised gating strategy (Figure 3.1) was adapted from Fernandes et. al [393] and populations represented as a percentage of parent population frequency. Data was not available for 1 GK rat due to Streck preservative associated coagulation and failure of the staining.

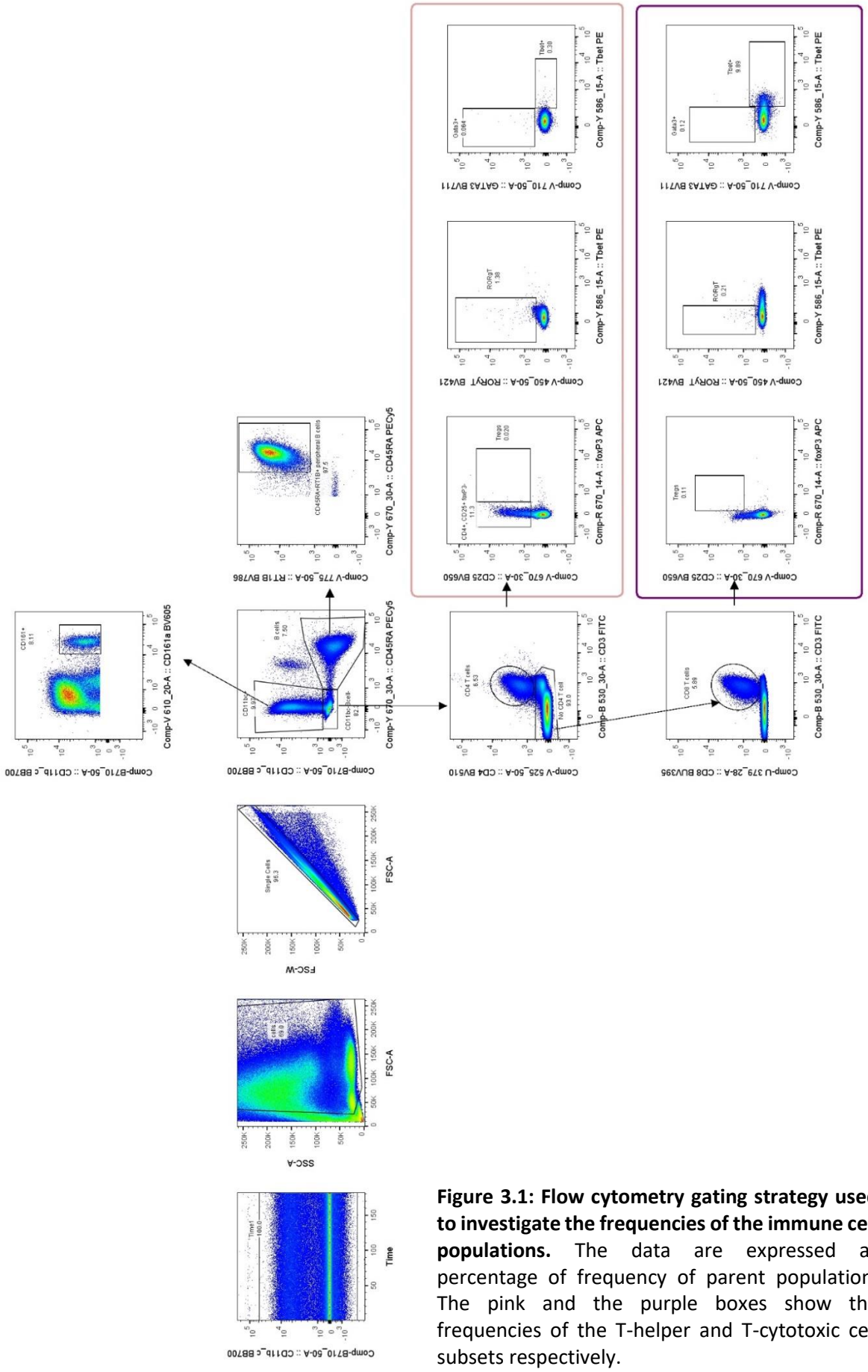


Figure 3.1: Flow cytometry gating strategy used to investigate the frequencies of the immune cell populations. The data are expressed as percentage of frequency of parent population. The pink and the purple boxes show the frequencies of the T-helper and T-cytotoxic cell subsets respectively.

3.3.4 Plasma Cytokines, glycaemia and insulin levels

Glucose, Insulin, and a panel of 27 plasma cytokines (Milliplex 27-plex kits; Eve Technologies, Calgary, Canada), were measured as described in Supplementary Materials.

3.3.5 Microarray Re-Analysis

Wistar and GK rat microarray data were downloaded from NCBI (GSE13271) for adipose tissue, gastrocnemius muscle, and liver from 20-week-old normal-diet fed animals [430]. Raw data were normalised, a linear model fitted, and differential gene expression calculated using limma (version 3.46.0) for genes expressed above a threshold (100) in all 10 samples per comparison using genefilter (version1.54.2).

3.3.6 Statistical analysis

All statistical analyses were performed using Graphpad Prism (version 8.2.0). Unless otherwise stated the unpaired Student's t-tests were used to compare normally distributed data from GK and Wistar rats when the variance was equal between the groups. Welch's t-test was used for normally distributed data with unequal variance between the groups, and the non-parametric unpaired Mann-Whitney test was used for non-normally distributed data. All data are the result of two biologically independent experiments. Pearson correlations were performed on the complete dataset and were used to determine the associations between immune cells profile and plasma cytokine levels. Microarray and pathway reanalysis was performed in R (version 4.0.2). Venn Diagrams were generated with "VennDiagram" (version 1.6.20). Statistical significance was set at $p < 0.05$. Data are expressed as means ± SEM.

3.4 Results

3.4.1 *GK rats show a limited pro-inflammatory immune-cell profile*

The numbers of circulating CD11b/c+ and CD45RA+ (B cells) cells in GK rats was ~2.5-fold ($p=0.0022$, Mann-Whitney test) and ~4-fold ($p=0.0022$, Mann-Whitney test) lower respectively, compared to Wistar rats (Figure 3.2 A-B) as previously reported [425, 426]. This suggests a decrease in the frequency of phagocytes such as macrophages, monocytes, dendritic cells, granulocytes and B-cells respectively.

GK rats have also been reported to have an increased frequency of total CD3+ T cells [422, 425, 426], although we did not observe this in our study (data not shown). The CD4+ T_h cells had a decreasing trend in the GK rats (~2.5-fold), compared to Wistar rats (p=0.0649, Mann-Whitney test, Figure 3.2 C).This was in line with Huda et. al., who reported a decreasing naive CD4+ T_h cell trend in diabetics [433].

We observed a significant ~5-fold decrease in the number of CD8+ cytotoxic T-lymphocytes (T-cytotoxic cells; T_c cells) (p=0.0022, Mann-Whitney test, Figure 3.2 D) in GK rats compared to control Wistar rats.

GK rats exhibit a ~2-fold increase in CD161a+ cells (p<0.0001, Mann-Whitney test, Figure 3.2 E), a "target specific receptor", which enables NK cells to activate and efficiently carry out their cytotoxic functions.

We also observed that despite the low percentage of T_c cells, majority of them were RORγT+ (p=0.0022, Mann-Whitney test, ~1.5-fold increase in GK rats, Figure 3.2 F) and GATA3+ (p=0.039, Mann-Whitney test, ~1.5-fold increase in GK rats, Figure 3.2 G) compared to Wistar rats. Furthermore, cell population frequencies of CD45RA+ RT1B+ peripheral B cells, Tregs (CD4+ CD25+ FoxP3+), Th1 cells (CD4+ Tbet+), Th2 cells (CD4+ GATA3+), Th17 cells (CD4+ RORγT+) and Tc1 cells (CD8+ Tbet+) were comparable between Wistar and GK rats (Figure 3.3). The cell populations that were most highly increased in the GK rats were unidentified CD11b/c- CD45RA- cells (Figure 3.3 H; p=0.0022; Mann-Whitney test). Thus, overall, GK rats show a very distinct immune profile that may be a direct consequence of the diabetic phenotype.

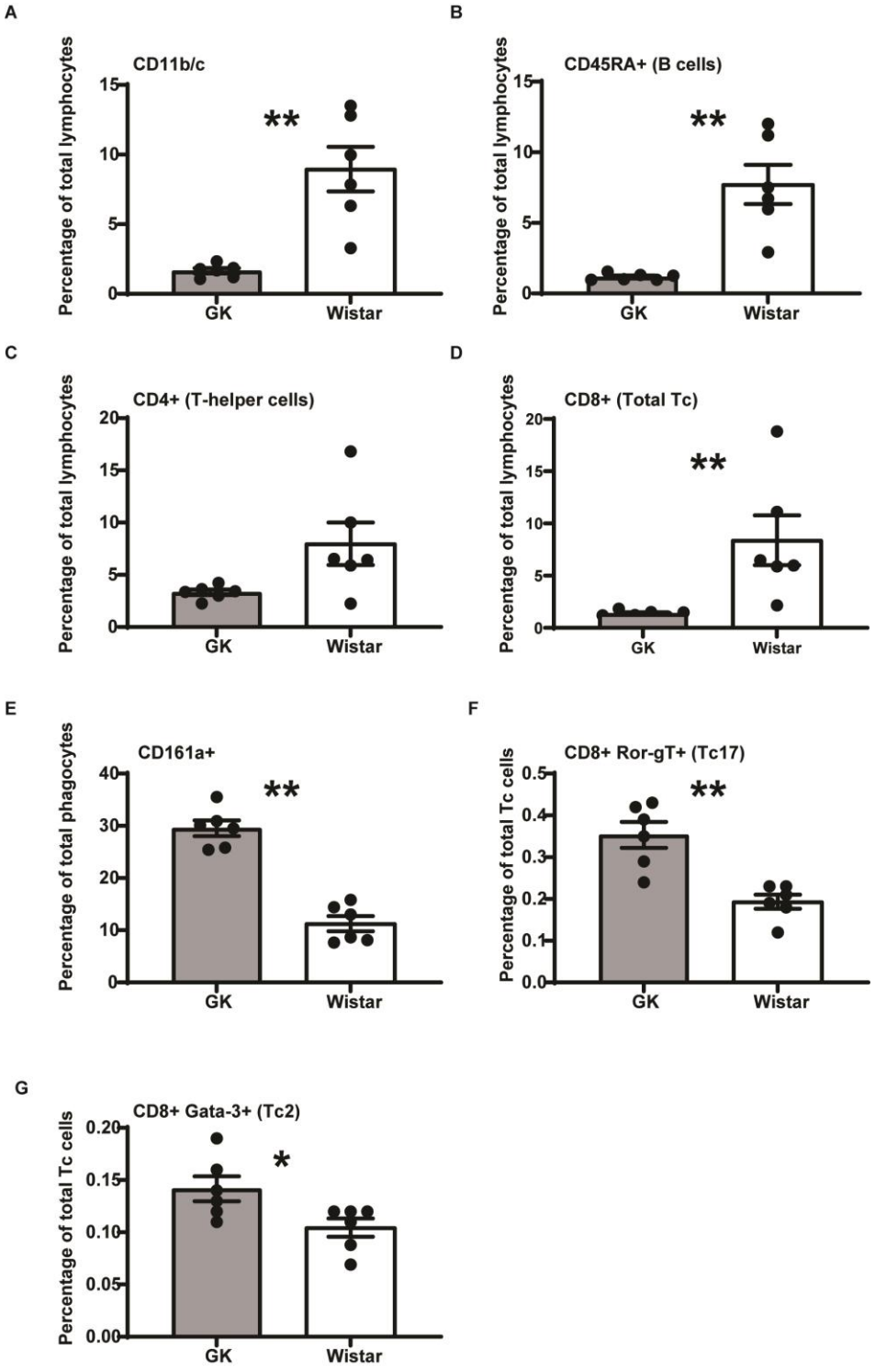


Figure 3.2: The diabetic milieu majorly alters the GK immune system. Significantly affected immune cells ($p<0.05$), expressed as percentage of parent population frequency are: A) CD11b/c+ phagocytes (macrophages, monocytes, dendritic cells, granulocytes), B) CD45RA+ B cells, C) CD4+ T-helper cells, D) CD8+ T-cytotoxic cells E) CD161a+ NK cells, T-cell subsets, activated monocytes, and dendritic cells F) RORγT+ Tc17 cells G) GATA3+ Tc2 cells. However, the T-helper cells narrowly missed the significance threshold ($p = 0.0649$). Data are mean +/- SEM; individual animals shown as dots. Grey bars: GK rats (n=6); white bars: Wistar rats. (n=6).

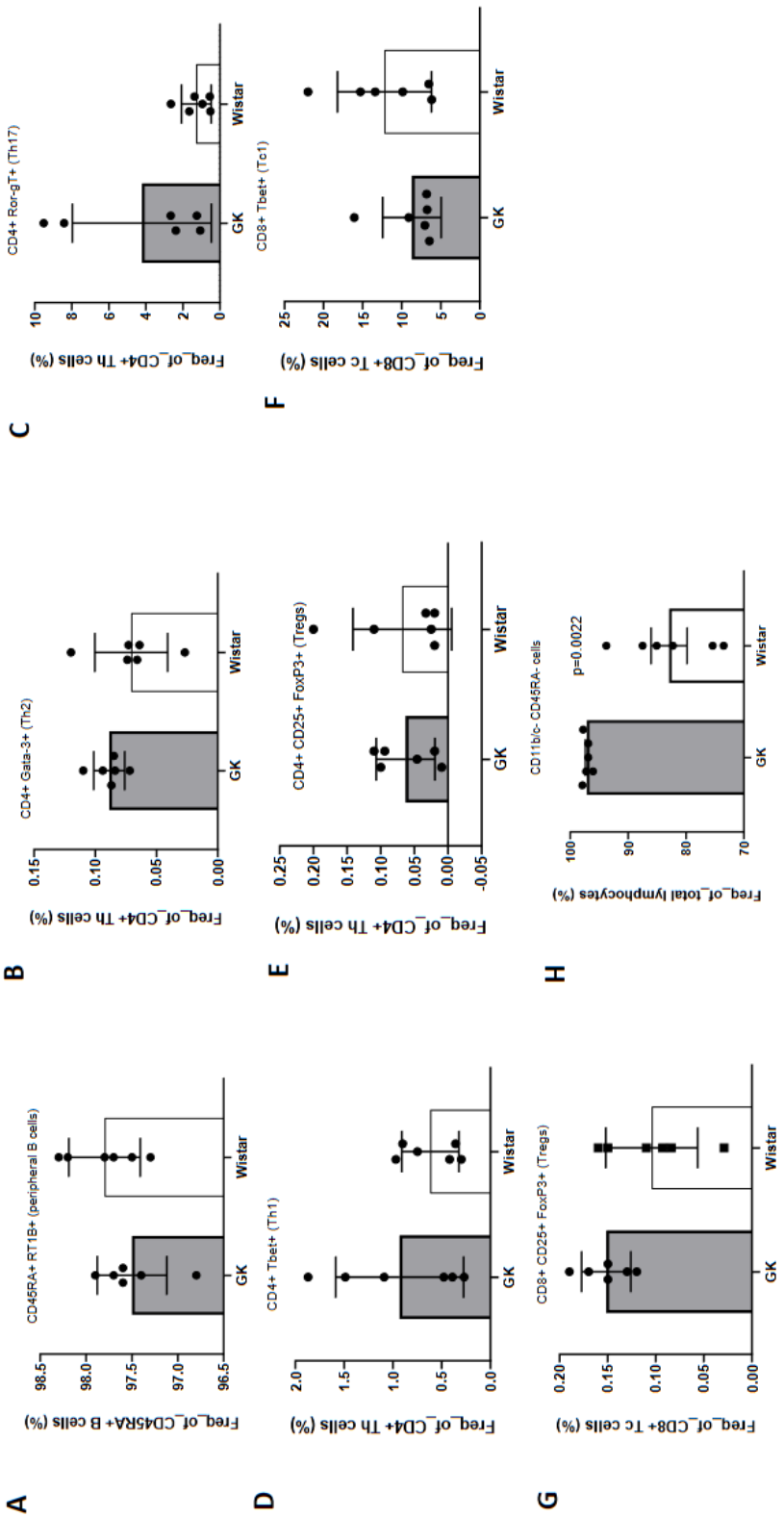


Figure 3.3: Immune cell populations that were not significantly different between the GK and Wistar rats (from the same panel as Figure 1). Data are displayed as means along with their SEM in frequency of parent (%). The colours of the bars represent the different strains (GK rats = grey, Wistar rats = white). Mann-Whitney test was used for all panels.

3.4.2 *Plasma cytokine profile of GK rats reflects the low percentage of the respective secretory immune cells*

Overall, our results revealed a strong impact of T2D on plasma cytokine levels. Surprisingly, most of the cytokines, regardless of its inflammatory role (pro- or anti- inflammatory) were significantly decreased in GK rats (Figure 3.4). Only 8 cytokines: EGF, Fractalkine IL-1β, IL-10, RANTES, VEGF, MIP-2 and GM-CSF levels were comparable in GK and Wistar rats (Figure 3.5). Of the 16 pro-inflammatory cytokines investigated, levels of 12 were significantly lower (Figure 3.4 A-L), in GK rats compared to Wistar rats (p-values between 0.0022 and 0.0115) as shown in Figure 3.4. The majority of these cytokines are secreted by myeloid-derived cells such as monocytes/macrophages including eotaxin, GRO/KC, IL-1α, IL-12(p70), LIX, MCP-1, TNF-α, and IL-18 [434-443]. Furthermore, levels of cytokines secreted by lymphoid derived cells such as B-cells and T_c cells including IL-1β [444], MIP-1α [445] and IFN-γ [446], IL-2 [447] respectively were lower in GK rats. Similarly, levels of IL-17A was also found to be lower in the GKs. Unfortunately, this was not consistent with the increased number of circulating T_c17 cells we observed. However, this can most probably be explained by reduced numbers of other primary sources of IL-17A such as T_h17, NK cells, or natural killer T cells [448]. Unlike other pro-inflammatory cytokines, IP-10 levels were significantly increased in GK rats ($p=0.0103$, Figure 3.4 M).

IL-6 bridges anti- and pro-inflammatory actions and is primarily secreted by macrophages and monocytes. IL-6 levels were lower in GK rats Figure 3.4 N; $p<0.05$). This was most likely due to the low percentage of macrophages/monocytes.

Three out of the four anti-inflammatory cytokines investigated (G-CSF, IL-13 and IL-4) were significantly decreased in GK rats (Figure 3.4 O-Q, $0.0001<p<0.02$). G-CSF is produced by monocytes and macrophages and their low levels in the GK plasma is consistent with our flow cytometry data (low percentage of CD11b/c+ cells). IL-13 is secreted by macrophages, B-cells and T_c cells, all of which show low frequency resulting in low secretion of these cytokines. IL-4 and IL-5 (Figure 3.4 Q-R, Mann-Whitney test for IL-5) has been reported to be secreted by T_h2 cells, basophils and eosinophils. Finally, leptin (Figure 3.4 S) was the unique plasma marker that was significantly upregulated in GK rats compared to Wistar animals ($p<0.001$). Elevated levels of leptin could correlate with increased adipose depots in the GK rats, as we previously described [431].

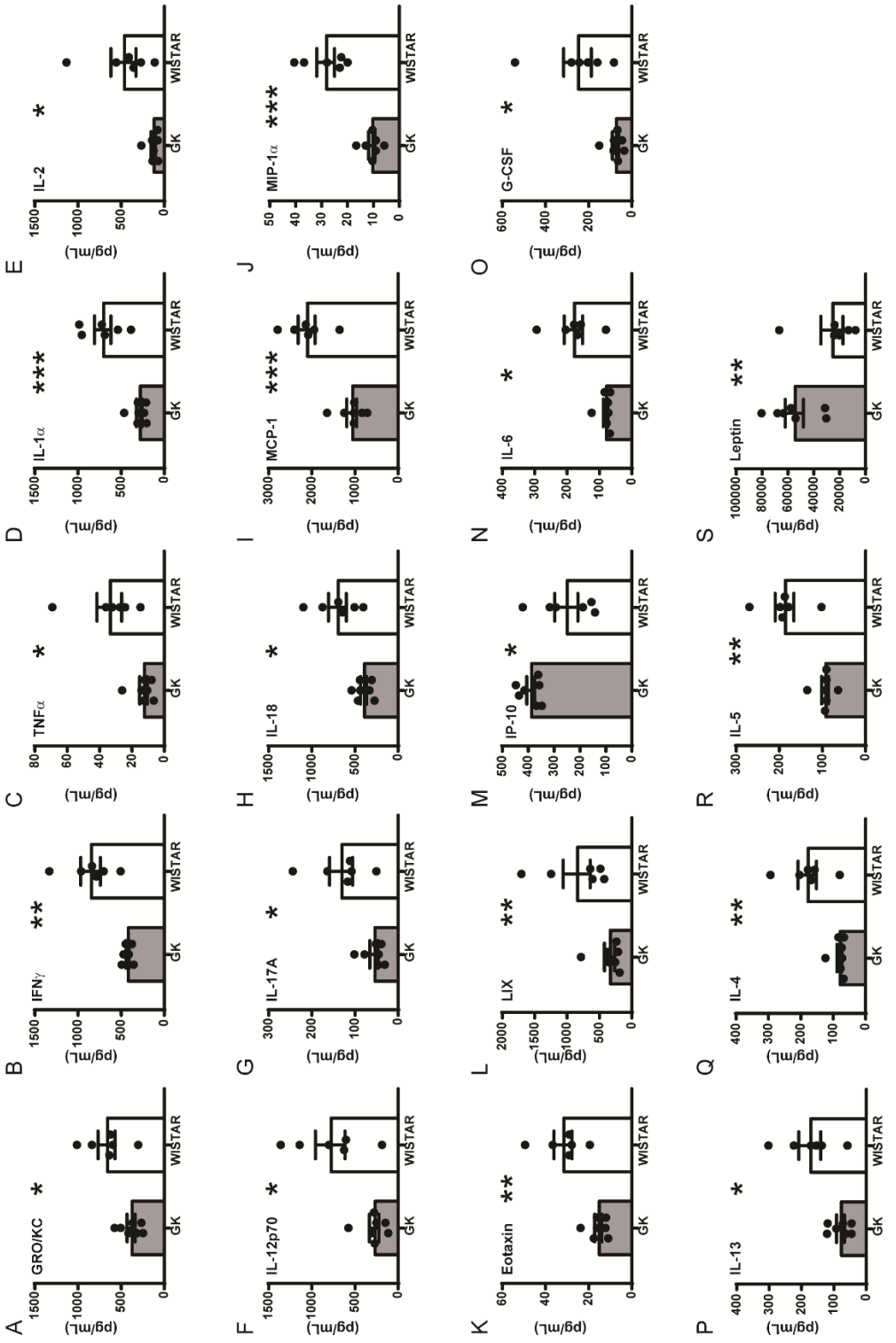


Figure 3.4: Overall cytokine profile shows limited peripheral inflammation in GK rats. All panels display the mean levels of plasma cytokines (mean +/- SEM) in pg/mL, that were significantly different ($p<0.05$) in the GK rats compared to the controls. Cytokines shown in panels A-M are proinflammatory, N) is both pro- and anti-inflammatory, O-Q are anti-inflammatory while R-S show no inflammatory role. Data are mean +/- SEM. Grey bars: GK rats (n=7); white bars: Wistar rats (n=6). $p<0.001$, $p<0.01$ and $p<0.05$ are reflected by ***, ** and * respectively. Student's t-test was used except for panels E (IL-2), L (LIX), and R (IL5), which were analysed using a Mann-Whitney test.

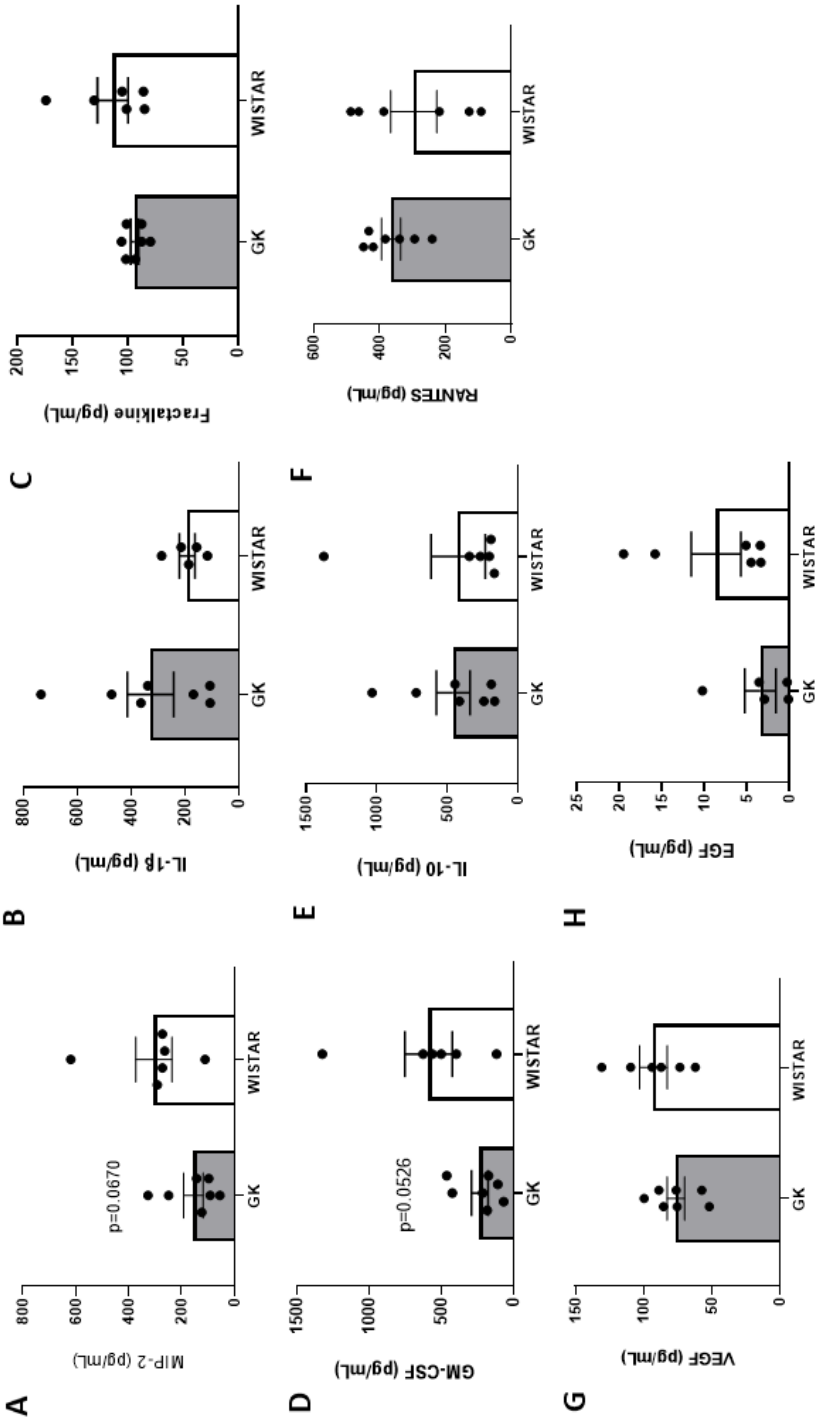


Figure 3.5: Cytokines that were not significantly different between the GK and Wistar rats (from the same panel as Figure 2). Data are displayed as means along with their SEM in pg/mL. Cytokines shown in Figures A-C are proinflammatory, D) is both pro and anti-inflammatory, E) is anti-inflammatiory and F-H show no inflammatory role. The colours of the bars represent the different strains (GK rats = grey, Wistar rats = white). Student's t-test was used except for panels H (EGF) and A (MIP-2).

Additionally, we also tested plasma glucose readings, which were ~2-fold ($p<0.0001$, Welch's t-test) higher in GK rats compared to the controls (Figure 3.6), while the insulin levels were comparable for the two groups.

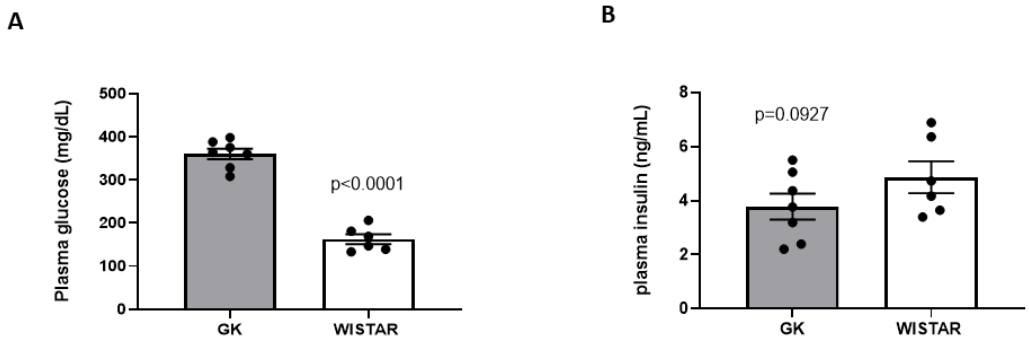


Figure 3.6: Plasma readouts for GK and Wistar rats. Panels A) and B) show glucose and insulin levels in GK rats compared to control Wistar rats respectively. Data are mean+/- SEM and the colours of the bars represent the different strains (GK rats = grey, Wistar rats = white).

We then investigated correlations between the circulating cytokines and immune cells along with plasma readouts such as glycaemia and insulin levels. Bravais Pearson correlations revealed several significant associations between the metabolic markers and cytokine levels. Glycaemia was negatively correlated with the majority of cytokines levels (Figure 3.7 A); whereas plasma insulin levels were positively correlated with cytokines levels (Figure 3.7 A). Most of the cytokines were positively correlated each other; in contrast IP-10 and leptin were strongly negatively correlated with majority of the cytokines (Figure 3.7 A). IL-1β levels were positively correlated with IL-10 levels but showed no association with any of the other cytokines ($p<0.001$). IP-10 correlations with cell numbers and cytokine levels were calculated, although we saw no correlation (Figure 3.7 A).

The correlation pattern for the immune cells was heterogeneous (Figure 3.7 B). B-cells, CD11b/c+ cells, T_c cells and T_h cells, all positively correlated with one another ($p<0.001$). CD161a+ cells were positively correlated with T_c17 and T_c2 cells ($0.01<p<0.05$). CD161a+ cells and T_c17 cells were negatively correlated with B-cells and CD11b/c+ cells ($0.01<p<0.05$). The associations between the immune cells and the cytokines also revealed some specific patterns (Figure 3.7 C). Glycaemia showed a strong positive association with CD161a+ cells and T_c17 cells; and negative correlation with B cells, CD11bc+ cells, cytotoxic T-cells and T_h cells. Interestingly, MIP-1α showed the exact opposite trend to glycaemia in terms of association. CD161a+ was negatively correlated with numerous cytokines. Thus, it is clear that the secretion pattern of most cytokines in our panel is dependent on the immune cell frequencies. Overall, the low levels of cytokines are consistent with the lower percentages of CD11b/c+ monocytes and macrophages, T_c cells and B-cells.

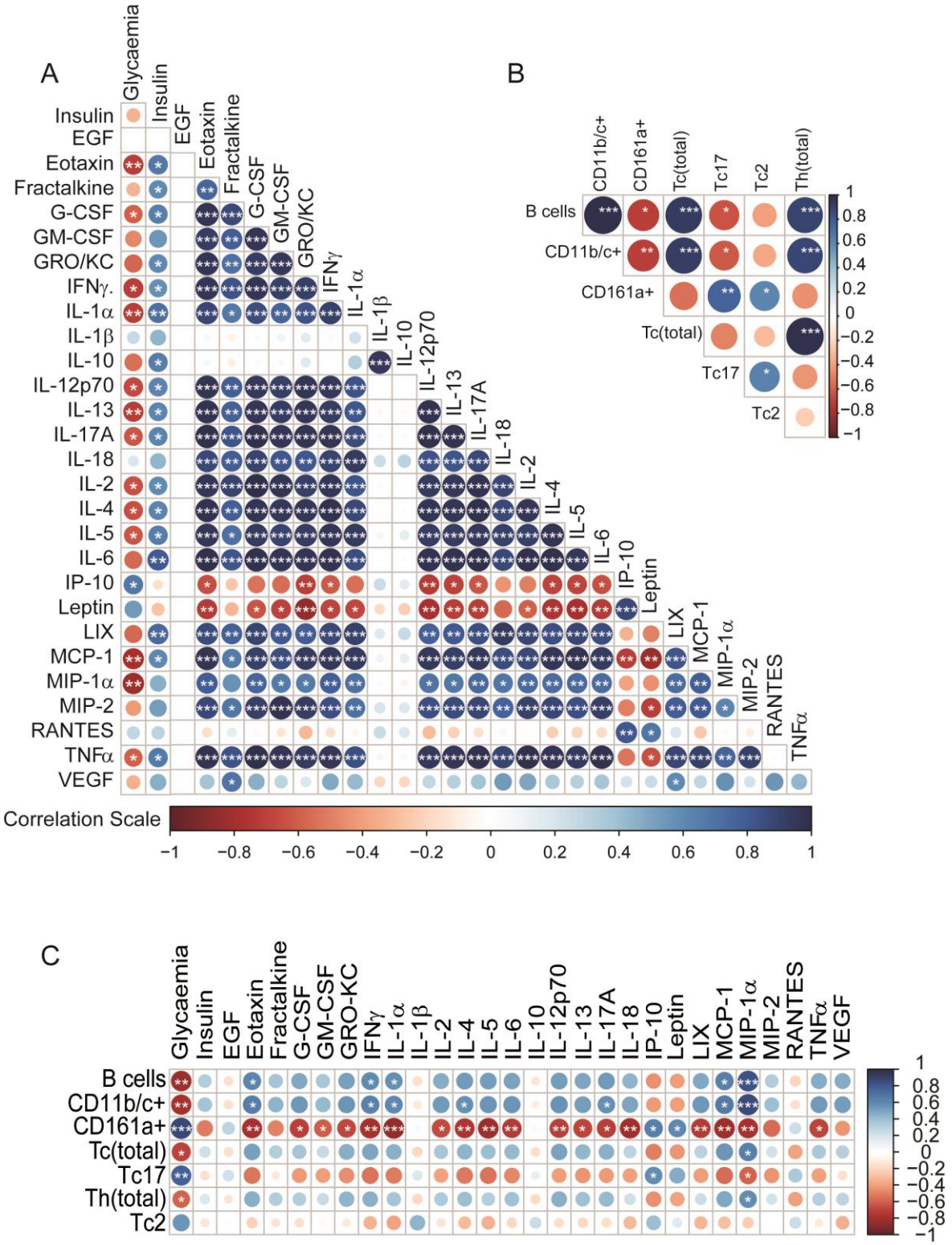


Figure 3.7: Integrative correlation analysis of the different immune parameters: A) Cytokine-cytokine B) Immune cell-immune cell C) Cytokine-immune cells. The sizes of the circles indicate the strength of association (Pearson's r^2), while the colours show negative (red) or positive (blue) correlation. p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively.

3.4.3 *GK rats show a distinct transcriptomic profile in key diabetic tissues, which also affects immune and diabetic biological pathways*

We re-analysed publicly available GK rat datasets and we observed 884, 665, and 1819 differentially expressed genes (DEGs; BH-adjusted $p<0.05$; log2 fold-change>2) in liver, muscle and adipose tissues respectively. Biological pathways were extracted from DEG profiles. Overall, we found 137 biological pathways common to all 3 tissues (Figure 3.8 A). Of these, 14 were specific to downstream signalling from cytokines, and 6 were common diabetes-related pathways as shown in Figure 3.8 B and 3.8 C for muscles and Figure 3.9 for liver and adipose tissue. Cytokine dependent pathways such as T-cell receptor signalling, Jak-STAT signalling, TGF-β signalling, and PDL1 signalling (Figures 3.8 D-G) were all significantly increased in Wistar compared to GK rats, consistent with the reduced circulating cytokine levels and numbers of cytokine secreting cells.

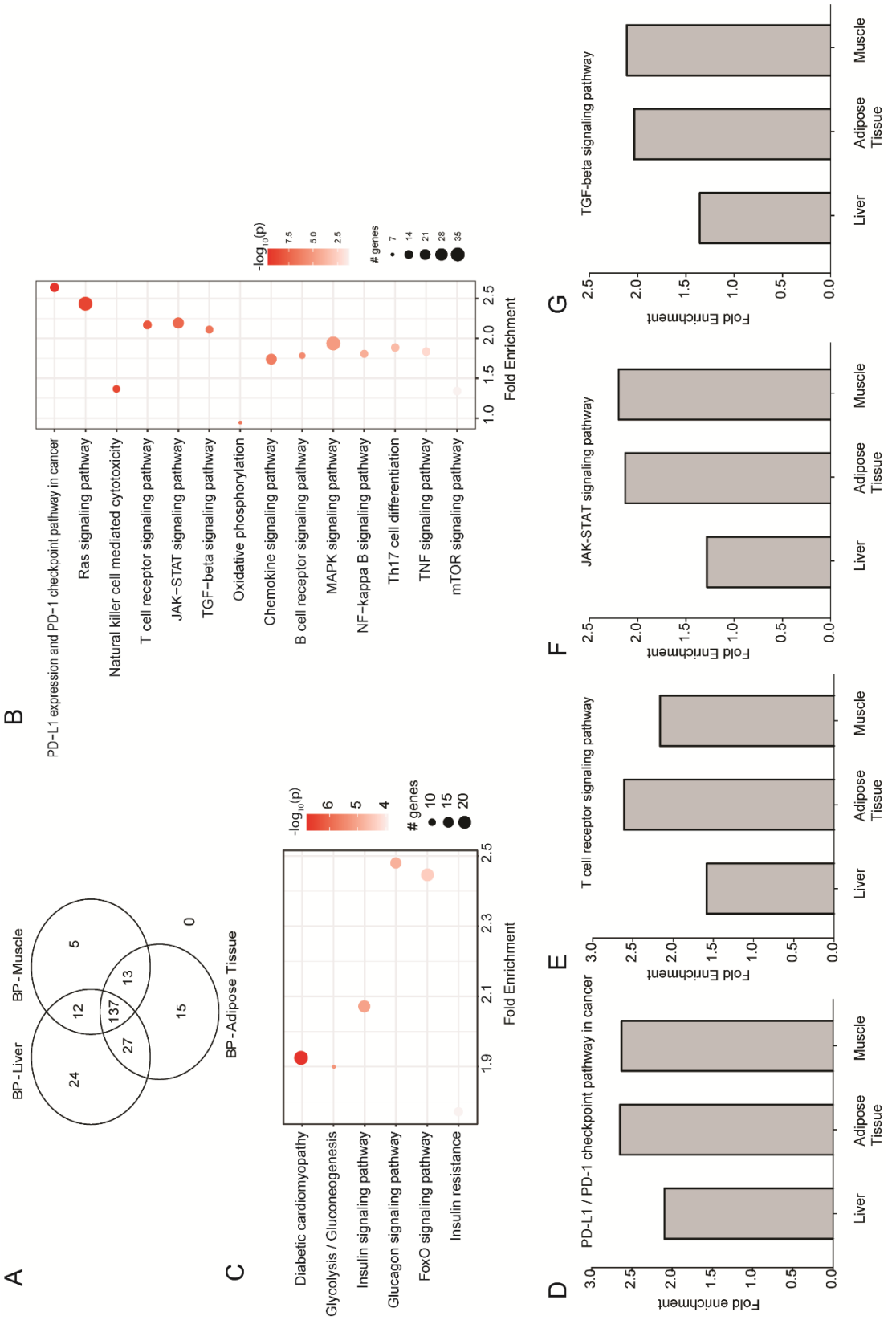


Figure 3.8: Transcriptomic re-analysis of 3 principal diabetic tissues (liver, muscle and adipose tissue) in Wistar rats compared to GK rats. A) Venn diagram showing the number of common biological pathways B) Biological pathways that are regulated by downstream signalling from

circulating cytokines analysed in the muscle. C) Biological pathways associated with diabetes and its complications analysed in the muscle. For B) and C), the intensity of the colour is proportional to the -$\log_{10}$ of P-value, sizes of the circles represent the number of genes involved, while placement of the circles on the x axis indicates the fold enrichment. D), E), F) and G) are T-cell receptor signalling, Jak-STAT signalling, TGF-β signalling and PDL1 signalling respectively in liver, muscle and adipose tissue. Data from GSE13271 [430].

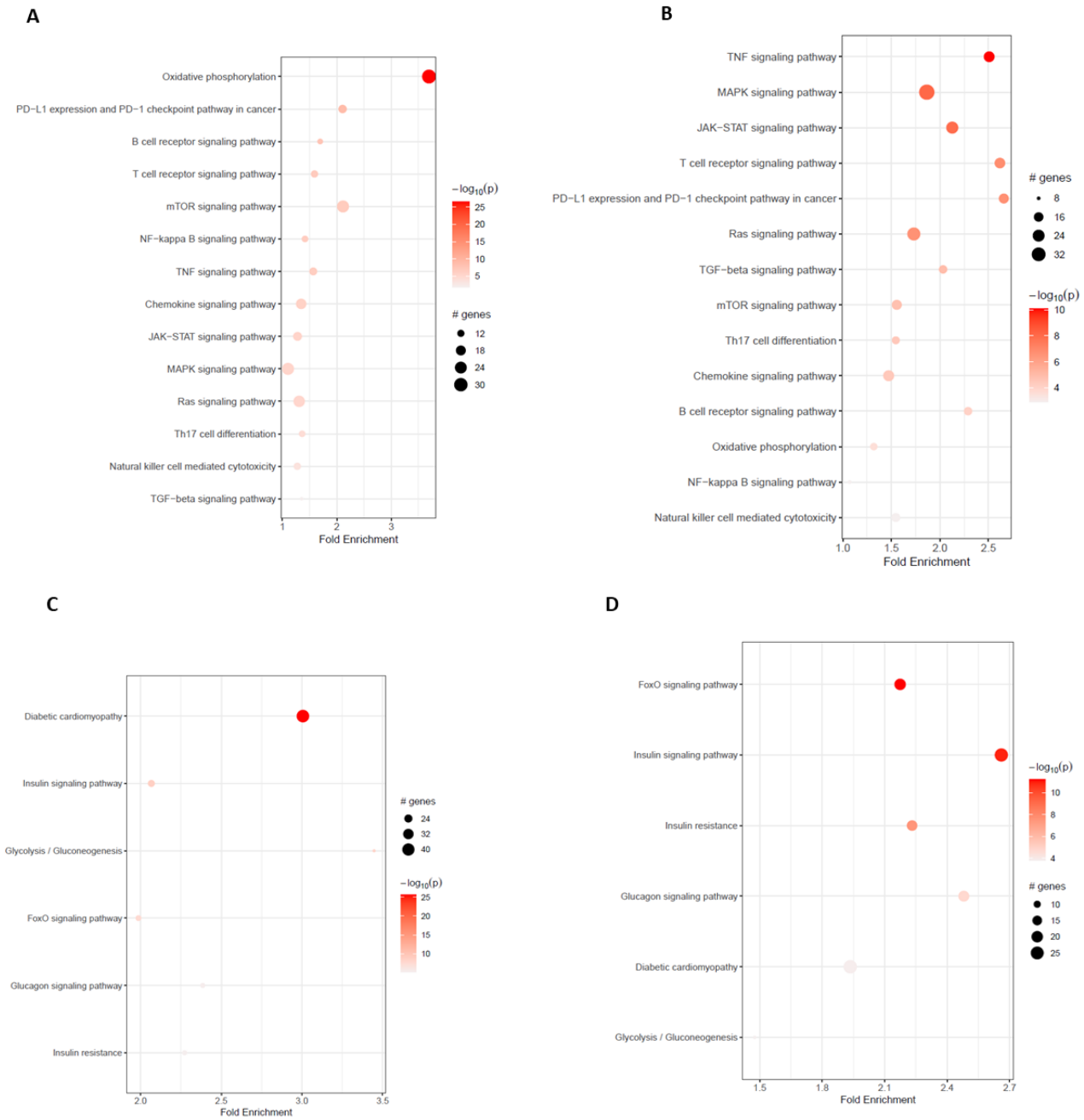


Figure 3.9: Transcriptomic analysis in Wistar rats compared to GK rats. Panels A) and B) show pathways regulated by downstream signalling from circulating cytokines analysed in the liver and adipose tissue respectively. Panels C) and D) show pathways associated with diabetes and its complications analysed in the liver and adipose tissue respectively. Data from GSE13271.

3.5 Discussion

T2D has been commonly associated with low grade inflammation, but it is not clear whether this inflammatory profile is related to diabetes or to obesity. The aim of the present study was to examine peripheral inflammatory status in an animal model of T2D without obesity. Surprisingly, our results suggest that GK rats do not show a low-grade inflammatory profile as reported in obese models, but instead exhibit a marked decrease of the examined immune markers. Beta cell dysfunction in the GK rats is the primary defect that leads to overt hyperglycaemia and marked islet inflammation [354, 428, 429]. Although, systemic inflammation is often associated with IR, it is not the sole contributor to the development of IR. Ycaza et. al reported that adipose insulin resistance did not correlate with markers of subcutaneous adipose inflammation [449]. GK rats develop hepatic insulin resistance at a young age [450], while whole-body insulin resistance (which is not associated with liver, adipose or muscle inflammation) appears later in life [429]. Pitasi et. al recently showed increased levels of GSK3B protein in the GK pancreas [429], which has been reported to be elevated in insulin target tissues in humans with T2D, and [451] is correlated with decreased insulin sensitivity [452]. Thus, we postulate that a possible over expression of GSK3 in insulin target tissues in the GK rat, could participate to the development of insulin resistance in this model.

Here, we provide a detailed description of the GK rat immune system, which shows limited peripheral inflammation, in addition to a lower percentage of phagocytes (macrophages, monocytes, dendritic cells, granulocytes and B-cells). This partially confirms impaired phagocytosis and antigen presentation as previously described in GK rats [422, 425, 426], however Zhai et. al reported a higher percentage of activated B cells in obese diabetics, which does not fit our observation [453], suggesting a major role of obesity in the inflammatory profile. The observed low percentage of T_h cells in the GK rats may also have an effect on the activation status of B- and cytotoxic T-cells, and may further explain their lower numbers in GK rats. Diabetes has also been known to disrupt monocyte recruitment to sites of injury, thereby impairing phagocytosis and impeding the switch from pro- to an anti-inflammatory state [454]. Interestingly, wound healing in diabetics is also known to be hindered as diabetes prompts neutrophils to cause tissue damage by NETosis [455], which further contributes to the inflammation causing a vicious pathological cycle [456].

We have seen a decrease in CD8+ T_c cells in our GK rats, which are normally responsible for eliminating viral-infected host cells and thus is also in line with the predisposition of T2D patients towards severe infections [457]. This agrees with the reported decrease in naive T_c cells, and accumulation of EMRA immunosenescent cells in man [233].

A higher CD4/CD8 ratio conventionally corresponds to a healthier immune system and estimates the likelihood of development of infections. In rats, this ratio is strain specific [458]. GK rats show a significantly higher CD4/CD8 ratio (Figure 3.10), which can be attributed to the low number of T_c cells and may not necessarily represent a healthier immune system compared to Wistar rats. Interestingly, a recent study also reported a higher CD4/CD8 ratio after a glucose bolus in both diabetics and non-diabetics [309], indicating a lymphocyte redistribution, which may be the case for our GK rats as well.

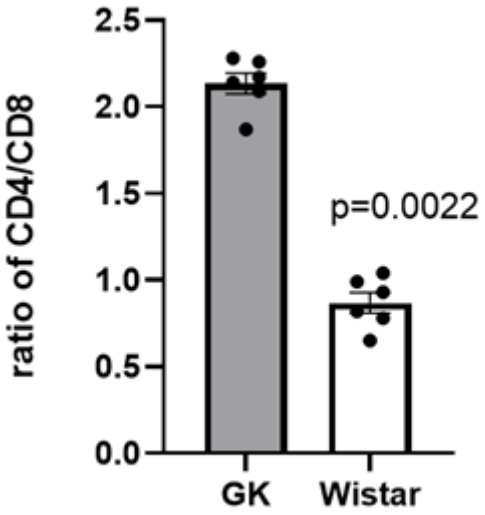


Figure 3.10: Ratio of CD4/CD8 cells in GK and Wistar rats shown as means along with the SEM. The colours of the bars represent the different strains (GK rats = grey, Wistar rats = white).

We also examined T_h1/T_h2, T_c1/T_c2 and T_h17/Treg ratio, which have been reported/reviewed to be impaired in T2D with comorbid obesity [459], inflammatory diseases such as Behcet's disease [460] and metabolic disorders [461] respectively. We did not see significant differences for any of these ratios in our GK rats ($p>0.05$, Mann-Whiney test, data not shown).

We interpret the overexpression of CD161a in GK rats as most likely to be an already activated defence system as a compensatory mechanism for their compromised immune system to combat an invading or existing pathogen/infection. It is logical to conclude that this is achieved by the

activation of monocytes and NK cells to target infected cells and by secretion of cytokines to further activate these cells or this can be indicative of healthy cell/tissue destruction. Altered immune functionality in both human B- and NK-cells has been reported to be mediated by global hypermethylation in obese (BMI>30) T2D patients [462]. The data on human NK cells is somewhat unclear. Meta-analysis of 13 NK cell studies identified 3 with decreased levels/activity, 2 reported elevated levels and 8 of them reported no differences between circulating NK cells in T2D patients and healthy controls [463].

RORγT+CD8+ lymphocytes are a somewhat underexplored population, but they are very pro-inflammatory [464]. GK rats show pancreatic islet inflammation [354] and in our data, peripheral inflammation appears to be limited to the observed increase in T_c17 cell population. T_c17 cells are produced under inflammatory conditions in a manner similar to their CD4+ counterparts, T_h17 cells [260], skewing the immune system towards a pro-inflammatory phenotype, although, as outlined above IL-17 levels are lower in GK rats. Interestingly, CD8+T_c17 cells have been reported to show a strong repression of *SOCS3* expression [465], a pivotal gene regulating insulin sensitivity [466] in addition to driving inflammation (as reviewed [467]). T_c17 cells have been shown to degrade pancreatic beta islet cells and induce hyperglycaemia in mice [467]. Thus, it is quite likely that this cell population contributes to the vicious diabetic and (peripheral) inflammatory aetiopathology of the GK rats.

Similar to the T_c17 cells, T_c2 cells have not been highly investigated in the light of diabetes but have been reported to be more diabetogenic compared to naive T_c cells [468]. However, in T2D with comorbid tuberculosis, there appear to be a clear role for both T_c17 and T_c2 cells [469]. Although, there is a preponderance of literature on immune changes in complicated diabetes and unfortunately, the immune status in uncomplicated human T2D is still ambiguous.

Circulating baseline cytokine levels have been studied in T2D for many decades [470]. In this study, we present the first account of multiplexing a panel of 27 cytokines simultaneously from the same samples to get a clearer, detailed overall picture of the GK immune system (Figure 3.4). The reduced levels of the cytokines in GK rats is most likely due to low number of circulating CD11b/c+ cells, principally monocytes/macrophages in addition to low percentages of CD45RA+ (B-cells) and CD8+ (T_c cells) cell populations. Interestingly, despite an overall decrease of both anti- and pro-inflammatory cytokines plasma levels, our results reveal a significant increase of interferon gamma-induced protein 10 (IP-10), a pro-inflammatory chemokine which plays an important role in the aetiology of inflammatory diseases. Previous studies suggest that IP-10 is associated with metabolic disorders. Accordingly, IP-10 levels were increased in the early stage of type 1 diabetes

[471] and were also predictive of insulin resistance and diabetes in patients suffering from nonalcoholic fatty liver disease [472]. Previous studies suggest a positive association between leptin levels and IP-10 in type 2 diabetic patients [473], which also fits our GK rat data (Supplementary Figure 5AA, data not shown in this thesis).

We also saw an increase in leptin levels in our GK rats. However, this does not necessarily indicate an increased secretion of proinflammatory cytokines from GK adipocytes due to tissue specific gene expression. Previously, we have shown that the expression of cytokines such as IL-6, IL-1β and TNF-α were increased in the GK islets, but not in the adipose tissues [429]. Thus, while we observe elevated levels of adipokines associated with the increased adiposity in GK rats, we do not see an increased pattern secreted proinflammatory cytokines.

Thus, it is clear from our study that the GK cytokine profile alone can be quite misleading, unless amalgamated with cognate immune date. However, there is no clear pro-inflammatory cytokine profile as previously thought. Our data suggests that the inflammatory milieu in GK rats is most likely due to T_c17 cells and activated monocytes, NK cells as represented by elevated CD161a+ cells. In the human context, T2D patients have been shown to have higher IL-17 levels compared to healthy controls, which contribute to the pro-inflammatory phenotype commonly observed in T2D [474, 475], thereby justifying our hypothesis.

This disproof of the proinflammatory profile in GK rats led us to investigate the gene expression pattern in liver, muscle and adipose tissues: all key tissues that not only play important roles in glucose homeostasis affecting glycaemic status but are also exposed to the circulating immune cells and cytokines. Therefore, we re-analysed previously published GK rat micro-array expression data for these three key diabetes related tissues. The extracted biological pathways from the gene expression profiles confirmed a downregulated pattern of most involved genes in the GK rats. The most prominent gene pathway was diabetic cardiomyopathy, fitting previous reports of ventricular hypertrophy, impaired diastolic function, and cardiomyopathy in GK rats [476, 477] that may be mediated by CRP [478]. This peripheral gene expression profile contrasts sharply with the inflammatory profile of the GK rat pancreatic islets, where similar to diabetic human islets [355], we consistently show increased pro-inflammatory cytokine levels, associated with increased pro-inflammatory immune cell numbers around and within the pancreatic islets [354, 428, 429]. This profound islet inflammation is a contributor to the impairment of beta cell growth and function, which constitutes the main characteristics of T2D in this model. GK rats serve as an excellent model to study the development of T2D, and the transition from prediabetic to a fully diabetic phenotype may yield insight into the development of T2D.

In conclusion, our study reports 3 lines of evidence proving that, outside the pancreatic islets, GK rats present a limited pro-inflammatory profile unlike previously thought. Additionally, non-obese GK rats exhibit a marked immune dysfunction as indicated by the overall blunted levels of cytokines and changes in the distribution of immune cells. Thus, it is also clear that there is a knowledge gap between uncomplicated human non-obese T2D, and the immune system that needs to be addressed in detail and that GK rats can serve as a good model for this.

Chapter 4: Prediabetes to diabetes: Immune profiling at the junction of stress and metabolism in Goto-Kakizaki rats

My contribution to this chapter: Conceptualisation; Literature review; Data collection and curation; experimentation and analysis; Interpretation of results and Writing of the article.

4.1 Abstract

Type- 2 diabetes is a complex disorder characterised by uncontrolled hyperglycaemia, and compromised insulin secretion and signalling and has been known to cause drastic immune changes often leading to low grade inflammation. However, T2D in the human clinical context is often, but not always accompanied by other complicated inflammatory comorbidities such as obesity. Most T2D (animal and human) immune studies do not dissect the exact deleterious impacts of T2D and obesity separately, thereby prompting the need for uncomplicated T2D models. In this longitudinal study, we used Goto-Kakizaki (GK) rats, a non-obese T2D model showing a prediabetic phase, to study immune changes occurring as the animals matured and when subjected to stress and glucotoxicity.

In addition to showing significant differences in weight and glycaemia, GK rats already showed significant immune differences in 13 out of the 21 immune cell populations investigated (including total T cells, T_h and T_c cells) in the early prediabetic phase. The global immune changes observed upon maturation, calculated using AUC_g measurements showed differences in circulating immune cells for almost 50% of the examined populations. Although stress has been known to induce immune cell trafficking in non-diabetic animals, little was known about what happens in uncomplicated type 2 diabetes. Using a simple restraint stress we showed that most of the cell populations showing stress induced differences included principle cytokine secreting cells such as T_c, B, monocytes, and dendritic cells, indicating a defective stress response. Moreover, we observed baseline differences in levels of IFN-γ which could be a rudimentary indication of the emergence of T2D induced inflammation. Upon stress, we saw differences in KC/GRO levels which likely prompted the stress induced immune trafficking. This is the first study to also identify cell mediated and humoral immune cell subsets that showed sensitivity and cell trafficking in response to glucotoxicity in the pre- and early diabetic phase. The role of IFN-γ and/or stress induced changes in KC/GRO levels may predict the transition of prediabetes to T2D at the immunological level, potentially refining clinical monitoring or diagnosis.

4.2 Introduction

Type- 2 diabetes (T2D) is a complex disorder characterised by uncontrolled hyperglycaemia, and compromised insulin secretion and signalling. T2D has been known to cause drastic immune changes (reviewed in [418]) and is often shown to produce low grade inflammation [479] leading to its recent classification as an inflammatory disorder [480]. Inflammation is the normal physiological reaction to an invading pathogen, injury or infection, which necessitates the shuttling of white blood cells from the blood stream to the location of the damaged/injured tissue (reviewed in [481]). This "lymphocyte trafficking" due to inflammation is orchestrated by mediating proteins such as cytokines, chemokines and adhesion molecules [482]. Therefore it is plausible that the proinflammatory component of T2D can also impact lymphocyte trafficking.

Numerous studies have reported T2D associated immune cell trafficking for both innate (monocytes/macrophages, dendritic cells (DCs), neutrophils) and adaptive (B cells, T-helper (T_h), T-cytotoxic (T_c), T-regulatory (Tregs) cells) immune cells (reviewed in [481]). Liu et. al recently described trafficking of immature monocytes in high fat diet (HFD) induced diabetic mice from the periphery to visceral adipose tissues, where they mature into macrophages [483]. Interestingly, adipose tissues of mice fed with HFD for 3 weeks showed higher expression of ICAM-1 (Intercellular adhesion molecule 1), a protein responsible for promoting migration of macrophages, consistent with the increase in macrophage infiltration [484].

Musilli et. al reported elevated numbers of conventional DCs in circulation in obese, diabetic women after menopause, compared to healthy controls [485]. Another study reported a diminished percentage of total DCs and an increase in plasmacytoid DCs in adipose tissues of T2D patients [486]. Chen et. al showed infiltration of CD11c+ conventional DCs in the adipose tissues of mice fed with HFD, which prompted naive T_h cells to differentiate into proinflammatory T_h17 cells [487], thereby setting off a vicious cycle of inflammation induced leukocyte trafficking. Two independent studies also reported neutrophil polarisation in adipose tissues of HFD fed mice after 3 days [488, 489]. Talukdar et. al reported that this neutrophil trafficking continued up to 3 months on HFD, and was correlated with an increased expression of elastase, which concomitantly increased proinflammatory processes [489], further supporting inflammation induced leucocyte trafficking.

Infiltration of B cells in adipose tissues of mice have been shown to peak around 21-28 days of HFD [490]. Data from several other studies indicate that during inflammation, B cells control migration of macrophages into adipose tissues, which results in insulin resistance (reviewed in

[481]). B cell infiltration is followed by T cell and monocyte trafficking [490] which further potentially exacerbates insulin resistance. Nishimura et. al have reported higher percentages of effector T_c cells and a decrease in T_h cells, especially Tregs in visceral adipose tissues of mice fed with HFD [304].

Interestingly, adipose tissues of (*RAG2*$^{-/-}$) knockout mouse models showed significant increase in NK cell and macrophage infiltration compared to controls, which has been deduced as a compensatory and exaggerated reaction of the adaptive immune system [490]. Feuerer et. al have shown that HFD increases trafficking of T_h1 cells into adipose tissue depots [491].

Thus it is clear that T2D in conjunction with obesity results in leucocyte trafficking. However, T2D in the human clinical context is often, but not always accompanied by other complicated inflammatory comorbidities such as obesity [351, 352]. Most T2D (animal and human) immune studies do not dissect the exact deleterious impacts of T2D and obesity separately, thereby prompting the need for uncomplicated T2D models. Goto-Kakizaki (GK) rats are non-obese T2D models that distinctly show a prediabetic (4 weeks old GK/Par colony) and a diabetic phase characterised by elevated hepatic glucose secretion and peripheral insulin resistance as reported in 4 and 8 weeks old GK/Par colony rats respectively, thereby mimicking the human uncomplicated (polygenic) T2D aetiology [351, 371]. However, GK/MolTac colony male rats have been reported to develop diabetes approximately around 14-16 weeks, indicating a longer prediabetic phase (up to week 13) [492]. Interestingly, even in the prediabetic phase, these GK rats showed slightly higher blood glucose levels compared to control Wistar (W) rats. In the life cycle of laboratory models such as mice and rats, light (Zeitgeber) plays an important role in influencing circadian rhythms, which in turn have been known to regulate energy homeostasis and therefore also impact blood glucose levels [493]. Zeitgeber 0 (Zt0) and Zeitgeber 12 (Zt12) corresponds to the light and dark phase respectively. In this study, we have also examined blood glucose levels in GK rats during the light (Zt3.5) and dark phase (Zt12.5).

We clearly see a knowledge gap in the literature with regards to T2D induced leucocyte trafficking. In order to achieve this, we explored lymphocyte trafficking patterns in non-obese GK rats after glucose tolerance test (GTT) in the prediabetic and diabetic phase.

Acute stress has also been known to induce immune cell trafficking [494, 495], thus in order to assess stress induced leucocyte migrations in diabetes, we also looked at lymphocyte trafficking after a stress test in the adult GK rats.

Previously, we have also described the immune and inflammatory status of adult (6 months old) GK rats [496]. However, there is no literature linking these changes to the exact time points

during which they arise. To our knowledge, this is the first longitudinal immune study conducted to understand when these changes occur in GK rats as they transition from a prediabetic to a diabetic state. Additionally, we also tested inflammation (cytokines) status between GK and W rats in the basal condition and after a 1-hour restraint stress test.

4.3 Materials and Methods

4.3.1 Animals

Adult male and female GK rats (GK/MolTac) from Taconic Biosciences (Leverkusen, Germany) were obtained and bred inhouse to acquire male GK pups (n=9) used for the study at age 5 weeks. Additionally, four weeks old, male W rats (n=10) were purchased from Janvier labs (Genest-Saint-Isle, France), left undisturbed for an acclimatisation period of 1 week and used for the protocol. Animals of the same sex, strain and age were housed in groups of 2 in 48 × 37.5 × 21 cm clear plastic isolator cages (Tecniplast, Varese, Italy) in a 12 h/12 h light:dark cycle (lights off at 07:00 p.m.), relative humidity: 49-54%, average temperature: 21°C, with access to food and water ad libitum (except for the glucose tolerance test, where the animals were fasted for 6 hours). Enrichment was provided as plastic tunnels and sterilised shredded paper in the form of nesting material. Routine husbandry was performed and the weights of animals were recorded weekly. All experiments were conducted in concertation with the European Union directive 2010/63/EU and the Animal Welfare Structure of the Luxembourg Institute of Health (protocol #DII-2020-04).

Blood sampling: Blood sampling for flow cytometry was done for three main experiments (Figure 4.1), ie, non-glucose tolerance (non-GTT) weeks (weeks 5, 7, 8, 9, 11, 12, 13, 15) performed in the morning between 10:15 am and 10:45 am and in the evening between 7:15 pm and 7:45 pm., glucose tolerance test (GTT) weeks (weeks 10 and 14 corresponding to the late prediabetic and early diabetic phase), and a 1h restraint stress test at week 16 (pre-stress and post-stress conditions). Animals were bled into EDTA-coated tubes by nicking one of the lateral tail veins. An intravenous catheter was used for blood sampling during GTT. Pre- and post-stress samples from week 16 were centrifuged at 2000g, for 10 minutes and plasma was collected and stored at −80 °C for corticosterone or cytokine assays.

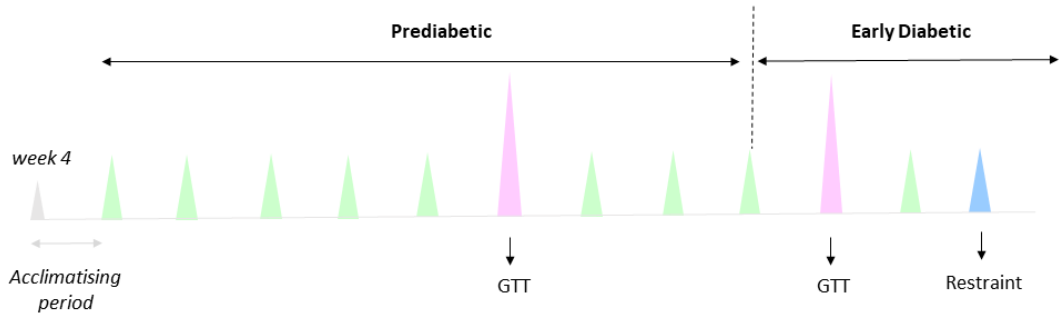


Figure 4.1: Overall experimental design. Each triangle represents a week in the rat's life cycle. Grey triangle represents arrival of the rats at week 4, followed by an acclimatisation period of 1 week. Green triangles represent timepoints (starting at week 5) for which immune, weight and blood glycaemia data was collected to assess changes as the rats matured. Pink triangles represent time points in the pre- and early diabetic phase (weeks 10 and 14 respectively) when rats were subjected to a metabolic challenge to assess lymphocyte trafficking. Blue triangle represents week 16 (early diabetic phase), when rats were subjected to a 1h-restraint stress to assess immune cell redistribution and cytokine profile.

Glucose Tolerance Test: At weeks 10 and 14, animals were fasted for 6 hours during the dark (active) phase. At the end of the fasting period, animals were orally administered a glucose bolus (1g/kg of body weight) and blood glucose was measured at time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively throughout the manuscript).

Restraint Test: All animals were subjected to a 1-hour restraint stress at week 16 during the light (inactive) phase between 9:00 am and noon. A dark coloured PVC tube (50mm diameter) with closed ends (adjustable from one end) was used as a restrainer to immobilize rats for the entire duration of stress test. Ventilation pattern of each animal was monitored throughout the process. Blood glucose was measured approximately after 3 minutes and 60 minutes of restraint.

4.3.2 Flow Cytometry

Fresh samples were stained extracellularly with antibodies against CD3, CD4, CD8, CD45RA, RT1B, CD11b/c ,CD161a, CD25 and intracellularly against RORγT, GATA3, Tbet and Foxp3 as reported previously [496]. Each experiment involved acquiring 500,000 events on an LSRFortessa (BD BioSciences, NJ, USA) using FACSDiva software (version 8.0.1, BD BioSciences, NJ, USA). Appendix 1 section 1 illustrates the gating strategy used to in the

study. Immune cell populations have been expressed as a percentage of parent population frequency**.**

4.3.3 Blood Glucose, Plasma Corticosterone and Cytokine measurement

Glucose levels were measured using an electronic glucometer (Accu-Chek, Roche) by placing a drop of blood on a fresh Accu-Chek glucose strip for all tests.

Total plasma corticosterone and 9 proinflammatory cytokines (IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13 and TNF-α) were measured from pre- and post-stress samples using ELISA (IBL International, Hamburg, Germany) and multiplexed proinflammatory panel 2 (rat) kit (Meso Scale Discovery, MD, USA) respectively, according to instructions from the manufacturer. Pre- and post-stress samples were diluted 10- and 20-fold respectively with assay diluent. For both ELISA, a 4-parameter curve was fit to the OD values of the standards, and the final concentration was deduced after accounting for dilution factor.

4.3.4 Data and Statistical analysis

Flow cytometry data analysis was done using FlowJo (version 10.6.1, BD BioSciences, NJ, USA) and GraphPad Prism (version 8.2, GraphPad Software. Inc, CA, USA).

Unpaired 2 sample unequal variance t-tests have been used to analyse the weight profile, Zeitgeber glucose profiles for non-GTT weeks, glucose profiles for GTT weeks, and both non-GTT and GTT immune profiles. Additionally AUC_g measurements were compared between W and GK rats for non GTT and GTT immune data (outliers higher/lower than means ± SD and animals with missing start/final value were removed from AUC_g calculation) and compared using unpaired 2 sample unequal variance t-test. For immune cell trafficking after stress, all populations were analysed using unpaired t-tests except CD161a+ CD8-, T_h2, T_h17, CD4+ Tregs and T_c17 (Mann-Whitney test). The stress-strain effects were analysed using a 2-way ANOVA. Glucose and corticosterone stress and delta changes were analysed using 2-way ANOVA and unpaired t-test respectively. Additionally, a regression modelling was constructed to explore associations between blood glucose-plasma corticosterone. Pre-stress cytokine levels were analysed using Mann-Whitney (IL-4, IL-6 and IL-10) or unpaired t-test (IFNγ, IL-1β, IL-5 and KC/GRO), while all stress induced cytokine changes were analysed using Mann-Whitney test. All data are shown as means ± SEM. Significance levels for all immune data were adjusted using the Bonferroni method.

4.4 Results

4.4.1 GK rats are significantly underweight and hyperglycaemic

We initially validated GK growth and glycaemia (Appendix 1, section 2) and, as expected, our GK rats were significantly underweight compared to W. Furthermore GK rats showed dysregulated glucose metabolism in comparison to W rats, as early as the prediabetic juvenile phase.

4.4.2 GK rats show overall significant immune differences during pre- and early diabetes

We followed the circulating immune cells as the GK rats transition from prediabetes to early diabetic phase using the gating strategy outlined in Appendix 1, section 1. Changes in all populations over time are shown in appendix 1 section 3, however, to identify global differences between GK and W rats we calculated the AUC_g for each of our 21 cell populations. Example curves are shown in Figure 4.2 A-C. These AUC_g measurements revealed significant differences between GK and W rats throughout the prediabetes-diabetes transition (Table 4.1).

CD3+ T-cells: Of the 14 CD3+ cell populations investigated, GK cell profiles were significantly and consistently higher than W rats for total CD3+ T cells (Table 4.1: $p < 0.01$) and CD3+ CD4+ T-helper (T_h) cells (Table 4.1: $p < 0.05$). A higher percentage of circulating CD3+ T cells has also been reported previously in 6 week old GK rats [425], likely contributed by the higher CD3+ CD4+ T-helper (T_h) cell numbers. Although not significant, CD3+ CD8+ T- cytotoxic (T_c) cell AUC_g (Table 4.1: $p > 0.05$) showed a decreasing trend in GK rats compared to control rats for all weeks.

CD3+ CD4+ cell subsets: Although the percentages of T_h cells were elevated in GK rats, the individual frequencies of CD4 cell subsets showed an opposite trend. CD4+ Tbet+ T-helper 1 cells (T_h1) showed a significant decrease in GK rats compared to control rats with advancement of age (and likely diabetic status), although the overall AUC_g was comparable between the 2 strains ($p > 0.05$). CD4+ GATA3+ T-helper 2 cells (T_h2) (Table 4.1: $p < 0.001$) showed a significant decrease in the diabetic rats, which disappeared for a short duration (weeks 11-12), before re-emerging towards the beginning of the late prediabetic phase (week 13). Overall CD4+ RORγT+ T-helper 17 cells (T_h17) AUC_g was comparable for the 2 strains ($p > 0.05$). Compared to other CD4 cell subsets, CD4+ CD25+ Foxp3+ regulatory T cells (Tregs) showed a statistically prominent decline in GK rats for all weeks (Table 4.1: $p < 0.001$). Additionally, we investigated an unidentified population of CD4+

CD25- Foxp3+ cells which showed a complex profile throughout the experiment, but the overall AUC_g was comparable between W and GK rats. In conclusion, we can hypothesise that the overall net increase in GK T_h cell numbers could be a result of increase in other similar unidentified populations.

CD3+ CD8+ cell subsets: Overall AUC_g measurements for CD8 cell subsets investigated i.e, CD8+ Tbet+ T-cytotoxic 1 cells (T_c1), CD8+ GATA3+ T-cytotoxic 2 cells (T_c2), CD8+ RORγT+ T-cytotoxic 17 cells (T_c17) and CD8+ CD25+ Foxp3+ regulatory T cells were comparable between the 2 rat strains (Table 4.1: $p > 0.05$).

CD3+ CD4+ CD8+ T cells: Overall AUC_g measurements for double positive (CD4+ CD8+) T cells showed a significantly lower GK profile compared to W rats (Table 4.1: $p < 0.001$). Furthermore, we saw a similar trend in GK rats for an unidentified population of CD4+ CD8+ CD25+ Foxp3+ (Table 4.1: $p < 0.05$) cells.

CD3- non T-cells: AUC_g measurements for CD3- non T-cells comprising of CD11b/c+ phagocytes, B cells and NK cells (described below), showed a general decrease in GK rats (Table 4.1: $p < 0.01$).

B cells: We have previously reported a significant decrease in CD45RA+ B cells in 6 months old GK rats [496]. In this study, we observe a similar overall AUC_g GK trend in CD45RA+ B cells (Table 4.1: $p < 0.05$). Furthermore, AUC_g trend for CD45RA+ RT1B+ peripheral B cells (Table 4.1: $p < 0.01$) in GK rats was also observed to be significantly lower than W rats.

CD11b/c+ cells: Overall AUC_g measurements for circulating phagocytes including monocytes were comparable between the 2 rat strains (Table 4.1: $p > 0.05$).

NK-Cells: Overall CD3- CD161a+ CD8+ NK cell percentages in GK rats were similar to that of W rats (Table 4.1: $p > 0.05$). However, CD3- CD161a+ CD8- cells indicative of activated monocytes and dendritic cells showed a marked decrease in GFTABLEK rats for all weeks (Table 4.1: $p < 0.01$).

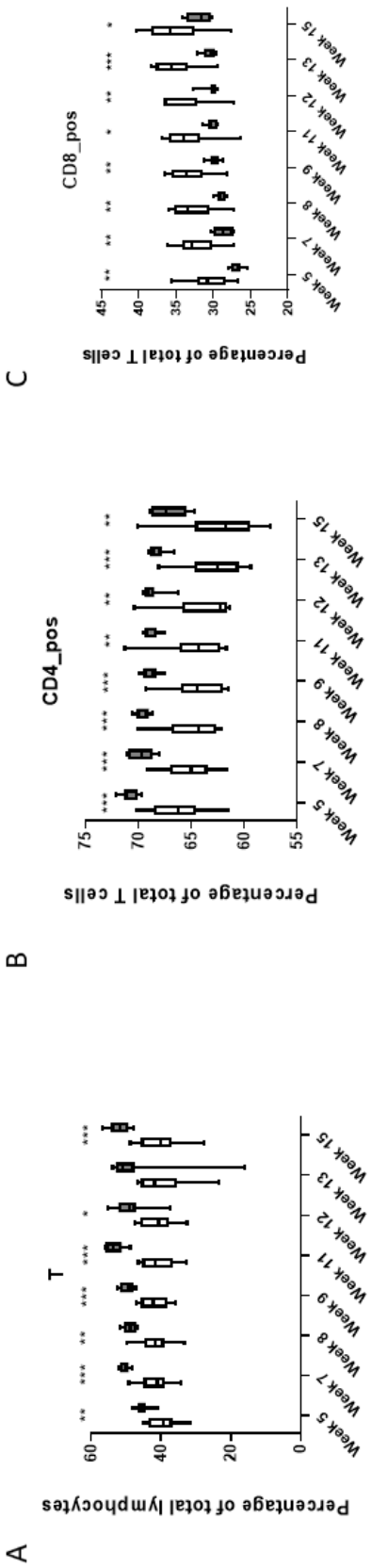


Figure 4.2: Example curves for immune changes in GK and Wistar rats as they mature. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; $p<0.001$, $p<0.01$ and $p<0.05$ are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data.

Table 4.1: AUC_g calculations to show global immune changes in GK and Wistar rats as they mature

Cell population	Wistar AUC_g mean (SEM)	GK AUC_g mean (SEM)	Bonferroni corrected p-value	Overall trend description	Fig.
CD3+ T cells	329.2 (11.1)	396.2 (3.5)	4.6×10^{-03}	W profile consistently stable; GK profile complex; overall GK profile consistently higher than W for all weeks and diverging over time, significant for all weeks except 13	4.2A
T_h cells	517.6 (6.9)	555.2 (1.2)	1.2×10^{-02}	GK + W profiles show slow drop until week 15; GK profile consistently and significantly higher than W for all weeks	4.2B
T_h1	5.1 (0.6)	2.6 (0.4)	-	GK profile significantly decreases after week 9 and strains start to diverge	S4.3A
T_h2	5.4 (0.3)	1.5 (0.2)	1.2×10^{-06}	Both GK + W profiles complex; overall GK profile consistently lower than W; intra-strain differences significant for all weeks except 11 and 12	S4.3B
T_h17	5.2 (0.4)	6.0 (0.9)	-	Both GK + W profiles complex; intra-strain differences significant only for weeks 11 and 15	S4.3C
CD4+Tregs	27.4 (1.2)	15.2 (0.8)	2.03×10^{-05}	Both GK + W profiles complex; GK profile consistently and significantly lower than W, diverging after week 9	S4.3D
CD25- FoxP3+	19.6 (0.9)	18.9 (1.1)	-	Both GK + W profiles complex; GK and W peak at week 7 and 13 respectively, strains start diverging week 13 onwards	S4.3E
T_C cells	263.9 (7.5)	234 (1.4)	-	Both GK + W profiles show linear increase until week 15; GK profile consistently and significantly lower than W for all weeks	4.2C
T_C1	24.2 (2.1)	22.4 (3.4)	-	Both GK + W profiles complex, although profiles diverge with GK decreasing and W increasing; GK and W peak at week 7 and 13 respectively	S4.3F
T_C2	1.6 (0.2)	0.9 (0.04)	-	Both GK + W profiles complex; GK profile lower than W for all weeks except 11 and 12; intra-strain significant differences only for weeks 7, 9 and 13	S4.3G
T_C17	6.2 (0.4)	4.0 (0.8)	-	Both GK + W profiles decrease sharply until week 7, with GK profile significantly lower than W; W profile significantly higher than GK profile at week 15; overall profiles complex, strains converge week 8 onwards	S4.3H
CD8+Tregs	4.4 (0.6)	2.4 (0.2)	-	Both GK + W profiles show linear decrease until week 15; GK profile consistently and significantly lower than W for all weeks	S4.3I

CD4+CD8+	13.6 (0.7)	8.2 (0.2)	5.7×10^{-04}	Both GK + W profiles show linear decrease until week 15; GK profile consistently and significantly lower than W for all weeks	S4.3J
CD25+ FoxP3+	450.4 (11.6)	383.7 (2.9)	1.1×10^{-02}	W profile shows slow rise until week 11 followed by a slow drop until week 15; GK profile more complex with peak at week 12; overall GK profile consistently and significantly lower than W	S4.3K
CD3- non T cells	461.8 (8.5)	345.6 (62.3)	2.4×10^{-03}	Both GK + W profiles consistently stable; overall GK profile consistently lower than W for all weeks except 13, strains diverge week 9 onwards	S4.3L
CD45RA+ B cells	378.1 (10.1)	322.8 (7.4)	1.2×10^{-02}	Both GK + W profiles complex; GK profile drops after week 11; overall GK profile significantly lower than W for all weeks except 5, 7 and 11, strains diverge week 11 onwards	S4.3M
Peripheral B cells	780.6 (0.7)	785 (0.5)	5.2×10^{-03}	Both GK + W profiles complex	S4.3N
CD11b/c+	245.3 (7.2)	230.4 (6.3)	-	Both GK + W profiles complex; overall GK profile lower than W for most weeks	S4.3O
monocytes	229.2 (19.0)	230.2 (3.9)	-	Both GK + W profiles complex	S4.3P
NK cells	40.4 (3.0)	47.7 (2.1)	-	Both GK + W show an increasing trend; overall GK profile higher than W for all weeks	S4.3Q
CD161a+ CD8- cells	4.1 (0.4)	1.6 (0.04)	2.3×10^{-03}	Both GK + W profiles consistently stable week 8 onwards; overall GK profile consistently and significantly lower than W	S4.3R

4.4.3 Metabolic challenges during prediabetic and early diabetic phases induce immune cell changes

We followed the impact of a metabolic challenge (glucose tolerance test) on the circulating immune cells during the prediabetic (week 10) and early diabetic (week 14) phases in GK rats. Changes in all populations over the GTT timepoints for weeks 10 and 14 are shown in appendix 1, sections 4 and 5. The overall global differences, calculated as AUC_g measurements and trends for the prediabetic and early diabetic phases have been described in Tables 2 and 3 respectively. Example curves are shown in Figure 4.3A-C and 4.4A-C for weeks 10 and 14 respectively. We saw significant changes during at least one GTT experiment for the following cell types:

CD3+ CD4+ T_h1: AUC_g measurements for CD4+ Tbet+ T-helper 1 cells (T_h1) were significantly decreased in GK rats compared to W rats for week 10 (Table 4.2: $p < 0.05$). The overall prediabetic trend for both strains was comparable and almost stable throughout the experiment, although the GK profile was consistently lower compared to the control rats, with a significant difference at T30 (Appendix 1, section 4). However, the observed difference in AUC_g measurements for the 2 strains dissipated in week 14 (Table 4.3: $p > 0.05$), likely due to progression of the diabetic phenotype.

CD3+ CD4+Tregs: Global AUC_g measurements for CD4+ CD25+ Foxp3+ regulatory T cells (Tregs) were significantly reduced in GK rats in comparison with W rats for both week 10 (Table 4.2: $p < 0.05$) and week 14 (Table 4.3: $p < 0.05$). Interestingly, both strains showed a similar and consistent drop in cell percentages post glucose administration (until T180) in the pre- and early diabetic phases (Appendix 1, section 4, and 5). The overall GK profile was significantly lower than control rats for all time points for both GTT experiments except week 10: T10 ($p > 0.05$).

CD3+ CD4+ CD8+ T cells: T cells that were double positive for both CD4 and CD8, showed a drastic decline in AUC_g measurements for both weeks 10 (Table 4.2: $p < 0.05$) and 14 (Table 4.3: $p < 0.05$) in GK rats, compared to euglycaemic W rats. The overall GK profile for both GTT weeks was significantly and consistently lower than W rats (Appendix 1, section 4 and 5). In the prediabetic phase, both strains showed a slow and linear decrease until the end of the experiment (T180), while in the diabetic phase both profiles were much more stable over the first 30 minutes, followed by a slow drop in cell percentages.

CD3+ CD4+ CD8+ CD25+ Foxp3+ cells: AUC_g measurements also showed a notable decrease for an unidentified T cell population in GK rats in comparison to W rats in both weeks 10 (Table 4.2: $p < 0.01$) and 14 (Table 4.3: $p < 0.05$). The overall trend in GK rats was consistently and significantly

lower than control rats for all timepoints for both GTT experiments (Appendix 1, section 4 and 5). At week 10, both strains showed a stable profile until T10, followed by a drop T30 onwards. At week 14, GK and W rats showed a plateaued profile up to 10 and 30 minutes post glucose administration respectively. This was followed by a decreasing profile at T30 and T60 for GK and W rats respectively.

B cells: GK rats showed an overall decrease of B cells compared to W rats, from T0 until the end of the GTT for both weeks 10 (Table 4.2: $p < 0.001$) and 14 (Table 4.3: $p < 0.05$). Interestingly, both GK and W profiles showed a similar and consistent drop until the end of the experiment, for each GTT week (Appendix 1, sections 4 and 5). The overall GK profile was lower than W rats for all timepoints for both weeks.

Peripheral B cells: AUC_g measurements for peripheral B cells were comparable between the 2 strains in week 10 (Table 4.2: $p > 0.05$), however in week 14, GK rats showed an overall decrease compared to W rats (Table 4.3: $p < 0.01$). Both strains exhibited a complex trend in the prediabetic phase (Appendix 1, section 5). The GK profile was stable until T10 with a slow drop T30 onwards, while the W profile showed a slow rise until T30, followed by a drop T60 onwards. The overall GK profile for week 10 was consistently higher than W rats for all timepoints, with intra-strain differences significant for all timepoints except T60. GK profile for week 14 was stable until T10 and dropped slowly T30 onwards, while the overall W profile remained stable for all timepoints except T60 (rise) as shown in Appendix 1, section 5. The GK profile was significantly lower than W rats, 30 and 180 minutes post glucose administration.

CD3- CD161a+ CD8- cells: Both weeks 10 (Table 4.2: $p < 0.05$) and 14 (Table 4.3: $p < 0.001$) showed a significant decrease in AUC_g measurements for GK rats in comparison to W controls. In the prediabetic phase, the GK profile rose and peaked at T10, followed by linear decrease until T180, while the W profile showed a slow rise until T30, followed by rapid decrease T60 onwards (Appendix 1, section 4). Overall GK profile was consistently and significantly lower than the controls. In the early diabetic phase, GK rats showed a slight increase at T10, followed by a decreasing trend, while the W profile remained almost stable (Appendix 1, section 5).Overall GK profile was significantly lower than W rats for all timepoints similar to week 10.

To sum up, CD4+ Tregs, CD4+ CD8+ T cells, CD3+ CD4+ CD8+ CD25+ Foxp3+ cells, CD45RA+ B cells and CD3- CD161a+ CD8- cells showed significant overall intra-strain differences for both GTT weeks, while T_h1 cells and peripheral B cells were different in weeks 10 and 14 respectively.

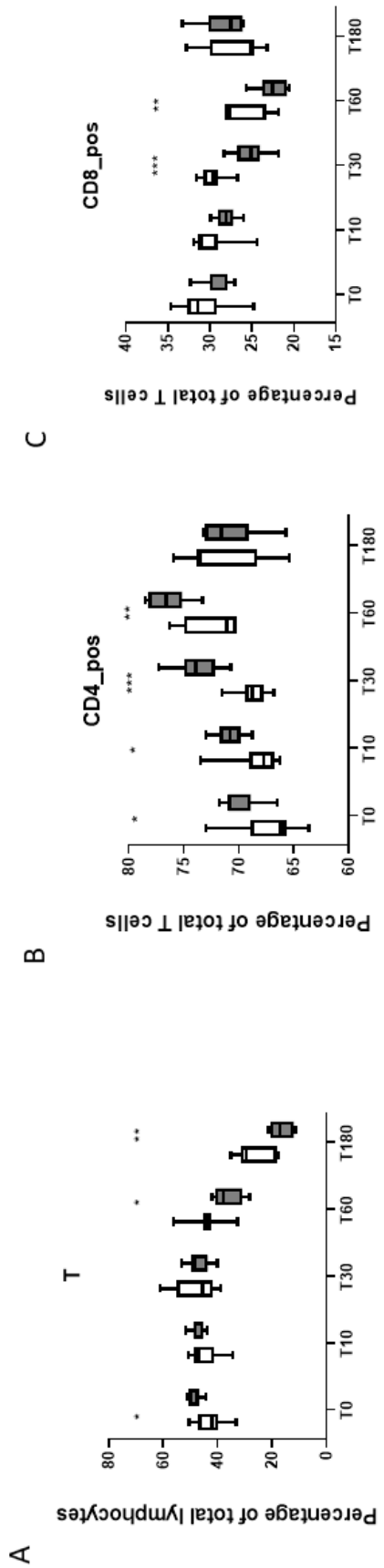


Figure 4.3: Example curves for immune changes in GK and Wistar rats after metabolic challenge in the prediabetic phase (week 10). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; $p<0.001$, $p<0.01$ and $p<0.05$ are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).

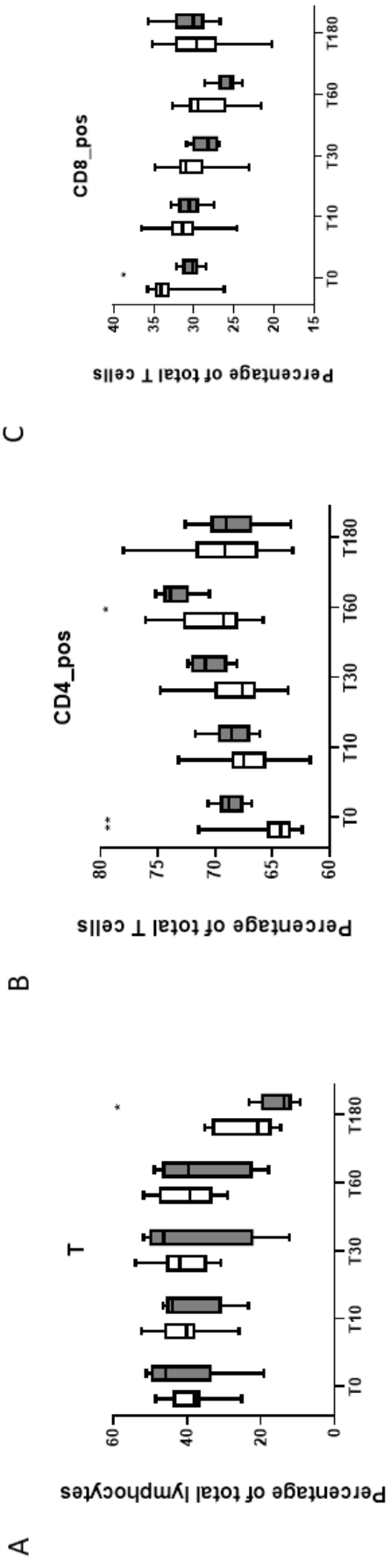


Figure 4.4: Example curves for immune changes in GK and Wistar rats after metabolic challenge in the early diabetic phase (week 14). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; $p<0.001$, $p<0.01$ and $p<0.05$ are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).

Table 4.2: AUC_g calculations to show global immune changes in GK and Wistar rats in the prediabetic phase (week 10)

Cell population	Wistar AUC_g mean (SEM)	GK AUC_g mean (SEM)	Bonferroni corrected p-value	Overall trend description	Fig.
CD3+ T cells	6813.4 (382.0)	5813.4 (242.8)	-	Profiles stable to T30; drop at T60 and 180; GK significantly faster drop, significant at T180	4.3A
T_h cells	12679.1 (162.5)	13314 (86.1)	-	GK profile significantly higher than W until T60; both rise and peak at T60; drop and equal at T180	4.3B
T_h1	94.4 (13.4)	29.0 (6)	2.9×10^{-02}	W levels stable; GK levels more heterogenous, but reducing over time, significantly lower at T30.	S4.4A
T_h2	75.2 (15.1)	19.6 (5.5)	-	GK + W profiles stable; GK profile consistently and significantly lower than W	S4.4B
T_h17	71.2 (5.4)	136.7 (26.4)	-	GK + W profiles stable throughout the experiment; overall GK profile higher than W, significant at T30.	S4.4C
CD4+Tregs	275.8 (27.5)	126.6 (20.7)	2.4×10^{-02}	Both GK + W profiles show a consistent drop until the end of the experiment; overall GK profile consistently and significantly lower than W; significant for all timepoints	S4.4D
CD25- FoxP3+	351 (38.4)	272.9 (44.4)	-	Both GK and Wistar profiles are comparable.	S4.4E
T_C cells	5054.6 (157.2)	4518.2 (82.6)	-	GK + W profiles drop until T60 and rise at T180; overall GK profile consistently lower than W up to T60; significant at T30 and T60	4.3C
T_C1	315.6 (69.2)	187.7 (51.5)	-	GK profile stable to T30 and slow drop T60 onwards; W profile stable to T60 and drop at T180. Overall GK + W profiles comparable	S4.4F
T_C2	17 (4.1)	17.3 (4.3)	-	GK + W profiles comparable and stable throughout the experiment	S4.4G
T_C17	51.9 (4.7)	136.9 (47.6)	-	GK + W profiles comparable and stable throughout the experiment; although not significant, overall GK profile consistently higher than W	S4.4H
CD8+Tregs	26.2 (3.8)	14.4 (3.6)	-	GK + W profiles stable to T10; drop T30 onwards;	S4.4I

				overall GK profile consistently lower than W, significant at T10 and T180	
CD4+CD8+	198.2 (15.8)	111.5 (4)	1.5×10^{-02}	GK + W profiles show slow and linear decrease until T180; overall GK profile consistently and significantly lower than W	S4.4J
CD25+ FoxP3+	7678.8 (277.8)	5400.4 (280.8)	1.9×10^{-03}	GK + W profiles stable to T10 and drop T30 onwards; overall GK profile consistently and significantly lower than W	S4.4K
CD3- non T cells	11178. 6 (382)	12183.6 (241.5)	-	GK + W profiles stable to T30; rise T60 onwards; significant intra-strain differences at all timepoints except T10 and T30	S4.4L
CD45RA+ B cells	4957.9 (277.7)	2028.1 (148.2)	4.3×10^{-05}	Both GK + W profiles show a consistent drop until the end of the experiment; overall GK profile consistently lower than W; significant for all timepoints	S4.4M
Peripheral B cells	17714.4 (20.9)	17778.8 (22.8)	-	GK profile stable to T10 and slow drop T30 onwards; W profile slow rise until T30 and drop T60 onwards; GK profile consistently higher than W for all timepoints; intra-strain differences significant for all timepoints except T60	S4.4N
CD11b/c+	9683.3 (396.6)	11100.4 (404.4)	-	Both GK + W profiles stable to T10, rise at T30 until the end of the experiment; overall GK profile higher than W, significant only at T180	S4.4O
monocytes	2227.9 (283.7)	1302.6 (127.1)	-	GK profile stable to T10 and drop T30 onwards; W profile shows linear drop until the end of the experiment; overall GK profile consistently lower than W, significant only for T0, T30 and T60	S4.4P
NK cells	1006.2 (75.0)	1233.3 (80.5)	-	GK profile rise and peak at T10, followed by linear decrease until T180; W profile shows slow drop until the end of the experiment; overall GK profile higher than Wistar rats for all timepoints except T180, intra-strain differences significant only for T10 and T30	S4.4Q
CD161a+ CD8- cells	72.3 (7.1)	34.6 (1.1)	3.5×10^{-02}	GK profile rise and peak at T10, followed by linear decrease until T180; W profile shows slow rise until T30, followed by rapid decrease T60 onwards; overall GK profile consistently and significantly lower than W	S4.4R

Table 4.3: AUC_g calculations to show global immune changes in GK and Wistar rats in the early diabetic phase (week 14)

Cell population	Wistar AUC_g mean (SEM)	GK AUC_g mean (SEM)	Bonferroni corrected p-value	Overall trend description	Fig.
CD3+ T cells	6280.4 (450.1)	5559.6 (608.8)	-	GK + W profiles stable to T30; drop at T60 and 180; GK significantly faster drop, significant at T180	4.4A
T_h cells	11836.9 (425.6)	12704.1 (117.7)	-	GK + W profiles rising up to T60, although GK profile significantly higher than W for T0 and T60, drop at T180	4.4B
T_h1	79.7 (13.0)	57.9 (28.8)	-	GK + W profiles comparable and stable throughout the experiment	S4.5A
T_h2	28.17 (6.3)	9.6 (1.6)	-	Overall GK profile lower than W; significantly lower for T0 and T60	S4.5B
T_h17	96.56 (5.5)	155.6 (22.3)	-	GK + W profiles comparable and stable to T60; GK significant rise at T180	S4.5C
CD4+Tregs	233.7 (29.8)	85.5 (11.0)	2.6×10^{-02}	Both GK + W profiles show a consistent drop until the end of the experiment; overall GK profile consistently lower than W; significant for all timepoints except T10	S4.5D
CD25- FoxP3+	311.8 (44.2)	270.1 (51.0)	-	Both GK + W profiles stable to T30 and drop at T60 and T180; inter-strain profile at each timepoint comparable.	S4.5E
T_C cells	5277.6 (227.0)	5142.1 (114.5)	-	GK + W profiles drop until T60 and rise at T180; overall GK profile consistently lower than W up to T60; significant at T0	4.4C

T_C1	608.8 (132.4)	319.8 (90.3)	-	Both GK + W profiles peak at T10; drop T30 onwards; although not significant, overall GK profile consistently lower than W	S4.5F
T_C2	9.4 (1.8)	12.5 (2.9)	-	GK + W profiles comparable and stable throughout the experiment	S4.5G
T_C17	34.0 (6.3)	53.2 (6.9)	-	GK + W profiles stable for all timepoints; GK profile consitently higher than Wistar profile although not significantly	S4.5H
CD8+Tregs	22.4 (3.8)	7.8 (1.1)	-	GK + W profiles stable to T30; drop at T60 and 180; GK significantly faster drop, significant at T30, T60 and T180; overall GK profile consistently lower than W profile	S4.5I
CD4+CD8+	166.3 (13.2)	94.1 (4.9)	1.4×10^{-02}	GK + W profiles stable to T30; slow drop at T60 and T180; GK profile consistently and significantly lower than W for all timepoints	S4.5J
CD25+ FoxP3+	8040.1 (656.0)	4803.1 (418.0)	3×10^{-02}	GK profile stable to T10 and drop T30 onwards; W profile stable to T30 and drop T60 onwards; GK cell profile significantly lower than W for all timepoints, strains converge T60 onwards	S4.5K
CD3- non T cells	11709.8 (450.5)	12430.4 (609.4)	-	GK + W profiles stable to T60; rise at T180; overall GK profile higher than W for all timepoints except T0; significant difference only at T180	S4.5L
CD45RA+ B cells	4253.4 (162.9)	1865.7 (319.5)	1.0×10^{-03}	Both GK + W profiles show a consistent drop until the end of the experiment; overall GK profile consistently lower than W; significant for all timepoints	S4.5M
Peripheral B cells	17789.1 (13.0)	17632.9 (26.5)	1.1×10^{-02}	GK profile stable to T10 and drops slowly T30 onwards; overall W profile stable for all timepoints except T60 (rise); GK profile significantly lower than W for T30 and T180, both strains diverge T30 onwards	S4.5N
CD11b/c+	9940.9 (274.2)	7609.1 (1096.4)	-	Both GK + W profiles show a consistent rise until the end of the experiment; overall GK profile consistently lower than W, significant at T0, T10 and T30; strains converge after T60	S4.5O

monocytes	1654.0 (232.8)	1357.6 (109.3)	-	Both GK + W profiles consistently drop until the end of the experiment; although not significant, GK profile always lower than W and become similar at T180	S4.5P
NK cells	1202.5 (89)	992.3 (121.3)	-	GK + W profiles stable to T60 and drop at T180, GK profile significantly lower than W at T180	S4.5Q
CD161a+ CD8- cells	66.4 (6.0)	22.4 (3.6)	1.1×10^{-03}	W profile stable but heterogenous at T30; GK profile rises at T10, followed by a decreasing trend; overall GK profile significantly lower than W for all timepoints	S4.5R

4.4.4 Stress impacts peripheral leucocyte trafficking

Given the dearth of literature on how stress impacts leukocyte trafficking in either diabetic patients or rodent models, we subjected both strains to a restraint stress at week 16. Unlike changes with age or over the metabolic challenge period we did not calculate the AUC_g, however the change from pre- stress to 1hour after stress. Cell populations significantly affected by the stressor are shown in Figure 4.5 and non-significant cell populations are included in appendix 1 section 6.

CD3+ T-cells: Pre-stress, CD3+ T cells were significantly higher in GK rats than W rats and became comparable in the 2 strains after stress, with a significantly larger change in cell numbers for W rats (Figure 4.5A, $p = 0.0002$). In a 2-way ANOVA (Appendix 1, section 7), there was a clear strain effect ($p = 0.0063$), although the stress effect was not significant ($p > 0.05$), with a highly significant strain*stress interaction ($p = 0.0092$). There was no difference between the two principal CD3+ subsets, CD3+CD4+ (Th cells) and CD3+CD8+ (Tc cells), in either the stress-induced change (Appendix 1, section 6, $p > 0.05$) or 2-way ANOVA (Appendix 1, section 7; CD3+CD4+: strain effect $p < 0.0001$; stress effect $p = 0.0035$; strain*stress interaction $p > 0.05$; CD3+CD8+: strain effect $p < 0.0001$; stress effect $p = 0.0065$; strain*stress interaction $p > 0.05$).

CD3+ CD4+ cell subsets: W rats showed a significant decrease in T_h1 cell numbers after stress in a 2-way ANOVA (Appendix 1, section 7) with a significant strain ($p < 0.0001$) and stress effect ($p = 0.0034$), although the strain*stress interaction ($p > 0.05$) and net stress induced change (Appendix 1, section 6: $p > 0.05$) was comparable between the 2 strains. Both T_h2 and T_h17 cells showed no stress-induced changes (Appendix 1, section 6, $p > 0.05$, Mann-Whitney test) or changes in the 2 way-ANOVA (Appendix 1, section 7: strain effect $p > 0.05$; stress effect $p > 0.05$; strain*stress interaction $p > 0.05$). W rats showed a significant decrease in CD4+ CD25+ Foxp3+ regulatory T cell (Tregs) numbers after stress in a 2-way ANOVA (Appendix 1, section 7) with a significant strain ($p < 0.0001$) and stress effect ($p = 0.0007$), although the strain*stress interaction and stress induced change (Appendix 1, section 6, Mann-Whitney test) was not significant ($p > 0.05$). Similar to T_h2 and T_h17, the unidentified population of CD4+ CD25- Foxp3+ cells showed no changes induced by stress (Appendix 1, section 6, $p > 0.05$) or a 2-way ANOVA (Appendix 1, section 7: strain effect $p > 0.05$; stress effect $p > 0.05$; strain*stress interaction $p > 0.05$).

CD3+ CD8+ cell subsets: None of the T_c cell subsets showed stress induced changes between the 2 strains (Appendix 1, section 6, $p > 0.05$, Mann-Whitney test for T_c17). CD8+ Tbet+ T-cytotoxic 1 cells (T_c1) were significantly decreased in the pre-stress condition for the GK rats and became comparable in the 2 strains after stress (Appendix 1, section 7: 2-way ANOVA), with a clear strain

effect ($p = 0.0008$), although the stress effect and strain*stress interaction were insignificant ($p > 0.05$). CD8+ GATA3+ T-cytotoxic 2 cells (T_c2), CD8+ RORγT+ T-cytotoxic 17 cells (T_c17) and CD8+ CD25+ Foxp3+ regulatory T cells showed similar stress effect ($p > 0.05$) and strain*stress interaction ($p > 0.05$) in a 2-way ANOVA (Appendix 1, section 7). However, the strain effect was significant for the T_c2 ($p = 0.04$) and CD8+ CD25+ Foxp3+ regulatory T cells ($p = 0.003$), while it was insignificant for T_c17 cells ($p > 0.05$).

CD3+ CD4+ CD8+ T cells: Double positive T cells showed no stress induced changes in cell numbers (Appendix 1, section 6, $p > 0.05$). Cell percentages in W rats was significantly higher than GK rats for both conditions in a 2-way ANOVA (Appendix 1, section 7), with a significant strain effect ($p < 0.0001$), while the stress effect and strain*stress interaction remained insignificant ($p > 0.05$). The unidentified CD4+ CD8+ CD25+ Foxp3+ population showed a significant overall stress-induced decrease in GK rats (Figure 4.5B, $p = 0.03$). A 2-way ANOVA (Appendix 1, section 7) showed significant decrease in cell numbers for GK rats post stress with a significant strain ($p = 0.0004$) and stress effect ($p = 0.02$), although the strain*stress interaction remained insignificant ($p > 0.05$).

CD3- non T-cells: GK rats showed a significant stress-induced increase in CD3- non T-cells compared to W rats (Figure 4.5C, $p = 0.0002$). In the pre-stress condition, cell numbers were significantly lower in the GK rats, which became comparable in the 2 strains after stress (Appendix 1, section 7: 2-way ANOVA), with a significant strain effect ($p = 0.0062$) and strain*stress interaction ($p = 0.0094$), although the stress effect was not significant ($p > 0.05$).

CD11b/c+ cells: There was no difference in either the stress-induced change (Appendix 1, section 6, $p > 0.05$) or 2-way ANOVA (Appendix 1, section 7: 2-way ANOVA; strain effect $p > 0.05$; stress effect $p > 0.05$; strain*stress interaction $p > 0.05$) for CD11b/c+ phagocytes. Monocytes were significantly lower in GK rats before stress, although they became comparable in the 2 strains post stress (Appendix 1, section 7: 2-way ANOVA), with a significant strain ($p = 0.0005$) and stress effect ($p = 0.0052$), although the strain*stress interaction and stress induced-changes (Appendix 1, section 6) were not significant ($p > 0.05$).

B cells: GK rats showed significant stress induced decrease in CD45RA+ B cells in comparison to W rats (Figure 4.5D, $p = 0.0021$). The 2-way ANOVA (Appendix 1, section 7) showed a significant decrease in cell numbers post stress in the GK rats, with significant strain effect ($p < 0.0001$), stress effect ($p < 0.0001$) and strain*stress interaction ($p = 0.0073$) Peripheral B cell numbers in the pre-stress condition was significantly higher in GK rats, although they became comparable post stress (Appendix 1, section 7: 2-way ANOVA), with a significant strain effect ($p = 0.0001$), while the stress

effect, strain*stress interaction and stress-induced changes (Appendix 1, section 6) remained insignificant ($p > 0.05$).

NK-Cells: There was no stress-induced changes in NK and CD3- CD161a+ CD8- cells in the 2 rat strains (Appendix 1, section 6: $p > 0.05$, Mann-Whitney test for CD3- CD161a+ CD8- cells). There was no change in the 2-way ANOVA (Appendix 1, section 7) for NK cells, although the strain effect was significant ($p = 0.0072$), while the stress effect and strain*stress interaction remained insignificant ($p > 0.05$). Despite CD3- CD161a+ CD8- cell numbers being significantly decreased in GK rats in the pre-stress condition, they become comparable in the 2 strains after stress (Appendix 1, section 7: 2-way ANOVA, with a significant strain effect ($p < 0.0001$), although the stress effect and strain*stress interaction remained unchanged ($p > 0.05$).

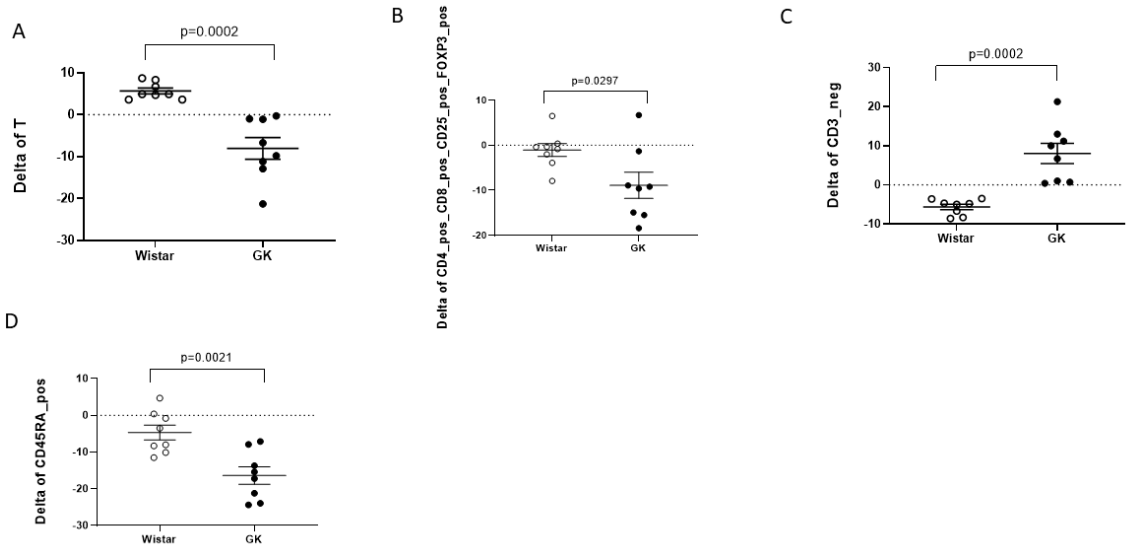


Figure 4.5: Stress-induced (significant) changes in immune cells in early diabetes (week 16). Data are presented as delta values (post stress values-pre-stress values for the percentage of parent population frequency). Data are mean +/- SEM; Each circle represents a single rat; Empty circles: Wistar rats, closed circles: GK rats; $p < 0.05$. Unpaired t-tests have been used to analyse the data.

4.4.5 Stress induces differences in blood glucose, corticosterone and proinflammatory cytokine profile

GK rats showed significantly higher pre-stress corticosterone levels compared to control rats (Figure 4.6A). Additionally, after a 1-hour restraint stress, both GK and W rats showed a significant stress

induced rise in plasma corticosterone (Figure 4.6A: $p < 0.0017$ for pre-stress corticosterone levels between the 2 strains, $p < 0.0001$ for W rats, $p < 0.0002$ for GK rats, 2-way ANOVA; strain effect $p = 0.0017$; stress effect $p < 0.0001$; strain*stress interaction $p = 0.04$). However, the overall stress induced corticosterone rise (value post stress - value pre-stress) in GK rats was comparable to W controls (Figure 4.6B: $p > 0.05$).

Similar to the corticosterone profile, GK rats showed significantly higher pre-stress glucose levels compared to the control rats (Figure 4.6C). Consistent with our previous findings [497], we also showed that a 1-hour restraint stress prompted a glucose rise in both strains (Figure 4.6C: $p < 0.0001$ for all groups except W post stress glucose levels, $p > 0.05$ for W post stress group, 2-way ANOVA; strain effect $p < 0.0007$; stress effect $p < 0.0001$; strain*stress interaction $p < 0.0001$), although this rise was insignificant in the control rats. Furthermore, the overall stress induced change in glucose (value post stress - value pre-stress) was also significantly higher in GK rats compared to the controls (Figure 4.6D: $p < 0.0001$). A simple linear regression modelling between blood glucose and plasma corticosterone showed no correlation, although the relationship between the 2 parameters was significant (Figure 4.6E: $R^2 = 0.31$, $p = 0.0006$). It is interesting to note that each group (except for the GK pre-stress data points), formed specific clusters highlighting that the 2 strains were significantly different in terms of their stress responses (Figure 4.6E).

In order to investigate the impact of physiological stress, we assessed a panel of 9 cytokines (detailed in Table 4.4) in the pre- and post-stress plasma from both strains. Here we exclusively report cytokines for which we had signals for both W and GK rats. GK rat signals for IL-13 and TNF-α were below detection limit.

Consistent with our previous findings, the GK rats exhibited significantly lower baseline levels of interferon gamma (IFNγ; Appendix 1, section 8, $p = 0.035$). However, pre-stress levels of interleukin-1 beta (IL-1β), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO) were comparable between GK and W rats in this study (Appendix 1, section 8). Furthermore, in GK rats, we observed an increase in KC/GRO levels after the restraint stress test (Appendix 1, section 9: $p < 0.0001$ for GK rats after stress, $p = 0.0003$ for both strains post stress, 2-way ANOVA, strain effect $p = 0.0010$; stress effect $p < 0.0001$; strain*stress interaction $p = 0.0042$). However, there was no stress induced differences in the levels of IFNγ, IL-1β, IL-4, IL-6 and IL-10 between the 2 strains (Appendix 1, section 6: $p > 0.05$ for all groups, 2-way ANOVA, strain effect $p > 0.05$; stress effect $p > 0.05$; strain*stress interaction $p > 0.05$).

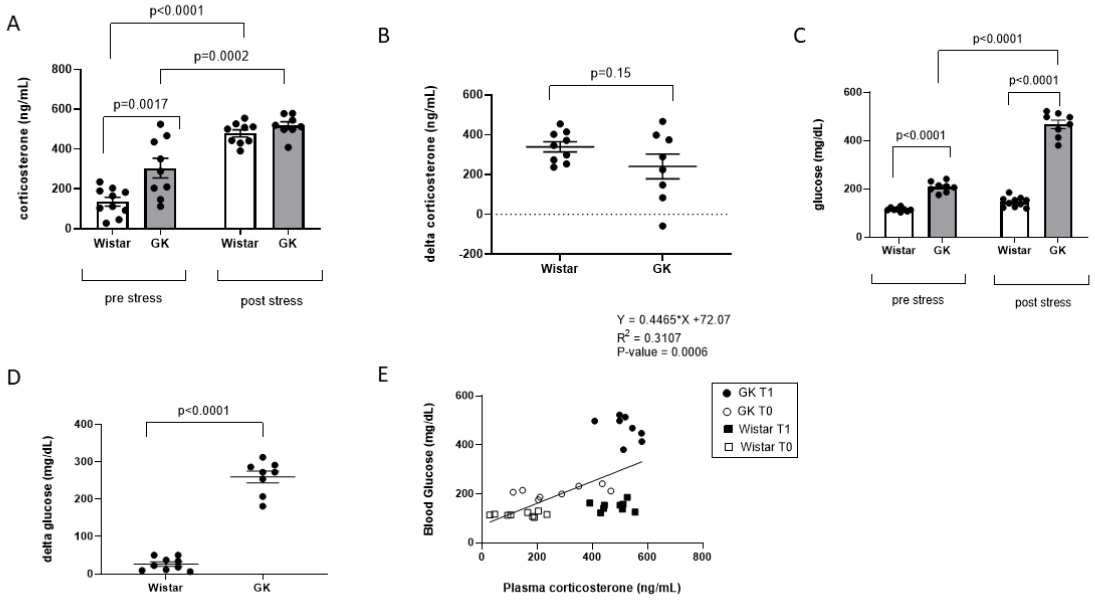


Figure 4.6: Stress-induced (2-way ANOVA and delta) changes in glucose and corticosterone in the early diabetic phase (week 16). Delta values indicate post stress values-pre-stress values. Data are mean +/- SEM; Each dot/square represents a single rat; Grey bars: GK rats, white bars: Wistar rats; For figure E: empty circle/square: pre-stress condition, closed circle/square: post-sress condition, circle: GK rats, square: Wistar rats. Unpaired t-test has been used to analyse delta changes in glucose and corticosterone.

Table 4.4: Cytokine data profile upon stress exposure in the early diabetic phase (week 16)

	Pre-stress (T0)		Post-stress (T1)	
	W mean (SEM)	**GK mean (SEM)**	**W mean (SEM)**	**GK mean (SEM)**
IFN-γ	4.39 (1.4)	3.2 (1.2)	5.26 (1.8)	8.8 (3.1)
IL-1β	12.72 (4.2)	4.3 (1.5)	8.02 (2.7)	15.1 (7.6)
IL-4	6.85 (3.1)	4.8 (2.4)	6.07 (2.3)	5.6 (2.0)
IL-5	90.51 (37.0)	43.7 (19.6)	54.46 (20.6)	80.4 (56.8)

IL-6	13.61 (6.1)	8.0 (4.0)	19.49 (8.0)	10.3 (3.6)
KC/GRO	23.39 (7.4)	26.9 (9.0)	33.45 (11.1)	78.3 (27.7)
IL-10	15.67 (5.2)	6.1 (2.0)	11.75 (3.9)	9.9 (3.5)
IL-13	28.62 (16.5)	-	4.55 (1.6)	-
TNF-α	-	-	-	-

4.5 Discussion

Using Goto-Kakizaki rats we showed that there are considerable immune changes in the transition from pre-diabetes to a clearly diabetic phenotype. Furthermore, once T2D was established, lymphocyte trafficking after a stressor or in response to a metabolic load was clearly influenced by diabetic status. Although, GK rats have been shown to have a limited pro-inflammatory profile which is largely restricted to the pancreatic islets, this still had considerable effects on the circulating immune cells. Using the AUC_g we were able to detect the principal cell populations that evolved differently over the transition from pre- to early diabetes as well as those that were significantly trafficked in response to the glucose bolus. Similarly, pre-post stress we identified the populations that were most affected.

When we examine the GK rat immune system, it is important to remember that even if the GK strain was developed by repeatedly inbreeding W rats that were glucose-intolerant, they are now considered to have a different genetic background to the parental W strain [498]. This difference is also pronounced between the many different sub-strains of GK rats. GK rats have been used to identify diabetes-associated genes [498]. As such, while it is common practice to compare GK rats to the parental W strain as we have done, it is not possible to determine if the differences we see in the immune cells are from the diabetic environment or are a produce of the genetic background. Nevertheless, as we follow the development of the GK rats from pre- to early-diabetes, there are clear changes in the circulating immune cells. We saw that 13 out of the 21 immune cell populations investigated (including total T cells, T_h and T_c cells) already showed a significant diffences between the 2 strains when the animals were 5 weeks old, however, without data from before the animals were weaned, it is not possible to know whether these were present from birth. When we took the AUC_g covering the entire pre- to early diabetes transition we saw differences in circulating immune cells for almost 50% of the examined populations including some that have been previously reported to correlate with the GK diabetic phenotype such as total T cells [425] and CD45RA+ B cells [425, 496]. Here, we also report some novel global changes in the GK immune cells such as total CD3- cells (all immune cells except T cells), T_h cells, T_h2, CD4+ Tregs, double positive T cells, unidentified population of CD4+ CD8+ CD25+ FoxP3+ cells, peripheral B cells and CD161a+ CD8- cells. It is interesting to note that one-third of the examined cell populations showed a diverging profile for cell subsets such as T cells, T_h1, CD4+ Tregs, CD3+ CD4+ CD25- FoxP3+ cells,

T_c1, CD3- non T-cells and CD45RA+ B cells, while T_c17 showed a converging profile between the 2 strains, as they attained maturity.

Furthermore, in this study we identified cells that show sensitivity upon administration of glucose in the form of a metabolic challenge in the pre- and early diabetic phase. In both phases, we saw common significant global differences between the 2 strains for CD4+ Tregs, double positive T cells, CD4+ CD8+ CD25+ FoxP3+ cells, CD45RA+ B cells and CD161a+ CD8- cells. We also saw inter-strain global differences in T_h1 and peripheral cells in the pre- and early diabetic phases respectively. It is interesting to note that although for most of these cell populations, the inter-strain differences after glucose infusion are similar to the differences observed upon maturation, we clearly see changes in cell numbers induced by the metabolic challenge. For example, CD45RA+ cells in the GK rats have been seen to be consistently lower than W rats for all weeks, however, after glucose administration, we see the exact same trend in addition to a linearly decreasing (significant) trend (even upto 180 minutes post glucose bolus). In the early diabetic phase, we also saw a glucotoxicity induced divergence in peripheral B cells, while CD3+ CD4+ CD25- FoxP3+ cells and CD11b/c+ cells showed a converging trend. Additionally, for both GTTs, the inter-strain AUC_g differences observed upon maturation for cell populations such as T, T_h cells and CD3- non T-cells briefly disappeared and resurfaced again for later timepoints (except T_h cell trend at wk14, which remained obliterated even after 3 hours post glucose bolus indicating an exaggerated reaction to the glucotoxicity). Overall, these differences are clearly indicative of glucose induced cell trafficking.

Stress hormones have also been accepted to orchestrate the trafficking and redistribution of immune cells as part of the natural reaction to a exogenous stressors or endogenous glucocorticoid release [495, 499]. In non-diabetic animals this has been regularly investigated, however, little was known about what happens in either type 1 or type 2 diabetes. We originally hypothesised that the pro-inflammatory nature of T2D would alter the stress-induced trafficking of the immune cells even though the peripheral immune cells do not appear to be overtly pro-inflammatory [496]. Indeed, despite this, here we show that each immune cell sub-population has specific and differential sensitivity to the stressor we applied. This is particularly important because stress affects immune cell redistribution [495, 499], and may underlie the aetiology of T2D [500]. In this study, we used a simple restraint stress to identify cell populations that are the most vulnerable to stress in early diabetes. We saw significant stress induced changes in total T cells, an unidentified population of CD4+ CD8+ CD25+ FoxP3+ cells, CD3- non T cells (consisting of monocytes, dendritic cells, B cells, NK cells, etc) and CD45RA+ B cells. One of the important functions performed by cytokines include immune cell trafficking [482] and it is interesting to note

that most of the cell populations showing stress induced differences include principle cytokine secreting cells such as T_c cells, phagocytes, B cells and dendritic cells. The inter-strain differences observed in these principle cytokine secreting cells upon exposure to stress could indicate defective stress (immune) response in the GK rats. It is interesting to note that different stressors have also been linked to insulin resistance and pancreatic β-cell dysfunction [500], thus we could hypothesise that the degree of dysregulation of the stress (immune) response depends on the type of stressor. Collectively, we see that a stressor in concertation with diabetes can alter immune as well as physiological parameters such as blood glucose and plasma corticosterone. In this study we have also shown significant stress-induced changes in KC/GRO levels. KC/GRO is a known a chemoattractant responsible for recruiting immune cells to specific sites [436], this could also explain the stress-induced lymphocyte trafficking observed in our study. It is likely that GK rats in more advanced stages of diabetes (age) could mediate stress-induced changes in other cytokines as well.

Previously, we have shown that 6 months old GK rats exhibit lower levels of basal cytokines compared to age matched W rats [496]. However, in this study, we saw baseline differences only for IFN-γ, while the other 8 pro-inflammatory cytokines remained comparable between the 2 strains. It is likely that we do not see differences in the other cytokines as here we used younger animals that are in the early diabetic phase rather than having been exposed to T2D for 2 more months. It is interesting to hypothesise that this basal difference in IFNγ levels could be used as a rudimentary indication of the emergence of T2D induced immune dysregulations that we have previously reported in 6 month old diabetic GK rats [496]. It is interesting to suggest that routine screening for baseline levels of IFN-γ and/or stress induced changes in KC/GRO levels may be used as a clinical tool to detect transition of prediabetes to T2D at the immunological level.

In man, T2D is often further complicated by concurrent obesity (reviewed in [231]). Obesity is one of the main human drivers of chronic low-level inflammation, and has been thought to drive oxidative stress, inflammation and immune cell infiltration in T2D (reviewed in [231]). Here, in the absence of obesity and overt inflammation, we see a very similar phenomenon, with clear lymphocyte trafficking. Our stress and metabolic challenges induced significant lymphocyte redistribution that may get exacerbated in conjuction to metabolic imbalances if left unchecked in diabetics, thereby giving rise to pathologies such as fatty liver disease, increased gut permeability, dysbiosis and higher predisposition to infections (reviewed in [231]).

Despite providing an insight into the immune component in uncomplicated diabetes and also in the presence of stress and glucotoxicity, some questions remain unanswered. We still do

not understand if the origin of these immune changes in the GK rats are rooted in the impaired energy metabolism or if they arose in a completely independent fashion [501]. If the former is true, it is interesting to further hypothesise that the excess glucose/glucotoxicity may impact the metabolic choices of the immune cells. This altered balance may also cause disturbances in the stress response (reviewed in [380]). Additionally the dependence of these immune cells on the freely available (excessive) glucose may cause metabolic and epigenetic reprogramming of the immune system thereby setting off a vicious cycle that may be transferred vertically [380].

Glucotoxicity has been shown to affect immune cells such as macrophages and neutrophils. Hepatic macrophages have been reported to have an involvement in the pathology of fatty liver disease through glucotoxicity by triggering the release of pro-inflammatory cytokines, thereby leading to impairments in the metabolic reprogramming [502]. Neutrophil functions and behaviour have been implied to be altered by glucotoxicity via excessive production of reactive oxygen species, which further initates NETosis (establishment of neutrophil extracellular traps that induce cell death [503]) and encourages inflammation [504]. It is interesting to note that insulin has been reported to have anti-inflammatory properties and was shown to favour a T_h2 type polarisation which was suggested to be induced by a reversal in the glucotoxicity [505].

Overall, our study showcases changes in the GK immune system induced by age and uncomplicated (early) diabetes, in addition to identifying immune cell subsets that are more sensitive to stress and glucotoxity. These results can potentially serve as early detection markers of T2D immune dysregulations and portrays immune changes that can happen in conjunction with stress in both pre- and early diabetes. However, since the human T2D aetiology is more complex, owing to differences in genetic, epigenetic and environmental exposures, we do not know how well our data translates to the clinical context. Since we were limited in data from before weaning of the rats, we were unable to dissect if the observed immune changes existed innately.

However, to sum up, our study shows that immune changes are already detectable in the prediabetic phase and that the immune system is significantly modifiable in the periphery with the presence of a stressor or glucotoxicity observed in uncontrolled diabetics.

Chapter 5: General Discussion

This section brings together key findings of Chapters 2-4, thereby connecting the dots between ELA, stress and the immune system, placing them in the context of T2D.

The connected dots: ELA, stress and the immune system

Although effects of ELA have been studied for decades, there are still numerous unanswered questions with regards to immunometabolism and the mechanisms through which they function. As detailed in Section 2.2, dissecting the exact contributions of each sub-cateogory of ELA and the stress associated with them is a challenge, especially in the human context. This thesis initially illustrates the basic stress response mechanism and glucose metabolism in healthy individuals across different species. We showed that psychophysiological stressors prompted a boost of readily available energy currency in the form of glucose by triggering gluconeogenic pathways, which eventually diminished after the stress dissipated. This glucose boost was aimed at providing subrates for glycolytic reactions and subsequent energy generation to orchestrate a fight/flight response, while the remaining glucose was most likely converted to glycogen by means of glycogenesis post stress. In addition to being an important regulator molecule in stress biology, cortisol plays an extremely crucial role in the sleep wake pattern known as the "cortisol awakening response" (CAR), where cortisol levels show a sharp increase approximately 30 minutes post awakening [506]. We know that stress results in elevated levels of cortisol (peaking approximately 30 minutes after stress onset, Fig. 2.3 A), similar to the glucose spike observed post stress in our studies. This stress induced cortisol rise is facilitated by 11-beta-hydroxysteroid dehydrogenase 1 (11-beta-HSD1), which converts inactive cortisone to its active form (cortisol) in majority of the tissues [507]. Once the stress wanes, the active cortisol is again reconverted in the pancreas and kidney into its inactive form, which enters into the circulation either bound to albumin or corticosteroid-binding globulin (CBG).

In our studies, we clearly show that glucose and cortisol rise are two independent stress linked phenomenon and that cortisol is not a diabetogenic hormone as previously thought. Instead, it would be interesting to hypothesise that similar to the CAR, the cortisol increase triggered by a stressor could actually be a mechanism to achieve a state of awakeness in the event of stress. Moreover, cortisol has been known to have inflammation suppressing properties [508] and studies have reported that both acute and chronic stress triggers inflammatory activities [509]. It is quite likely that during stress, cortisol also plays a compensatory role to counterbalance the inflammatory reactions.

Endogenous production of cortisol during stress is known to have a pulsatile/rhythmic nature and is secreted approximately every 90 minutes as part of an ultradian cycle [510]. Thus, cortisol, unlike glucose, shows a refractory (non-response) period. It is interesting to note that the socially evaluated cold pressor test in healthy individuals (Figure 2.3 A and Figure 2.3 B), showed (mean)

27.5% of the participants as non responders for cortisol, who were possible in the refractory phase, while only (mean) 2.5% of the participants were non responders for glucose. This absence of refractory period for glucose makes it a more reliable measure/indicator of stress.

A unique feature of our study was the use of different types of ELA models: perinatal infection in mice, maternal deprivation in rats and institutionalization-adoption in humans. The institutionalization-adoption setting served as more real-world hybrid ELA model comprising maternal deprivation and perinatal exposure to infections. The latter was evidenced by a higher seroprevalence of cytomegalovirus (CMV) in EpiPath participants which was most likely responsibe for driving "T-cell specific immunosenescence" [511]. Another study reported higher herpes simplex virus (HSV) type 1 viral loads in institutionalized individuals comared to controls [512]. Both CMV and HSV are persistent infections that are characterised by a period of latency post initial infection and are reactivated upon incapacitation of the immune system [511].

Thus, we examined glucose paramaters at baseline and post stress, in all our ELA models. We have shown that ELA individuals have an innate dysregulation of glucose metabolism that presents itself in both pre- and post stress states. It is interesting to note that the pre stress glucose trends in the mice perinatal infection model was similar to the human mixed infection/MD model characterised by an institutionalization-adoption setting, while the rat MD model showed the exact opposite trend (Figure 2.1). It is quite possible that glucose metabolism is impacted differently depending on the type of ELA. Building on this theory further, it is quite likely that in the human hybrid ELA model, perinatal infection confers a more overpowering impact compared to the MD element, thereby giving rise to a net glucose profile that matches the perinatal infection model thereby providing a first insight into the relative contribution of different stressors to the overall phenotype in the human mixed model.

Overall, the main proof of concept encompassing this study was that 1) ELA and metabolism are intimately connected and 2) in order to fully comprehend an individual's metabolic contours, their early life exposome has to be reviewed.

In line with the stress induced glucose release in healthy rats, mice and EpiPath control participants (Figure 2.2), we have previously validated endogenous production of cortisol in response to stressors from these individuals/animals [389, 513, 514]. In order to mimic the stress exposure and to further test if cortisol prompted stress-glucose production, we administered escalating doses of (biologically functional) exogenous cortisol to a healthy cohort (ESCO) and studied glucose parameters from the same biological samples. An important finding in terms of mechanism was that the rapid stress induced release of glucose via gluconeogenesis was not

mediated by cortisol and was instead hypothesized to be under neuronal control. This may provide a novel perspective to understand metabolic and stress related diseases, and to design more effective therapeutic strategies to neutralise the ELA induced stress-glucose changes. The key findings from Chapter 2 have been summarised below:

Key findings: ELA, HPA axis and metabolic stress response

- Knowledge related to how stressors affect energy homeostasis is sparse.
- Acute stress induces rapid glucose release that could be impacted by a negative early life environment.
- The impact of early life stress on carbohydrate (glucose) metabolism differs according to the type of stressor (psychosocial or immune) and sex.
- Stress induced glucose rise is a hormone (glucocorticoid) independent process and instead may be result of a direct hepatic innervation.
- HPA axis and metabolic stress responses are independently programmed by the quality of the early life environment.

The overall ELA exposome of an individual is often a conglomeration of several different types of negative physical, psychosocial and psychological experiences such as early life infection, maternal separation, adversities faced in an institutionalization-adoption setting and nutritional stress. In order to understand the exact impacts of these sub-categories on an individual's health, these have to be modelled in laboratory animals. This thesis predominantly focused on T2D as a form of early life life nutritional stress in GK rats to address the knowledge gaps existing in immune and stress biology. This approach not only gave us an insight into the overall status of the immune system in uncomplicated diabetes, but also helped us understand the exact deleterious effects of nutritional stress in the absence of other ELA subtypes. Additionally, this study also helped us identify immune cell populations that were the most susceptible to changes that arose due to stress in the form of exposure to a hyperglycaemic in utero environment. The immune profile of fully diabetic GK rats have been described in Chapter 3 and the key findings have been listed below:

Key findings: what does the adult GK immune system look like?

- Outside the pancreatic islets, GK rats show a limited pro-inflammatory profile as corroborated by 3 lines of evidence:

- marked decrease of the examined immune markers (lower numbers of circulating phagocytes)
- reduced levels of the cytokines most likely due to low numbers of associated secretory cells
- cytokine dependent biological pathways constructed from gene expression profiles confirmed a downregulated pattern of most involved genes

- GK rats can serve as a good model to address the knowledge gap between uncomplicated non-obese T2D and the immune system.
- GK cytokine profile alone can be quite misleading, unless amalgamated with cognate immune date.

The T2D aetiology is known to present a prediabetic phase before transitioning into the fully diabetic phase [351]. Thus, having fully characterised the adult phenotype, we examined the exact time points at which these immune changes emerged using a longitudinal assessment of the circulating immune cells.

Significant immune signatures observed for GK rats in the early prediabetic phase, could be used as early detection markers for immune dysregulation, which could have emerged as a result of their distinct genetic composition. Inflammation has been reported to orchestrate lymphocyte trafficking (reviewed in [481]) and GK rats have been known to show pancreatic inflammation [354, 428, 429], thus we investigated the influence of glucotoxicity induced by a glucose bolus, on the immune cell distribution in the pre- and early diabetic phase. Similarly stress has been shown to induce lymphocyte trafficking [495, 499]. As described previously in section 1.3.4, T2D can also act as a source of (early-life) stress and since we have limited knowledge about how stress-immune components are affected in the (uncomplicated) diabetic context, we also examined stress-induced immune cell shuttling and stress-cytokine profiles in early diabetes.

However, this study was unable to shed light on whether these changes arose as a result of dysregulated metabolism in the GK rats or was developed in a completely independent manner. Despite this caveat, findings from this study could serve as a fruitful approach to be integrated into current immunotherapeutic approaches in T2D, which are limited. These include immune system modulators such as Metformin, that have been reported to downregulate IL-1β and NK-kB expression, thereby improving insulin sensitivity [515] and inducing reactive oxygen species expression in macrophages, prompting a shift towards the anti-inflammatory M2 phenotype [516]. Results from our study will help toward developing more effective immunotherapeutic approaches

in the future, targeting a broader range of immune cells such as T cells and its subsets, B cells, NK cells, monocytes, etc. The key results from Chapter 4 have been summarised below:

Key findings: GK immune changes from prediabetes to diabetes

- GK rats are consistently underweight and show significant differences in blood glucose since the prediabetic phase.
- GK rats show evident global changes for most immune cell subsets during early prediabetes.
- Both stress and glucotoxicity in GK rats induce lymphocyte redistribution
- Stress induced lymphocyte trafficking was mostly observed for cytokine producing cells, thereby indicating impaired stress-immune response in GK rats.
- Baseline and stress-induced changes were observed in the early diabetic phase for IFN-γ and KC/GRO levels respectively.

Overall strengths and limitations

The evidence from our primary study on stress and glucose was generated using several cross species models and different types of ELA. This unique feature of our study provides robust and convincing proof that ELA alters hepatic gluconeogenesis and metabolism, which can eventually lead to disorders such as T2D. ELA in humans is a collective experience of every adverse condition faced in the early formative years such as early life infection, nutritional stress, maternal separation, pollutant exposure, other exposures due to low SES, etc. Thus, it becomes near impossible to dissect these adversities in humans and to understand their exact impact on the various physiological systems. Thus, modelling the different sub-categories of ELA in animals helped us overcome this additive effect. Our ELA laboratory models helped us control for confounding human variables such as genetic variation, diet and lifestyle which tends to confer either protective or deleterious impacts to pathogenic phenotypes.

Additionally, studying the T2D aetiology and changes in the immune system in GK rats was especially advantageous due to their short lifespan during which the disease manifests and progresses to the fully advanced stages. In humans, differences in age of T2D onset can further complicate the comprehension of the existing phenotype. GK rats on the other hand have clearly defined prediabetic and diabetic phases depending on the age and colony of rats providing a more uniform study design.

Despite these strengths, our studies had some limitations. The ELA MD rat model for our first stress-glucose study lacked statistical power in terms of sex to validate some of the trends observed for the other ELA models and therefore just missed statistical significance (Fig. 2.4 C, $p = 0.06$; paired t-test). Our results clearly proved that the stress induced glucose rise was not GC mediated and instead we hypothesised it to be under hepatic nervous control. However, we were limited by a lack of experimental apparatus required to test this hypothesis. Furthermore, we lacked data on vagal tone and splanchnic nerve activity from the EpiPath cohort.

The immune system related findings stated in Chapter 3, were tested in Streck-preserved blood samples from the GK Paris colony. As stated in Section 1.6.1. there exists subtle phenotypic differences between different GK rat clonies. Thus, results obtained from this study, may show some variation in replication studies depending on the colony of GK rats used. Additionally, the Streck lymphoctye preservative may affect some immune cell markers. One of the main drawbacks of the longitudinal immune study in the GK/MolTac colony rats was the lack of stress induced lymphocyte redistribution data in the prediabetic phase to investigate the effect of stress in the (near) absence of the T2D phenotype.

For decades, mice have been the favoured choice of models to understand the functioning of the immune system [517] as evidenced by the vast availability of mice specific antibodies as opposed to rat specific ones. As detailed in section 1.6.1, GK rats are the 'closest-to-human' models to study uncomplicated T2D. Thus, in our GK rat immune system studies, we have tried to make use of majority of the commercially available rat specific immune markers to get a general overview of their immune system.

Although findings of Chapter 4 clearly showed detectable immune differences in the early GK prediabetic phase, we were limited in data collection from before weaning, and thus, it was not possible to dissect if the differences we saw were from the (pre) diabetic environment or were a produce of the genetic background. Additionally, it is known that the human T2D pathology is much more complicated due to differences in genetic, epigenetic and environmental exposures. Therefore, we are unsure about how well our GK rat data successfully describes the human clinical scenario.

Furthermore, it is interesting to note that, Beura et al. have reported laboratory rodents as being born and raised in environments that are "too clean", resulting in their immune systems being poorly educated [518] and may also not ideally mimic real life environments of humans in their early life. Thus, it is quite plausible that despite having immense advantages of using laboratory models in conjunction with advances in scientific technologies, we still don't have answers to

numerous mechanistic enquiries in the field of immunolgy. Nevertheless, our animal studies provide a good starting point to answer some of the most basic ELA-immune research questions that can eventually be translated into the wider human context.

Conclusion and prospectives

Response to environmental signals is inevitable for survival and is generally manifested as structural and functional alterations in physiological systems [519] such as the nervous and immune system. One of the lesser known factors influencing the adult nervous system has been hypothesized to be parental exposure to environmental cues preceding conception [519]. This theory is in line with Hales and Barker's thrifty phenotype hypothesis and suggests vertical transfer of information mediated via the germline from parents to their offspring, educating them about their immediate environment, even before conception [519]. Using "olfactory fear conditioning" in the parental generation of mice before conception of offspring, followed by cross-fostering of F1 pups, Dias et. al have demonstrated that the associated behavioral and structural alterations were transgenerationally transmitted [519]. In Chapter 2, we have proposed that the stress induced glucose release is most likely mediated by a hepatic innervation. Thus, the hereditary pattern of neuronal changes across generations reported by Dias et. al could also be exptrapolated to changes in stress-glucose patterns as well as to other physiological systems such as the immune system.

Therefore, like other forms of ELA (reviewed in [225]), we can hypothesize that T2D also reprograms the immune system in addition to metabolism, by means of epigenetic alterations that can be passed on through succeeding generations. This also raises the question whether there are common mechanisms between the different sources of ELA and the resultant immunophenotype. Although it is outside the scope of this thesis, it would be interesting to perform a detailed comparison of the commonalities between the Epipath imstitutionalisation-adoption, rat maternal separation, murine early life infection and now the T2D model immunophenotype. We propose future study designs in the next section, targeting parameters that influence the immune system, and answering the question of common mechanistic elements between the different early-life exposure modalities.

Future study designs to further understand the uncomplicated T2D aetiology:

- Although a cross fostering and embryo-transfer paradigm using GK rats have not been successful in ameliorating impaired metabolic parameters in GK rats as described in section 1.6.1, it would be interesting to see what effects it would have on the circulating immune cells and also if these changes would persist through time as the rats mature. This experiment would help us understand the exact role of the genetic and epigenetic factors contributing to the diabetes related immune changes.
- Additionally, we can also design experiments to dissect out if there is a parental sex bias in terms of transmittance of the immune pathogenic component. This would also help us understand the much less explored paternal exposure transmission to the offspring (POHAD- paternal origins of health and disease), mediated by the sperm epigenome.
- As previously discussed, the gut microflora and the immune system are intimately tied. We can design a fecal microbiota transfer experiment using GK and Wistar rats and then conduct a full characterization of the immune profile (immune cell percentages and cytokine level assessment) to see if we can (at least partially) rescue the GK immune dysregulations described in Chapters 3 and 4.
- Additionally, we can also explore if the feeding of fibre rich diet to GK mothers and/or pups can have a positive impact on the immune profile.
- Haematopoietic stem cells act as precursors to all blood cells including immune cells. Thus it could be hypothesized that the differences in the immune system have their origins in the immune progenitor cells of the bone marrow that are "programmed" by the exposure *in utero* to a diabetic hyperglycaemic environment. Investigation of this theory will likely help us alleviate/curb the (pre)diabetic phenotype and therefore strategize effective therapeutic approaches in the future. This may explain commonalities in immune phenotype between different types of early life adversity.

Overall, it is clear that different types of ELA, including T2D deeply impacts stress response, metabolism and also the immune system. Thus, as a precautionary measure individuals who have been subjected to ELA and are potentially at a higher risk of developing T2D, should have their immune age (different from chronological age) checked using IMM-AGE, a recent technology that scores the competency of their immune system regardless of age. This technique will help identify individuals who are genuinely at a higher risk of T2D, while taking into account their immune age as opposed to their chronological age, which might not be a good risk predictor due to inter-

individual variations [520] [521]. Hence, it is clear that ELA is the prologue to understanding lifelong metabolic trajectories and changes associated with them to physiologically critical systems. Thus to sum up, the full extent of the composite aetiology presented by T2D can only be understood by connecting all the dots, including ELA, the immune and the stress response system.

References

1. International_Diabetes_Federation, *IDF Diabetes Atlas*. 8th Edition ed, ed. N.H. Cho. 2017, Watermael-Boitsfort, Belgium: International Diabetes Foundation.
2. Zheng, Y., S.H. Ley, and F.B. Hu, *Global aetiology and epidemiology of type 2 diabetes mellitus and its complications.* Nat Rev Endocrinol, 2018. **14**(2): p. 88-98.
3. Ling, C. and L. Groop, *Epigenetics: a molecular link between environmental factors and type 2 diabetes.* Diabetes, 2009. **58**(12): p. 2718-25.
4. Daskalakis, N.P., et al., *The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome.* Psychoneuroendocrinology, 2013. **38**(9): p. 1858-73.
5. Li, X., et al., *"Three Hits" Hypothesis for Developmental Origins of Health and Diseases in View of Cardiovascular Abnormalities.* Birth Defects Res, 2017. **109**(10): p. 744-757.
6. Whitaker, R.C., et al., *Predicting obesity in young adulthood from childhood and parental obesity.* N Engl J Med, 1997. **337**(13): p. 869-73.
7. Meigs, J.B., L.A. Cupples, and P.W. Wilson, *Parental transmission of type 2 diabetes: the Framingham Offspring Study.* Diabetes, 2000. **49**(12): p. 2201-7.
8. Hemminki, K., et al., *Familial risks for type 2 diabetes in Sweden.* Diabetes Care, 2010. **33**(2): p. 293-7.
9. Hanson, R.L., et al., *Strong parent-of-origin effects in the association of KCNQ1 variants with type 2 diabetes in American Indians.* Diabetes, 2013. **62**(8): p. 2984-91.
10. Karter, A.J., et al., *Excess maternal transmission of type 2 diabetes. The Northern California Kaiser Permanente Diabetes Registry.* Diabetes Care, 1999. **22**(6): p. 938-43.
11. Arfa, I., et al., *Familial aggregation and excess maternal transmission of type 2 diabetes in Tunisia.* Postgrad Med J, 2007. **83**(979): p. 348-51.
12. Al-Sinani, S., et al., *Familial Clustering of Type 2 Diabetes among Omanis.* Oman Med J, 2014. **29**(1): p. 51-4.
13. Kwak, S.H. and K.S. Park, *Recent progress in genetic and epigenetic research on type 2 diabetes.* Exp Mol Med, 2016. **48**(3): p. e220.
14. Drong, A.W., C.M. Lindgren, and M.I. McCarthy, *The genetic and epigenetic basis of type 2 diabetes and obesity.* Clin Pharmacol Ther, 2012. **92**(6): p. 707-15.
15. Cuschieri, S., *The genetic side of type 2 diabetes - A review.* Diabetes Metab Syndr, 2019. **13**(4): p. 2503-2506.
16. Prasad, R.B. and L. Groop, *Genetics of type 2 diabetes-pitfalls and possibilities.* Genes (Basel), 2015. **6**(1): p. 87-123.
17. Sparso, T., et al., *G-allele of intronic rs10830963 in MTNR1B confers increased risk of impaired fasting glycemia and type 2 diabetes through an impaired glucose-stimulated insulin release: studies involving 19,605 Europeans.* Diabetes, 2009. **58**(6): p. 1450-6.
18. Ali, O., *Genetics of type 2 diabetes.* World J Diabetes, 2013. **4**(4): p. 114-23.
19. Bego, T., et al., *Association of FTO Gene Variant (rs8050136) with Type 2 Diabetes and Markers of Obesity, Glycaemic Control and Inflammation.* J Med Biochem, 2019. **38**(2): p. 153-163.
20. Xi, B., et al., *Common polymorphism near the MC4R gene is associated with type 2 diabetes: data from a meta-analysis of 123,373 individuals.* Diabetologia, 2012. **55**(10): p. 2660-2666.
21. Small, K.S., et al., *Regulatory variants at KLF14 influence type 2 diabetes risk via a female-specific effect on adipocyte size and body composition.* Nat Genet, 2018. **50**(4): p. 572-580.
22. Sharaf, S.A., et al., *Altered expression of WFS1 and NOTCH2 genes associated with diabetic nephropathy in T2DM patients.* Diabetes Res Clin Pract, 2018. **140**: p. 304-313.
23. Tang, L., et al., *BCL11A gene DNA methylation contributes to the risk of type 2 diabetes in males.* Exp Ther Med, 2014. **8**(2): p. 459-463.
24. Mahajan, A., et al., *Fine-mapping type 2 diabetes loci to single-variant resolution using high-density imputation and islet-specific epigenome maps.* Nat Genet, 2018. **50**(11): p. 1505-1513.
25. Spracklen, C.N., et al., *Identification of type 2 diabetes loci in 433,540 East Asian individuals.* Nature, 2020. **582**(7811): p. 240-245.
26. Pearson, E.R., *Type 2 diabetes: a multifaceted disease.* Diabetologia, 2019. **62**(7): p. 1107-1112.
27. Mattis, K.K. and A.L. Gloyn, *From Genetic Association to Molecular Mechanisms for Islet-cell Dysfunction in Type 2 Diabetes.* J Mol Biol, 2020. **432**(5): p. 1551-1578.

28. Cordell, H.J., *Detecting gene-gene interactions that underlie human diseases.* Nat Rev Genet, 2009. **10**(6): p. 392-404.
29. Lawlor, N. and M.L. Stitzel, *(Epi)genomic heterogeneity of pancreatic islet function and failure in type 2 diabetes.* Mol Metab, 2019. **27S**(Suppl): p. S15-S24.
30. Kaprio, J., et al., *Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland.* Diabetologia, 1992. **35**(11): p. 1060-7.
31. Newman, B., et al., *Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins.* Diabetologia, 1987. **30**(10): p. 763-8.
32. Poulsen, P., et al., *Heritability of type II (non-insulin-dependent) diabetes mellitus and abnormal glucose tolerance--a population-based twin study.* Diabetologia, 1999. **42**(2): p. 139-45.
33. Almgren, P., et al., *Heritability and familiality of type 2 diabetes and related quantitative traits in the Botnia Study.* Diabetologia, 2011. **54**(11): p. 2811-9.
34. Delicano, R.A., et al., *The shared risk of diabetes between dog and cat owners and their pets: register based cohort study.* BMJ, 2020. **371**: p. m4337.
35. Maher, B., *Personal genomes: The case of the missing heritability.* Nature, 2008. **456**(7218): p. 18-21.
36. Diabetes Genetics Initiative of Broad Institute of, H., et al., *Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels.* Science, 2007. **316**(5829): p. 1331-6.
37. Zeggini, E., et al., *Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes.* Science, 2007. **316**(5829): p. 1336-41.
38. Takeuchi, F., et al., *Confirmation of multiple risk Loci and genetic impacts by a genome-wide association study of type 2 diabetes in the Japanese population.* Diabetes, 2009. **58**(7): p. 1690-9.
39. Perry, J.R., et al., *Stratifying type 2 diabetes cases by BMI identifies genetic risk variants in LAMA1 and enrichment for risk variants in lean compared to obese cases.* PLoS Genet, 2012. **8**(5): p. e1002741.
40. Grarup, N., et al., *Studies of association of variants near the HHEX, CDKN2A/B, and IGF2BP2 genes with type 2 diabetes and impaired insulin release in 10,705 Danish subjects: validation and extension of genome-wide association studies.* Diabetes, 2007. **56**(12): p. 3105-11.
41. Chandak, G.R., et al., *Common variants in the TCF7L2 gene are strongly associated with type 2 diabetes mellitus in the Indian population.* Diabetologia, 2007. **50**(1): p. 63-7.
42. Holmkvist, J., et al., *The type 2 diabetes associated minor allele of rs2237895 KCNQ1 associates with reduced insulin release following an oral glucose load.* PLoS One, 2009. **4**(6): p. e5872.
43. Kasuga, M., *KCNQ1, a susceptibility gene for type 2 diabetes.* J Diabetes Investig, 2011. **2**(6): p. 413-4.
44. Musambil, M. and K. Siddiqui, *Genetics and genomics studies in type 2 diabetes: A brief review of the current scenario in the Arab region.* Diabetes Metab Syndr, 2019. **13**(2): p. 1629-1632.
45. Tuerxunyiming, M., et al., *Association of rs7754840 G/C polymorphisms in CDKAL1 with type 2 diabetes: a meta-analysis of 70141 subjects.* Int J Clin Exp Med, 2015. **8**(10): p. 17392-405.
46. Yan, S.T., et al., *Association of calpain-10 rs2975760 polymorphism with type 2 diabetes mellitus: a meta-analysis.* Int J Clin Exp Med, 2014. **7**(10): p. 3800-7.
47. Makhzoom, O., Y. Kabalan, and F. Al-Quobaili, *Association of KCNJ11 rs5219 gene polymorphism with type 2 diabetes mellitus in a population of Syria: a case-control study.* BMC Med Genet, 2019. **20**(1): p. 107.
48. Mahmutovic, L., et al., *Association of IRS1 genetic variants with glucose control and insulin resistance in type 2 diabetic patients from Bosnia and Herzegovina.* Drug Metab Pers Ther, 2019. **34**(1).
49. Staiger, H., et al., *Novel meta-analysis-derived type 2 diabetes risk loci do not determine prediabetic phenotypes.* PLoS One, 2008. **3**(8): p. e3019.
50. Ding, X.Y., et al., *Ubiquitination of NOTCH2 by DTX3 suppresses the proliferation and migration of human esophageal carcinoma.* Cancer Sci, 2020. **111**(2): p. 489-501.
51. Kretowski, A., et al., *The rs340874 PROX1 type 2 diabetes mellitus risk variant is associated with visceral fat accumulation and alterations in postprandial glucose and lipid metabolism.* Genes Nutr, 2015. **10**(2): p. 4.
52. Wang, J., et al., *Prox1 activity controls pancreas morphogenesis and participates in the production of "secondary transition" pancreatic endocrine cells.* Dev Biol, 2005. **286**(1): p. 182-94.
53. Hodson, D.J., et al., *ADCY5 couples glucose to insulin secretion in human islets.* Diabetes, 2014. **63**(9): p. 3009-21.
54. Roman, T.S., et al., *A Type 2 Diabetes-Associated Functional Regulatory Variant in a Pancreatic Islet Enhancer at the ADCY5 Locus.* Diabetes, 2017. **66**(9): p. 2521-2530.
55. Tuomi, T., et al., *Increased Melatonin Signaling Is a Risk Factor for Type 2 Diabetes.* Cell Metab, 2016. **23**(6): p. 1067-1077.

56. Matsuba, R., et al., *Replication Study in a Japanese Population to Evaluate the Association between 10 SNP Loci, Identified in European Genome-Wide Association Studies, and Type 2 Diabetes.* PLoS One, 2015. **10**(5): p. e0126363.
57. Harada, N. and N. Inagaki, *Role of GIP receptor signaling in beta-cell survival.* Diabetol Int, 2017. **8**(2): p. 137-138.
58. Parra, E.J., et al., *Genome-wide association study of type 2 diabetes in a sample from Mexico City and a meta-analysis of a Mexican-American sample from Starr County, Texas.* Diabetologia, 2011. **54**(8): p. 2038-46.
59. Bai, H., et al., *Association Analysis of Genetic Variants with Type 2 Diabetes in a Mongolian Population in China.* J Diabetes Res, 2015. **2015**: p. 613236.
60. Gao, K., et al., *Polymorphisms in Four Genes (KCNQ1 rs151290, KLF14 rs972283, GCKR rs780094 and MTNR1B rs10830963) and Their Correlation with Type 2 Diabetes Mellitus in Han Chinese in Henan Province, China.* Int J Environ Res Public Health, 2016. **13**(3).
61. Cheng, L., et al., *Association between SLC30A8 rs13266634 Polymorphism and Type 2 Diabetes Risk: A Meta-Analysis.* Med Sci Monit, 2015. **21**: p. 2178-89.
62. Fall, C.H.D. and K. Kumaran, *Metabolic programming in early life in humans.* Philos Trans R Soc Lond B Biol Sci, 2019. **374**(1770): p. 20180123.
63. Hales, C.N. and D.J. Barker, *Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis.* Diabetologia, 1992. **35**(7): p. 595-601.
64. Hales, C.N., et al., *Fetal and infant growth and impaired glucose tolerance at age 64.* BMJ, 1991. **303**(6809): p. 1019-22.
65. Horner, E.M., et al., *Investigating the Early Life Determinants of Type-II Diabetes Using a Project Talent-Medicare Linked Data-set.* SSM Popul Health, 2018. **4**: p. 189-196.
66. Hostinar, C.E., et al., *Early-Life Socioeconomic Disadvantage and Metabolic Health Disparities.* Psychosom Med, 2017. **79**(5): p. 514-523.
67. Needham, B.L., et al., *Life course socioeconomic status and DNA methylation in genes related to stress reactivity and inflammation: The multi-ethnic study of atherosclerosis.* Epigenetics, 2015. **10**(10): p. 958-69.
68. Elwenspoek, M.M.C., et al., *Proinflammatory T Cell Status Associated with Early Life Adversity.* J Immunol, 2017. **199**(12): p. 4046-4055.
69. Elwenspoek, M.M.C., et al., *The effects of early life adversity on the immune system.* Psychoneuroendocrinology, 2017. **82**: p. 140-154.
70. Elwenspoek, M.M.C., et al., *T Cell Immunosenescence after Early Life Adversity: Association with Cytomegalovirus Infection.* Front Immunol, 2017. **8**(1263): p. 1263.
71. Holuka, C., et al., *The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity?* Int J Mol Sci, 2020. **21**(14).
72. Dancause, K.N., et al., *Prenatal stress due to a natural disaster predicts insulin secretion in adolescence.* Early Hum Dev, 2013. **89**(9): p. 773-6.
73. van Abeelen, A.F., et al., *Famine exposure in the young and the risk of type 2 diabetes in adulthood.* Diabetes, 2012. **61**(9): p. 2255-60.
74. Shanes, E.D., et al., *Placental Pathology in COVID-19.* Am J Clin Pathol, 2020. **154**(1): p. 23-32.
75. Knofler, M., et al., *Human placenta and trophoblast development: key molecular mechanisms and model systems.* Cell Mol Life Sci, 2019. **76**(18): p. 3479-3496.
76. Rocha, V.Z. and P. Libby, *Obesity, inflammation, and atherosclerosis.* Nat Rev Cardiol, 2009. **6**(6): p. 399-409.
77. Cieslak, M., A. Wojtczak, and M. Cieslak, *Role of pro-inflammatory cytokines of pancreatic islets and prospects of elaboration of new methods for the diabetes treatment.* Acta Biochim Pol, 2015. **62**(1): p. 15-21.
78. Qing, H., et al., *Origin and Function of Stress-Induced IL-6 in Murine Models.* Cell, 2020. **182**(2): p. 372-387 e14.
79. Senn, J.J., et al., *Interleukin-6 induces cellular insulin resistance in hepatocytes.* Diabetes, 2002. **51**(12): p. 3391-9.
80. van Tilburg, J., et al., *Defining the genetic contribution of type 2 diabetes mellitus.* J Med Genet, 2001. **38**(9): p. 569-78.
81. Hu, F.B., et al., *Diet, lifestyle, and the risk of type 2 diabetes mellitus in women.* N Engl J Med, 2001. **345**(11): p. 790-7.
82. Rosmond, R., *Stress induced disturbances of the HPA axis: a pathway to Type 2 diabetes?* Med Sci Monit, 2003. **9**(2): p. RA35-9.

83. Turner, J.D., *Holistic, personalized, immunology? The effects of socioeconomic status on the transcriptional milieu of immune cells.* Pediatr Pulmonol, 2018. **53**(6): p. 696-697.
84. Rao, X., et al., *Air pollution as a risk factor for type 2 diabetes.* Toxicol Sci, 2015. **143**(2): p. 231-41.
85. Chambers, J.C., et al., *Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study.* Lancet Diabetes Endocrinol, 2015. **3**(7): p. 526-534.
86. Zhou, Z., et al., *DNA methylation landscapes in the pathogenesis of type 2 diabetes mellitus.* Nutr Metab (Lond), 2018. **15**: p. 47.
87. Hudec, M., et al., *Epigenetic Regulation of Circadian Rhythm and Its Possible Role in Diabetes Mellitus.* Int J Mol Sci, 2020. **21**(8).
88. Ronn, T., et al., *A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue.* PLoS Genet, 2013. **9**(6): p. e1003572.
89. Sandi, C. and J. Haller, *Stress and the social brain: behavioural effects and neurobiological mechanisms.* Nat Rev Neurosci, 2015. **16**(5): p. 290-304.
90. Herzig, S., *Liver: a target of late diabetic complications.* Exp Clin Endocrinol Diabetes, 2012. **120**(4): p. 202-4.
91. de Guia, R.M., A.J. Rose, and S. Herzig, *Glucocorticoid hormones and energy homeostasis.* Horm Mol Biol Clin Investig, 2014. **19**(2): p. 117-28.
92. Nuttall, F.Q., A. Ngo, and M.C. Gannon, *Regulation of hepatic glucose production and the role of gluconeogenesis in humans: is the rate of gluconeogenesis constant?* Diabetes Metab Res Rev, 2008. **24**(6): p. 438-58.
93. Konig, M., S. Bulik, and H.G. Holzhutter, *Quantifying the contribution of the liver to glucose homeostasis: a detailed kinetic model of human hepatic glucose metabolism.* PLoS Comput Biol, 2012. **8**(6): p. e1002577.
94. ter Horst, G.J. and P.G. Luiten, *The projections of the dorsomedial hypothalamic nucleus in the rat.* Brain Res Bull, 1986. **16**(2): p. 231-48.
95. Cui, A., et al., *Dexamethasone-induced Kruppel-like factor 9 expression promotes hepatic gluconeogenesis and hyperglycemia.* J Clin Invest, 2019. **129**(6): p. 2266-2278.
96. Grundy, S.M., et al., *Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition.* Circulation, 2004. **109**(3): p. 433-8.
97. Delpierre, C., et al., *The early life nutritional environment and early life stress as potential pathways towards the metabolic syndrome in mid-life? A lifecourse analysis using the 1958 British Birth cohort.* BMC Public Health, 2016. **16**(1): p. 815.
98. Turner, J.D., *Childhood adversity from conception onwards: are our tools unnecessarily hindering us?* J Behav Med, 2018. **41**(4): p. 568-570.
99. Suglia, S.F., et al., *Childhood and Adolescent Adversity and Cardiometabolic Outcomes: A Scientific Statement From the American Heart Association.* Circulation, 2018. **137**(5): p. e15-e28.
100. Jaaskelainen, P., et al., *Childhood nutrition in predicting metabolic syndrome in adults: the cardiovascular risk in Young Finns Study.* Diabetes Care, 2012. **35**(9): p. 1937-43.
101. Huang, J.Y., et al., *Maternal Education in Early Life and Risk of Metabolic Syndrome in Young Adult American Females and Males: Disentangling Life Course Processes Through Causal Models.* Epidemiology, 2019. **30 Suppl 2**: p. S28-S36.
102. Afifi, T.O., et al., *Child abuse and physical health in adulthood.* Health Rep, 2016. **27**(3): p. 10-8.
103. Tomasdottir, M.O., et al., *Self Reported Childhood Difficulties, Adult Multimorbidity and Allostatic Load. A Cross-Sectional Analysis of the Norwegian HUNT Study.* PLoS One, 2015. **10**(6): p. e0130591.
104. Danese, A. and M. Tan, *Childhood maltreatment and obesity: systematic review and meta-analysis.* Mol Psychiatry, 2014. **19**(5): p. 544-54.
105. Huang, H., et al., *Adverse childhood experiences and risk of type 2 diabetes: A systematic review and meta-analysis.* Metabolism, 2015. **64**(11): p. 1408-18.
106. Zhang, S., et al., *Maternal obesity and the early origins of childhood obesity: weighing up the benefits and costs of maternal weight loss in the periconceptional period for the offspring.* Exp Diabetes Res, 2011. **2011**: p. 585749.
107. Chrousos, G.P. and P.W. Gold, *The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis.* JAMA, 1992. **267**(9): p. 1244-52.

108. Tsigos, C. and G.P. Chrousos, *Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders.* Endocrinol Metab Clin North Am, 1994. **23**(3): p. 451-66.
109. Rotenberg, S. and J.J. McGrath, *Inter-relation between autonomic and HPA axis activity in children and adolescents.* Biol Psychol, 2016. **117**: p. 16-25.
110. Jones, B.E. and T.Z. Yang, *The efferent projections from the reticular formation and the locus coeruleus studied by anterograde and retrograde axonal transport in the rat.* J Comp Neurol, 1985. **242**(1): p. 56-92.
111. Lewis, D.I. and J.H. Coote, *Excitation and inhibition of rat sympathetic preganglionic neurones by catecholamines.* Brain Res, 1990. **530**(2): p. 229-34.
112. Unnerstall, J.R., T.A. Kopajtic, and M.J. Kuhar, *Distribution of alpha 2 agonist binding sites in the rat and human central nervous system: analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents.* Brain Res, 1984. **319**(1): p. 69-101.
113. Reiche, E.M., S.O. Nunes, and H.K. Morimoto, *Stress, depression, the immune system, and cancer.* Lancet Oncol, 2004. **5**(10): p. 617-25.
114. Ito, R. and A.C.H. Lee, *The role of the hippocampus in approach-avoidance conflict decision-making: Evidence from rodent and human studies.* Behav Brain Res, 2016. **313**: p. 345-357.
115. Fee, C., et al., *Chronic Stress-induced Behaviors Correlate with Exacerbated Acute Stress-induced Cingulate Cortex and Ventral Hippocampus Activation.* Neuroscience, 2020. **440**: p. 113-129.
116. Jankord, R. and J.P. Herman, *Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress.* Ann N Y Acad Sci, 2008. **1148**: p. 64-73.
117. Nixon, M., et al., *ABCC1 confers tissue-specific sensitivity to cortisol versus corticosterone: A rationale for safer glucocorticoid replacement therapy.* Sci Transl Med, 2016. **8**(352): p. 352ra109.
118. Wang, M., *The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome.* Nutr Metab (Lond), 2005. **2**(1): p. 3.
119. Reppert, S.M. and D.R. Weaver, *Coordination of circadian timing in mammals.* Nature, 2002. **418**(6901): p. 935-41.
120. Ulrich-Lai, Y.M. and J.P. Herman, *Neural regulation of endocrine and autonomic stress responses.* Nat Rev Neurosci, 2009. **10**(6): p. 397-409.
121. Antoni, F.A., *Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor.* Endocr Rev, 1986. **7**(4): p. 351-78.
122. Tsigos, C. and G.P. Chrousos, *Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress.* J Psychosom Res, 2002. **53**(4): p. 865-71.
123. Stavreva, D.A., et al., *Ultradian hormone stimulation induces glucocorticoid receptor-mediated pulses of gene transcription.* Nat Cell Biol, 2009. **11**(9): p. 1093-102.
124. Trifonova, S.T., et al., *The use of saliva for assessment of cortisol pulsatile secretion by deconvolution analysis.* Psychoneuroendocrinology, 2013. **38**(7): p. 1090-101.
125. Lightman, S.L., et al., *The significance of glucocorticoid pulsatility.* Eur J Pharmacol, 2008. **583**(2-3): p. 255-62.
126. Walker, J.J., J.R. Terry, and S.L. Lightman, *Origin of ultradian pulsatility in the hypothalamic-pituitary-adrenal axis.* Proc Biol Sci, 2010. **277**(1688): p. 1627-33.
127. Sapolsky, R.M. and M.J. Meaney, *Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period.* Brain Res, 1986. **396**(1): p. 64-76.
128. Romeo, R.D., *The metamorphosis of adolescent hormonal stress reactivity: A focus on animal models.* Front Neuroendocrinol, 2018. **49**: p. 43-51.
129. Kuo, T., et al., *Regulation of Glucose Homeostasis by Glucocorticoids.* Adv Exp Med Biol, 2015. **872**: p. 99-126.
130. Thorens, B. and M. Mueckler, *Glucose transporters in the 21st Century.* Am J Physiol Endocrinol Metab, 2010. **298**(2): p. E141-5.
131. Huang, S. and M.P. Czech, *The GLUT4 glucose transporter.* Cell Metab, 2007. **5**(4): p. 237-52.
132. Gartner, K., et al., *Stress response of rats to handling and experimental procedures.* Lab Anim, 1980. **14**(3): p. 267-74.
133. Nirupama, R., M. Devaki, and H.N. Yajurvedi, *Chronic stress and carbohydrate metabolism: persistent changes and slow return to normalcy in male albino rats.* Stress, 2012. **15**(3): p. 262-71.
134. Wu, P., et al., *Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart.* Biochem J, 1998. **329 (Pt 1)**(Pt 1): p. 197-201.

135. Sato, T., et al., *Restraint stress alters the duodenal expression of genes important for lipid metabolism in rat.* Toxicology, 2006. **227**(3): p. 248-61.
136. Foley, P. and C. Kirschbaum, *Human hypothalamus-pituitary-adrenal axis responses to acute psychosocial stress in laboratory settings.* Neurosci Biobehav Rev, 2010. **35**(1): p. 91-6.
137. Kirschbaum, C., et al., *Effects of fasting and glucose load on free cortisol responses to stress and nicotine.* J Clin Endocrinol Metab, 1997. **82**(4): p. 1101-5.
138. Gonzalez-Bono, E., et al., *Glucose but not protein or fat load amplifies the cortisol response to psychosocial stress.* Horm Behav, 2002. **41**(3): p. 328-33.
139. Choi, S., et al., *The hypothalamic ventromedial nuclei couple activity in the hypothalamo-pituitary-adrenal axis to the morning fed or fasted state.* J Neurosci, 1996. **16**(24): p. 8170-80.
140. Rosmond, R., G. Holm, and P. Bjorntorp, *Food-induced cortisol secretion in relation to anthropometric, metabolic and haemodynamic variables in men.* Int J Obes Relat Metab Disord, 2000. **24**(4): p. 416-22.
141. Bergendahl, M., et al., *Short-term fasting selectively suppresses leptin pulse mass and 24-hour rhythmic leptin release in healthy midluteal phase women without disturbing leptin pulse frequency or its entropy control (pattern orderliness).* J Clin Endocrinol Metab, 2000. **85**(1): p. 207-13.
142. Sherwin, R.S. and L. Sacca, *Effect of epinephrine on glucose metabolism in humans: contribution of the liver.* Am J Physiol, 1984. **247**(2 Pt 1): p. E157-65.
143. Burgess, S.C., et al., *Cytosolic phosphoenolpyruvate carboxykinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver.* Cell Metab, 2007. **5**(4): p. 313-20.
144. Opherk, C., et al., *Inactivation of the glucocorticoid receptor in hepatocytes leads to fasting hypoglycemia and ameliorates hyperglycemia in streptozotocin-induced diabetes mellitus.* Mol Endocrinol, 2004. **18**(6): p. 1346-53.
145. Barthel, A. and D. Schmoll, *Novel concepts in insulin regulation of hepatic gluconeogenesis.* Am J Physiol Endocrinol Metab, 2003. **285**(4): p. E685-92.
146. Petersen, M.C. and G.I. Shulman, *Mechanisms of Insulin Action and Insulin Resistance.* Physiol Rev, 2018. **98**(4): p. 2133-2223.
147. Liu, Y., et al., *Increased glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type 1 expression in hepatocytes may contribute to the phenotype of type 2 diabetes in db/db mice.* Diabetes, 2005. **54**(1): p. 32-40.
148. Lee, D., J. Le Lay, and K.H. Kaestner, *The transcription factor CREB has no non-redundant functions in hepatic glucose metabolism in mice.* Diabetologia, 2014. **57**(6): p. 1242-8.
149. Shukla, R., et al., *11beta Hydroxysteroid dehydrogenase - 1 activity in type 2 diabetes mellitus: a comparative study.* BMC Endocr Disord, 2019. **19**(1): p. 15.
150. Granner, D.K., *In pursuit of genes of glucose metabolism.* J Biol Chem, 2015. **290**(37): p. 22312-24.
151. Schacke, H., W.D. Docke, and K. Asadullah, *Mechanisms involved in the side effects of glucocorticoids.* Pharmacol Ther, 2002. **96**(1): p. 23-43.
152. Blondeau, B., et al., *Novel transgenic mice for inducible gene overexpression in pancreatic cells define glucocorticoid receptor-mediated regulations of beta cells.* PLoS One, 2012. **7**(2): p. e30210.
153. Buren, J., et al., *Insulin action and signalling in fat and muscle from dexamethasone-treated rats.* Arch Biochem Biophys, 2008. **474**(1): p. 91-101.
154. Karnia, M.J., et al., *BST Stimulation Induces Atrophy and Changes in Aerobic Energy Metabolism in Rat Skeletal Muscles-The Biphasic Action of Endogenous Glucocorticoids.* Int J Mol Sci, 2020. **21**(8).
155. Chiodini, I., et al., *Cortisol secretion in patients with type 2 diabetes: relationship with chronic complications.* Diabetes Care, 2007. **30**(1): p. 83-8.
156. Nouwen, A., et al., *Type 2 diabetes mellitus as a risk factor for the onset of depression: a systematic review and meta-analysis.* Diabetologia, 2010. **53**(12): p. 2480-6.
157. Mosili, P., et al., *The dysregulation of the hypothalamic-pituitary-adrenal axis in diet-induced prediabetic male Sprague Dawley rats.* Nutr Metab (Lond), 2020. **17**(1): p. 104.
158. Herman, J.P., et al., *Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response.* Compr Physiol, 2016. **6**(2): p. 603-21.
159. Swierczynska, M.M., et al., *Changes in morphology and function of adrenal cortex in mice fed a high-fat diet.* Int J Obes (Lond), 2015. **39**(2): p. 321-30.
160. Shu, H.J., et al., *Expression of fructose sensitive glucose transporter in the brains of fructose-fed rats.* Neuroscience, 2006. **140**(3): p. 889-95.
161. Harrell, C.S., et al., *High-fructose diet during periadolescent development increases depressive-like behavior and remodels the hypothalamic transcriptome in male rats.* Psychoneuroendocrinology, 2015. **62**: p. 252-64.

162. Centers for Disease, C. and Prevention, *Adverse childhood experiences reported by adults --- five states, 2009.* MMWR Morb Mortal Wkly Rep, 2010. **59**(49): p. 1609-13.
163. van Bodegom, M., J.R. Homberg, and M. Henckens, *Modulation of the Hypothalamic-Pituitary-Adrenal Axis by Early Life Stress Exposure.* Front Cell Neurosci, 2017. **11**: p. 87.
164. Gutteling, B.M., C. de Weerth, and J.K. Buitelaar, *Prenatal stress and children's cortisol reaction to the first day of school.* Psychoneuroendocrinology, 2005. **30**(6): p. 541-9.
165. O'Connor, T.G., et al., *Prenatal anxiety predicts individual differences in cortisol in pre-adolescent children.* Biol Psychiatry, 2005. **58**(3): p. 211-7.
166. Davies, P.T., et al., *Children's patterns of emotional reactivity to conflict as explanatory mechanisms in links between interpartner aggression and child physiological functioning.* J Child Psychol Psychiatry, 2009. **50**(11): p. 1384-91.
167. Bugental, D.B., G.A. Martorell, and V. Barraza, *The hormonal costs of subtle forms of infant maltreatment.* Horm Behav, 2003. **43**(1): p. 237-44.
168. Carlson, M. and F. Earls, *Psychological and neuroendocrinological sequelae of early social deprivation in institutionalized children in Romania.* Ann N Y Acad Sci, 1997. **807**: p. 419-28.
169. Bernard, K., J. Zwerling, and M. Dozier, *Effects of early adversity on young children's diurnal cortisol rhythms and externalizing behavior.* Dev Psychobiol, 2015. **57**(8): p. 935-47.
170. Hengesch, X., et al., *Blunted endocrine response to a combined physical-cognitive stressor in adults with early life adversity.* Child Abuse Negl, 2018. **85**: p. 137-144.
171. Pesonen, A.K., et al., *Childhood separation experience predicts HPA axis hormonal responses in late adulthood: a natural experiment of World War II.* Psychoneuroendocrinology, 2010. **35**(5): p. 758-67.
172. Fries, E., et al., *A new view on hypocortisolism.* Psychoneuroendocrinology, 2005. **30**(10): p. 1010-6.
173. Yehuda, R., et al., *Alterations in cortisol negative feedback inhibition as examined using the ACTH response to cortisol administration in PTSD.* Psychoneuroendocrinology, 2006. **31**(4): p. 447-51.
174. Elwenspoek, M.M.C., et al., *Glucocorticoid receptor signaling in leukocytes after early life adversity.* Dev Psychopathol, 2019: p. 1-11.
175. Teicher, M.H., A. Tomoda, and S.L. Andersen, *Neurobiological consequences of early stress and childhood maltreatment: are results from human and animal studies comparable?* Ann N Y Acad Sci, 2006. **1071**: p. 313-23.
176. Andersen, S.L., et al., *Preliminary evidence for sensitive periods in the effect of childhood sexual abuse on regional brain development.* J Neuropsychiatry Clin Neurosci, 2008. **20**(3): p. 292-301.
177. Maercker, A., et al., *Age of traumatisation as a predictor of post-traumatic stress disorder or major depression in young women.* Br J Psychiatry, 2004. **184**: p. 482-7.
178. Caballero, A., R. Granberg, and K.Y. Tseng, *Mechanisms contributing to prefrontal cortex maturation during adolescence.* Neurosci Biobehav Rev, 2016. **70**: p. 4-12.
179. Starr, L.R., et al., *Childhood adversity moderates the influence of proximal episodic stress on the cortisol awakening response and depressive symptoms in adolescents.* Dev Psychopathol, 2017. **29**(5): p. 1877-1893.
180. Fogelman, N. and T. Canli, *Early life stress and cortisol: A meta-analysis.* Horm Behav, 2018. **98**: p. 63-76.
181. Bunea, I.M., A. Szentagotai-Tatar, and A.C. Miu, *Early-life adversity and cortisol response to social stress: a meta-analysis.* Transl Psychiatry, 2017. **7**(12): p. 1274.
182. Lorenzo, C., et al., *The metabolic syndrome as predictor of type 2 diabetes: the San Antonio heart study.* Diabetes Care, 2003. **26**(11): p. 3153-9.
183. Rich-Edwards, J.W., et al., *Abuse in childhood and adolescence as a predictor of type 2 diabetes in adult women.* Am J Prev Med, 2010. **39**(6): p. 529-36.
184. Boynton-Jarrett, R., et al., *Child and adolescent abuse in relation to obesity in adulthood: the Black Women's Health Study.* Pediatrics, 2012. **130**(2): p. 245-53.
185. Reid, B.M., et al., *Persistent skewing of the T-cell profile in adolescents adopted internationally from institutional care.* Brain Behav Immun, 2019. **77**: p. 168-177.
186. Slopen, N., et al., *Early life adversity and inflammation in African Americans and whites in the midlife in the United States survey.* Psychosom Med, 2010. **72**(7): p. 694-701.
187. Hostinar, C.E., et al., *Additive contributions of childhood adversity and recent stressors to inflammation at midlife: Findings from the MIDUS study.* Dev Psychol, 2015. **51**(11): p. 1630-44.
188. Klassen, S.A., et al., *Linking systemic arterial stiffness among adolescents to adverse childhood experiences.* Child Abuse Negl, 2016. **56**: p. 1-10.

189. Su, S., et al., *Adverse childhood experiences and blood pressure trajectories from childhood to young adulthood: the Georgia stress and Heart study.* Circulation, 2015. **131**(19): p. 1674-81.
190. Chandan, J.S., et al., *Increased Cardiometabolic and Mortality Risk Following Childhood Maltreatment in the United Kingdom.* J Am Heart Assoc, 2020. **9**(10): p. e015855.
191. Joung, K.E., et al., *Early life adversity is associated with elevated levels of circulating leptin, irisin, and decreased levels of adiponectin in midlife adults.* J Clin Endocrinol Metab, 2014. **99**(6): p. E1055-60.
192. Mantzoros, C.S., et al., *Leptin in human physiology and pathophysiology.* Am J Physiol Endocrinol Metab, 2011. **301**(4): p. E567-84.
193. Yang, W.S., et al., *Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin.* J Clin Endocrinol Metab, 2001. **86**(8): p. 3815-9.
194. Spranger, J., et al., *Adiponectin and protection against type 2 diabetes mellitus.* Lancet, 2003. **361**(9353): p. 226-8.
195. Bostrom, P., et al., *A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis.* Nature, 2012. **481**(7382): p. 463-8.
196. Naumova, O.Y., et al., *Effects of early social deprivation on epigenetic statuses and adaptive behavior of young children: A study based on a cohort of institutionalized infants and toddlers.* PLoS One, 2019. **14**(3): p. e0214285.
197. Suderman, M., et al., *Childhood abuse is associated with methylation of multiple loci in adult DNA.* BMC Med Genomics, 2014. **7**: p. 13.
198. Wu, L.H., et al., *Loss of toll-like receptor 3 function improves glucose tolerance and reduces liver steatosis in obese mice.* Metabolism, 2012. **61**(11): p. 1633-45.
199. Truax, A.D., et al., *The Inhibitory Innate Immune Sensor NLRP12 Maintains a Threshold against Obesity by Regulating Gut Microbiota Homeostasis.* Cell Host Microbe, 2018. **24**(3): p. 364-378 e6.
200. Jackson, M., et al., *The genetic basis of disease.* Essays Biochem, 2018. **62**(5): p. 643-723.
201. Long, S.A., et al., *Partial exhaustion of CD8 T cells and clinical response to teplizumab in new-onset type 1 diabetes.* Sci Immunol, 2016. **1**(5).
202. Zhang, H., et al., *M2-specific reduction of CD1d switches NKT cell-mediated immune responses and triggers metaflammation in adipose tissue.* Cell Mol Immunol, 2018. **15**(5): p. 506-517.
203. Salonen, J.T., et al., *Type 2 diabetes whole-genome association study in four populations: the DiaGen consortium.* Am J Hum Genet, 2007. **81**(2): p. 338-45.
204. Sidibeh, C.O., et al., *FKBP5 expression in human adipose tissue: potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes.* Endocrine, 2018. **62**(1): p. 116-128.
205. Carroll, H.A. and L.J. James, *Hydration, Arginine Vasopressin, and Glucoregulatory Health in Humans: A Critical Perspective.* Nutrients, 2019. **11**(6).
206. Pisto, L., et al., *Childhood Adversities are Associated with Diabetes Management in Working Age in Finland.* Int J Family Med, 2014. **2014**: p. 864572.
207. Selye, H., *A syndrome produced by diverse nocuous agents. 1936.* J Neuropsychiatry Clin Neurosci, 1998. **10**(2): p. 230-1.
208. Dhabhar, F.S., et al., *Reflections on Bruce S. McEwen's contributions to stress neurobiology and so much more.* Stress, 2020. **23**(5): p. 499-508.
209. Lopez-Cepero, A., et al., *Changes in Glycemic Load Are Positively Associated with Small Changes in Primary Stress Markers of Allostatic Load in Puerto Rican Women.* J Nutr, 2020. **150**(3): p. 554-559.
210. Young, J.B. and L. Landsberg, *Stimulation of the sympathetic nervous system during sucrose feeding.* Nature, 1977. **269**(5629): p. 615-7.
211. Fernandez-Twinn, D.S. and S.E. Ozanne, *Early life nutrition and metabolic programming.* Ann N Y Acad Sci, 2010. **1212**: p. 78-96.
212. Heijmans, B.T., et al., *Persistent epigenetic differences associated with prenatal exposure to famine in humans.* Proc Natl Acad Sci U S A, 2008. **105**(44): p. 17046-9.
213. Ravelli, A.C., et al., *Glucose tolerance in adults after prenatal exposure to famine.* Lancet, 1998. **351**(9097): p. 173-7.
214. Li, C. and L.H. Lumey, *Exposure to the Chinese famine of 1959-61 in early life and long-term health conditions: a systematic review and meta-analysis.* Int J Epidemiol, 2017. **46**(4): p. 1157-1170.
215. Lumey, L.H., M.D. Khalangot, and A.M. Vaiserman, *Association between type 2 diabetes and prenatal exposure to the Ukraine famine of 1932-33: a retrospective cohort study.* Lancet Diabetes Endocrinol, 2015. **3**(10): p. 787-94.

216. Poulsen, P., et al., *Low birth weight is associated with NIDDM in discordant monozygotic and dizygotic twin pairs.* Diabetologia, 1997. **40**(4): p. 439-46.
217. Harder, T., et al., *Birth weight and subsequent risk of type 2 diabetes: a meta-analysis.* Am J Epidemiol, 2007. **165**(8): p. 849-57.
218. Berends, L.M., et al., *Programming of central and peripheral insulin resistance by low birthweight and postnatal catch-up growth in male mice.* Diabetologia, 2018. **61**(10): p. 2225-2234.
219. Dunn, G.A. and T.L. Bale, *Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice.* Endocrinology, 2009. **150**(11): p. 4999-5009.
220. Dunn, G.A. and T.L. Bale, *Maternal high-fat diet effects on third-generation female body size via the paternal lineage.* Endocrinology, 2011. **152**(6): p. 2228-36.
221. El Hajj, N., et al., *Epigenetics and life-long consequences of an adverse nutritional and diabetic intrauterine environment.* Reproduction, 2014. **148**(6): p. R111-20.
222. Kim, C., K.M. Newton, and R.H. Knopp, *Gestational diabetes and the incidence of type 2 diabetes: a systematic review.* Diabetes Care, 2002. **25**(10): p. 1862-8.
223. Zhu, Z., et al., *Gestational diabetes mellitus alters DNA methylation profiles in pancreas of the offspring mice.* J Diabetes Complications, 2019. **33**(1): p. 15-22.
224. Perkins, J.M., J.P. Dunn, and S.M. Jagasia, *Perspectives in Gestational Diabetes Mellitus: A Review of Screening, Diagnosis, and Treatment.* 2007. **25**(2): p. 57-62.
225. Batra, A., et al., *Early adversity and insulin: neuroendocrine programming beyond glucocorticoids.* Trends Endocrinol Metab, 2021. **32**(12): p. 1031-1043.
226. King, S. and D.P. Laplante, *The effects of prenatal maternal stress on children's cognitive development: Project Ice Storm.* Stress, 2005. **8**(1): p. 35-45.
227. Cao-Lei, L., et al., *DNA methylation mediates the effect of maternal cognitive appraisal of a disaster in pregnancy on the child's C-peptide secretion in adolescence: Project Ice Storm.* PLoS One, 2018. **13**(2): p. e0192199.
228. Tsai, P.S., et al., *Pregnancy environment, and not preconception, leads to fetal growth restriction and congenital abnormalities associated with diabetes.* Sci Rep, 2020. **10**(1): p. 12254.
229. Moloney, R.D., et al., *The microbiome: stress, health and disease.* Mamm Genome, 2014. **25**(1-2): p. 49-74.
230. Wang, J., W.D. Chen, and Y.D. Wang, *The Relationship Between Gut Microbiota and Inflammatory Diseases: The Role of Macrophages.* Front Microbiol, 2020. **11**: p. 1065.
231. Daryabor, G., et al., *The Effects of Type 2 Diabetes Mellitus on Organ Metabolism and the Immune System.* Front Immunol, 2020. **11**: p. 1582.
232. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.
233. Lau, E.Y.M., et al., *Type 2 diabetes is associated with the accumulation of senescent T cells.* Clin Exp Immunol, 2019. **197**(2): p. 205-213.
234. Weaver, I.C., et al., *Epigenetic programming by maternal behavior.* Nat Neurosci, 2004. **7**(8): p. 847-54.
235. Witzmann, S.R., et al., *Epigenetic regulation of the glucocorticoid receptor promoter 1(7) in adult rats.* Epigenetics, 2012. **7**(11): p. 1290-301.
236. Zhu, Y., et al., *Enhanced neuroinflammation mediated by DNA methylation of the glucocorticoid receptor triggers cognitive dysfunction after sevoflurane anesthesia in adult rats subjected to maternal separation during the neonatal period.* J Neuroinflammation, 2017. **14**(1): p. 6.
237. Reid, B.M., et al., *Persistent Skewing of the T-Cell Profile in Adolescents Adopted Internationally from Institutional Care.* Brain Behav Immun, 2019.
238. Pedicino, D., et al., *Adaptive immunity, inflammation, and cardiovascular complications in type 1 and type 2 diabetes mellitus.* J Diabetes Res, 2013. **2013**: p. 184258.
239. Lee, Y.H., et al., *Senescent T Cells Predict the Development of Hyperglycemia in Humans.* Diabetes, 2019. **68**(1): p. 156-162.
240. McEwen, B.S., *Stressed or stressed out: what is the difference?* J Psychiatry Neurosci, 2005. **30**(5): p. 315-8.
241. Barboza Solis, C., et al., *Adverse childhood experiences and physiological wear-and-tear in midlife: Findings from the 1958 British birth cohort.* Proc Natl Acad Sci U S A, 2015. **112**(7): p. E738-46.
242. Sinha, R. and A.M. Jastreboff, *Stress as a common risk factor for obesity and addiction.* Biol Psychiatry, 2013. **73**(9): p. 827-35.
243. Steptoe, A., et al., *Disruption of multisystem responses to stress in type 2 diabetes: investigating the dynamics of allostatic load.* Proc Natl Acad Sci U S A, 2014. **111**(44): p. 15693-8.

244. Ceriello, A., *The emerging challenge in diabetes: the "metabolic memory".* Vascul Pharmacol, 2012. **57**(5-6): p. 133-8.
245. Drzewoski, J., J. Kasznicki, and Z. Trojanowski, *The role of "metabolic memory" in the natural history of diabetes mellitus.* Pol Arch Med Wewn, 2009. **119**(7-8): p. 493-500.
246. Testa, R., et al., *The "Metabolic Memory" Theory and the Early Treatment of Hyperglycemia in Prevention of Diabetic Complications.* Nutrients, 2017. **9**(5).
247. Rana, K.S., et al., *Plasma irisin is elevated in type 2 diabetes and is associated with increased E-selectin levels.* Cardiovasc Diabetol, 2017. **16**(1): p. 147.
248. Ridout, K.K., et al., *Early life adversity and telomere length: a meta-analysis.* Mol Psychiatry, 2018. **23**(4): p. 858-871.
249. Upadhyaya, S. and G. Banerjee, *Type 2 diabetes and gut microbiome: at the intersection of known and unknown.* Gut Microbes, 2015. **6**(2): p. 85-92.
250. Jeyachandran, A., et al., *Type I Vs. Type II Cytokine Levels as a Function of SOD1 G93A Mouse Amyotrophic Lateral Sclerosis Disease Progression.* Front Cell Neurosci, 2015. **9**: p. 462.
251. Lim, J.J., S. Grinstein, and Z. Roth, *Diversity and Versatility of Phagocytosis: Roles in Innate Immunity, Tissue Remodeling, and Homeostasis.* Front Cell Infect Microbiol, 2017. **7**: p. 191.
252. Gaudino, S.J. and P. Kumar, *Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis.* Front Immunol, 2019. **10**: p. 360.
253. Panda, S.K. and M. Colonna, *Innate Lymphoid Cells in Mucosal Immunity.* Front Immunol, 2019. **10**: p. 861.
254. Alberts, B., et al., *Molecular Biology of the Cell*. Fourth ed. 2002, New York: Garland Science.
255. Roitt, I., J. Brostoff, and D. Male, *Immunology*. Fifth ed. 1998, London: Mosby-Year Book.
256. Krangel, M.S., *Mechanics of T cell receptor gene rearrangement.* Curr Opin Immunol, 2009. **21**(2): p. 133-9.
257. Fietta, P. and G. Delsante, *The effector T helper cell triade.* Riv Biol, 2009. **102**(1): p. 61-74.
258. Haghshenas, M.R., et al., *Helper and cytotoxic T-cell subsets (Th1, Th2, Tc1, and Tc2) in benign and malignant salivary gland tumors.* Oral Dis, 2016. **22**(6): p. 566-72.
259. Kondelkova, K., et al., *Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders.* Acta Medica (Hradec Kralove), 2010. **53**(2): p. 73-7.
260. Huber, M., et al., *A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity.* Eur J Immunol, 2009. **39**(7): p. 1716-25.
261. Niederlova, V., et al., *CD8(+) Tregs revisited: A heterogeneous population with different phenotypes and properties.* Eur J Immunol, 2021. **51**(3): p. 512-530.
262. Coe, C.L. and G.R. Lubach, *Prenatal origins of individual variation in behavior and immunity.* Neurosci Biobehav Rev, 2005. **29**(1): p. 39-49.
263. Coe, C.L. and C.M. Erickson, *Stress decreases lymphocyte cytolytic activity in the young monkey even after blockade of steroid and opiate hormone receptors.* Dev Psychobiol, 1997. **30**(1): p. 1-10.
264. Coe, C.L. and G.R. Lubach, *Critical periods of special health relevance for psychoneuroimmunology.* Brain Behav Immun, 2003. **17**(1): p. 3-12.
265. Coe, C.L., J.W. Kemnitz, and M.L. Schneider, *Vulnerability of placental antibody transfer and fetal complement synthesis to disturbance of the pregnant monkey.* J Med Primatol, 1993. **22**(5): p. 294-300.
266. Tourneur, E. and C. Chassin, *Neonatal immune adaptation of the gut and its role during infections.* Clin Dev Immunol, 2013. **2013**: p. 270301.
267. Zheng, D., T. Liwinski, and E. Elinav, *Interaction between microbiota and immunity in health and disease.* Cell Res, 2020. **30**(6): p. 492-506.
268. Bailey, M.T. and C.L. Coe, *Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys.* Dev Psychobiol, 1999. **35**(2): p. 146-55.
269. de Steenhuijsen Piters, W.A.A., et al., *Early-life viral infections are associated with disadvantageous immune and microbiota profiles and recurrent respiratory infections.* Nat Microbiol, 2022. **7**(2): p. 224-237.
270. Neu, J. and J. Rushing, *Cesarean versus vaginal delivery: long-term infant outcomes and the hygiene hypothesis.* Clin Perinatol, 2011. **38**(2): p. 321-31.
271. Fiebiger, U., S. Bereswill, and M.M. Heimesaat, *Dissecting the Interplay Between Intestinal Microbiota and Host Immunity in Health and Disease: Lessons Learned from Germfree and Gnotobiotic Animal Models.* Eur J Microbiol Immunol (Bp), 2016. **6**(4): p. 253-271.
272. Dowling, D.J. and O. Levy, *Ontogeny of early life immunity.* Trends Immunol, 2014. **35**(7): p. 299-310.

273. Kollmann, T.R., et al., *Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny.* Immunity, 2017. **46**(3): p. 350-363.
274. Danese, A. and J.L. S, *Psychoneuroimmunology of Early-Life Stress: The Hidden Wounds of Childhood Trauma?* Neuropsychopharmacology, 2017. **42**(1): p. 99-114.
275. Alexandre-Silva, G.M., et al., *The hygiene hypothesis at a glance: Early exposures, immune mechanism and novel therapies.* Acta Trop, 2018. **188**: p. 16-26.
276. Okada, H., et al., *The 'hygiene hypothesis' for autoimmune and allergic diseases: an update.* Clin Exp Immunol, 2010. **160**(1): p. 1-9.
277. Strachan, D.P., *Hay fever, hygiene, and household size.* BMJ, 1989. **299**(6710): p. 1259-60.
278. *Stress Challenges and Immunity in Space.* . Second ed. 2020, Cham: Springer Cham.
279. Donath, M.Y. and S.E. Shoelson, *Type 2 diabetes as an inflammatory disease.* Nat Rev Immunol, 2011. **11**(2): p. 98-107.
280. Atashzar, M.R., et al., *Pyrin and Hematopoietic Interferon-Inducible Nuclear Protein Domain Proteins: Innate Immune Sensors for Cytosolic and Nuclear DNA.* Crit Rev Immunol, 2019. **39**(4): p. 275-288.
281. Fang, X., et al., *Glycemic reduction alters white blood cell counts and inflammatory gene expression in diabetes.* J Diabetes Complications, 2018. **32**(11): p. 1027-1034.
282. Seifarth, C.C., et al., *Reduced frequency of peripheral dendritic cells in type 2 diabetes.* Exp Clin Endocrinol Diabetes, 2008. **116**(3): p. 162-6.
283. Gilardini Montani, M.S., et al., *High glucose and hyperglycemic sera from type 2 diabetic patients impair DC differentiation by inducing ROS and activating Wnt/beta-catenin and p38 MAPK.* Biochim Biophys Acta, 2016. **1862**(4): p. 805-813.
284. Khanna, S., et al., *Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice.* PLoS One, 2010. **5**(3): p. e9539.
285. Mirza, R.E., et al., *Sustained inflammasome activity in macrophages impairs wound healing in type 2 diabetic humans and mice.* Diabetes, 2014. **63**(3): p. 1103-14.
286. Mirza, R.E., et al., *Macrophage PPARgamma and impaired wound healing in type 2 diabetes.* J Pathol, 2015. **236**(4): p. 433-44.
287. Inoue, H., et al., *Signaling between pancreatic beta cells and macrophages via S100 calcium-binding protein A8 exacerbates beta-cell apoptosis and islet inflammation.* J Biol Chem, 2018. **293**(16): p. 5934-5946.
288. Tessari, P., et al., *Effects of insulin on methionine and homocysteine kinetics in type 2 diabetes with nephropathy.* Diabetes, 2005. **54**(10): p. 2968-76.
289. Berrou, J., et al., *Natural killer cell function, an important target for infection and tumor protection, is impaired in type 2 diabetes.* PLoS One, 2013. **8**(4): p. e62418.
290. Zhou, T., et al., *Role of Adaptive and Innate Immunity in Type 2 Diabetes Mellitus.* J Diabetes Res, 2018. **2018**: p. 7457269.
291. McLaughlin, T., et al., *T-cell profile in adipose tissue is associated with insulin resistance and systemic inflammation in humans.* Arterioscler Thromb Vasc Biol, 2014. **34**(12): p. 2637-43.
292. Harford, K.A., et al., *Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue.* Proc Nutr Soc, 2011. **70**(4): p. 408-17.
293. Nekoua, M.P., et al., *Modulation of immune cells and Th1/Th2 cytokines in insulin-treated type 2 diabetes mellitus.* Afr Health Sci, 2016. **16**(3): p. 712-724.
294. Lobo, T.F., et al., *Impaired Treg and NK cells profile in overweight women with gestational diabetes mellitus.* Am J Reprod Immunol, 2018. **79**(3).
295. Cortez-Espinosa, N., et al., *CD39 expression on Treg and Th17 cells is associated with metabolic factors in patients with type 2 diabetes.* Hum Immunol, 2015. **76**(9): p. 622-30.
296. Sell, H., C. Habich, and J. Eckel, *Adaptive immunity in obesity and insulin resistance.* Nat Rev Endocrinol, 2012. **8**(12): p. 709-16.
297. Sireesh, D., et al., *Association of NF-E2 Related Factor 2 (Nrf2) and inflammatory cytokines in recent onset Type 2 Diabetes Mellitus.* Sci Rep, 2018. **8**(1): p. 5126.
298. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.
299. Mathis, D., *Immunological goings-on in visceral adipose tissue.* Cell Metab, 2013. **17**(6): p. 851-859.
300. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.

301. Jagannathan-Bogdan, M., et al., *Elevated proinflammatory cytokine production by a skewed T cell compartment requires monocytes and promotes inflammation in type 2 diabetes.* J Immunol, 2011. **186**(2): p. 1162-72.
302. Yuan, N., et al., *Expression of CD4+CD25+Foxp3+ Regulatory T Cells, Interleukin 10 and Transforming Growth Factor beta in Newly Diagnosed Type 2 Diabetic Patients.* Exp Clin Endocrinol Diabetes, 2018. **126**(2): p. 96-101.
303. Zeng, L., et al., *Noninferiority effects on glycemic control and beta-cell function improvement in newly diagnosed type 2 diabetes patients: basal insulin monotherapy versus continuous subcutaneous insulin infusion treatment.* Diabetes Technol Ther, 2012. **14**(1): p. 35-42.
304. Nishimura, S., et al., *CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity.* Nat Med, 2009. **15**(8): p. 914-20.
305. Pindjakova, J., et al., *Gut Dysbiosis and Adaptive Immune Response in Diet-induced Obesity vs. Systemic Inflammation.* Front Microbiol, 2017. **8**: p. 1157.
306. Menart-Houtermans, B., et al., *Leukocyte profiles differ between type 1 and type 2 diabetes and are associated with metabolic phenotypes: results from the German Diabetes Study (GDS).* Diabetes Care, 2014. **37**(8): p. 2326-33.
307. Rausch, M.E., et al., *Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration.* Int J Obes (Lond), 2008. **32**(3): p. 451-63.
308. Monteiro-Sepulveda, M., et al., *Jejunal T Cell Inflammation in Human Obesity Correlates with Decreased Enterocyte Insulin Signaling.* Cell Metab, 2015. **22**(1): p. 113-24.
309. Miya, A., et al., *Impact of Glucose Loading on Variations in CD4(+) and CD8(+) T Cells in Japanese Participants with or without Type 2 Diabetes.* Front Endocrinol (Lausanne), 2018. **9**: p. 81.
310. Winer, D.A., et al., *B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies.* Nat Med, 2011. **17**(5): p. 610-7.
311. Winer, S., et al., *Normalization of obesity-associated insulin resistance through immunotherapy.* Nat Med, 2009. **15**(8): p. 921-9.
312. van Beek, L., et al., *Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance.* Metabolism, 2014. **63**(4): p. 492-501.
313. Cao, Y.L., F.Q. Zhang, and F.Q. Hao, *Th1/Th2 cytokine expression in diabetic retinopathy.* Genet Mol Res, 2016. **15**(3).
314. DeFuria, J., et al., *B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile.* Proc Natl Acad Sci U S A, 2013. **110**(13): p. 5133-8.
315. Zhou, J., et al., *Non-obese type 2 diabetes patients present intestinal B cell dysregulations associated with hyperactive intestinal Tfh cells.* Mol Immunol, 2018. **97**: p. 27-32.
316. Farnsworth, C.W., et al., *Exacerbated Staphylococcus aureus Foot Infections in Obese/Diabetic Mice Are Associated with Impaired Germinal Center Reactions, Ig Class Switching, and Humoral Immunity.* J Immunol, 2018. **201**(2): p. 560-572.
317. Mathews, C.E., et al., *Impaired function of antibodies to pneumococcal surface protein A but not to capsular polysaccharide in Mexican American adults with type 2 diabetes mellitus.* Clin Vaccine Immunol, 2012. **19**(9): p. 1360-9.
318. Schmidt, M.V., et al., *Metabolic signals modulate hypothalamic-pituitary-adrenal axis activation during maternal separation of the neonatal mouse.* J Neuroendocrinol, 2006. **18**(11): p. 865-74.
319. von Dawans, B., P. Zimmer, and G. Domes, *Effects of glucose intake on stress reactivity in young, healthy men.* Psychoneuroendocrinology, 2021. **126**: p. 105062.
320. Grova, N., et al., *Epigenetic and Neurological Impairments Associated with Early Life Exposure to Persistent Organic Pollutants.* Int J Genomics, 2019. **2019**: p. 2085496.
321. de Jong, M., et al., *Components of the metabolic syndrome in early childhood in very-low-birth-weight infants.* Horm Res Paediatr, 2014. **81**(1): p. 43-9.
322. Finken, M.J., et al., *Glucocorticoid Programming in Very Preterm Birth.* Horm Res Paediatr, 2016. **85**(4): p. 221-31.
323. Vargas, J., et al., *Early Life Stress Increases Metabolic Risk, HPA Axis Reactivity, and Depressive-Like Behavior When Combined with Postweaning Social Isolation in Rats.* PLoS One, 2016. **11**(9): p. e0162665.
324. Yau, Y.H. and M.N. Potenza, *Stress and eating behaviors.* Minerva Endocrinol, 2013. **38**(3): p. 255-67.
325. Cohen, J.I., *Stress and mental health: a biobehavioral perspective.* Issues Ment Health Nurs, 2000. **21**(2): p. 185-202.

326. Serchov, T., D. van Calker, and K. Biber, *Sucrose Preference Test to Measure Anhedonic Behaviour in Mice.* Bio-Protocol, 2016. **6**(19): p. e1958.
327. Fang, J.Y., et al., *In Vivo Rodent Models of Type 2 Diabetes and Their Usefulness for Evaluating Flavonoid Bioactivity.* Nutrients, 2019. **11**(3).
328. King, A.J., *The use of animal models in diabetes research.* Br J Pharmacol, 2012. **166**(3): p. 877-94.
329. Wang, X., G. Ye, and J. Sun, *[Leptin and the feedback regulation of body weight].* Wei Sheng Yan Jiu, 1999. **28**(5): p. 318-20.
330. Ingalls, A.M., M.M. Dickie, and G.D. Snell, *Obese, a new mutation in the house mouse.* J Hered, 1950. **41**(12): p. 317-8.
331. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-32.
332. Hummel, K.P., M.M. Dickie, and D.L. Coleman, *Diabetes, a new mutation in the mouse.* Science, 1966. **153**(3740): p. 1127-8.
333. Chen, H., et al., *Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice.* Cell, 1996. **84**(3): p. 491-5.
334. Pick, A., et al., *Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat.* Diabetes, 1998. **47**(3): p. 358-64.
335. Srinivasan, K. and P. Ramarao, *Animal models in type 2 diabetes research: an overview.* Indian J Med Res, 2007. **125**(3): p. 451-72.
336. Leiter, E.H. and P.C. Reifsnyder, *Differential levels of diabetogenic stress in two new mouse models of obesity and type 2 diabetes.* Diabetes, 2004. **53 Suppl 1**: p. S4-11.
337. Clee, S.M. and A.D. Attie, *The genetic landscape of type 2 diabetes in mice.* Endocr Rev, 2007. **28**(1): p. 48-83.
338. Chakraborty, G., et al., *Age dependence of glucose tolerance in adult KK-Ay mice, a model of non-insulin dependent diabetes mellitus.* Lab Anim (NY), 2009. **38**(11): p. 364-8.
339. Kim, J.H., et al., *Type 2 diabetes mouse model TallyHo carries an obesity gene on chromosome 6 that exaggerates dietary obesity.* Physiol Genomics, 2005. **22**(2): p. 171-81.
340. Kawano, K., et al., *OLETF (Otsuka Long-Evans Tokushima Fatty) rat: a new NIDDM rat strain.* Diabetes Res Clin Pract, 1994. **24 Suppl**: p. S317-20.
341. Kanemoto, N., et al., *Genetic dissection of "OLETF", a rat model for non-insulin-dependent diabetes mellitus.* Mamm Genome, 1998. **9**(6): p. 419-25.
342. Hirata, T., et al., *Pathological and gene expression analysis of a polygenic diabetes model, NONcNZO10/LtJ mice.* Gene, 2017. **629**: p. 52-58.
343. Cho, Y.R., et al., *Hyperglycemia, maturity-onset obesity, and insulin resistance in NONcNZO10/LtJ males, a new mouse model of type 2 diabetes.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E327-36.
344. Furman, B.L., *Streptozotocin-Induced Diabetic Models in Mice and Rats.* Curr Protoc, 2021. **1**(4): p. e78.
345. Graham, M.L., et al., *The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources.* Comp Med, 2011. **61**(4): p. 356-60.
346. Murea, M., L. Ma, and B.I. Freedman, *Genetic and environmental factors associated with type 2 diabetes and diabetic vascular complications.* Rev Diabet Stud, 2012. **9**(1): p. 6-22.
347. Asthana, S., et al., *IAPP in type II diabetes: Basic research on structure, molecular interactions, and disease mechanisms suggests potential intervention strategies.* Biochim Biophys Acta Biomembr, 2018. **1860**(9): p. 1765-1782.
348. de la Monte, S.M. and J.R. Wands, *Alzheimer's disease is type 3 diabetes-evidence reviewed.* J Diabetes Sci Technol, 2008. **2**(6): p. 1101-13.
349. Hoppener, J.W., et al., *Molecular physiology of the islet amyloid polypeptide (IAPP)/amylin gene in man, rat, and transgenic mice.* J Cell Biochem, 1994. **55 Suppl**: p. 39-53.
350. Goto, Y., M. Kakizaki, and N. Masaki, *Production of spontaneous diabetic rats by repetition of selective breeding.* Tohoku J Exp Med, 1976. **119**(1): p. 85-90.
351. Portha, B., et al., *The GK rat: a prototype for the study of non-overweight type 2 diabetes.* Methods Mol Biol, 2012. **933**: p. 125-59.
352. Katakura, M., et al., *Primacy of beta-cell dysfunction in the development of hyperglycemia: a study in the Japanese general population.* Metabolism, 2004. **53**(7): p. 949-53.
353. Portha, B., et al., *The GK rat beta-cell: a prototype for the diseased human beta-cell in type 2 diabetes?* Mol Cell Endocrinol, 2009. **297**(1-2): p. 73-85.

354. Homo-Delarche, F., et al., *Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat.* Diabetes, 2006. **55**(6): p. 1625-33.
355. Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes.* Diabetes, 2007. **56**(9): p. 2356-70.
356. Giroix, M.H., et al., *Hypercholesterolaemia, signs of islet microangiopathy and altered angiogenesis precede onset of type 2 diabetes in the Goto-Kakizaki (GK) rat.* Diabetologia, 2011. **54**(9): p. 2451-62.
357. Ghanaat-Pour, H., et al., *Global expression profiling of glucose-regulated genes in pancreatic islets of spontaneously diabetic Goto-Kakizaki rats.* J Mol Endocrinol, 2007. **39**(2): p. 135-50.
358. Atef, N., B. Portha, and L. Penicaud, *Changes in islet blood flow in rats with NIDDM.* Diabetologia, 1994. **37**(7): p. 677-80.
359. Svensson, A.M., et al., *Inhibition of nitric oxide synthase by NG-nitro-L-arginine causes a preferential decrease in pancreatic islet blood flow in normal rats and spontaneously diabetic GK rats.* Endocrinology, 1994. **135**(3): p. 849-53.
360. Svensson, A.M., C.G. Ostenson, and L. Jansson, *Age-induced changes in pancreatic islet blood flow: evidence for an impaired regulation in diabetic GK rats.* Am J Physiol Endocrinol Metab, 2000. **279**(5): p. E1139-44.
361. Lacraz, G., et al., *Diabetic beta-cells can achieve self-protection against oxidative stress through an adaptive up-regulation of their antioxidant defenses.* PLoS One, 2009. **4**(8): p. e6500.
362. Movassat, J., et al., *Impaired development of pancreatic beta-cell mass is a primary event during the progression to diabetes in the GK rat.* Diabetologia, 1997. **40**(8): p. 916-25.
363. Portha, B., et al., *beta-cell function and viability in the spontaneously diabetic GK rat: information from the GK/Par colony.* Diabetes, 2001. **50 Suppl 1**: p. S89-93.
364. Giroix, M.H., C. Saulnier, and B. Portha, *Decreased pancreatic islet response to L-leucine in the spontaneously diabetic GK rat: enzymatic, metabolic and secretory data.* Diabetologia, 1999. **42**(8): p. 965-77.
365. Ostenson, C.G., et al., *Abnormal insulin secretion and glucose metabolism in pancreatic islets from the spontaneously diabetic GK rat.* Diabetologia, 1993. **36**(1): p. 3-8.
366. Ling, Z.C., et al., *Glucose metabolism in Goto-Kakizaki rat islets.* Endocrinology, 1998. **139**(6): p. 2670-5.
367. MacDonald, M.J., S. Efendic, and C.G. Ostenson, *Normalization by insulin treatment of low mitochondrial glycerol phosphate dehydrogenase and pyruvate carboxylase in pancreatic islets of the GK rat.* Diabetes, 1996. **45**(7): p. 886-90.
368. Serradas, P., et al., *Mitochondrial deoxyribonucleic acid content is specifically decreased in adult, but not fetal, pancreatic islets of the Goto-Kakizaki rat, a genetic model of noninsulin-dependent diabetes.* Endocrinology, 1995. **136**(12): p. 5623-31.
369. Giroix, M.H., et al., *Metabolic, ionic, and secretory response to D-glucose in islets from rats with acquired or inherited non-insulin-dependent diabetes.* Biochem Med Metab Biol, 1993. **50**(3): p. 301-21.
370. Bisbis, S., et al., *Insulin resistance in the GK rat: decreased receptor number but normal kinase activity in liver.* Am J Physiol, 1993. **265**(5 Pt 1): p. E807-13.
371. Movassat, J., et al., *Follow-up of GK rats during prediabetes highlights increased insulin action and fat deposition despite low insulin secretion.* Am J Physiol Endocrinol Metab, 2008. **294**(1): p. E168-75.
372. Gill-Randall, R., et al., *Type 2 diabetes mellitus--genes or intrauterine environment? An embryo transfer paradigm in rats.* Diabetologia, 2004. **47**(8): p. 1354-9.
373. Calderari, S., et al., *Is defective pancreatic beta-cell mass environmentally programmed in Goto-Kakizaki rat model of type 2 diabetes?: insights from crossbreeding studies during suckling period.* Pancreas, 2006. **33**(4): p. 412-7.
374. Barker, D.J. and C. Osmond, *Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales.* Lancet, 1986. **1**(8489): p. 1077-81.
375. McEwen, B.S., *Understanding the potency of stressful early life experiences on brain and body function.* Metabolism, 2008. **57 Suppl 2**(Suppl 2): p. S11-5.
376. Alastalo, H., et al., *Cardiovascular health of Finnish war evacuees 60 years later.* Ann Med, 2009. **41**(1): p. 66-72.
377. Eriksson, M., K. Raikkonen, and J.G. Eriksson, *Early life stress and later health outcomes--findings from the Helsinki Birth Cohort Study.* Am J Hum Biol, 2014. **26**(2): p. 111-6.
378. Gardner, R., et al., *Adverse childhood experiences are associated with an increased risk of obesity in early adolescence: a population-based prospective cohort study.* Pediatr Res, 2019. **86**(4): p. 522-528.

379. Deschenes, S.S., et al., *Adverse Childhood Experiences and the Risk of Diabetes: Examining the Roles of Depressive Symptoms and Cardiometabolic Dysregulations in the Whitehall II Cohort Study.* Diabetes Care, 2018. **41**(10): p. 2120-2126.
380. Seal, S.V. and J.D. Turner, *The 'Jekyll and Hyde' of Gluconeogenesis: Early Life Adversity, Later Life Stress, and Metabolic Disturbances.* Int J Mol Sci, 2021. **22**(7).
381. Gunnar, M.R., *A commentary on deprivation-specific psychological patterns: effects of institutional deprivation.* Monogr Soc Res Child Dev, 2010. **75**(1): p. 232-47.
382. Koss, K.J., et al., *Social deprivation and the HPA axis in early development.* Psychoneuroendocrinology, 2014. **50**: p. 1-13.
383. Kroupina, M.G., et al., *Adoption as an intervention for institutionally reared children: HPA functioning and developmental status.* Infant Behav Dev, 2012. **35**(4): p. 829-37.
384. van, I.M.H., et al., *Children in Institutional Care: Delayed Development and Resilience.* Monogr Soc Res Child Dev, 2011. **76**(4): p. 8-30.
385. Hengesch, X., et al., *Blunted endocrine response to a combined physical-cognitive stressor in adults with early life adversity.* Child Abuse Negl, 2018. **85**(1873-7757 (Electronic)): p. 137-144.
386. Elwenspoek, M.M.C., et al., *Glucocorticoid receptor signaling in leukocytes after early life adversity.* Dev Psychopathol, 2020. **32**(3): p. 853-863.
387. Plotsky, P.M. and M.J. Meaney, *Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats.* Brain Res Mol Brain Res, 1993. **18**(3): p. 195-200.
388. Rincel, M., et al., *Maternal high-fat diet prevents developmental programming by early-life stress.* Transl Psychiatry, 2016. **6**(11): p. e966.
389. Merz, M.P., *'A TALE OF ADVERSITY: THE IMPACT OF EARLY LIFE INFECTION BEYOND THE IMMUNE SYSTEM'*. 2022, University of Luxembourg: Esch-sur-Alzette, Luxembourg. p. 146.
390. Yurtsever, T., et al., *The acute and temporary modulation of PERIOD genes by hydrocortisone in healthy subjects.* Chronobiol Int, 2016. **33**(9): p. 1222-1234.
391. Schilling, T.M., et al., *For whom the bell (curve) tolls: cortisol rapidly affects memory retrieval by an inverted U-shaped dose-response relationship.* Psychoneuroendocrinology, 2013. **38**(9): p. 1565-72.
392. Charalambous, E.G., et al., *Early-Life Adversity Leaves Its Imprint on the Oral Microbiome for More Than 20 Years and Is Associated with Long-Term Immune Changes.* Int J Mol Sci, 2021. **22**(23).
393. Fernandes, S.B., et al., *Unbiased Screening Identifies Functional Differences in NK Cells After Early Life Psychosocial Stress.* Front Immunol, 2021. **12**: p. 674532.
394. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
395. Schote, A.B., et al., *Nuclear receptors in human immune cells: expression and correlations.* Mol Immunol, 2007. **44**(6): p. 1436-45.
396. Pruessner, J.C., et al., *Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change.* Psychoneuroendocrinology, 2003. **28**(7): p. 916-31.
397. Guzylack-Piriou, L. and S. Menard, *Early Life Exposure to Food Contaminants and Social Stress as Risk Factor for Metabolic Disorders Occurrence?-An Overview.* Biomolecules, 2021. **11**(5).
398. Clore, J.N. and L. Thurby-Hay, *Glucocorticoid-induced hyperglycemia.* Endocr Pract, 2009. **15**(5): p. 469-74.
399. Kirschbaum, C., K.M. Pirke, and D.H. Hellhammer, *The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting.* Neuropsychobiology, 1993. **28**(1-2): p. 76-81.
400. Kirschbaum, C. and D.H. Hellhammer, *Salivary cortisol in psychoneuroendocrine research: recent developments and applications.* Psychoneuroendocrinology, 1994. **19**(4): p. 313-33.
401. Barker, D.J.P., *Editorial: The Developmental Origins of Adult Disease.* European Journal of Epidemiology, 2003. **18**(8): p. 733-736.
402. Hochberg, Z., et al., *Child health, developmental plasticity, and epigenetic programming.* Endocr Rev, 2011. **32**(2): p. 159-224.
403. Lesage, J., et al., *Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat.* J Endocrinol, 2004. **181**(2): p. 291-6.
404. Ilchmann-Diounou, H., et al., *Early life stress induces type 2 diabetes-like features in ageing mice.* Brain Behav Immun, 2019. **80**: p. 452-463.

405. Stimson, R.H., et al., *Acute physiological effects of glucocorticoids on fuel metabolism in humans are permissive but not direct.* Diabetes Obes Metab, 2017. **19**(6): p. 883-891.
406. Dekkers, O.M., et al., *Multisystem morbidity and mortality in Cushing's syndrome: a cohort study.* J Clin Endocrinol Metab, 2013. **98**(6): p. 2277-84.
407. Mazziotti, G., C. Gazzaruso, and A. Giustina, *Diabetes in Cushing syndrome: basic and clinical aspects.* Trends Endocrinol Metab, 2011. **22**(12): p. 499-506.
408. Wellen, K.E. and G.S. Hotamisligil, *Inflammation, stress, and diabetes.* J Clin Invest, 2005. **115**(5): p. 1111-9.
409. Yi, C.X., et al., *The role of the autonomic nervous liver innervation in the control of energy metabolism.* Biochim Biophys Acta, 2010. **1802**(4): p. 416-31.
410. Li, L., et al., *Acute psychological stress results in the rapid development of insulin resistance.* J Endocrinol, 2013. **217**(2): p. 175-84.
411. Pocai, A., et al., *A brain-liver circuit regulates glucose homeostasis.* Cell Metab, 2005. **1**(1): p. 53-61.
412. Matsuhisa, M., et al., *Important role of the hepatic vagus nerve in glucose uptake and production by the liver.* Metabolism, 2000. **49**(1): p. 11-6.
413. Li, J.H., et al., *Hepatic muscarinic acetylcholine receptors are not critically involved in maintaining glucose homeostasis in mice.* Diabetes, 2009. **58**(12): p. 2776-87.
414. Li, X., et al., *Intracerebroventricular leptin infusion improves glucose homeostasis in lean type 2 diabetic MKR mice via hepatic vagal and non-vagal mechanisms.* PLoS One, 2011. **6**(2): p. e17058.
415. Huang, F., et al., *Effect of transcutaneous auricular vagus nerve stimulation on impaired glucose tolerance: a pilot randomized study.* BMC Complement Altern Med, 2014. **14**: p. 203.
416. Cardin, S., et al., *Involvement of the vagus nerves in the regulation of basal hepatic glucose production in conscious dogs.* Am J Physiol Endocrinol Metab, 2002. **283**(5): p. E958-64.
417. Harp, J.B., G.D. Yancopoulos, and J. Gromada, *Glucagon orchestrates stress-induced hyperglycaemia.* Diabetes Obes Metab, 2016. **18**(7): p. 648-53.
418. Berbudi, A., et al., *Type 2 Diabetes and its Impact on the Immune System.* Curr Diabetes Rev, 2020. **16**(5): p. 442-449.
419. de Candia, P., et al., *Type 2 Diabetes: How Much of an Autoimmune Disease?* Front Endocrinol (Lausanne), 2019. **10**: p. 451.
420. Ahlqvist, E., R.B. Prasad, and L. Groop, *Subtypes of Type 2 Diabetes Determined From Clinical Parameters.* Diabetes, 2020. **69**(10): p. 2086-2093.
421. Kietsiriroje, N., et al., *Double diabetes: A distinct high-risk group?* Diabetes Obes Metab, 2019. **21**(12): p. 2609-2618.
422. Takeda, Y., et al., *Relationship between Immunological Abnormalities in Rat Models of Diabetes Mellitus and the Amplification Circuits for Diabetes.* J Diabetes Res, 2017. **2017**: p. 4275851.
423. Lasselin, J. and L. Capuron, *Chronic low-grade inflammation in metabolic disorders: relevance for behavioral symptoms.* Neuroimmunomodulation, 2014. **21**(2-3): p. 95-101.
424. Capuron, L., J. Lasselin, and N. Castanon, *Role of Adiposity-Driven Inflammation in Depressive Morbidity.* Neuropsychopharmacology, 2017. **42**(1): p. 115-128.
425. Takeda, Y. and I. Wakabayashi, *Alteration in lymphocyte population and humoral immune response in type 2 diabetic Goto-Kakizaki rats.* Life Sci, 2012. **90**(13-14): p. 545-52.
426. Takeda, Y., M. Marumo, and I. Wakabayashi, *Attenuated phagocytic activity of monocytes in type 2 diabetic Goto-Kakizaki rats.* Immunobiology, 2011. **216**(10): p. 1094-102.
427. Galvez, I., et al., *Anti-inflammatory effect of beta2 adrenergic stimulation on circulating monocytes with a pro-inflammatory state in high-fat diet-induced obesity.* Brain Behav Immun, 2019. **80**: p. 564-572.
428. Ehses, J.A., et al., *IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13998-4003.
429. Pitasi, C.L., et al., *Implication of glycogen synthase kinase 3 in diabetes-associated islet inflammation.* J Endocrinol, 2020. **244**(1): p. 133-148.
430. Xue, B., et al., *Effects of High Fat Feeding on Adipose Tissue Gene Expression in Diabetic Goto-Kakizaki Rats.* Gene Regul Syst Bio, 2015. **9**: p. 15-26.
431. Bourgneuf, C., et al., *The Goto-Kakizaki rat is a spontaneous prototypical rodent model of polycystic ovary syndrome.* Nat Commun, 2021. **12**(1): p. 1064.
432. Xue, B., et al., *Adipose tissue deficiency and chronic inflammation in diabetic Goto-Kakizaki rats.* PLoS One, 2011. **6**(2): p. e17386.

433. Huda, N., et al., *Genetic variation of the transcription factor GATA3, not STAT4, is associated with the risk of type 2 diabetes in the Bangladeshi population.* PLoS One, 2018. **13**(7): p. e0198507.
434. Katherine A. Fitzgerald, L.A.J.O.N., ... Robin E. Callard, *The Cytokine FactsBook and Webfacts*. second ed. 2001.
435. Greaves, D.R., et al., *Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions.* Arterioscler Thromb Vasc Biol, 2001. **21**(6): p. 923-9.
436. Sawant, K.V., et al., *Chemokine CXCL1-Mediated Neutrophil Trafficking in the Lung: Role of CXCR2 Activation.* J Innate Immun, 2015. **7**(6): p. 647-58.
437. Richard A. Polin, W.W.F.a.S.H.A., *Fetal and Neonatal Physiology*. 4th ed. 2011.
438. Liu, J., et al., *Interleukin-12: an update on its immunological activities, signaling and regulation of gene expression.* Curr Immunol Rev, 2005. **1**(2): p. 119-137.
439. Rousselle, A., et al., *CXCL5 limits macrophage foam cell formation in atherosclerosis.* J Clin Invest, 2013. **123**(3): p. 1343-7.
440. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.
441. Parameswaran, N. and S. Patial, *Tumor necrosis factor-alpha signaling in macrophages.* Crit Rev Eukaryot Gene Expr, 2010. **20**(2): p. 87-103.
442. Qin, C.C., et al., *Macrophage inflammatory protein-2 as mediator of inflammation in acute liver injury.* World J Gastroenterol, 2017. **23**(17): p. 3043-3052.
443. Yasuda, K., K. Nakanishi, and H. Tsutsui, *Interleukin-18 in Health and Disease.* Int J Mol Sci, 2019. **20**(3).
444. *Encyclopedia of Immunobiology*. 2016.
445. Sa, V.C., et al., *The pattern of immune cell infiltration in chromoblastomycosis: involvement of macrophage inflammatory protein-1 alpha/CCL3 and fungi persistence.* Rev Inst Med Trop Sao Paulo, 2007. **49**(1): p. 49-53.
446. Bhat, P., et al., *Interferon-gamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity.* Cell Death Dis, 2017. **8**(6): p. e2836.
447. Nelson, A.J., W.R. Staines, and W.E. McIlroy, *Tactile stimulus predictability modulates activity in a tactile-motor cortical network.* Exp Brain Res, 2004. **154**(1): p. 22-32.
448. Kuen, D.S., B.S. Kim, and Y. Chung, *IL-17-Producing Cells in Tumor Immunity: Friends or Foes?* Immune Netw, 2020. **20**(1): p. e6.
449. Espinosa De Ycaza, A.E., et al., *Adipose Tissue Inflammation Is Not Related to Adipose Insulin Resistance in Humans.* Diabetes, 2022. **71**(3): p. 381-393.
450. Berthelier, C., M. Kergoat, and B. Portha, *Lack of deterioration of insulin action with aging in the GK rat: a contrasted adaptation as compared with nondiabetic rats.* Metabolism, 1997. **46**(8): p. 890-6.
451. Eldar-Finkelman, H., et al., *Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice.* Diabetes, 1999. **48**(8): p. 1662-6.
452. Nikoulina, S.E., et al., *Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes.* Diabetes, 2000. **49**(2): p. 263-71.
453. Zhai, X., et al., *Elevated B Cell Activation is Associated with Type 2 Diabetes Development in Obese Subjects.* Cell Physiol Biochem, 2016. **38**(3): p. 1257-66.
454. den Dekker, A., et al., *Targeting epigenetic mechanisms in diabetic wound healing.* Transl Res, 2019. **204**: p. 39-50.
455. Wong, S.L., et al., *Diabetes primes neutrophils to undergo NETosis, which impairs wound healing.* Nat Med, 2015. **21**(7): p. 815-9.
456. Lauková, L. and B. Konečná, *NETosis - Dr. Jekyll and Mr. Hyde in inflammation.* Journal of Applied Biomedicine, 2018. **16**(1): p. 1-9.
457. Casqueiro, J., J. Casqueiro, and C. Alves, *Infections in patients with diabetes mellitus: A review of pathogenesis.* Indian J Endocrinol Metab, 2012. **16 Suppl 1**(Suppl1): p. S27-36.
458. Damoiseaux, J.G., et al., *A dominant role for the thymus and MHC genes in determining the peripheral CD4/CD8 T cell ratio in the rat.* J Immunol, 1999. **163**(6): p. 2983-9.
459. Mahlangu, T., et al., *A systematic review on the functional role of Th1/Th2 cytokines in type 2 diabetes and related metabolic complications.* Cytokine, 2020. **126**: p. 154892.
460. Houman, H., et al., *Tc1/Tc2 ratio in the inflammatory process in patients with Behcet's disease.* Mediators Inflamm, 2004. **13**(4): p. 247-53.

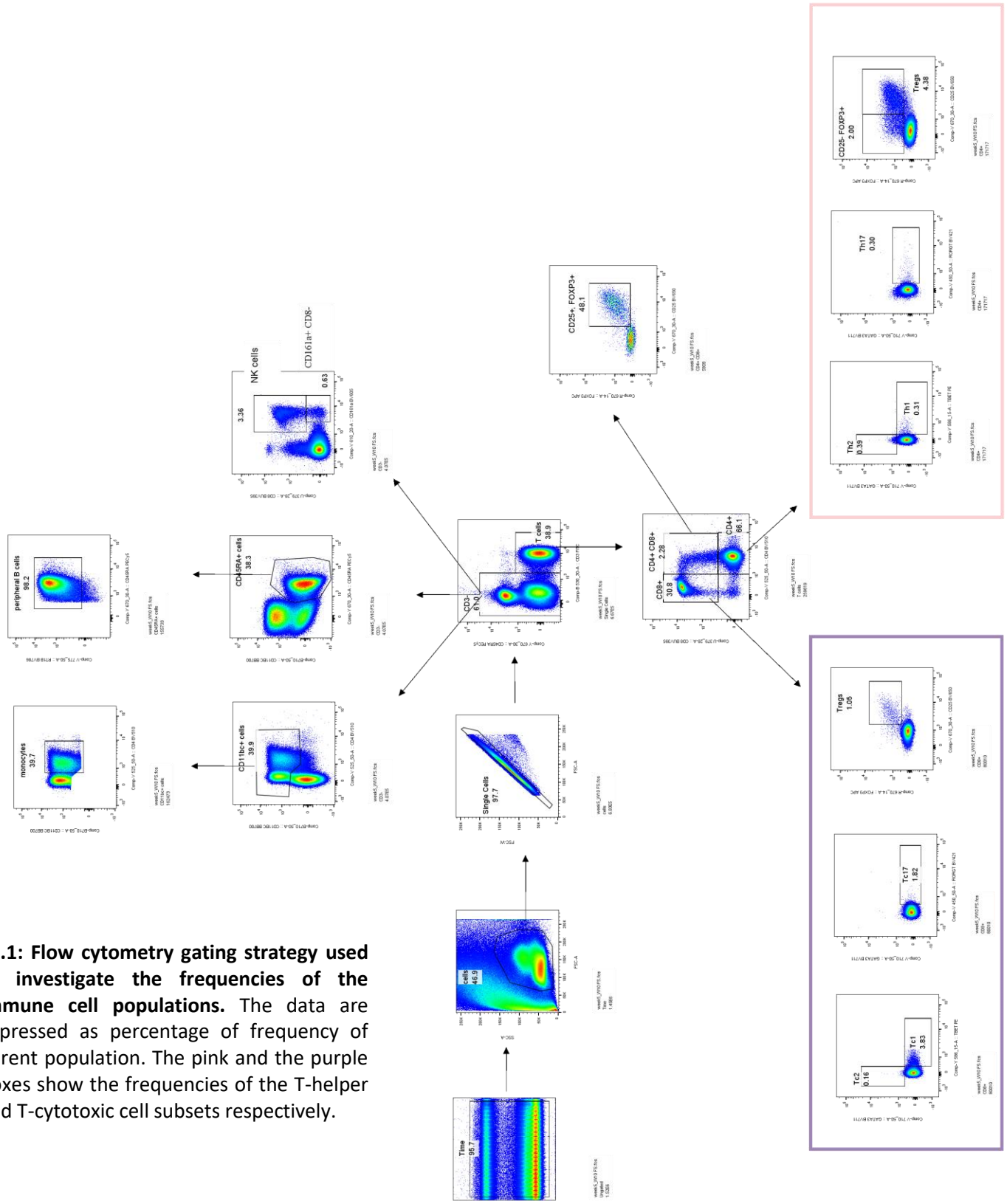
461. Zhang, S., et al., *The Alterations in and the Role of the Th17/Treg Balance in Metabolic Diseases.* Front Immunol, 2021. **12**: p. 678355.
462. Simar, D., et al., *DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human.* Metabolism, 2014. **63**(9): p. 1188-97.
463. Mxinwa, V., et al., *Natural killer cell levels in adults living with type 2 diabetes: a systematic review and meta-analysis of clinical studies.* BMC Immunol, 2020. **21**(1): p. 51.
464. Chellappa, S., et al., *CD8+ T Cells That Coexpress RORgammat and T-bet Are Functionally Impaired and Expand in Patients with Distal Bile Duct Cancer.* J Immunol, 2017. **198**(4): p. 1729-1739.
465. Satoh, T., et al., *The development of IL-17/IFN-gamma-double producing CTLs from Tc17 cells is driven by epigenetic suppression of Socs3 gene promoter.* Eur J Immunol, 2012. **42**(9): p. 2329-42.
466. Palanivel, R., et al., *Reduced Socs3 expression in adipose tissue protects female mice against obesity-induced insulin resistance.* Diabetologia, 2012. **55**(11): p. 3083-93.
467. Liang, Y., H.F. Pan, and D.Q. Ye, *Tc17 Cells in Immunity and Systemic Autoimmunity.* Int Rev Immunol, 2015. **34**(4): p. 318-31.
468. Vizler, C., et al., *Relative diabetogenic properties of islet-specific Tc1 and Tc2 cells in immunocompetent hosts.* J Immunol, 2000. **165**(11): p. 6314-21.
469. Kumar, N.P., et al., *Prediabetes is associated with the modulation of antigen-specific Th1/Tc1 and Th17/Tc17 responses in latent Mycobacterium tuberculosis infection.* PLoS One, 2017. **12**(5): p. e0178000.
470. Pfeiffer, A., et al., *Circulating tumor necrosis factor alpha is elevated in male but not in female patients with type II diabetes mellitus.* Horm Metab Res, 1997. **29**(3): p. 111-4.
471. Nicoletti, F., et al., *Serum concentrations of the interferon-gamma-inducible chemokine IP-10/CXCL10 are augmented in both newly diagnosed Type I diabetes mellitus patients and subjects at risk of developing the disease.* Diabetologia, 2002. **45**(8): p. 1107-10.
472. Chang, C.C., et al., *Interferon gamma-induced protein 10 is associated with insulin resistance and incident diabetes in patients with nonalcoholic fatty liver disease.* Sci Rep, 2015. **5**: p. 10096.
473. Jain, S.K., et al., *The effect of sleep apnea and insomnia on blood levels of leptin, insulin resistance, IP-10, and hydrogen sulfide in type 2 diabetic patients.* Metab Syndr Relat Disord, 2012. **10**(5): p. 331-6.
474. Chen, C. and Y. Shao, *Elevated Interleukin-17 Levels in Patients with Newly Diagnosed Type 2 Diabetes Mellitus.* Biochemistry & Physiology: Open Access, 2016. **5**(2).
475. Parhi, A., *The Level and Role of Interleukin-17 in Patients of Type 2 Diabetes Mellitus with and without Complications* Journal of Diabetes Mellitus, 2019. **9**.
476. El-Omar, M.M., et al., *Cardiac dysfunction in the Goto-Kakizaki rat. A model of type II diabetes mellitus.* Basic Res Cardiol, 2004. **99**(2): p. 133-41.
477. Meagher, P., et al., *The Goto Kakizaki rat: Impact of age upon changes in cardiac and renal structure, function.* PLoS One, 2021. **16**(6): p. e0252711.
478. Park, J.M., et al., *Postprandial hypoglycemic effect of mulberry leaf in Goto-Kakizaki rats and counterpart control Wistar rats.* Nutr Res Pract, 2009. **3**(4): p. 272-8.
479. Tsalamandris, S., et al., *The Role of Inflammation in Diabetes: Current Concepts and Future Perspectives.* Eur Cardiol, 2019. **14**(1): p. 50-59.
480. Calle, M.C. and M.L. Fernandez, *Inflammation and type 2 diabetes.* Diabetes Metab, 2012. **38**(3): p. 183-91.
481. Pezhman, L., A. Tahrani, and M. Chimen, *Dysregulation of Leukocyte Trafficking in Type 2 Diabetes: Mechanisms and Potential Therapeutic Avenues.* Front Cell Dev Biol, 2021. **9**: p. 624184.
482. Wright, R.D. and D. Cooper, *Glycobiology of leukocyte trafficking in inflammation.* Glycobiology, 2014. **24**(12): p. 1242-51.
483. Liu, Y., et al., *High-fat diet triggers obesity-related early infiltration of macrophages into adipose tissue and transient reduction of blood monocyte count.* Mol Immunol, 2020. **117**: p. 139-146.
484. Brake, D.K., et al., *ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice.* Am J Physiol Cell Physiol, 2006. **291**(6): p. C1232-9.
485. Musilli, C., et al., *Characterization of circulating and monocyte-derived dendritic cells in obese and diabetic patients.* Mol Immunol, 2011. **49**(1-2): p. 234-8.
486. Mraz, M., et al., *Dendritic Cells in Subcutaneous and Epicardial Adipose Tissue of Subjects with Type 2 Diabetes, Obesity, and Coronary Artery Disease.* Mediators Inflamm, 2019. **2019**: p. 5481725.
487. Chen, Y., et al., *Adipose tissue dendritic cells enhances inflammation by prompting the generation of Th17 cells.* PLoS One, 2014. **9**(3): p. e92450.

488. Elgazar-Carmon, V., et al., *Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding.* J Lipid Res, 2008. **49**(9): p. 1894-903.
489. Talukdar, S., et al., *Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase.* Nat Med, 2012. **18**(9): p. 1407-12.
490. Duffaut, C., et al., *Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity.* Biochem Biophys Res Commun, 2009. **384**(4): p. 482-5.
491. Feuerer, M., et al., *Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters.* Nat Med, 2009. **15**(8): p. 930-9.
492. Cao, Y., et al., *Modeling diabetes disease progression and salsalate intervention in Goto-Kakizaki rats.* J Pharmacol Exp Ther, 2011. **339**(3): p. 896-904.
493. Kalsbeek, A., S. la Fleur, and E. Fliers, *Circadian control of glucose metabolism.* Mol Metab, 2014. **3**(4): p. 372-83.
494. Viswanathan, K. and F.S. Dhabhar, *Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation.* Proc Natl Acad Sci U S A, 2005. **102**(16): p. 5808-13.
495. Dhabhar, F.S., et al., *Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner.* Psychoneuroendocrinology, 2012. **37**(9): p. 1345-68.
496. Seal, S.V., et al., *A Holistic View of the Goto-Kakizaki Rat Immune System: Decreased Circulating Immune Markers in Non- Obese Type 2 Diabetes.* Front Immunol, 2022. **13**: p. 896179.
497. Seal, S.V., et al., *Stressing Glucose: At the Crossroads of Early Life Adversity, HPA Axis Reactivity and Carbohydrate Metabolism.* Preprints, 2022: p. 2022070311.
498. Liu, T., et al., *Comparative Genome of GK and Wistar Rats Reveals Genetic Basis of Type 2 Diabetes.* PLoS One, 2015. **10**(11): p. e0141859.
499. Trifonova, S.T., et al., *Diurnal redistribution of human lymphocytes and their temporal associations with salivary cortisol.* Chronobiol Int, 2013. **30**(5): p. 669-81.
500. Sunena and D.N. Mishra, *Stress Etiology of Type 2 Diabetes.* Curr Diabetes Rev, 2022. **18**(9): p. e240222201413.
501. John, A., et al., *Alterations in Energy Metabolism, Mitochondrial Function and Redox Homeostasis in GK Diabetic Rat Tissues Treated with Aspirin.* Life (Basel), 2022. **12**(1).
502. Xu, L., et al., *Hepatic Macrophage as a Key Player in Fatty Liver Disease.* Front Immunol, 2021. **12**: p. 708978.
503. Vorobjeva, N.V. and B.V. Chernyak, *NETosis: Molecular Mechanisms, Role in Physiology and Pathology.* Biochemistry (Mosc), 2020. **85**(10): p. 1178-1190.
504. Johnson, J., et al., *Oxidative Stress in Neutrophils: Implications for Diabetic Cardiovascular Complications.* Antioxid Redox Signal, 2022. **36**(10-12): p. 652-666.
505. Viardot, A., et al., *Potential antiinflammatory role of insulin via the preferential polarization of effector T cells toward a T helper 2 phenotype.* Endocrinology, 2007. **148**(1): p. 346-53.
506. Clow, A., F. Hucklebridge, and L. Thorn, *The cortisol awakening response in context.* Int Rev Neurobiol, 2010. **93**: p. 153-75.
507. Seckl, J.R., *11beta-hydroxysteroid dehydrogenases: changing glucocorticoid action.* Curr Opin Pharmacol, 2004. **4**(6): p. 597-602.
508. Hench, P.S., E.C. Kendall, and et al., *The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis.* Proc Staff Meet Mayo Clin, 1949. **24**(8): p. 181-97.
509. Maydych, V., *The Interplay Between Stress, Inflammation, and Emotional Attention: Relevance for Depression.* Front Neurosci, 2019. **13**: p. 384.
510. Fink, G., *Stress: Neuroendocrinology and Neurobiology*. 2017.
511. Elwenspoek, M.M.C., et al., *T Cell Immunosenescence after Early Life Adversity: Association with Cytomegalovirus Infection.* Front Immunol, 2017. **8**: p. 1263.
512. Shirtcliff, E.A., C.L. Coe, and S.D. Pollak, *Early childhood stress is associated with elevated antibody levels to herpes simplex virus type 1.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2963-7.
513. FERNANDES, S.B.G., *DNA METHYLATION: CONDUCTING THE ORCHESTRA FROM EXPOSURE TO PHENOTYPES*. 2021, University of Luxembourg DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG EN BIOLOGIE PhD thesis 2021: Esch-sur-Alzette, Luxembourg.
514. Martha M. C. Elwenspoek, *Phenotype and Mechanisms of Altered Immune Functions induced by Early Life Adversity*. 2017, UNIVERSITÄT TRIER: Trier, Germany.

515. Xu, X., et al., *The Effect of Metformin on the Expression of GPR109A, NF-kappaB and IL-1beta in Peripheral Blood Leukocytes from Patients with Type 2 Diabetes Mellitus.* Ann Clin Lab Sci, 2017. **47**(5): p. 556-562.
516. Buldak, L., et al., *Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages.* Pharmacol Rep, 2014. **66**(3): p. 418-29.
517. Mestas, J. and C.C. Hughes, *Of mice and not men: differences between mouse and human immunology.* J Immunol, 2004. **172**(5): p. 2731-8.
518. Beura, L.K., et al., *Normalizing the environment recapitulates adult human immune traits in laboratory mice.* Nature, 2016. **532**(7600): p. 512-6.
519. Dias, B.G. and K.J. Ressler, *Parental olfactory experience influences behavior and neural structure in subsequent generations.* Nat Neurosci, 2014. **17**(1): p. 89-96.
520. Alpert, A., et al., *A clinically meaningful metric of immune age derived from high-dimensional longitudinal monitoring.* Nat Med, 2019. **25**(3): p. 487-495.
521. Moura, J., et al., *Immune aging in diabetes and its implications in wound healing.* Clin Immunol, 2019. **200**: p. 43-54.

Appendix 1

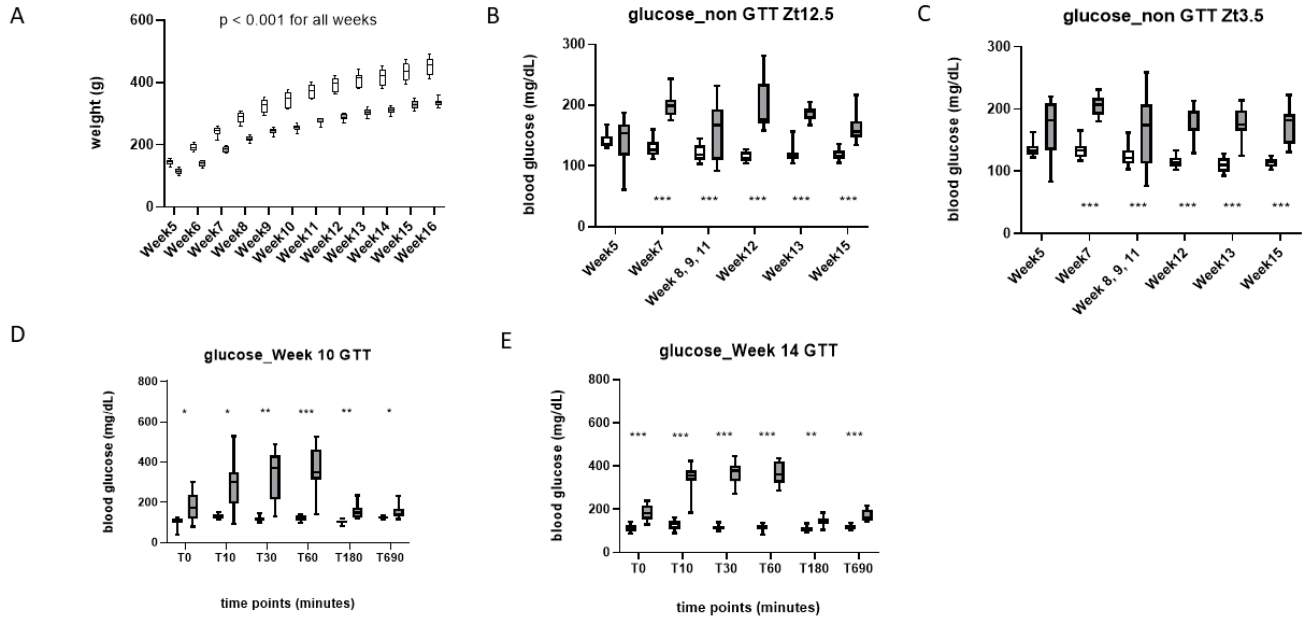
Appendix 1 – Section 1 for Chapter 4



S4.1: Flow cytometry gating strategy used to investigate the frequencies of the immune cell populations. The data are expressed as percentage of frequency of parent population. The pink and the purple boxes show the frequencies of the T-helper and T-cytotoxic cell subsets respectively.

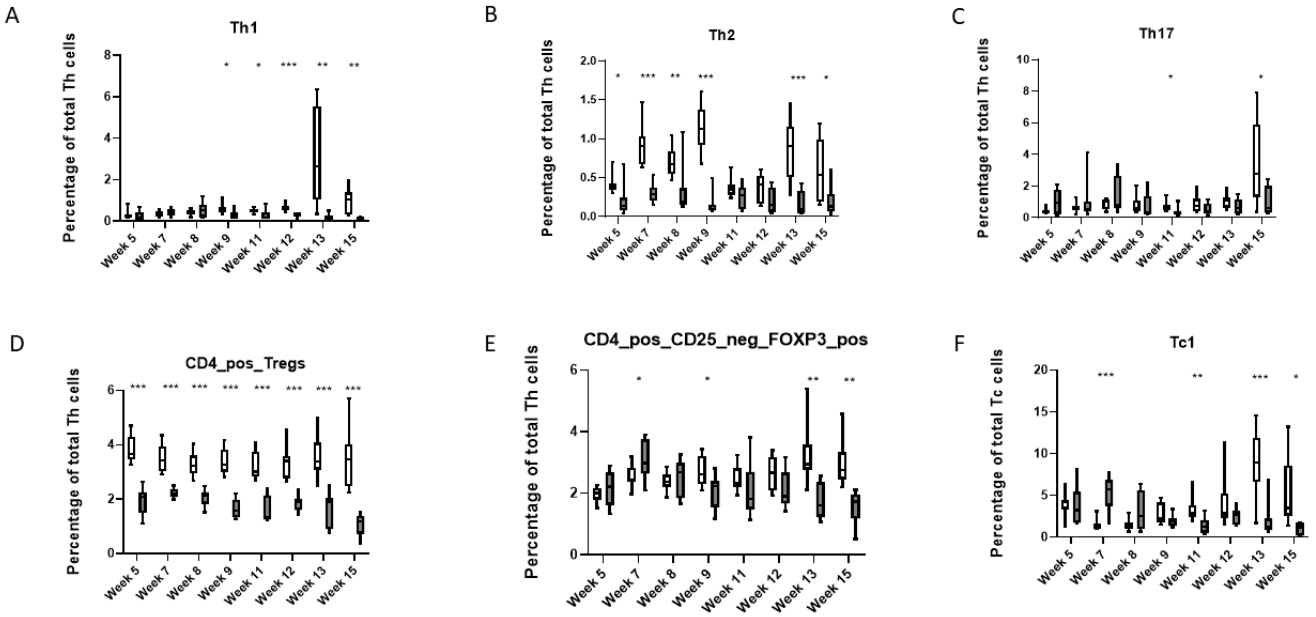
Appendix 1 – Section 2 for Chapter 4

S4.2: Weight (A) and glycaemia profiles during ZT12.5 (B), ZT3.5 (C) and metabolic challenge in the pre- (D) and early diabetic (E) phases for GK and Wistars rats as they mature. Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats. Unpaired 2 sample unequal variance t-tests have been used to analyse the data.

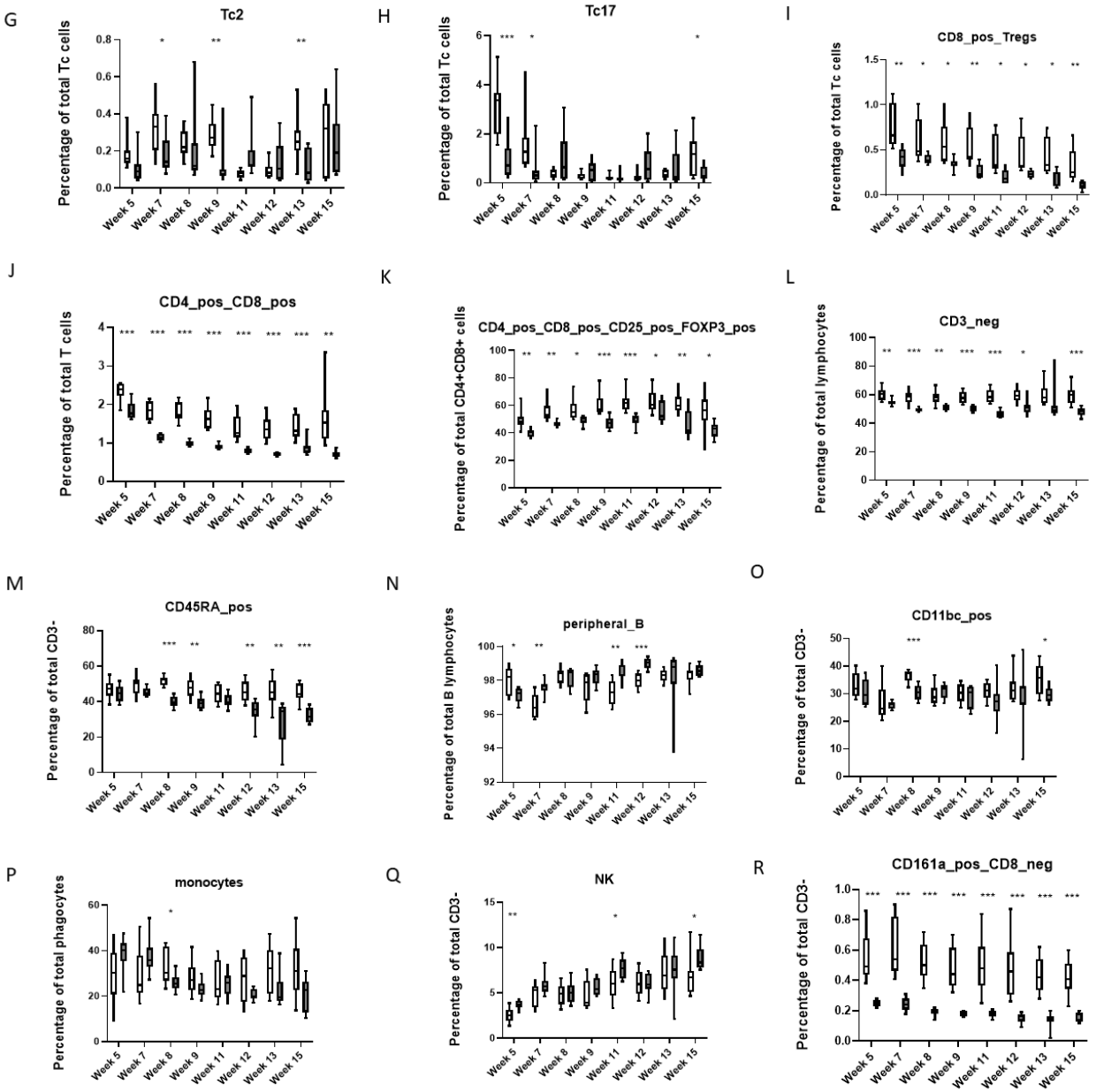


Appendix 1 – Section 3 for Chapter 4

S4.3: Immune changes in GK and Wistar rats as they mature. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data.

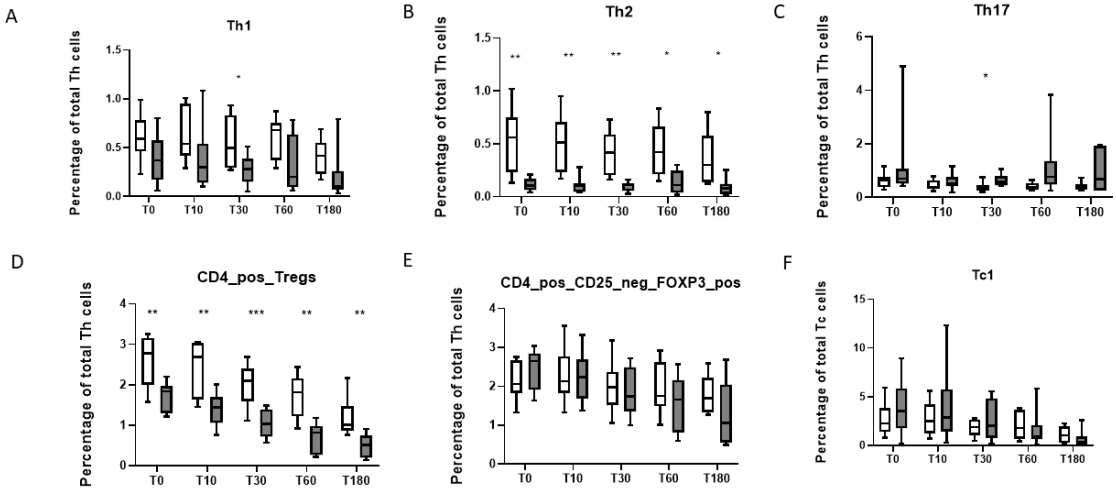


S4.3: Immune changes in GK and Wistar rats as they mature (contd). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data.

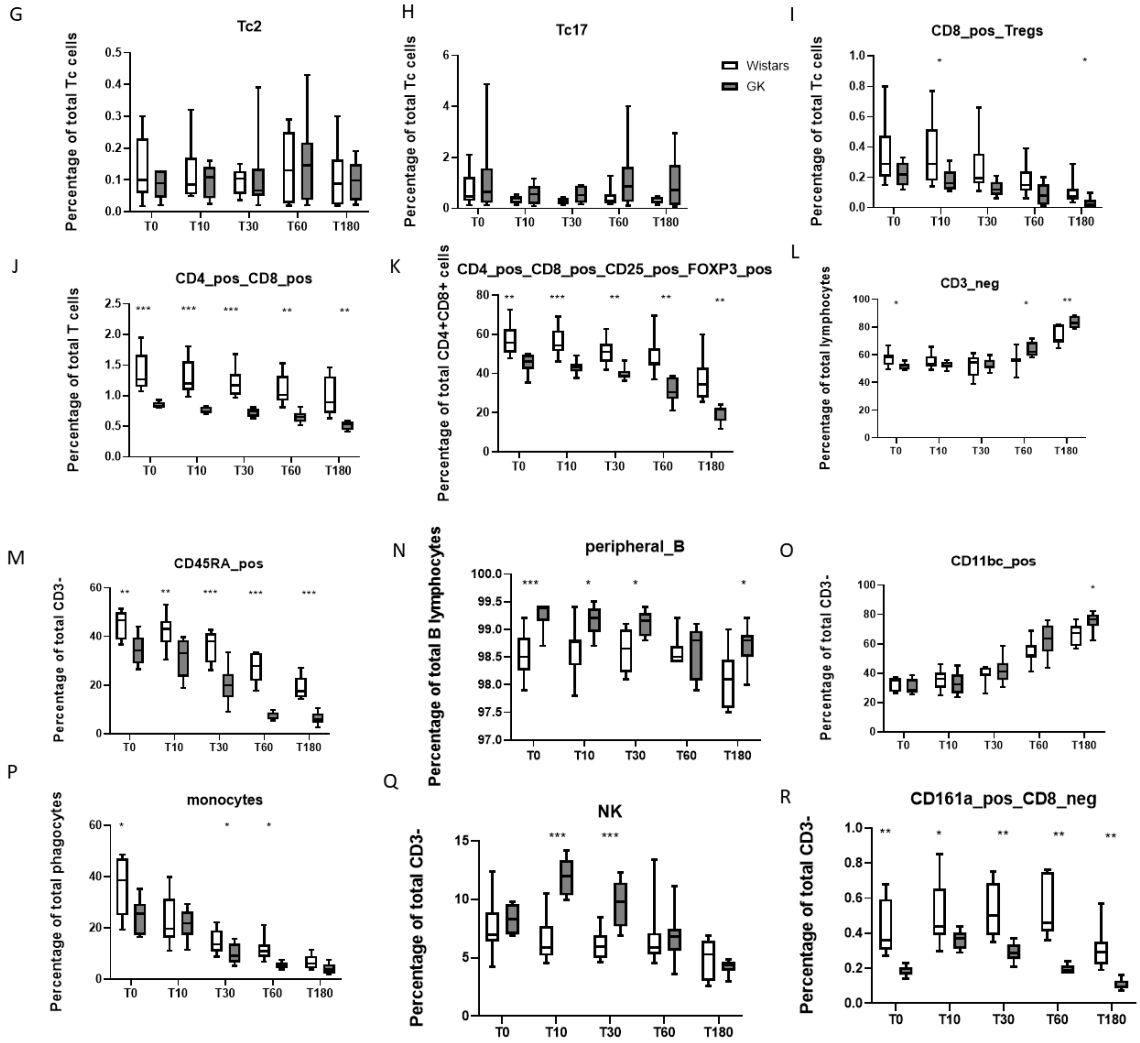


Appendix 1 – Section 4 for Chapter 4

S4.4: Immune changes in GK and Wistar rats after metabolic challenge in the prediabetic phase (week 10). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).

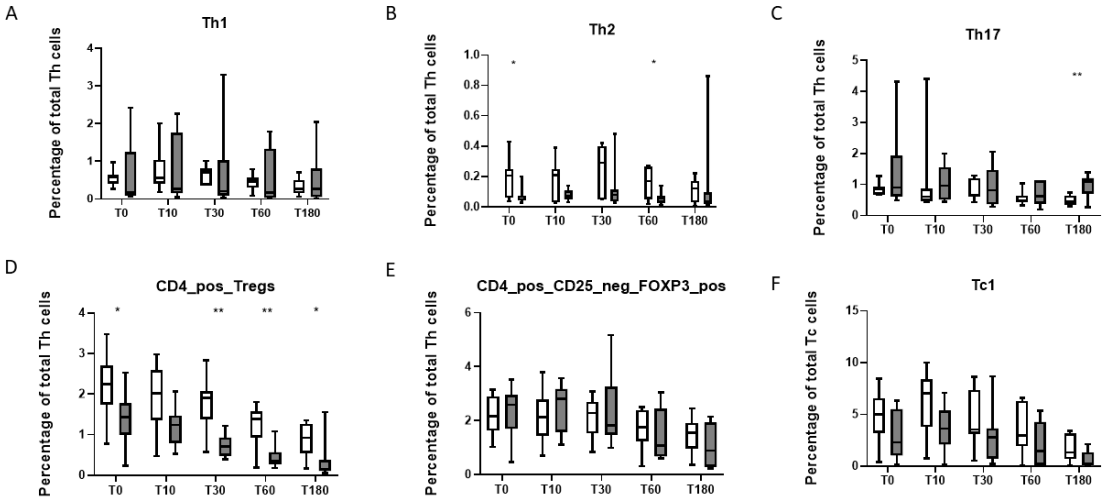


S4.4: Immune changes in GK and Wistar rats after metabolic challenge in the prediabetic phase (week 10) contd. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).

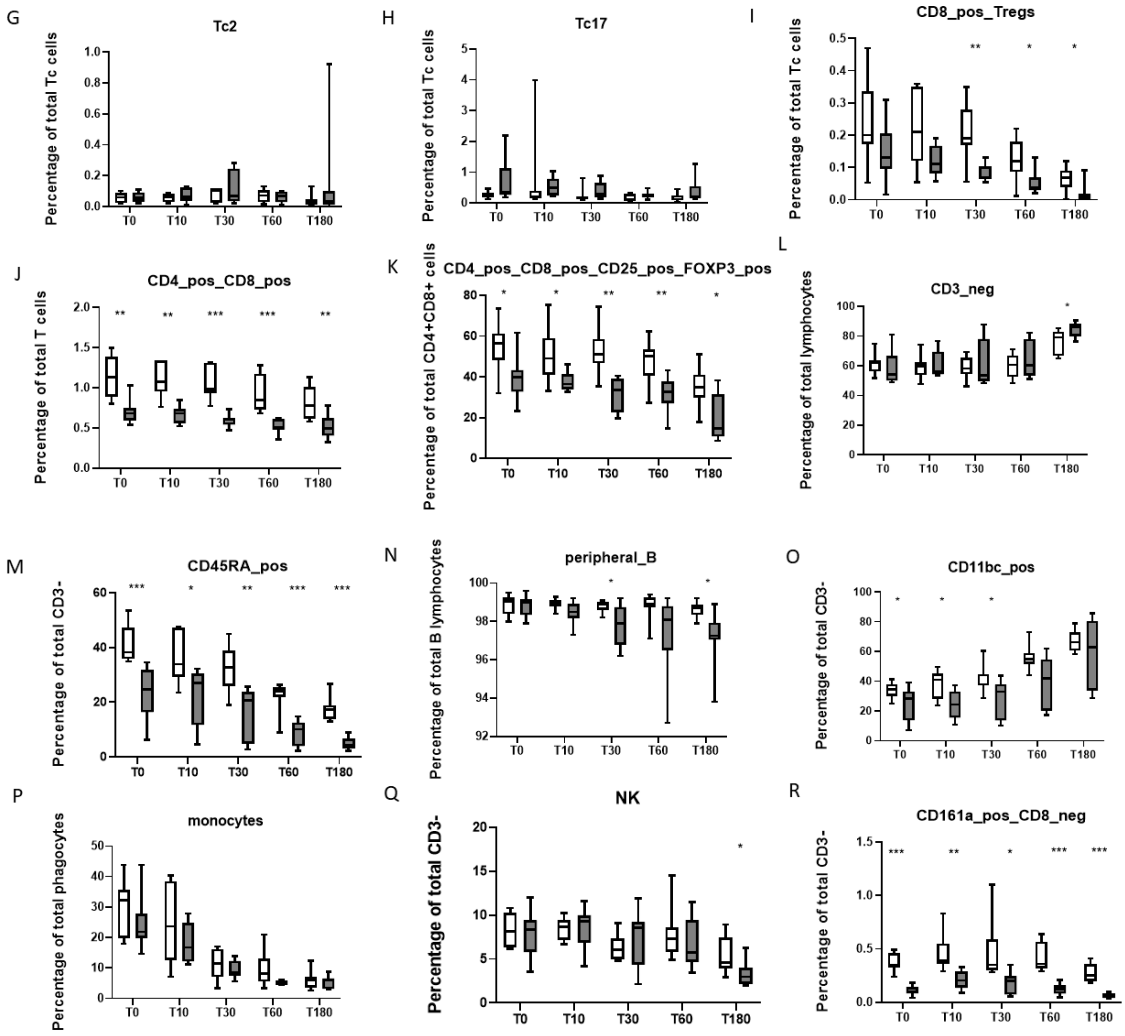


Appendix 1 – Section 5 for Chapter 4

S4.5: Immune changes in GK and Wistar rats after metabolic challenge in the early diabetic phase (week 14). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).

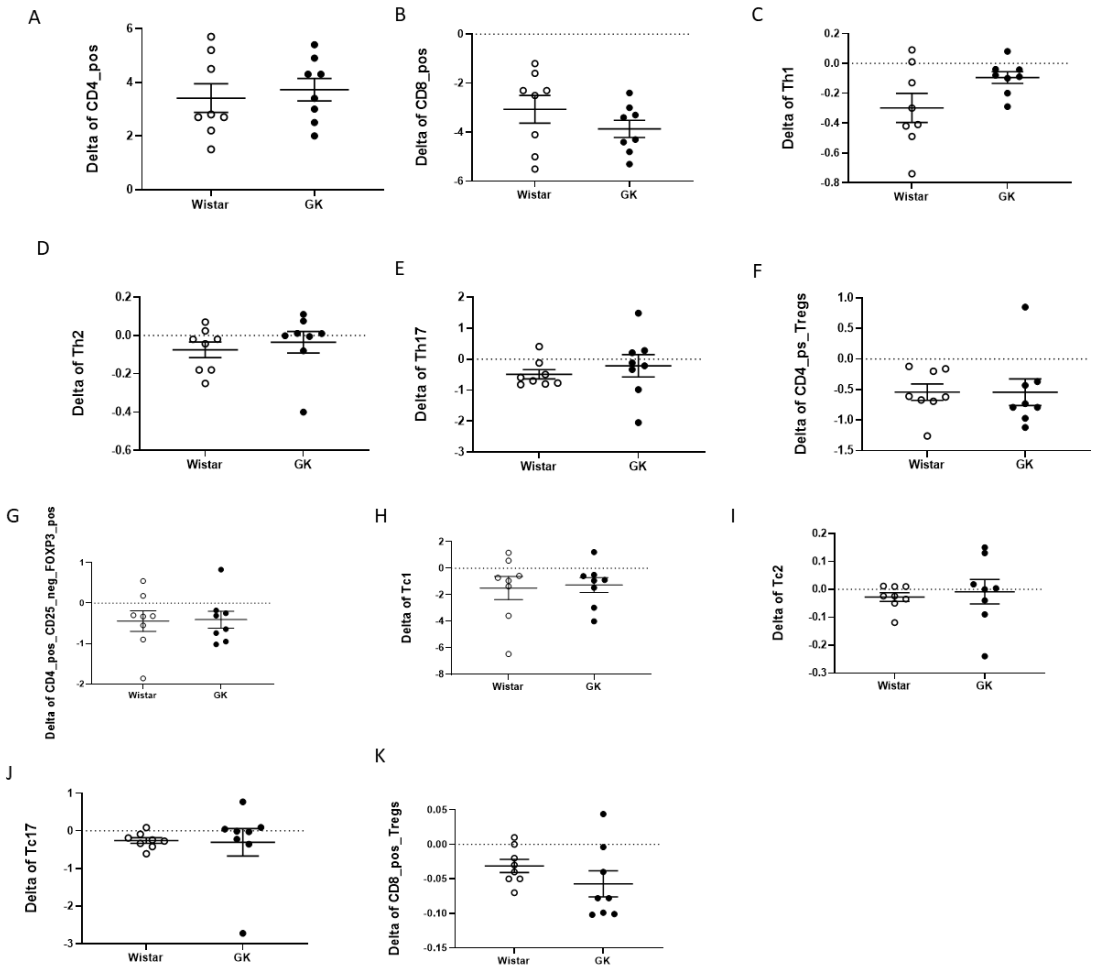


S4.5: Immune changes in GK and Wistar rats after metabolic challenge in the early diabetic phase (week 14) contd. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).

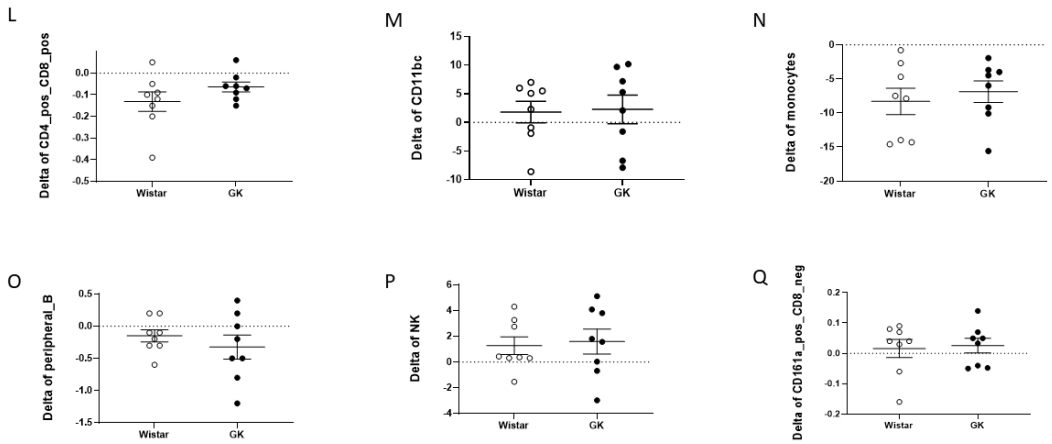


Appendix 1 – Section 6 for Chapter 4

S4.6: Stress-induced (non-significant) changes in immune cells in early diabetes (week 16). Data are presented as delta values (post stress values-pre-stress values for the percentage of parent population frequency). Data are mean +/- SEM; Each circle represents a single rat; Empty circles: Wistar rats, closed circles: GK rats; $p > 0.05$. For immune cell trafficking after stress, all populations were analysed using unpaired t-tests except CD161a+ CD8-, T_h2, T_h17, CD4+ Tregs and T_c17 (Mann-Whitney test).

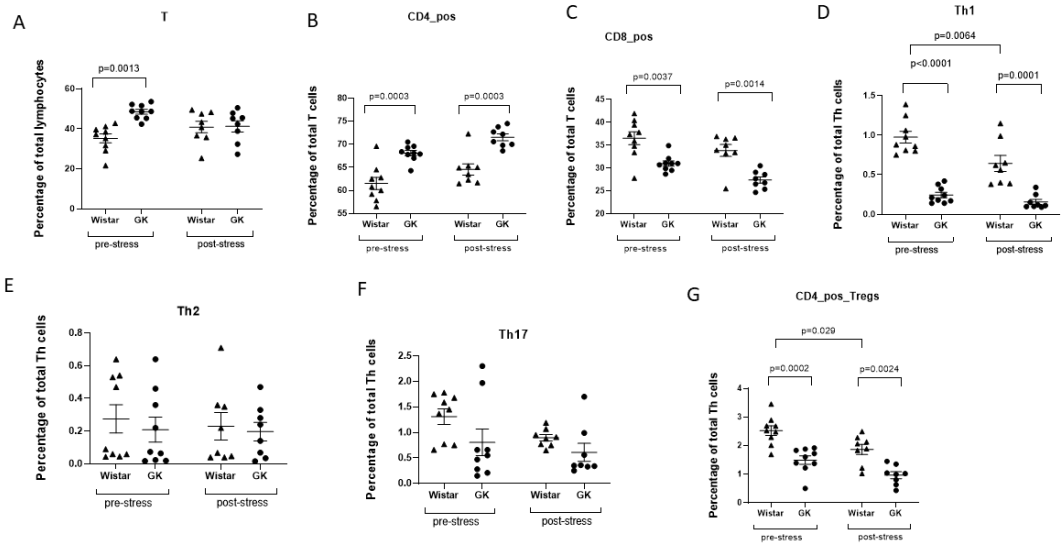


S4.6: Stress-induced (non-significant) changes in immune cells in early diabetes (week 16) contd. Data are presented as delta values (post stress values-pre-stress values for the percentage of parent population frequency). Data are mean +/- SEM; Each circle represents a single rat; Empty circles: Wistar rats, closed circles: GK rats; $p > 0.05$. For immune cell trafficking after stress, all populations were analysed using unpaired t-tests except CD161a+ CD8-, T_h2, T_h17, CD4+ Tregs and T_c17 (Mann-Whitney test).

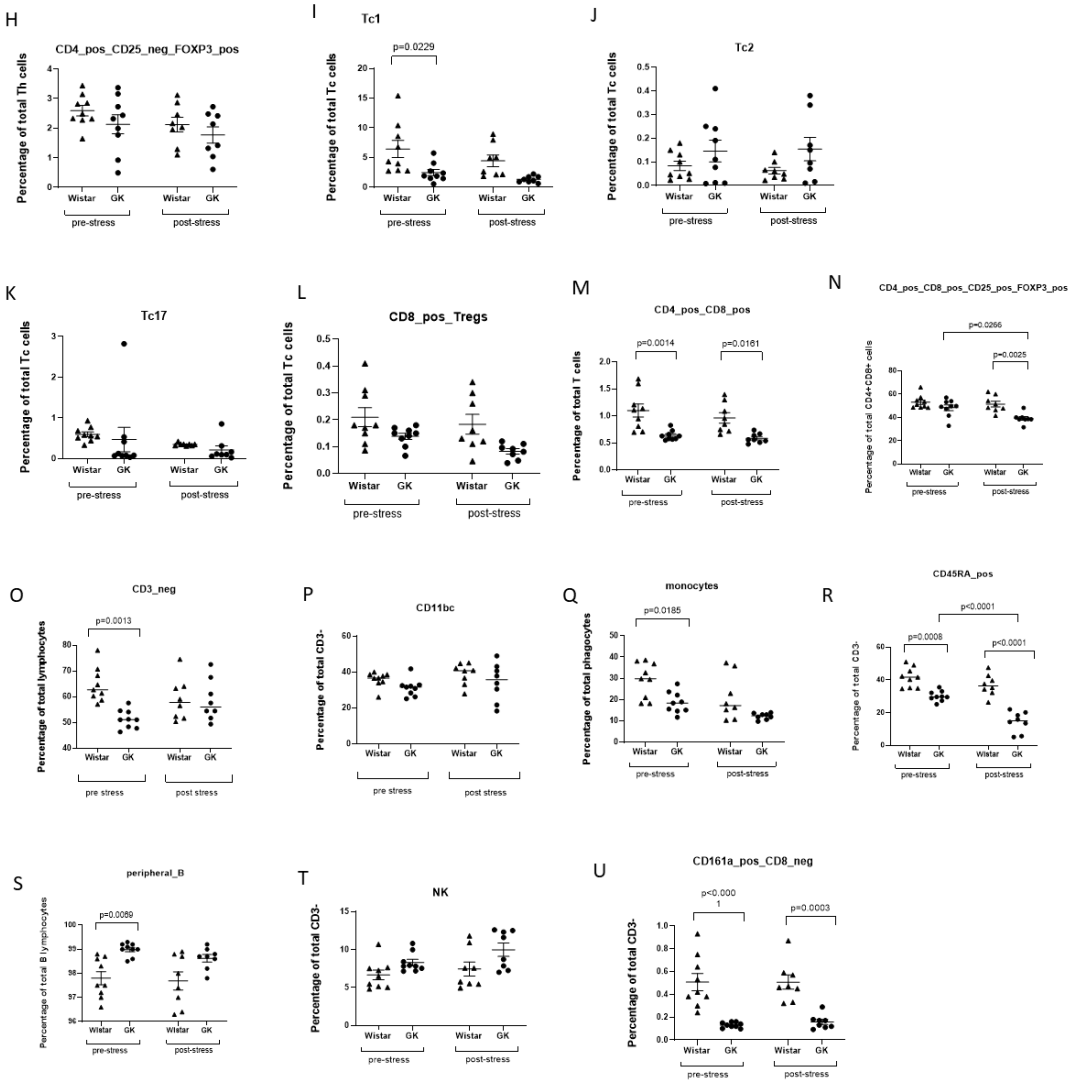


Appendix 1 – Section 7 for Chapter 4

S4.7: Stress-induced 2-way ANOVA changes in immune cells in early diabetes (week 16). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; Each circle/triangle represents a single rat; Triangles: Wistar rats, Circles: GK rats.

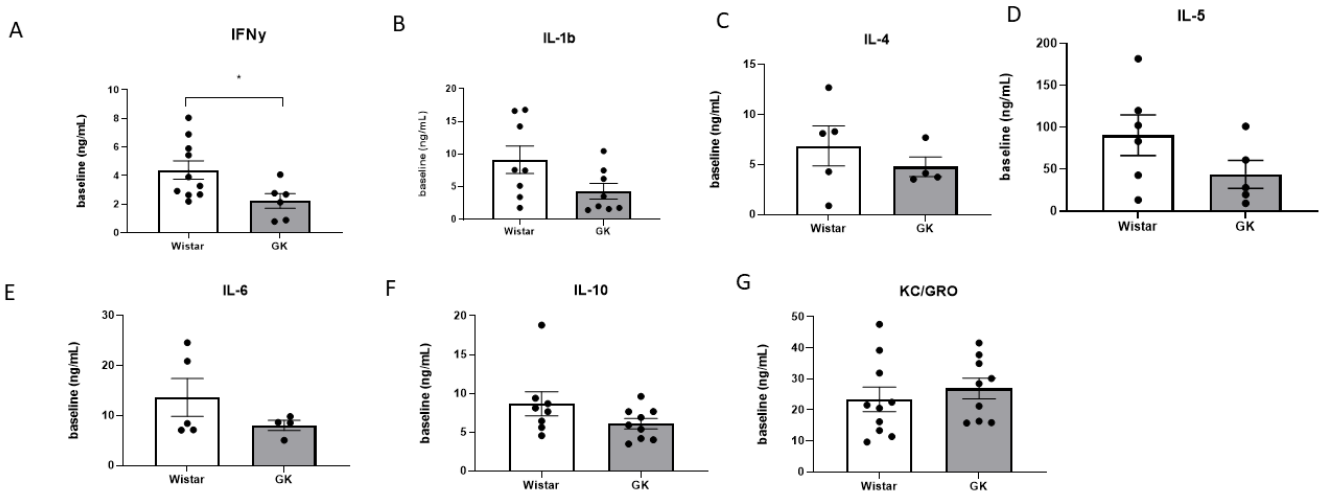


S4.7: Stress-induced 2-way ANOVA changes in immune cells in early diabetes (week 16) contd. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; Each circle/triangle represents a single rat; Triangles: Wistar rats, Circles: GK rats.



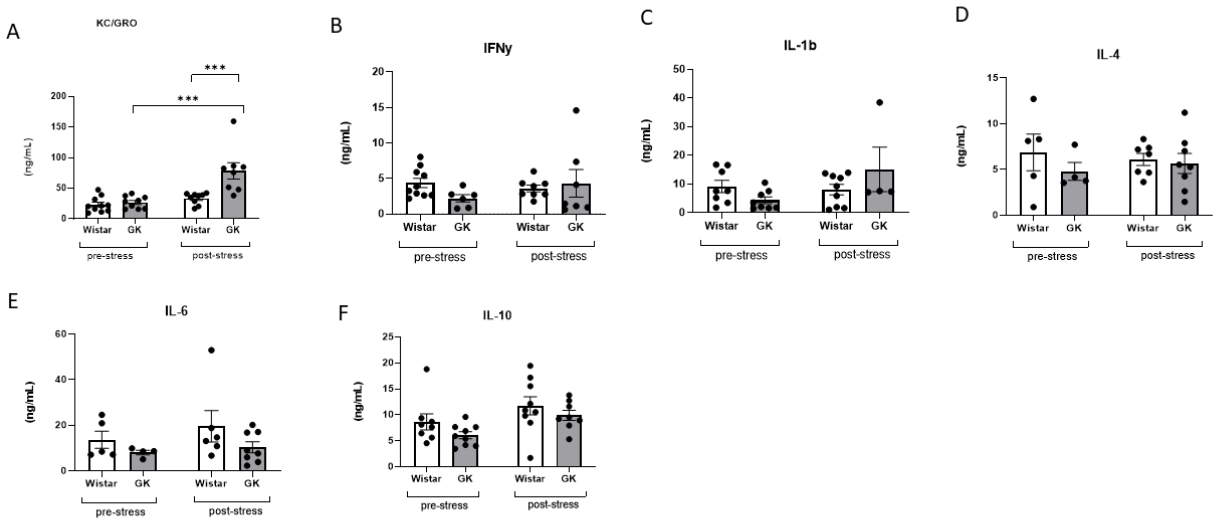
Appendix 1 – Section 8 for Chapter 4

S4.8: Baseline cytokine profile upon exposure to stress in the early diabetic phase (week 16). All panels display the mean levels of plasma cytokines (mean +/- SEM) in ng/mL; Each dot represents a single rat; Grey bars: GK rats; white bars: Wistar rats. * represents $p < 0.05$. For cytokine profile after stress, all populations were analysed using unpaired t-tests except IL-4, IL-6 and IL-10 (Mann-Whitney test).

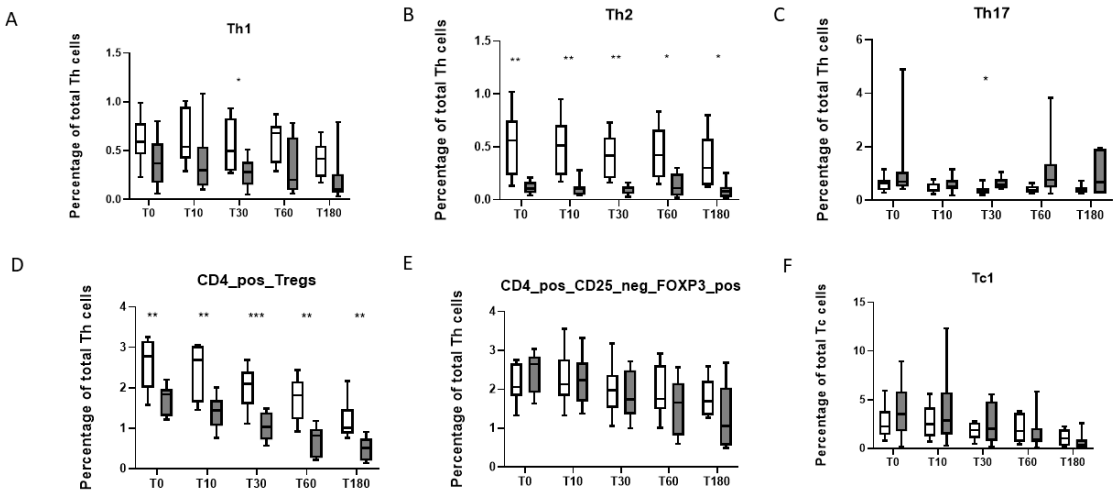


Appendix 1 – Section 9 for Chapter 4

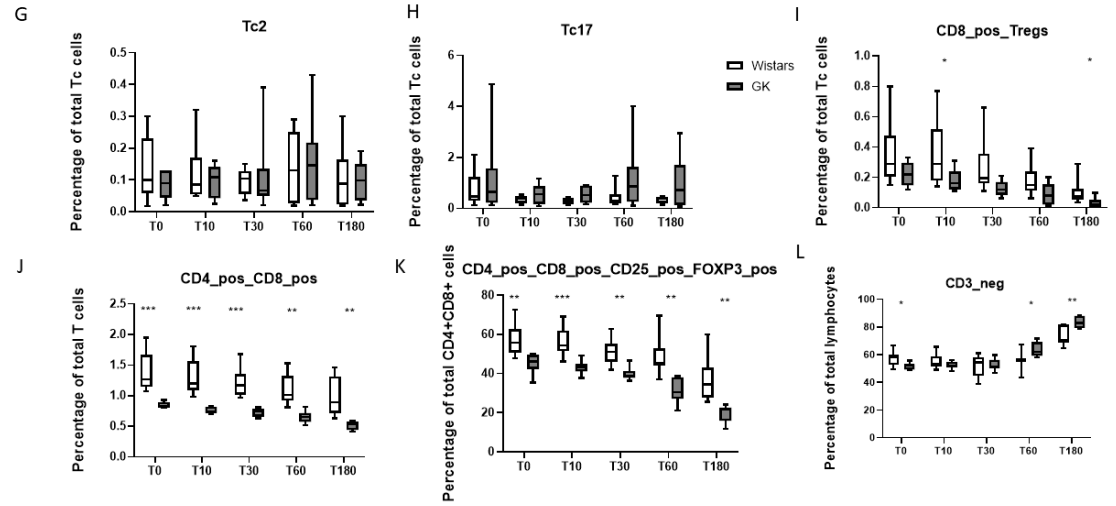
S4.9: 2 way ANOVA changes in cytokines upon exposure to stress in the early diabetic phase (week 16). All panels display the mean levels of plasma cytokines (mean +/- SEM) in ng/mL; Each dot represents a single rat; Grey bars: GK rats; white bars: Wistar rats. *** represents p < 0.001.



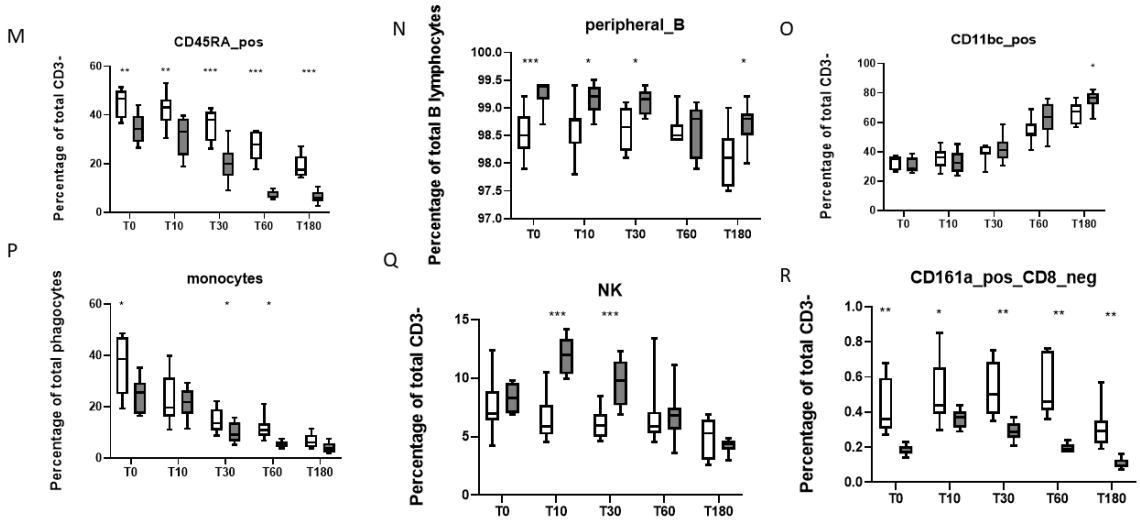
S4.4: Immune changes in GK and Wistar rats after metabolic challenge in the prediabetic phase (week 10). Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).



S4.4: Immune changes in GK and Wistar rats after metabolic challenge in the prediabetic phase (week 10) contd. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).



S4.4: Immune changes in GK and Wistar rats after metabolic challenge in the prediabetic phase (week 10) contd. Data for immune cells expressed as percentage of parent population frequency; Data are mean +/- SEM; White bars: Wistar rats, grey bars: GK rats; p<0.001, p<0.01 and p<0.05 are reflected by ***, ** and * respectively. Unpaired 2 sample unequal variance t-tests have been used to analyse the data; Time = 0, 10, 30, 60 and 180 minutes post administration of glucose (referred to as T0, T10, T30, T60 and T180 respectively).



Appendix 2 – List of Publications

Seal, S.V.; Turner, J.D. *The 'Jekyll and Hyde' of Gluconeogenesis: Early Life Adversity, Later Life Stress, and Metabolic Disturbances. Int. J. Mol. Sci.* 2021, *22*, 3344. https://doi.org/10.3390/ijms22073344. doi: 10.3390/ijms22073344 . PMID: 33805856; PMCID: PMC8037741.

Seal, S.V., et al., *A Holistic View of the Goto-Kakizaki Rat Immune System: Decreased Circulating Immune Markers in Non- Obese Type 2 Diabetes.* Frontiers in Immunology, 2022, 13, https://doi.org/10.3389/fimmu.2022.896179 . PMID: 35677049**;** PMCID: PMC9168276.

Holuka C, **Seal SV**, […] Turner JD. *The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity?* Int J Mol Sci. 2020 Jul 19;21(14):5094. doi: 10.3390/ijms21145094. PMID: 32707661; PMCID: PMC7404093.

Online preprints:

Seal, S.V., et al., *Stressing Glucose: At the Crossroads of Early Life Adversity, HPA Axis Reactivity and Carbohydrate Metabolism,* doi: 10.20944/preprints202207.0311.v1

In preparation:

Myriam P. Merz, Snehaa V. Seal, Nathalie Grova, Sophie Meriaux, Pauline Guebels, Georgia Kanli, Elise Mommaerts, Nathalie Nicot, Tony Kamoa, Olivier Keunen, Petr Nazarov, and Jonathan D. Turner, Early-life Influenza A (H1N1) infection independently programs HPA and endocrine tissues.

Appendix 3 – Contribution to co-authored publications

International Journal of *Molecular Sciences*

MDPI

Review

The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity?

Cyrielle Holuka [1], Myriam P. Merz [1], Sara B. Fernandes [1], Eleftheria G. Charalambous [1], Snehaa V. Seal [1], Nathalie Grova [1,2] and Jonathan D. Turner [1,*]

SVS contributed approximately 20% to the writing of the review, along with 15% of the final revising.

Myriam P. Merz, Snehaa V. Seal, Nathalie Grova, Sophie Meriaux, Pauline Guebels, Georgia Kanli, Elise Mommaerts, Nathalie Nicot, Tony Kamoa, Olivier Keunen, Petr Nazarov, and Jonathan D. Turner, Early-life Influenza A (H1N1) infection independently programs HPA and endocrine tissues.

SVS performed approximately 80% of the MRI scans, 100% of the bioinformatics skull stripping, 50% of manual co-registration of atlas to MRI scans and downstream DTI analysis, verified co-registration for every scan, reported the misaligned slices for further correction, wrote the relevant Materials and Methods, and participated in the writing and revision of the manuscript.