Type 2 diabetes & inflammation: The role of Toll-like receptors signalling in pathophysiology of diabetes & complications related to diabetes

Saket Gupta
Type 2 diabetes & inflammation: The role of Toll-like receptors signalling in pathophysiology of diabetes & complications related to diabetes

Dr. Saket Gupta
Department of Endocrinology and Diabetes
RCSI

A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfillment of the degree of Doctor of Medicine

Supervisor(s): Dr Shu Hoashi
Dr Sinead Miggin

October 2014
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Student Number  9110097

Date 24 October 2014
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<td>Delta Delta CT method</td>
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<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<td>ACR</td>
<td>Albumin-creatinine ratio</td>
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<td>ADA</td>
<td>American Diabetes Association</td>
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<tr>
<td>AdipoR1</td>
<td>Adiponectin receptor 1</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>Adiponectin receptor 2</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<td>AGE</td>
<td>Advanced glycation end product</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Antigen producing cell</td>
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<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
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<tr>
<td>AT</td>
<td>Adipose tissue</td>
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<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BSF-2</td>
<td>B cell stimulating factor-2</td>
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<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
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<tr>
<td>CpG</td>
<td>&quot;—C—phosphate—G—&quot; dinucleotides</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CSIF</td>
<td>Cytokine synthesis inhibitory factor</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<td>DBP</td>
<td>Diastolic blood pressure</td>
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<td>Dendritic cell</td>
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<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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<tr>
<td>DDC</td>
<td>Diabetes Day Centre</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DN</td>
<td>Diabetic nephropathy</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
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<td>FFA</td>
<td>Free fatty acid</td>
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<td>FPG</td>
<td>Fasting plasma glucose</td>
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<td>g</td>
<td>Gravity</td>
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<td>h</td>
<td>Hour</td>
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<td>HbA1c</td>
<td>Glycosylated haemoglobin</td>
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<td>High fat diet</td>
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<td>HOMA-IR</td>
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<td>HPRT</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<td>IFG</td>
<td>Impaired fasting glucose</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Interferon beta</td>
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<td>Interferon gamma</td>
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<td>IFNRI</td>
<td>Type I interferon receptor</td>
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<td>IGT</td>
<td>Impaired glucose tolerance</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>Interleukin 1 beta</td>
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<td>IL-1RA</td>
<td>Interleukin-1 receptor antagonist</td>
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<td>IR</td>
<td>Insulin resistance</td>
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<td>IRF-3</td>
<td>Interferon regulatory factor 3</td>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<td>ISL</td>
<td>Immune Signal Laboratory</td>
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<td>IκB</td>
<td>Inhibitor of kappa B</td>
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<td>LCN2</td>
<td>Lipocalin-2</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LRP</td>
<td>Leucocyte-rich plasma</td>
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<td>Major histocompatibility complex</td>
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<td>min</td>
<td>Minute</td>
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<td>MRH</td>
<td>Midland Regional Hospital</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
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<td>MSD</td>
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<td>n</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NGT</td>
<td>Normal glucose tolerant (healthy control)</td>
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<td>NGAL</td>
<td>Neutrophil gelatinase-associated lipocalin</td>
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<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
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<tr>
<td>IkB-NS or IkB-d</td>
<td>Nuclear factor of k light polypeptide gene enhancer or Nfkbid in B- cells inhibitor delta</td>
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<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
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<td>p</td>
<td>Probability</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PAD</td>
<td>Peripheral arterial disease</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>pDC</td>
<td>Plasmacytoid DC</td>
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<td>PC</td>
<td>Type 2 diabetes with poor glycaemic control &amp; no micro/macrovacular complications</td>
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<td>PCC</td>
<td>Type 2 diabetes with poor glycaemic control &amp; micro/macrovacular complications</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
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<tr>
<td>polyIC</td>
<td>Polyinosinepolycytidylic acid</td>
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<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>qPCR or RT-PCR</td>
<td>Quantitative/real-time polymerase chain reaction</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<td>RAGE</td>
<td>Receptor for advanced glycation end product</td>
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<td>RANTES</td>
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<td>Retinol binding protein 4</td>
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<td>RR</td>
<td>Relative risk</td>
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<td>Room temperature</td>
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<td>SA</td>
<td>Serum amyloid</td>
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<td>SAA</td>
<td>Serum amyloid A</td>
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<tr>
<td>SARM</td>
<td>Sterile alpha and HEAT-Armadillo motifs containing protein</td>
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<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
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<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
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<td>SOCS3</td>
<td>Suppressor of cytokine signalling 3</td>
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<td>SR-A</td>
<td>Scavenger receptor-A</td>
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<td>ssRNA</td>
<td>Single-stranded RNA</td>
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<td>STEPS</td>
<td>STEPwise Approach to Surveillance</td>
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T1D  Type 1 Diabetes Mellitus
T2D  Type 2 Diabetes Mellitus
TAC  Tetrameric Antibody Complexes
TGF-β Transforming growth factor beta
Th1  Type 1 T helper cells
Th2  Type 2 T helper cells
Th cell  T helper cell
TIA  Transient ischaemic stroke
TIR  Toll-IL-1 receptor
TIRAP  Toll/interleukin-1-receptor (TIR)-domain-containing adaptor protein
TLR  Toll-like receptor
TNFα  Tumor necrosis factor-alpha
TRAM  TRIF-related adapter molecule
TRIF  TIR-domain containing adaptor inducing protein inducing interferon-β
2hPG  Two-hour plasma glucose
UB  Ubiquitination
VCAM-1  Vascular cell adhesion molecule-1
WAT  White adipose tissue
WHO  World Health Organisation
WHR  Waist-to-hip ratio
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Abstract

Aims:
The aim of this study was to determine association between inflammation, glucose control and type 2 diabetes (T2D) related complications.

Methods:
We determined cytokines, adipokines & Toll-like receptors (TLR) mRNA expression in monocytes (M) and neutrophils (N) using QPCR in a cohort of 146 subjects: non-diabetic controls [NGT; n=34, BMI 26 (24-30) kg/m$^2$, HbA1c 5.6 (5.4-5.7)%] and compared them with T2D with four different profiles: good glycaemic control without complications [GC; n=27, BMI 32 (29-38) kg/m$^2$, HbA1c 6.4 (6.0-6.9)%], good glycaemic control with complications [GCC; n=32, BMI 31 (28-35) kg/m$^2$, HbA1c 6.9 (6.4-7.4)%], poor glycemic control without complications [PC; n=21, BMI 35 (31-37) kg/m$^2$, HbA1c 10.2 (9.2-11)%] and poor glycaemic control with complications [PCC; n=32, BMI 34 (29-37) kg/m$^2$, HbA1c 9.6 (8.9-10.8)%]. Data are expressed as median (Inter quartile range).

Results:
The highest expressions of IL-6, TNFα, Rantes & IFNβ mRNA in neutrophils, and IL-6 and IFNβ mRNA in monocytes were seen in GC, compared to NGT. In contrast, IL-1β mRNA expression was significantly decreased in monocytes from GCC, PC and PCC.

Retinol binding protein 4 (RBP4), Lipocalin 2 and adiponectin mRNA in neutrophils, and RBP4 mRNA in monocytes were significantly overexpressed in GC, compared to NGT. In contrast, Lipocalin 2 and adiponectin mRNA were significantly suppressed in monocytes from PC and PCC.
Similarly, TLRs mRNA 1-10, except TLR 2 in neutrophils, and TLR1, 3, 5, 7, 9 & 10 in monocytes were overexpressed from GC group compared to NGT.

**Conclusions:**
The overexpressed cytokine, adipokine & TLR mRNA levels in monocytes and neutrophils from GC group indicate an inflammatory milieu in T2D with good glycaemic control with no complications. In contrast, suppressed levels of inflammatory markers in PC, GCC & PCC indicate compromised innate immune state i.e. “burnt out” disease in poor glycaemic control, and in patients with complications.
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Chapter 1

Introduction
1.1 Type 2 Diabetes: an inflammatory disease

Type 2 Diabetes (T2D) is one of the world's most important diseases with a global epidemic ensuing, fuelled by population growth, aging, urbanisation and increasing obesity and inactivity. It is estimated that, in many countries, including those, such as India, China and the USA, that have the highest numbers of diabetic patients currently (>90% of these with T2D), the prevalence will double in the next 20 years or so. The disease is, in short, common, costly and associated with devastating complications. T2D is caused by a combination of insulin resistance (IR) at skeletal muscle, liver and adipose tissues (AT) and impaired insulin secretion from the pancreatic islets. It is thought that environmental trigger factors, such as overeating and underactivity cause a decrease in insulin release and action in individuals with a susceptibility to the disease (Leahy et al., 2005). Passing first through a stage of impaired glucose tolerance and/or impaired fasting glucose concentration, frank T2D eventually develops, in association with a varying number of other clinical and biochemical features, which are themselves cardiovascular risk factors and are together called the metabolic syndrome (Festa et al., 2000a). Overall, T2D occurs when beta-cell function fails to compensate for insulin resistance, partly because of beta-cell demise through apoptosis.

In the last decade, increasing evidence suggests that patients with T2D display features of inflammation years before the disease onset (Alexandraki et al., 2006). Moreover, low-grade inflammation has been proposed to be involved in the pathogenetic processes causing T2D (Festa et al., 2000a; Navarro & Mora, 2006a). In fact, numerous studies have demonstrated a strong link between inflammatory markers and carbohydrate and lipid metabolism abnormalities and obesity (Festa et al., 2000b; Duncan et al., 2003). Given that T2D is a polygenic disease, defects in many molecular pathways have been demonstrated or
implicated. Recent evidence suggests common molecular mechanisms between inflammatory and insulin signalling pathways both of which cause insulin resistance (Rotter et al., 2003). Also, there is evidence that inflammatory mediators may not only represent markers of metabolic aberrations in T2D (Navarro & Mora, 2005; Navarro & Mora, 2006a) but may also contribute to β-cell death due to impaired function and progressive decline in β-cell function and mass (Alexandraki et al., 2006). In fact, apoptotic cells alone can activate the innate immune system and hyperglycaemia can induce β-cell expression of several molecules involved in immunological processes including Interleukin (IL)-1β itself (Maedler et al., 2002). Regarding the investigation of the origin of the inflammatory process in T2D, recent studies have demonstrated that an accumulation of macrophages in the AT of obese subjects as well as their participation in the inflammatory pathways activated in adipocytes (Weisberg et al., 2003). Macrophages reside in human AT and their number has been found positively related with body mass index (BMI), suggesting their contribution to the dysregulation of AT as well as to the impairment of adipocytes function. Moreover, these macrophages are a source of proinflammatory cytokines such as tumor necrosis factor-alpha (TNFα) and interleukin 6 (IL-6). Also, another source of the circulating proinflammatory cytokines may be the mononuclear cells as these cells are the precursors to tissue macrophages. The peripheral blood mononuclear cells (PBMC) of obese subjects have been shown to be in an inflammatory state, expressing increased amounts of proinflammatory cytokines and related factors with an increase in the transcription of proinflammatory genes regulated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Ghanim et al., 2004), the central proinflammatory transcription factor as well as an increase in the intranuclear expression of p65 (Rel A), the major protein component of NF-κB. These cells also express diminished amounts of the inhibitor of kappa B (IκB) β, which is the inhibitor of NF-κB activation (Dandona et al., 2004). Interestingly, it has been shown that macrophages and adipocytes are quite similar and functional capability of these two cells overlap.
Macrophages can take up and store lipid to become atherosclerotic foam cells (Wellene et al., 2005). Preadipocytes, the progenitors of mature adipocytes can exhibit phagocytic and antimicrobial properties and differentiate into macrophages under some conditions (Cousin et al., 1999; Charrière et al., 2003). Gene expression is also highly similar; macrophages express many adipocyte gene products such as the adipocyte/macrophage fatty-acid-binding protein (FABP) aP2 (also known as FABP4) and PPARγ, while adipocytes can express many macrophage proteins such as TNF-α, IL-6, and matrix metalloproteinases (MMP) (Hotamisligil et al., 1993; Makowski et al., 2001; Tontonoz et al., 1998; Bouloumie et al., 2001). Furthermore, it has been observed that monocyte chemoattractant protein-1 (MCP-1), a proinflammatory chemokine mainly produced by macrophages and endothelial cells, can be secreted from isolated adipocytes (Alexandraki et al., 2006).

A few years ago, a hypothesis was proposed which suggested that elements of the innate immune system contribute to the development of T2D (Navarro & Mora, 2006a). In this model, the idea was forwarded that the innate immune system modulates the effects of many factors including genes, ethnicity, foetal programming, nutrition and age upon the later development of metabolic sequelae associated with insulin resistance. The innate immune system, which is phylogenetically older than the so-called acquired or adaptive immunity, is a rapid first line defence mechanism based on non-lymphoid tissue components, including macrophages and polymorphonuclear cells (PMNs). A major component of innate immunity is a series of sentinel cells (classically macrophages, antigen-presenting B-cells, and dendritic cells (DC), but probably also intestinal epithelial cells, endothelium, Kupffer cells in the liver, adipocytes, and others) that act as “trouble detectors.” A number of germ line-encoded (i.e., nonclonal) pattern recognition receptors (PRRs) on and in these cells recognize conserved molecular structures, pathogen-associated molecular patterns (PAMP), that are characteristic of a class of harmful agents.
(Miggin and O’Neill, 2006). The most studied PRRs are probably the family of at least 10 Toll-like receptors (TLRs) (named after the toll receptor, first identified in the fruit fly, drosophila) that are present at the cell surface as transmembrane receptors. TLR-4, for example, recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, in conjunction with associated accessory molecules (Cluster of differentiation (CD) 14, MD-2). Other cell surfaces PRRs include the macrophage scavenger receptors, the mannose receptor, and the receptor for advanced glycation end products (RAGE). There are also intracellular PRRs, e.g., for double-stranded RNA (present in viruses). Binding to PRR activates NF-κB signaling pathways that induce immune response genes, especially those for inflammatory cytokines (including IL-1α, TNFα, IL-6), which are the main mediators of inflammation and the acute-phase response (Miggin and O’Neill, 2006). Secreted and circulating PRRs such as C-reactive protein (CRP) and mannan-binding lectin function as opsonins, binding to microbial cell components and flagging them for recognition by the complement system and phagocytes. An important second function of innate immunity, which has only recently been appreciated, is to control the adaptive immune response. T-cells require two signals to be activated: the complex of presented antigen and the major histocompatibility complex class II molecule on the surface of an antigen-presenting cell and costimulatory molecules (CD80 and CD86), which are invoked by the innate immune system and the binding of pathogen-associated molecular patterns to PRRs. Thus, the innate immune system ensures that the adaptive immune system responds only to harmful antigens and that the biological context of a threat is recognized.

The acute phase response is part of the innate immune system and results in pronounced changes in the concentration of plasma proteins in response to a variety of stresses including infection, tissue injury or inflammation. Some of these proteins increase (positive acute phase proteins) such as CRP, fibrinogen and SAA, whilst others decrease
(negative acute phase proteins) such as albumin and transferrin. The acute phase proteins are synthesized in the liver, stimulated by certain proinflammatory cytokines including IL-1β, IL-6 and TNFα, which are released from macrophages, monocyte and endothelium (Figure 1). Interestingly, it has also been shown that AT can release cytokines and, thus, subclinical low-grade chronic inflammation may in part be due to obesity. Several studies have shown that elevated levels of inflammatory and endothelial cell markers predict diabetes. Acute phase reactants and certain cytokines are involved in a plethora of metabolic pathways, such as insulin regulation, reactive oxygen species, lipoprotein lipase action and adipocyte function, all that could be relevant to IR.
Figure 1.1 The components of the innate immune system

Sentinel cells such as macrophages, intestinal epithelial cells, endothelium, Kupffer cells in the liver and adipocytes detect potential environmental threats from infection, chemicals, and foods by specific pattern recognition receptors (PRRs) called Toll like receptors (TLRs) that activate signalling pathways and release proinflammatory cytokines (IL-6 and TNFα). For example, TLR4 senses bacterial LPS and the receptor for advanced glycation end products (RAGE) detects advanced glycation end products (AGEs). Cytokines stimulate acute-phase protein production from the liver and also act on the brain to release adrenocorticotropic hormone (and thereby cortisol from the adrenal gland) and activate the sympathetic nervous system with the release of catecholamines. Psychological stress can cause an acute-phase response via innervation of cytokine-producing cells and via activation of the sympathetic nervous system and adrenergic receptors on macrophages. The central cytokines induced "sickness behaviour" includes lethargy, sleep changes and depression. The innate immune system also controls the adaptive (acquired) immune system via costimulatory molecule expression that is necessary for antigen presentation. SAA, serum amyloid A.
TLRs are members of the IL-1 receptor family, an evolutionary conserved signalling system against invading pathogens (Miggin and O'Neill, 2006). Due to their ability to recognize microbial components, mammalian TLRs are among the most important components of the innate immunity pathway. The first described and best-known member of this family is TLR4, identified as the signalling receptor for LPS. TLR4 also interacts with endogenous ligands such as heat shock proteins, fibronectin, fibrinogen, minimally modified and oxidized low-density lipoprotein (LDL) and free fatty acids (FFA), which are elevated in diabetes (Kolz et al., 2008; Siednienko & Miggin, 2009a). TLR4 ligation activates several intracellular signalling pathways, with TLR4/NF-κB pathway being the most important one, leading to the synthesis and release of inflammatory cytokines including IL-1β, IL-6 and TNFα and other co-stimulatory molecules that provide a link to adaptive immunity (Figure 1.2). Given that TLRs are expressed in macrophages and mononuclear cells (Siednienko & Miggin, 2009a) and that TLR engagement elicits the production of pro-inflammatory cytokines such as TNFα and IL-6 (Miggin and O'Neill, 2006), it is plausible to speculate that TLRs may play a role in the pathogenesis of T2D.
Figure 1.2 TLR3 and TLR4 mediated signalling pathways. Whereas TLR4 activates the MyD88-dependent and the Trif-dependent pathways, TLR3 activates the Trif-dependent pathway only. MyD88 recruits IRAK4 and TRAF6 upon ligand stimulation. TRAF6 activates TAK1/TAB1/TAB2/TAB3 complex via K63-linked ubiquitination (UB). Activated TAK1 complex then activates the IKK complex, which catalyzes IkB phosphorylation and degradation by the proteasome pathway, thus allowing NF-κB to translocate into nuclei. NF-κB and AP-1 control inflammatory responses by inducing proinflammatory cytokines such as TNF, IL-1β and IL-6. TLR4 also recruits TRAM and Trif, which interacts with TBK1. TBK1 together with IKK-i mediates phosphorylation of IRF3. Phosphorylated IRF3 is dimerized and translocated into nucleus to bind DNA. Trif also interacts with TRAF6 and RIP1, which mediate NF-κB activation. Activation of IRF3, NF-κB and AP-1 is required for induction of type I interferon, particularly interferon (IFN) β. TLR3, which resides in endosomal vesicles, utilizes Trif but not MyD88, mediating the induction of type 1 interferons.
Our hypothesis is that long-term innate immune system activation, resulting in chronic inflammation, elicits disease instead of repair in individuals who develop T2D. Interestingly, chronic infections, including periodontal disease, Helicobacter pylori or Chlamydia pneumoniae can also evoke an inflammatory response (Nishimura et al., 2000; Cassell, 1998). In the case of T2D, this may be a two-way relationship. It is, of course, plausible to speculate that the very factors that the innate immune system modulates may also cause its long-term activation.

The chronic inflammatory hypothesis has been strongly supported by various research groups throughout the world. One study by the ARIC Investigators showed that increased inflammatory markers, including white blood count, plasma fibrinogen and SA, were associated with the risk of developing T2D (Schmidt et al., 1999; Duncan et al., 2003). The Cardiovascular Health Study reported serum CRP concentrations were associated with the development of diabetes in the elderly (Barzilay et al., 2001). Additionally, increased serum gamma globulin concentrations, another expression of the acute phase response, predict risk of developing T2D in the Pima Indian population, as did raised leukocyte count (Lindsay et al., 2001 & 2002). In a Nurses's Health Study, elevated inflammatory markers, namely serum CRP, TNF-alpha receptor 2 and IL-6, were associated with the development of T2D in healthy middle-aged women (Hu et al., 2004). A supportive observation was made in the West of Scotland Coronary Prevention Study where CRP was shown to be an independent predictor of risk for the development of T2D in middle-aged men (Freeman et al., 2002). These were similar findings to the MONICA Augsburg Cohort Study that reported low-grade inflammation being associated with increased T2D risk in middle-aged men. Further confirmation of the 'chronic inflammatory' hypothesis has also come from the 'Insulin Resistance Atherosclerosis Study' where those individuals that converted to T2D had higher base-line levels of inflammatory proteins, including plasma fibrinogen, CRP and plasminogen activator inhibitor-1.
(PAI-1) than non-convertors. After adjustment for body fat (BMI or waist circumference), this association was attenuated, albeit less for PAI-1 than the other acute phase proteins Festa et al., 2000a & 2002). The authors also concluded that chronic inflammation is a risk factor for the development of T2D. Also pertinent is that elevated serum SA is higher in women developing gestational T2D.

In the present study, the expression of various inflammatory marker, cytokines, chemokines, adipokines and TLRs, that are purported to play a role in T2D, were examined relative to glucose control levels in the absence and presence of complications. The role of the inflammatory markers in the context of diabetes and micro/macro vascular complications, obesity and IR are summarized in Table 1.
Table 1: Inflammatory markers that were evaluated in healthy and T2D subjects

<table>
<thead>
<tr>
<th>No</th>
<th>Acute phase reactant</th>
<th>Cytokine</th>
<th>Chemokine</th>
<th>Cytokine</th>
<th>Chemokine</th>
<th>Adipokine</th>
<th>TLRs</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>CRP</td>
<td>TNF-α</td>
<td>RANTES*</td>
<td>TNF-α</td>
<td>RANTES</td>
<td>RBP4</td>
<td>TLR 1</td>
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<tr>
<td>2.</td>
<td>IL-10</td>
<td>IL-8**</td>
<td>IL-1β</td>
<td>LCN2</td>
<td></td>
<td></td>
<td>TLR 2</td>
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<tr>
<td>3.</td>
<td>IL-1β</td>
<td>IL-6</td>
<td></td>
<td>Adiponectin</td>
<td></td>
<td></td>
<td>TLR 3</td>
</tr>
<tr>
<td>4.</td>
<td>IL-6</td>
<td>IFN-β</td>
<td></td>
<td>TLR 4</td>
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</tr>
<tr>
<td>5.</td>
<td>IFN-β</td>
<td></td>
<td></td>
<td>TLR 5</td>
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<tr>
<td>6.</td>
<td>IFN-γ</td>
<td></td>
<td></td>
<td>TLR 6</td>
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<tr>
<td>7.</td>
<td>IL-12p70</td>
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<td>TLR 7</td>
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<tr>
<td>8.</td>
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<td>TLR 8</td>
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<tr>
<td>9.</td>
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<td>TLR 9</td>
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<tr>
<td>10.</td>
<td></td>
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<td></td>
<td>TLR 10</td>
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</tbody>
</table>

*RANTES, Regulated upon Activation, Normal T-cell Expressed, and Secreted
**IL-8, a cytokine sub classified as chemokine
CRP, C-reactive protein, TNF-α, Tumour necrosis factor-alpha, IL, Interleukin, IFN, Interferon, RBP4, Retinol binding protein 4, LCN2, Lipocalin-2, TLR, Toll like receptors
1.2 Acute phase reactant

1.2a CRP

CRP, a member of the pentraxin family of oligomeric proteins is an acute phase reactant produced mainly by the liver in response to IL-6 and TNFα. It is involved in PRRs activation and is a common marker for inflammation (Pearson et al., 2003).

It is associated with β-cell dysfunction and IR and plays a critical role in T2D (Pfutzner et al., 2006). It has been shown that it inhibit cell proliferation and increases the rates of apoptotic cell death (Nabata et al., 2008).

Numerous prospective studies have shown an independent positive association of CRP with the risk of developing T2D (Pradhan et al., 2001; Spranger et al., 2003; Hu et al., 2004; Doi et al., 2005), while others show no association after adjustment for adiposity and IR (Thorand et al., 2003; Duncan et al., 2003; Krakoff et al., 2003). A meta-analysis of a 10 prospective studies showed a positive association between serum CRP and T2D independent of obesity (Dehgan et al., 2007). Another meta-analysis of 16 published studies with 3,920 T2D cases and 24,914 controls demonstrated a relative risk (RR) of T2D of 1.72 (95% CI: 1.54–1.92) for subjects with high CRP levels (Lee et al., 2009).

It has been shown that CRP levels are higher in obese subjects who are also IR and levels decrease with weight loss and improvement of insulin sensitivity (Klover et al., 2005; McLaughlin et al., 2002). Circulating levels of CRP may decrease following bariatric surgery (Vazquez et al., 2005; Schernthaner et al., 2006; Kopp et al., 2005). Several single nucleotide polymorphisms (SNPs) were identified in the CRP gene and have been linked to elevated levels of serum CRP, low insulin sensitivity, and incidence of T2D (Hage & Szalai, 2007; Obisesan et al., 2006).
1.3 Cytokines

Cytokines are a group of small and pharmacologically active polypeptides produced by cells that exert autocrine and paracrine functions. In the context of inflammation individual cytokines may trigger several biological activities, and different cytokines may function through the same transcription factors and signal transduction pathways. Pro-inflammatory and anti-inflammatory cytokines are important to host defense and innate immunity because they respond to multiple stresses very early, trigger the local inflammation cascade and allow the immune cells to get access to injured tissues (Alexandraki et al., 2006).

1.3a TNFα

It is a pleiotropic cytokine secreted both by immune cells and adipocytes. It is found in extracellular matrix, endothelium, and vessel walls of fibrovascular tissue. TNFα is known to stimulate the production of other cytokines such as IL-8 and IL-6 (Bruun et al., 2001; Barnes & Karin, 1997) and has been shown to play a role in obesity and particularly in the development of IR (Hotamisligil et al., 1993). While some studies have reported increases in serum TNFα in human subjects with IR (Dandona et al., 1998; Katsuki et al., 1998), others have reported the reverse (Muller et al., 2002; Bruun et al 2003b). It has been suggested that TNFα downregulates the tyrosine kinase activity of the insulin receptor (Hotamisligil et al., 1994 & 1996). The infusion of anti-TNFα antibodies in T2D patients, however, showed no effect on insulin sensitivity (Ofei et al., 1996).

The patients with T2D have 3-4 times greater serum levels of TNF-α compared to non diabetic subjects, and these levels are higher in diabetic patients with microalbuminuria compared with those that have normoalbuminuria (Moriwaki et al., 2003; Navarro et al., 2003). The urinary TNFα excretion correlates well with the clinical markers of diabetic nephropathy (DN) and progression of disease (Navarro et al., 2006b). The widespread distribution of TNFα noticed in the retina of diabetic subjects
from both animals (Zhang et al., 2006) and humans (Limb et al., 1996) suggested that it also plays an important role in the pathogenesis of proliferative diabetic retinopathy. This cytokine is reported to be a key player in the development of T1D, contributing to beta cell dysfunction and death. The effects of TNFα in vivo, however, are age-dependent and there is evidence that TNFα can also have anti-diabetogenic effects (Meagher et al., 2001; Rabinovitch et al., 2001).

1.3b IL-10

IL-10 (initially characterized as cytokine synthesis inhibitory factor (CSIF)) is a pleiotropic type 2 helper T cells (Th2)-type cytokine that is produced by a wide range of immunological cell types, including B-cells, T-cells, monocytes and macrophages. It is a centrally operating anti-inflammatory cytokine that plays a crucial role in the regulation of the innate immune system (Akdis et al., 2001). Immunosuppressive effects of IL-10 involve both inhibition of cytokine synthesis (e.g., TNF-α, IL-6, IFN-γ) and their biological activities on target cells (Pestka et al., 2004)).

It has been shown that polymorphisms and haplotypes of the IL-10 promoter are associated with obesity and IR (Scarpelli et al., 2006) and co-treatment with IL-10 attenuates IR following acute lipid infusion (Kim et al., 2004). Lumeng et al. (2007) demonstrated that IL-10 was overexpressed in adipose tissue macrophages from lean animals and these adipocytes showed polarization toward an alternatively activated state. This study further showed that IL-10 increases glucose uptake and protects against TNFα-mediated IR in isolated adipocytes. Another study showed that IL-10 improved insulin sensitivity and protected skeletal muscle from obesity-associated macrophage infiltration in transgenic IL-10–overexpressed mice following high fat diet (Hong et al., 2009).

The Leiden 85-Plus Study showed that low IL-10 production capacity (i.e., a pro-inflammatory cytokine response) was associated with high plasma glucose, high glycosylated haemoglobin (HbA1c), T2D, and dyslipidemia
In another study, Yaghini et al. (2011) showed that IL-10 serum levels were low in T2D patients compared to healthy controls and suggested that low levels of IL-10 is a risk factor in T2D. It appears that IL-10 levels prevent the development of the metabolic syndrome and T2D by counter regulating the effects of pro-inflammatory cytokines such as TNF-α and IL-6 and causes an upregulation of tyrosine kinase activity of the insulin receptor and decreases lipolysis (Hotamisligil et al., 1994 & 1996; Feingold et al., 1992).

IL-10 is crucial in the pathogenesis of T1D as well (Yang et al., 2002). IL-10 was shown to increase pancreatic β-cell functions in response to glucose in vitro, and IL-10 plasmid therapy significantly reduced insulitis and prevented T1D in nonobese diabetic mice (Pennline et al., 1994).

1.3c IL-1β

IL-1β is the prototypical inflammatory cytokine and a critical early mediator of inflammation (Koh et al., 2005). The primary sources of IL-1β are blood monocytes, tissue macrophages, and DCs. B-lymphocytes and natural killer (NK) cells also produce IL-1β (Dinarello et al., 2009).

It has been shown that IL-1β impair insulin release, induces Fas expression and enables Fas-triggered apoptosis in rodent and human islets (Giannoukakis et al., 2000; Maedler et al., 2001; Stasis et al., 1997), and share similarities with glucose-induced apoptosis.

A low concentration of IL-1β stimulates insulin release and proliferation in rat and human islets (Maedler et al., 2006; Spinas et al., 1986). The beneficial IL-1β effects appear to be partly mediated by the increased secretion of the naturally occurring anti-inflammatory cytokine and antagonist of IL-1α and IL-1β, the IL-1 receptor antagonist (IL-1RA) (Dinarello, 2000).
In a prospective study, it was shown that elevated concentrations of both IL-6 and IL-1β were associated with a threefold increased risk of developing diabetes compared to the control group, but when only IL-6 levels were increased and IL-1β levels undetectable, no increased risk was documented (Yuan et al., 2001; Kim et al., 2001).

1.3d IL-6

IL-6, a 26kD glycoprotein, is an interleukin that acts as both a pro-inflammatory and an anti-inflammatory cytokine. It is secreted by T cells and induces B cells to form antibody-forming plasma cells, and is also known as B cell stimulating factor-2 (BSF-2). Additionally, endothelial cells, skeletal and smooth muscle cells, islet β-cells, hepatocytes, microglial cells, astrocytes etc. also express and secrete IL-6 (Kamimura et al., 2003).

Few studies demonstrated that elevated levels of IL-6 predict future risk of T2D development (Spranger et al., 2003; Hu et al., 2004; Pradhan et al., 2001). However, CRP was found to be a stronger predictor than IL-6 of future T2D in two of the studies investigating both parameters (Hu et al., 2004; Pradhan et al., 2001). These studies showed an association but not the causation.

Adipose tissue is a major source of circulating IL-6 and that in obese subjects with or without T2D, adipose tissue IL-6 content correlates with impaired whole-body insulin-mediated glucose uptake and glucose tolerance (Bastard et al., 2002; Kern et al., 2001). The weight loss and bariatric surgery leads to decrease of IL-6 concentrations and improvement of IR (Kopp et al., 2003). IL-6 production in visceral adipose tissue is 3-fold higher compared with subcutaneous adipose tissue, indicating that IL-6 might be one of the factors that make visceral adipose tissue a high risk factor for the development of IR (Fried et al., 1998).
The role of IL-6 in IR is debatable. In humans, circulating IL-6 levels may (Ridker et al., 2000; Koukkunen et al., 2001; Bastard 2002) or may not (Carey et al., 2004; Petersen et al., 2007) be associated with IR. Interventional studies, using acute or chronic application of IL-6, confirmed its potential to induce IR (Stith & Luo, 1994; Klover et al., 2003) and antibody neutralization experiments of IL6 also showed reversal of IL-6 induced IR (Klover et al., 2005).

However, IL-6 application to healthy volunteers not only failed to initiate IR (Krogh-Madsen et al., 2006), but also improved muscular glucose disposal and decreased endogenous glucose production (Weigert et al., 2006). These studies were however limited by a confined observation period of a maximum 3 hours, which was probably not sufficient to detect deleterious effects of IL-6 on insulin sensitivity. In humans, infusion of a physiological IL-6 concentration in healthy subjects, as well as T2D patients, increases lipolysis and enhances glucose infusion rates during euglycemic–hyperinsulinemic clamp (Carey et al., 2006; Van Hall et al., 2003; Petersen et al., 2005).

Mechanistically, the role of IL-6 in inducing IR is thought to be due to its ability to impair insulin signalling primarily in the liver by induction of suppressor of cytokine signalling 3 (SOCS3) and inhibitory IRS-1 phosphorylation (Weigert et al., 2006). On the other hand, the role of IL-6 in improving insulin sensitivity was attributed to IL-6 dependent activation of AMP-activated protein kinase (AMPK) (Weigert et al., 2006).

Skeletal muscle contraction during exercise improves skeletal muscle insulin sensitivity (Holloszy et al., 2005; O’Gorman & Krook, 2011) and also increases IL-6 mRNA expression and subsequently the circulating IL-6 concentration (Steensberg et al., 2000; Febbraio et al., 2004). Acute IL-6 exposure increases insulin action in cultured human skeletal muscle (Al-Khalili et al., 2006; Weigert et al., 2005; Carey et al., 2006). Recent evidence suggests IL-6 is a crucial exercise-dependent signal and
increases circulating glucagon-like peptide 1 (GLP-1) and enhances β-cell function (Ellingsgaard et al., 2011).

1.3e IFN-γ
It is a Th1 cytokine and plays a role in defense against viruses and intracellular pathogens and in the induction of immune-mediated inflammatory responses. It is produced predominantly by natural killer and natural killer T (NKT) cells as part of the innate immune response, and by T helper type 1 lymphocytes (Th1) CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (Schoenborn & Wilson, 2007). There is evidence that B cells and antigen-producing cells (APC) also produce IFN-γ (Frucht et al., 2001). Its production is controlled by cytokines secreted by APCs, most notably interleukin IL-12 and IL-18. In macrophages, NK and T cells, the combination of IL-12 and IL-18 stimulation further increases IFN-γ production. Negative regulators of IFN-γ production include IL-4, IL-10, transforming growth factor (TGF)-β, and glucocorticoids (Munder et al., 1998; Schindler et al., 2001).

IFN-γ plays an important role in type 1 diabetes and lower levels of IFN-γ and IL-4 were noticed in newly diagnosed diabetic patients (Halminen et al., 2001; Avanzini et al., 2005). It has been proposed that absence of IFN-γ production makes beta cells highly susceptible to viral infection and subsequent attack by natural killer cells lead to hyperglycaemia and diabetes mellitus. Low IFN-γ and IL-12 levels has also been observed in T2D patients (Tsiavou et al., 2004 & 2005). The frequency of the low IFN-γ production allele (IFN-γ 874*A) was significantly higher in T2D, and CD4+ and CD8+ cells obtained from T2D released significantly lower amounts of IFN-γ in the intracellular space. Based on a combination of molecular and immunological observations it appears that IFN-γ also contributes in development of T2D.
IL-1β along with TNFα and IFN γ has been shown to induce β-cell apoptosis in both types of diabetes. In vitro, IL-1β appeared the most β-cell cytotoxic cytokine sufficient to cause inhibition of β-cell function and often sufficient to promote an apoptotic response. However, massive induction of apoptosis in β-cells usually requires a combination of IL-1β plus IFN-γ and/or TNFα. (Maedler et al., 2001 & 2002; Giannoukakis et al., 1999; Stassi et al., 1997; Cnop et al., 2005).

1.3f IFN-β

IFN-β is generally produced in response to a viral infection, and the IFN-β dependent upregulation of genes increase presentation of viral peptides by major histocompatibility complex (MHC) class I molecules to facilitate CD8 T cell recognition and destruction of infected cells. IFN-β activates and induces proliferation in NK cells (Janeway et al., 1999).

IFN-β has been shown to immunomodulate the Th1/Th2 cytokine profile by attenuating the secretion of IFN-γ and IL-12 and augmenting IL-4 and IL-10 secretion (Sellner et al., 2008). Literature search did not reveal any study on IFN-β in relation to T2D. However, in an animal study, transgenic mice, expressing IFN-β in β-cells, presented with impaired β-cell function, hypoinsulinemia, and altered glucose tolerance test, all features of a prediabetic state, and it was demonstrated that there might be an association between IFN-β and T1DM (Pelegrin et al., 1998).

1.3g IL-12p70

It is a pro-inflammatory cytokine, produced by antigen presenting cells like DCs, macrophages and NK cells. It plays a critical role in cell-mediated immunity (CMI)-IL12p70 induces NK cells and T cells to produce pro-inflammatory cytokines, such as IFN-γ, IL-2, IL-3 and TNFα. It contributes
to NK cell maturation (Trinchieri et al., 1997) and, along with other pro-inflammatory factors; it stimulates CD4+CD25− T cell activation in the presence of regulatory T cells (King et al., 2005).

IL-12p70 also regulates naïve T cell differentiation into type 1 T helper cells (Th1), and inhibits differentiation into Th2 (Kang et al., 2005; Stern et al., 1996). IL-12 and Th1 cell infiltration has emerged as an important pathway for autoimmune diabetes as well as chronic inflammation associated with atherosclerosis (Alleva et al., 2000; Fernandes et al., 2004).

It is involved in the pathogenesis of T1D (Kang et al., 2005; Skarsvik et al., 2005), but its role in T2D is uncertain. It has been shown that IL-12 plasma levels are elevated in T2D (Winkler et al., 1998; Wegner et al., 2008), and it contributes in atherosclerotic plaque formation and probably augments macrovascular complications in T2D (Uyemura et al., 1996; Hauer et al., 2005). Wegner et al. (2008) also demonstrated that the IL-12 serum concentration in T2D primarily is dependent upon fasting serum proinsulin concentration.

1.4 Chemokines

Chemokines /chemotactic cytokines are low molecular weight signaling proteins that regulate the migration of different types of cells by interacting with corresponding G protein coupled transmembrane receptors present on the surfaces of their target cells and play a fundamental role in host and innate immunity. The KORA Survey S4 study population showed not only upregulated chemokines IL-10 and RANTES in T2D but also raised RANTES in impaired glucose tolerance (IGT) and supported the fact that these immune abnormalities precede T2D by several years (Herder et al.,
Chemokines have been hypothesized to be involved in macrophage infiltration into adipose tissue in obesity and might therefore play an important role in the development of obesity-related disorders like T2D. Recently, CCX140-B, an inhibitor of the chemokine receptor known as CCR2, has been shown to have beneficial effect on glycaemic control in Phase 2 clinical trial in T2D (Hanefield et al., 2012).

1.4a RANTES

RANTES is a protein encoded by CCL5 gene. It belongs to the β-subgroup of the chemokine family with pro-inflammatory actions. It is expressed by a large variety of cells, such as T-cells, endothelial cells, gestational tissues (Wender-Ozegowska et al., 2008) as well as adipocytes and has been hypothesized to mediate leukocyte infiltration of adipose tissue in obesity (Wu et al., 2007; Poulain-Godefroy et al., 2007).

Circulating RANTES concentrations are elevated in obesity, IGT and T2D (Normura et al., 2000; Herder et al., 2005). In the Finnish Diabetes Prevention Study, high RANTES levels were associated with resistance to lifestyle intervention and higher incidence of T2D in the intervention group (Herder et al., 2006). It has been shown in a large cohort of subjects that the CCL5 gene variant and RANTES serum concentration were not causally related to T2D and it was argued that the elevated level of RANTES in both T2D and IGT group might be a consequent of hyperglycaemia (Herder et al., 2008) instead of the cause of hyperglycaemia.

1.4b IL-8

IL-8 is a potent chemoattractant (chemokine) and its main sources are macrophages, endothelial and epidermal cells. It induces recruitment of
neutrophils and T-cells into the subendothelial space, as well as adhesion of monocytes to endothelium (Gerzten et al., 1999). It seems that the PMN activation in diabetic patients is an important pathogenic link in long-term complications of the disease (Huseynova et al., 2009).

IL-8 is stimulated by high glucose concentrations in endothelial cells in vitro and has a chemotactic activity for PMN, lymphocyte T and smooth muscle cells (Urakaze et al., 1996.) Plasma levels of IL-8 have been found to be significantly increased in patients with both T1D and T2D compared with healthy subjects (Zozulinska et al., 1999; Esposito et al., 2003). A study showed increased levels of serum IL-8, TNF-α and TGF-β1 levels in T2D patients with a significant increase in the stage of decompensation, suggesting that these cytokines may participate in the development and progression of diabetic complications (Huseynova et al., 2009). Another study revealed an increase in IL-8 concentrations in the vitreous of patients with diabetic retinopathy and suggested that IL-8 participates in the pathogenesis of diabetic retinopathy (Elner et al., 1995). It has been reported that urinary levels of IL-8 were significantly increased in patients with T2D nephropathy (Tashiro et al., 2002).

It has been reported that circulating IL-8 correlates with measures of adiposity and insulin sensitivity, suggesting its involvement obesity-related health complications (Bruun et al., 2003b). The plasma levels of IL-8 in abnormally obese subjects is noticed higher than in lean subjects (Straczkowski et al., 2002). Brunn et al. (2004) further demonstrated that there was increased release of IL-8 from visceral adipose tissue compared with subcutaneous AT and primarily the release was from nonfat cells from AT. IL-8 has also been reported to play a role in plaque destabilization and to predict cardiovascular death in patients with coronary artery disease (CAD) (Blankenberg at al., 2002).
1.5 Adipokines

Adipokines are biologically active substances that are secreted by the adipocytes of white adipose tissue (WAT). Notably, these factors may be synthesised at other sites within the body and participate in functions unrelated to those within WAT (Lago et al., 2007). They play a key role in modulating not only inflammation, but also immune and autoimmune reactivity. Adipokines include a variety of pro-inflammatory peptides including TNF and anti-inflammatory factors such as IL-1RA, which binds competitively to the IL-1 receptor without triggering activity within the cell, and IL-10. We measured the following adipokines in the current study:

1.5a Retinol binding protein

RBP4 is an adipokine associated with obesity and comorbidities, especially IR, T2D, and certain components of the metabolic syndrome (Graham et al., 2006). Liver has the highest expression level of RBP4; however, AT has the second highest rate of expression, i.e. 20–40% of that found in the liver (Tsutsumi et al., 1992). The RBP4 gene is located on chromosome 10 (10q23–q24) near the region that has been linked to increased fasting glucose levels in European Caucasians and to T2D in Mexican–Americans (Duggirala et al., 1999).

RBP4 is preferentially expressed in visceral fat when compared with subcutaneous fat (Kloting et al., 2007). Omental adipose tissue is an important source of RBP4 in severely obese patients (Kelly et al., 2010). In a recent study, higher waist circumference and waist-to-hip ratio were associated with higher RBP4 levels and markers of systemic inflammation (Hermsdorff et al., 2010). Few studies have shown that significant decrease in weight, achieved by diet, exercise, or bariatric surgery, leads to a decrease in circulating and/or adipose tissue RBP4 levels. The changes in RBP4 levels in non-diabetic subjects during weight loss were significantly correlated with the amount of visceral fat loss but were not associated with the amount of total body fat loss or abdominal subcutaneous fat loss (Lee et al., 2008). A decrease in serum RBP4 levels...
achieved by exercise training predicts the improvement in insulin sensitivity with greater specificity than leptin, adiponectin, IL-6, or CRP (Graham et al., 2006). Serum RBP4 levels are increased in subjects with impaired glucose tolerance, T2D, and correlate inversely with insulin sensitivity in non-diabetic subjects with a family history of T2D (Yang et al., 2005; Graham et al., 2006). Circulating RBP4 levels correlate with the degree of IR in these subjects and relationship is independent of obesity. Weight loss and exercise decrease RBP4 levels in subjects with IR and T2D (Graham et al., 2006). Therapy with insulin sensitizing agent rosiglitazone also decreases circulating RBP4 levels (Jia et al., 2007). The RBP4 haplotypes were related to an increased risk of T2D (Craig et al., 2007).

However, in several clinical studies, associations and/or causality of observed RBP4 expression changes with these states could not be shown (Yao-Borengasser et al., 2007; Broch et al., 2007; Ulgen et al., 2010; Promintzer et al., 2007).
Figure 1.3 Role of adipocytokines in the modulation of glucose metabolism. Increased adipose tissue mass is associated with decreased adiponectin levels and increased RBP4, IL-6, and TNFα production, which in turn stimulates LCN2 synthesis. LCN2 upregulates adiponectin & downregulates TNFα. The circulating levels of FFAs are increased in obesity. Decreased adiponectin and increased RBP4 levels leads to decreased fatty acid oxidation, increased gluconeogenesis in the liver and accounts for IR. Adiponectin suppresses expression of ICAM-1, E-selectin, and VCAM-1, macrophage-associated SR-A and decreases foam cell formation and exerts antiatherosclerotic effects. FFA, free fatty acid; ICAM-1, intercellular adhesion molecule-1; IR, insulin receptor; IRS, insulin receptor substrate; PPARγ, peroxisome proliferator-activated receptor gamma; SR-A, scavenger receptor-A; VCAM-1, vascular cell adhesion molecule-1. (Reproduced from ref. Esteve et al., 2009).
1.5b Lipocalin-2 (LCN2)

LCN2, also known as oncogene 24p3 or neutrophil gelatinase-associated lipocalin (NGAL), is an adipokine that belongs to the superfamily of lipocalins (such as RBP4) and in humans is encoded by the LCN2 gene. It is expressed in neutrophils and in low levels in the kidney, adipocytes, prostate, and epithelia of the respiratory and alimentary tracts (Cowland et al., 1997) and also used as a biomarker of kidney function (Devarajan et al., 2010). LCN2 is implicated in innate immunity and recently the LCN2 gene was identified as a novel IL-17-induced gene (Shen et al., 2006). LPS and TNFα are two of the strongest inducers of LCN2 production. The TLRs, expressed on immune cells, stimulate the synthesis and secretion of LCN2 upon exposure to bacteria and LCN2 can bind to the bacterial siderophores and so limit bacterial growth (Yang et al., 1997; Flo et al., 2004). LCN2 deficiency resulted in an increased susceptibility to bacterial infection in a mice experiment (Berger et al., 2006).

LCN2 is highly expressed in adipose tissue levels and circulating levels are increased in obese animals and human subjects with T2D (Wang et al., 2007; Yan et al., 2007). The agents that promote IR including TNFα, glucocorticoids, and hyperglycaemia increased the expression of LCN2, and thiazolidinediones, an insulin-sensitizing agent reduced it (Wang et al., 2007; Yan et al., 2007). Yan et al. (2007) further demonstrated that LCN2 2 promotes IR in cultured adipocytes. These data suggest that LCN2 acts as an adipocyte-derived mediator of insulin resistance in obesity and inflammation (Jun et al., 2011). However, studies revealed discordant results on the role of LCN2 in the modulation of insulin sensitivity. One group showed that Lcn2−/− mice were leaner and more insulin sensitive and the other showed that Lcn2−/− mice were more obese and insulin resistant (Guo et al., 2010; Law et al., 2010). Contrary to this, Jun et al. (2011) found that only male Lcn2−/− mice on a high-fat diet (HFD) had a small improvement in glucose tolerance, but that ablation of LCN2 had no effect on insulin sensitivity in the chow-fed mice of either sex or in female HFD-fed mice.
LCN2 has been shown to antagonise the detrimental effects of inflammatory molecules on inflammation and metabolism in adipocytes and macrophages. Moreover, LCN2 upregulated peroxisome proliferator–activated receptor (PPAR)-γ and its target genes, adiponectin, leptin, fatty acid synthase, and lipoprotein lipase in adipocytes and suppression of LCN2 expression resulted in decreased expression of PPAR-γ (Zhang et al., 2008). LCN2 antagonized the effect of TNFα on adipocytes and macrophages, protected adipocytes from TNF-α–induced production of IL-6 and MCP-1, attenuated TNFα effect on glucose uptake, and completely reversed TNFα mediated inhibition of leptin and adiponectin secretion from adipocytes (Zhang et al., 2008). The stimulatory effect of LPS on cytokine gene expression in macrophages was also significantly attenuated by LCN2 (Zhang et al., 2008). This suggests that the anti-inflammatory effect of LCN2 is associated with modulation of PPARγ activity via direct or indirect mechanisms, may be through the inhibition NF-κB activity (Zhang et al., 2008). These findings suggests that anti-inflammatory is the primary role of LCN2 and the increased LCN2 levels in obesity and IR is reflection of protective mechanism against inflammation (Esteve et al., 2009)).

### 1.5c Adiponectin

Adiponectin is the most abundant protein secreted by adipocytes and acts as a hormone with anti-inflammatory and insulin sensitizing properties (Esteve et al., 2009). The animal models and humans studies suggest that adiponectin decreases the risk of T2D by lowering hepatic gluconeogenesis, enhancing fatty acid oxidation in the liver, stimulating fatty acid oxidation and glucose uptake in skeletal muscle, and improving insulin secretion (Kadowaki et al., 2006; Rabe et al., 2008).

Adiponectin exerts its effect through two distinct receptors termed adiponectin receptor 1 (AdipoR1) and adiponectin receptor2 (AdipoR2) and leads to phosphorylation and activation of AMPK and increased expression of PPAR-α. TNFα and IL-6 regulate adiponectin production by
reducing gene expression whereas insulin sensitizers and PPAR-γ agonists increase adiponectin levels in mice and humans (Kadowaki et al., 2005). Adiponectin secretion, in contrast to secretion of other adipokines, is paradoxically decreased in obesity (Bruun et al., 2003a). This may be attributable to inhibition of adiponectin gene transcription by inflammatory and angiogenic factors secreted by hypertrophic adipocytes (Hajer et al., 2008).

In intervention studies, circulating adiponectin levels increased after weight loss in humans. Diet, gastric bypass surgery, orlistat, or selective cannabinoid receptor type 1 (CB1) receptor blockade with rimonabant significantly increased adiponectin levels in parallel to reduced body weight (Despres et al., 2005). Thiazolidinediones increased adiponectin expression and circulating levels in rodents, nondiabetic subjects, and patients with T2D. Exercise without significant weight loss did not affect circulating adiponectin, but improved insulin resistance, suggesting different pathways for the insulin-sensitive mechanism of exercise other than adipokine modulation (Kadowaki et al., 2006).

A meta-analysis of thirteen prospective studies with a total of 14,598 participants and 2,623 incident cases of T2D showed that higher adiponectin levels are associated with a lower risk of T2D across diverse populations, consistent with a dose-response relationship (Li et al., 2009).

Despite the well-documented negative association between adiponectin and several inflammatory factors, adiponectin was found increased in chronic inflammation associated with autoimmune diseases, such as T1D, systemic lupus erythematosus (SLE), RA, and inflammatory bowel disease (IBD), suggesting that adiponectin levels increase in chronic inflammatory conditions that are not associated with obesity. It is possible that adiponectin could have a protective role in inflammation related to adipose tissue loss during starvation and fasting, but not in inflammation unassociated with obesity (Esteve et al., 2009).
1.6 Toll-like receptors

TLRs are a conserved family of pattern recognition receptors that play a fundamental role in the innate immune system by triggering proinflammatory-signalling pathways in response to microbial pathogens. Thirteen mammalian TLRs, TLR1-13, have been identified to date, which includes 11 human (Miggin and O’Neill et al., 2006). Different microbial structures are recognized by different TLRs (Fig. 1). LPS from Gram-negative bacteria is recognized by TLR4. Gram-positive bacteria activate TLR2, while bacterial components, such as lipopeptides and lipoteichoic acid (LTA) are recognized by TLR2 in cooperation with TLR1 or TLR6. Viral and/or bacterial nucleic acids are recognized by TLR3, TLR7, TLR8 and TLR9 (Quereshi et al., 2003; Schwandner et al., 1999; Okusawa et al., 2004). TLR3 recognizes viral dsRNA and synthetic polynosinepolycytidylic acid (polyIC), while viral single-stranded RNA (ssRNA) and the antiviral compounds imiquimod and resiquimod (R848) are ligands for TLR7 and TLR8 (Alexopoulou et al., 2001; Diebold et al., 2004).

Bacterial DNA and the synthetic unmethylated oligonucleotides containing CpG dinucleotides (CpG) are recognized by TLR9 (Hemmi et al., 2000). TLR5 is activated by flagellin from bacterial flagella (Hayashi et al., 2001). TLR11 recognizes uropathogenic bacteria and a protozoan-derived profilin-like protein (Yarovinsky et al., 2005). The ligands for TLR10, TLR12 and TLR13 are currently unknown.

1.6a TLR signalling

TLRs 1, 2, 4, 5, 6 are trans-membrane proteins, while TLRs 3, 7, 8 and 9 are predominantly localized intracellularly. Their common structure consists of an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic domain, sharing homology with the mammalian IL-1 receptor (Takeda et al., 2003). The LRR domain binds ligands, while the cytoplasmic Toll-IL-1 receptor (TIR) domain initiates intracellular signalling.
pathways through homotypic protein-protein interaction with TIR-adapter molecules (O’Neill et al., 2007). Four TIR-adapter molecules have been shown to mediate TLR signalling; myeloid differentiation factor 88 (MyD88), myeloid adapter-like protein (Mal) also known as Toll/interleukin-1-receptor (TIR)-domain-containing adaptor protein (TIRAP), TIR-domain containing adaptor inducing protein inducing IFN-β (TRIF) and TRIF-related adapter molecule (TRAM) (Figure 1). In TLR4 signalling MyD88 dependent signaling is facilitated by the adaptors, MyD88 and Mal, resulting in the activation of NF-κB and production of inflammatory cytokines (Fitzgerald et al., 2001). MyD88-independent signalling, facilitated by the adaptors TRIF and TRAM, leads to activation of interferon regulatory factor 3 (IRF-3) and induction of Type I IFNs (Yamamoto et al., 2003a). TLR2 signaling utilizes both Mal and MyD88 in a manner similar to TLR4, while TLR7/8 and TLR9 require only MyD88 for signalling (Akira et al., 2006). TLR3 signalling requires TRIF alone, and is the only TLR known to signal in a MyD88-independent manner (Yamamoto et al., 2003b). A fifth TIR-adapter SARM (sterile alpha and HEAT-Armadillo motifs containing protein) may interact with TRIF and inhibit its function (Carty et al., 2006). However, cells from animals deficient in SARM do not display altered TLR signaling (Carty et al., 2006). Interestingly, SARM has been shown to contribute to neuronal death in viral infections (Peterson et al., 2012).
Figure 1.4 Overview of selected members of the TLR family, ligands and the TIR-adapters. TLRs 1, 2, 4, 5, and 6 are located primarily in the plasma membrane and interact with components of microbial pathogens while TLRs 3, 7, 8, and 9 are situated in the membranes of endosomes and lysosomes. TLR4 activates two distinct signalling pathways: one pathway is activated by the adaptors MAL and MyD88 leading to activation of NF-κB and finally production of inflammatory cytokines, and the second pathway is activated by the adaptors TRIF and TRAM leading to the induction of IRF-3 and type I IFNs. TLR2 signalling utilises both Mal and MyD88, while TLR7/8 and TLR9 require only MyD88 for signalling. TLR3 signalling requires TRIF alone. TLR, Toll like receptors, MAL, myeloid adapter-like protein, MyD88, myeloid differentiation factor 88, TRIF, TIR-domain containing adaptor inducing protein inducing IFN-β, TRAM, TRIF-related adapter molecule, NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells, IRF-3, interferon regulatory factor 3 and IFN, Interferon.
1.6b TLR responses

Activation of the MyD88-dependent pathway results in the induction of many genes including IκB protein IκBζ, which functions as an inducible coactivator for the NF-κB p50 subunit to facilitate IL-6 and IL-12p40 induction (Yamamoto et al., 2004). Also, C/EBPδ, which acts together with NF-κB serves to maximize IL-6 production (Litvak et al., 2009). IκB-NS (also known as IκB-d, or Nfkbid: nuclear factor of k light polypeptide gene enhancer in B-cells inhibitor, delta), a TLR-inducible nuclear IκB protein, suppresses the induction of both IL-6 and TNF by modulating the DNA-binding activity of the NF-κB p65 subunit (Kuwata et al., 2006). Also, activating transcription factor 3 (ATF3) restricts NF-κB activity by recruiting histone deacetylase (Gilchrist et al., 2006) In addition to NF-κB activation; the TRIF dependent pathway also activates IRF3 and interferon-β transcription (Kawai et al., 2010).

The immune response is determined by the actual TLRs that are activated (Agrawal et al., 2003; Seya et al., 2006). Distinct TLR ligands instruct human DCs to induce distinct T helper (Th) cell responses by differentially modulating mitogen-activated protein kinase signalling (Agrawal et al., 2003). TLR3, TLR8 and surface-expressed TLRs are expressed in antigen-presenting myeloid dendritic cells (mDCs) while TLR7 and 9 are expressed in human plasmacytoid DCs (pDCs) (Liu et al., 2006). Thus, TLR3, TLR8 and surface-expressed TLRs such as TLR2, 4 and 5 are mainly involved in the modulation of antigen-presentation in mDC. DCs activated by TLR9 and TLR7/8 ligands yield IL-12, IFNα and induce strong Th1 and CTL responses in a MyD88-dependent manner (Blasius et al., 2010). DCs triggered via TLR3 yield mostly IFNα and also induce Th 1 and CTL responses (Blasius & Beutler, 2010). TLR4 ligands predominantly induce IL-12, IFNα and drive a Th1 response. LPS and poly (I:C) additionally induce upregulation of costimulatory molecules such as CD40, CD80 and CD86 in an IFN-β dependent manner, via type I interferon.
receptor (IFNRI), on macrophages and DC (Hoebe et al., 2003).

The activation of DCs by TLR ligands is necessary for their maturation and consequent ability to initiate adaptive immune responses. mDCs are central to T/B cell activation and facilitate production of antibodies through the induction of differentiation of B lymphocytes. T lymphocytes are differentiated by matured mDCs into Th1, Th2 and CTL. mDCs process antigens, alter their function and migrate to draining lymph nodes (Fig. 1).

The disruption in negative regulators of TLRs results in persistent inflammation. These include splice variants for adaptors or their related proteins, ubiquitin ligases, deubiquitinases, transcriptional regulators and microRNAs. The negative regulators are helpful in suppressing inflammation and might play an important role in immunity (Kawai et al., 2010).
Figure 1.5 Role of human TLRs in mDC maturation followed by activation of various lymphocytes. Immature dendritic cells (mDC) residing in local tissue phagocytose exogenous antigen (Ag) and pattern molecule (namely adjuvant) and initiate the maturation process. During maturation mDC induce IFNs, cytokines and chemokines, allow the upregulation of co-stimulators, NK-activating ligands (ULBP, MIC, etc.) and MHC, and activate a variety of lymphocytes. These maturation events are largely dependent on adjuvant properties. Also, adjuvant may participate in switching on of some unknown mechanisms, which are essential in induction of CD8+ CTL by mDCs. Adapted from ref. Seya et al., 2006.
1.6c TLRs and diabetes

Both human and rodent pancreatic islets (Wen et al., 2004) express TLRs and TLRs been shown to play a multifaceted role in the development of diabetes, which include β-cell immune response and metabolic disorder associated inflammation.

Emerging evidence indicates that the pro-inflammatory pathways may become activated due to metabolic syndrome, which includes insulin resistance, hyperinsulinemia, dyslipidemia, obesity and hypertension, and lead to consequential interference with insulin signalling and cause insulin insensitivity in the target organs, such as endothelium, adipose tissue, liver, skeletal muscle and most recently podocytes (Shrivastava et al., 2013). The LPS-derived saturated FFA which is present in abundance in metabolic abnormalities plays a key role in modulating the IR and it is postulated that the TLRs signaling cascade may become activated by the FFA and thereby affect the pathogenesis of obesity and metabolic syndrome (Schaeffler et al., 2009). Increased expression of TLR2 and TLR4 along with IL-1β, IL-6, IL-8 and NF-κB nuclear binding has been demonstrated in metabolic syndrome patients without diabetes or CAD (Jilal et al., 2012).

The mechanism by which TLR2 and TLR4 modulate insulin resistance was studied in different tissues (Mohammad et al., 2006). In adipose tissue, TLRs/NF-κB pathway may be directly activated by nutrients, especially the saturated fatty acid, and so may mediate inflammation through interaction between adipocytes and infiltrated macrophages (Nguyen et al., 2007; Shi et al., 2006; Suganami et al., 2007; Vitseva et al., 2008; Yeop Han et al., 2010). The functional expression of TLR2 and
TLR4 has also been confirmed in human AT (Song et al., 2006; Zhang et al., 2009). Elevated TLR4 expression, but not TLR2, was detected in muscle biopsies from obese and T2D patients, and correlated with homeostatic model assessment, HOMA-IR and fasting plasma FFA concentrations (Reyna et al., 2008).

The role of PBMC TLRs in diabetes has been extensively studied using a series of clinical and animal study. In T1DM patients, the increased TLR2 and TLR4 expression in monocytes was associated with increased HbA1c levels (Devaraj et al., 2008), while in newly-diagnosed T2D subjects, the upregulation of TLR2 and TLR4 was correlated with HOMA-IR (Dasu, et al., 2010). Another study showed that TLR4 expression and signalling was elevated in IR patients (Reyna et al., 2008), which may be caused by elevated plasma FFA levels and contributed to the pathogenesis of IR in humans. In another study, TLR4 deficient 10ScN mice were exposed to high saturated fat and it showed that TLR4 deficiency, protected mice against the obesigenic effects of SFA and altered obesity-related inflammatory responses in adipose tissue (Davis et al., 2008). Interestingly, insulin infusion significantly suppressed TLR1, 2, 4, 7 and 9 mRNA expressions in mononuclear cells within 2 h (Ghanim et al., 2008), whereas high glucose induces the expression of TLR mRNA (Dasu et al., 2008). In vitro, hyperglycaemia induced TLR2 and TLR4 expression via protein kinase C (PKC) and NADPH oxidase activation, and knocking down of TLR2 and TLR4 significantly downregulated the high glucose-induced NF-κB activation. Additionally, high glucose also induces TLR2 receptor dimerisation with TLR6 and activate downstream MyD88-dependent signaling pathway (Dasu, et al., 2008). In vivo, PPARγ agonist could exert its anti-inflammatory effect via modulating the TLR2 and TLR4 signaling in db/db mice. Therefore, the role of TLR2 and TLR4 in monocytes and macrophages provides further evidence of systemic proinflammatory state in diabetes.
Recent results from TULIP and METSIM studies showed that TLR4 genetic variation (D299G/T399I) correlated with increased total body fat, visceral fat and impaired insulin sensitivity, and they may contribute to the risk factor for diabetes and metabolic syndrome in more than 6000 non-diabetic Caucasians (Weyrich et al., 2010). This result was consistent with the data of other studies, which showed that TLR4 polymorphism was associated with dyslipidemia, vascular inflammation, and diabetic neuropathy in T2D patients (Buraczynska et al., 2009; Kolek et al., 2004; Rudofsky et al., 2004). However, a number of other studies found no correlation between TLR2 or TLR4 polymorphism and diabetes or metabolic syndrome (Illig et al., 2003; Santin et al., 2006). On the other hand, TLR2 (Ht4 and rs3804100 T) variation was reported to be strongly associated with T1DM (Bjornvold et al., 2009; Park et al., 2004), and (593 C/T, 2642 C/Am and 2690 A/G) polymorphism in TLR3 was associated with T1D in South African Blacks with T1DM (Pirie et al., 2005). However, a recent study evaluated the association of TLR4 +3725G/C and +11367G/C polymorphisms with T2D in the Chinese population and it was demonstrated that people carrying 11367CC genotype may have 46% less risk of developing T2D compared to those with GG genotype (Jiang et al., 2013a).

Collectively, we noticed that there is paucity of data examining adipokine and TLR expression in T2D. In particular, the role of glycaemic control in modulating adipokine and TLR expression is uncertain. With this concern, we carried out a pilot study to investigate mRNA expressions of TLR2, TLR6 and TLR4 in non-diabetic subjects (n=10, HbA1c 5.3±0.2%) and compared to T2D subjects with good (n=9, HbA1c 6.7±0.7%) and poor glucose control (n=8, HbA1c 10.9±2.2%) by qPCR (*p<0.05 vs. control). Interestingly, our data showed that TLR2, TLR4 and TLR6 mRNA expressions were suppressed in T2D subjects (presented as Late breaking abstract in ADA in 2009, Siednienko et al., 2009b). This was in contrast to previous studies, which had shown overexpressed TLR2 and
TLR4 in T2D (Reyna et al., 2008; Dasu et al., 2010). The T2D subjects in our pilot study were not classified according to the presence or absence of diabetes-related complications and it was conceivable that complications and treatment could have affected the TLRs result in this study. To explore the role of glycaemic control and diabetes-related micro/macrovacular complications in TLRs expression, we decided to divide the T2D cohort in 4 groups i.e. GC, T2D with good glycaemic control (HbA1c <7.5%) and no macrovascular complications (heart disease, Transient ischaemic stroke (TIA)/stroke or peripheral arterial disease (PAD)) and microvascular complications (retinopathy, neuropathy or nephropathy); GCC, T2D with good glycaemic control and complications; PC, T2D with poor glycaemic control (HbA1c >7.5%) without complications, and PCC, T2D with poor glycaemic control and complications, and compare them with the healthy controls (NGT, normal glucose tolerant). It has been shown that monocytes and neutrophils differ in the surface expression of TLR mRNAs (Muzio et al., 2000) and with this in mind, we aimed to evaluate mRNA expressions in both monocytes and neutrophils. Knowing that adipokines data are lacking in T2D, we aimed to investigate the adipokines mRNA expression in these 4 different T2D study groups.

Previous studies have shown altered cytokines level in T2D subjects. In our pilot study we also measured serum levels of pro-inflammatory cytokines IL-6, IL-1β and TNF-α by ELISA and showed elevated IL-6 in T2D subjects compared to healthy controls (presented as Late breaking abstract in ADA in 2009, Siednienko et al., 2009b). There has been no prior study in relation to IFNβ and T2D. IFN-β has been shown to immunomodulate the Th1/Th2 cytokine profile by attenuating the secretion of IFN-γ and IL-12 and augmenting IL-4 and IL-10 secretion (Sellner et al., 2008). With the aim of exploring the inflammatory and anti-inflammatory milieu in T2D, we sought to investigate pro-inflammatory cytokines; IL-6, TNF-α and IL-1β and IFNβ, anti-inflammatory cytokines;
IL-10, IL-8 and Rantes, and Th1 cytokines: IL-12 and IFN-γ in 4 different T2D study groups and comparing with NGT subjects.
Aims of Study

The overall aim of this study was to determine whether T2D is a chronic inflammatory condition and is associated with raised inflammatory markers. Our aim was to investigate if the degree of inflammation varies with glycaemic control and with diabetes associated long-term microvascular and macrovascular complications. To investigate this, we determined the serum concentration of various inflammatory markers including CRP, pro-inflammatory and anti-inflammatory cytokines and chemokine in different T2D profile subjects, those with good and poor glycaemic control with or without complications and compare them with the healthy controls. We sought to evaluate cytokines, adipokines and TLR’s gene expression in the monocytes and neutrophils of T2D subjects and compare them with the healthy controls. In particular our objectives were as follows:

1. To compare the circulating pro-inflammatory cytokine levels in the blood of T2D subjects and healthy controls by employing biochemical techniques

2. To perform comparative analysis of cytokines expression in subjects with T2D (with either good or poor glucose control, +/-complications) relative to normal individuals.

3. To perform comparative analysis of adipokine expression in subjects with T2D (with either good or poor glucose control, +/-complications) relative to normal individuals.

4. To perform comparative analysis of TLR expression in subjects with T2D (with either good or poor glucose control, +/-complications) relative to normal individuals.
Chapter 2

Materials and Methods
2.1 Patients and Methods

The local research ethics committee, Department of Public Health, Health Service Executive, Midland area and Ethical Review Board, National University of Ireland Maynooth, Co. Kildare, Ireland, approved the research project of this study.

Research participants were identified following inspection of the lists of patients due to attend the Diabetes Day Centre (DDC) and outpatient endocrinology clinic at the Midland Regional Hospital (MRH), Mullingar. Potential research participants were approached during their clinic visit.

If the potential research participant gave informed consent, a subject contact sheet and an anonymous case report form was created and completed for each research participant and used to ensure optimal data collection. All participant data were kept anonymous.

Given that immune function is affected by both metabolic control and intercurrent illness, participants who had evidence of infection were excluded. The inclusion and exclusion criteria were as follows-

2.1a Inclusion criteria
- An age of 18 years or more
- Type 2 diabetic subjects diagnosed according to American Diabetes Association criteria

2.1b Exclusion criteria
- Signs of current infection,
- Current treatment with antibiotics,
- Neutropenia (a leucocyte count of less than 2000 per cubic millimeter)
- Pregnancy or breast-feeding (contraception for at least 3 months before inclusion was required for fertile women)
- Liver or renal disease (a level of asparate aminotransferase or alanine aminotransferase of more than three times the upper limit of the normal range)
- Ongoing or previous cancer: the use of oral glucocorticoid medication and immunosuppressive treatment or immunodeficiency.

2.1c Healthy control subjects

Healthy adults were recruited as control subjects from laboratory, secretarial, medical, porter staff and ambulance crew from the Midland regional Hospital, Mullingar catchment area. Each healthy control participant was screened for diabetes and pre-diabetes using the standard oral glucose tolerance test (American Diabetes Association (ADA) reference). Participants with abnormal results were excluded from the study.

2.2 Oral Glucose Tolerance Test (OGTT)

The OGTT was performed according to World Health Organisation (WHO) criteria. Participants were asked not to eat or drink, except water for 10 h prior to the test. The first fasting venous blood sample was collected by venepuncture at 08.00 h and then the participants were given 410 ml of Lucozade from a standard Lucozade bottle (70 kcal/100mmls) or equivalent drink containing 75 grams of anhydrous glucose. Participants were asked to consume the glucose load within 5 min and second blood sample was collected at 120 min (10.00 h). Participants remain seated throughout the test in the department and were not allowed to smoke during the test.
2.3 ADA criteria for diagnosis of diabetes

The ADA recommends following criteria for the diagnosis of diabetes and pre-diabetes -

1. A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the Diabetes Control and Complications Trial (DCCT) assay.

2. Fasting plasma glucose (FPG) ≥7mM. Fasting is defined as 8 hours or more without caloric intake.

3. Two-hour plasma glucose (2hPG) ≥11.1mM during performance of an oral OGTT.

4. In a patient with classic Symptoms of hyperglycaemia or hyperglycaemic crisis a random plasma glucose ≥11.1mM. In absence of symptoms, result should be confirmed by repeat testing.

Table 2.1. Criteria for diagnosis of diabetes

<table>
<thead>
<tr>
<th>Normal</th>
<th>IFG or IGT</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG &lt; 5.6mM</td>
<td>FPG 5.6-6.9mM</td>
<td>FPG ≥ 7mM</td>
</tr>
<tr>
<td>2hPG &lt; 7.8mM</td>
<td>2hPG 7.8-11.0mM</td>
<td>2hPG ≥ 11.1mM</td>
</tr>
<tr>
<td></td>
<td>A1C 5.7-6.4%</td>
<td>A1C ≥ 6.5%</td>
</tr>
</tbody>
</table>

IFG – Impaired fasting glucose, IGT – Impaired glucose tolerance
2.4 The study design

Based on these criteria, 146 participants were recruited from a hospital based diabetes clinic and diabetes day centre. Among these 34 healthy volunteers without diabetes acted as control, normal glucose tolerant (NGT) and 112 as T2D subjects. The T2D subjects (mean duration 95 months) were classified in four different profiles based on glycaemic control and complications: T2D with good glycaemic control (HbA1c <7.5%) and no macrovascular complications (heart disease, Transient ischaemic stroke (TIA)/stroke or peripheral arterial disease (PAD)) and microvascular complications (retinopathy, neuropathy or nephropathy): (GC, n=27), T2D with good glycaemic control and complications (GCC, n=32), T2D with poor glycaemic control (HbA1c >7.5%) without complications (PC, n=21), and T2D with poor glycaemic control and complications (PCC, n=32).

Table 2.2. The study design showing T2D groups

<table>
<thead>
<tr>
<th>T2D subjects (n = 112)</th>
<th>Good glycaemic control (HbA1c &lt; 7.5%)</th>
<th>Poor glycaemic control (HbA1c &gt; 7.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Complications</td>
<td>GC = 27</td>
<td>PC = 21</td>
</tr>
<tr>
<td>Complications</td>
<td>GCC = 32</td>
<td>PCC = 32</td>
</tr>
</tbody>
</table>

HbA1c – Glycosylated haemoglobin, T2D – Type 2 diabetes mellitus
Clinical data that were collected include:

1. Signs detected by the recruiting personnel
2. Medications taken prior to hospital admission
3. Known medical illnesses
4. Family history of diabetes
5. Smoking habits and alcohol consumption
6. General physical examination
7. Results of blood tests and other diagnostic procedures.

The research participants were invited to attend the diabetes centre in a fasting state for blood tests. The fasting state involved fasting overnight for at least 12 hours. The personal and medical data were obtained by patient interview, by using hospital medical notes, and from using hospital blood test results. The demographic information was obtained from research participants and their weight, height, blood pressure & waist and hip circumferences were determined. BMI was calculated as body weight (in kilograms) divided by body height (in meters) squared. Waist-to-hip ratio (WHR) was calculated as per WHO criteria.

2.4a Waist hip ratio

Waist and hip circumference was measured as per the WHO STEPwise Approach to Surveillance (STEPS) (WHO, 2008b). Waist circumference was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest, using a stretch resistant tape that provides a constant 100 g tension. Hip circumference was measured around the widest portion of the buttocks, with the tape parallel to the floor. For both measurements, the subject stand with feet close together, arms at the side and body weight evenly distributed, and wore little clothing. The subject was relaxed, and the measurements were taken at the end of a normal expiration. Each measurement was repeated twice; if the measurements were within 1 cm of one another, the average was calculated. If the difference between the two measurements exceeds 1
cm, the two measurements were repeated. The WHO recommendations for waist circumference and WHR are as follows:

Table 2.3 World Health Organization cut-off points and risk of metabolic complications

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Cut-off points</th>
<th>Risk of metabolic complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>&gt;94 cm (M); &gt;80 cm (W)</td>
<td>Increased</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>&gt;102 cm (M); &gt;88 cm (W)</td>
<td>Substantially increased</td>
</tr>
<tr>
<td>WHR</td>
<td>≥0.90 cm (M); ≥0.85 cm (W)</td>
<td>Substantially increased</td>
</tr>
</tbody>
</table>

M, men; W, women

2.4b Retinopathy assessment

This MRH, Mullingar has an excellent ophthalmology department and diabetic patients are followed up and treated closely based on severity of retinopathy. The digital retinal examination and fundus photographs are used as a screening tool in this centre. The fundus examination and retinopathy staging including history of laser treatment was noted from ophthalmology assessment part of medical notes.

2.4c Neuropathy assessment

Subjects with diabetes were examined for presence of distal symmetric polyneuropathy by using following tests:

1. Vibration perception (using a 128-Hz tuning fork)
2. 10-g mono-filament pressure sensation at the distal plantar aspect of both great toes and metatarsal joints, and
3. Assessment of ankle reflexes

In T2D subjects with neuropathy, particularly when severe, causes other than diabetes, such as neurotoxic mediations, alcohol abuse, vitamin B12 deficiency (especially in those taking metformin for prolonged periods),
renal disease, chronic inflammatory demyelinating neuropathy, inherited neuropathies, and vasculitis were ruled by taking proper history, examination and vitamin B12 blood level check up. History of autonomic neuropathy was noted from medical notes.

2.4d Nephropathy assessment

An early morning urine sample for measurement of albumin-creatinine ratio (ACR) and urine microalbumin was collected. The urine albumin concentrations and urine creatinine were determined using an automated immunoturbidity method and alkaline picrate method respectively. ACR was then categorized as normoalbuminuria, microalbuminuria and macroalbuminuria (Table 3). As per ADA recommendations, ACR was considered abnormal, if two of three specimens collected within a 3- to 6-month period were abnormal (> 2.5 mg/mmol for men, > 3.5 mg/mmol for women).

<table>
<thead>
<tr>
<th>Category</th>
<th>Spot collection urine (ACR in mg/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.00 – 2.50</td>
</tr>
<tr>
<td>Micro albuminuria</td>
<td>2.5 – 25</td>
</tr>
<tr>
<td>Macro (clinical)-albuminuria</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

ACR - Albumin-Creatinine Ratio
The National Kidney Foundation classification based on level of GFR was used for staging chronic kidney disease (CKD) (Levey et al., 2003).

Table 2.5 Stages of CKD

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (ml/min per 1.73 m² body surface area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Kidney damage* with normal or increased GFR</td>
<td>≥90</td>
</tr>
<tr>
<td>2.</td>
<td>Kidney damage* with mildly decreased GFR</td>
<td>60–89</td>
</tr>
<tr>
<td>3.</td>
<td>Moderately decreased GFR</td>
<td>30–59</td>
</tr>
<tr>
<td>4.</td>
<td>Severely decreased GFR</td>
<td>15–29</td>
</tr>
<tr>
<td>5.</td>
<td>Kidney failure</td>
<td>&lt;15 or dialysis</td>
</tr>
</tbody>
</table>

* Kidney damage defined as abnormalities on pathological, urine, blood, or imaging tests. (Adapted from Levey et al., 2003)

2.4e Foot examination

All participants were examined for active foot ulcers, amputations, structural abnormality and Charcot foot. History of previous ulcer was noted from medical notes.

The foot was examined for dorsalis pedis and posterior tibial artery pulsations to look for PAD. Few patients already had PAD and were
attending vascular consultant. The foot was assessed for neuropathy (mentioned above).

2.5 Lab techniques

2.5a Blood sampling procedures

Fasting blood sample was used to measure various biochemical parameters and cytokines. About 40 ml peripheral blood was collected from each participant including healthy volunteers.

A haemoglobin analyser HA-8160 (Menarini Pharmaceuticals, Ireland) was used for HbA1C (HPLC Chromatography Method) and Advia analysers were used to measure the whole blood count measurement. Lipid profile (Enzymatic Colourmetric Methods), urea (Kinetic UV Method), creatinine (Jaffe rate-blanked compensated kinetic colourimetric Method), sodium, potassium, aspartate aminotransferase, alanine aminotransferase, bilirubin, alkaline phosphatase, gamma glutamyl transferase, CRP (Particle-enhanced immunoturbidimetric), ferritin, coagulation screen, thyroid profile (competitive immunoassay using direct chemiluminescent technology) & plasma glucose (Hexokinase) were measured using Roche Modular 1800 analyser. Erythrocyte Sedimentation Rate (ESR) was measured manually using an automated Westergren equivalent (Sedimentation Method). An early morning urine sample for measurement of ACR (Immunoturbidimetric assay for urine microalbumin & Jaffe rate blanked compensated kinetic colourmetric for urine creatinine) was also collected at the same time. The blood and urine samples were assayed at the MRH, Mullingar.

Analysis of cytokines, chemokines, adipokines and TLR expression

Analysis of cytokines, chemokines, adipokines and TLR mRNA expression was performed at the Immune Signal Laboratory (ISL), National University of Ireland Maynooth, Co. Kildare, supervised by Dr Sinead Miggin. Fresh
blood sample was transported in ice cold packs from MRH, Mullingar to ISL, Maynooth and processed the same day for mRNA assay.

2.5b Preparation of Optiprep reagent
A working solution of Optiprep was prepared by dissolving 4 vol of OptiPrep (density 1.320±0.001 g/ml) with 2 vol of Dulbecco's Modified Eagle Medium (DMEM). Next, 9 vol of DMEM was added to the working solution of Optiprep to achieve the required density of 1.080 g/ml.

2.5c Preparation of buffy coat
Peripheral blood samples (30 ml) was collected in five Ethylenediaminetetraacetic acid (EDTA) vacutainer tubes and was transferred to 50 ml Falcon tubes and centrifuged at 500g for 20 min at 20°C at RAD 180 & RCF 350 using a Hettich Mikro 120 centrifuge. The plasma supernatant was aspirated and erythrocytes were allowed to settle at the bottom. The buffy coat i.e. Leucocyte-rich plasma (LRP) (10 ml) was collected and transferred into a fresh 50 ml polypropylene conical tube. The buffy coat was under layered with 1.080 g/ml solution of OptiPrep. Care was taken to avoid mixing the layers. The buffy coat/OptiPrep mixture was centrifuged at 800 g for 30 min at 18-22°C using a Hettich Mikro 120 centrifuge. No brake was applied during deceleration.

2.5d Isolation of Peripheral Blood Mononuclear Cell (PBMC)
The PBMCs were harvested from the upper interface into a 15 ml tube followed by centrifugation at 350 g for 10 min at 20°C using a Hettich Mikro 120 centrifuge. The supernatant was discarded and the cell pellet was used for monocyte isolation (described in section 2.6.4).
The polymorphonuclear (PMN) cell pellet was isolated from the lower interface and transferred to a new 50 ml Falcon tubes to avoid contamination with remaining mononuclear cells left in the tube. Next, an ammonium chloride solution (NH₄Cl) 0.8% (RBC lysis buffer, Catalog # 07850, Stem cell) was added to the PMN cell pellet at a ratio of 9:1 to lyse the red blood cell (RBC) pellet. About 45 ml of NH₄Cl was added to 5 ml of pellet and kept on ice for 10 min.

The tube was then centrifuged at 500 g for 10 min at 4°C using a Hettich Mikro 120 centrifuge and the brake speed was set to low. The supernatant was then removed. Next, to remove the platelets, the pellet was washed with 15 ml of phosphate Buffered Saline (PBS) containing 2% FBS and 1 mM EDTA and centrifuged at 120 g for 10 min at 4°C using a Hettich Mikro 120 centrifuge with the brake off. This step was repeated once.

Next, the supernatant was discarded and cells were resuspended in 5 ml of PBS containing 2% FBS and 1 mM EDTA and transferred to a 5 ml polystyrene round-bottom tube. The tube was then centrifuged at 500 g for 10 min at 20°C using an Eppendorf centrifuge 5810R refrigerated ultracentrifuge. The supernatant was discarded and the cell pellet was used for isolation of neutrophils.

2.5e Monocyte extraction

Human CD14 positive monocytes were isolated from PBMCs using the EasySep® Human CD14 Positive Selection Cocktail. The CD14 positive monocytes were specifically labeled with dextran-coated magnetic nanoparticles using bispecific Tetrameric Antibody Complexes (TAC). These complexes recognise both dextran and the target cell surface antigen (Figure 2.1). The small size of the magnetic dextran iron particle permitted efficient binding to the TAC-labeled cells, and did not interfere
with subsequent FACS analysis. Magnetically labeled cells were then separated from unlabeled cells using the EasySep® procedure.

### 2.5e.1 Component description and principle

The EasySep® Human CD14 Positive Selection Cocktail contained a combination of monoclonal antibodies purified from hybridoma culture supernatant by affinity chromatography using Protein A or Protein G Sepharose. These antibodies were then bound in bispecific TAC, which are directed against CD14 and dextran. The mouse monoclonal antibody subclass is IgG1. The cocktail was supplied in phosphate buffered saline. The EasySep® Magnetic Nanoparticles were supplied as a suspension of magnetic dextran iron particles in water. Where indicated, PBS containing 2% FBS and 1 mM EDTA was utilised and was calcium and magnesium free.

### 2.5e.2 Experimental protocol

The PBMCs were resuspended in 5 ml PBS containing 2% FBS and 1 mM EDTA and transferred to a 5 ml polystyrene round-bottom tubes. The tube was centrifuged at 350 g for 10 min at 20°C. The supernatant was discarded and the cells were resuspended in 100 µL of PBS containing 2% FBS and 1 mM EDTA. Next, 10 µL of EasySep® Positive Selection Cocktail was then added to the cell suspension. The solution was mixed well and incubated at RT for 15 min. Next, 10 µL of EasySep® Magnetic nanoparticles was added to the suspension followed by vigorously pipetting up and down 5 times, as vortexing was not recommended. The solution was incubated at RT for 10 min. Next, PBS containing 2% FBS and 1 mM EDTA was added to the cell suspension to bring the volume total to 2.5 ml. The cells in the tube were then mixed gently by pipetting up and down 2-3 times. The tube (without cap) was then placed into the magnet for 5 min. The magnet was then picked up and in one continuous motion; the magnet and tube were inverted then permitting the
supernatant to be removed. Thus, the cells that were not bound to the magnetic particles were removed by inversion of the magnet. The magnet containing the tube was maintained in the inverted position for 2-3 s and then returned to upright position. Drops of liquid that remained on the edge of the tube were not removed. Next, the tube was removed from the magnet and 2.5 ml of PBS containing 2% FBS and 1 mM EDTA was added. The cell suspension was then mixed by gently pipetting up and down 2-3 times. The tube was then returned to the magnet and incubated at RT for 5 min. The washing and incubation step was repeated once more. The tube then removed from the magnet and the cells were resuspended in an appropriate amount of desired medium.
Figure 2.1: Schematic drawing of EasySep® Human CD14 Positive Selection Cocktail. The CD14 positive monocytes were specifically labeled with dextran-coated magnetic nanoparticles using bispecific Tetrameric Antibody Complexes (TAC). These complexes recognise both dextran and the target cell surface antigen and are composed of a specific anti-cell-antibody and an anti-dextran antibody (a pair of mouse monoclonal antibody IgG1). Adapted from Human CD14 selection kit catalog #18058 (www.stemcell.com).
2.5f Neutrophil extraction

To isolation neutrophils, the EasySep® Human Neutrophil Enrichment Kit (catalog # 19257; Stem Cell) was used whereby neutrophil enrichment was facilitated by depletion of non-neutrophil. Unwanted cells were specifically labeled with dextran-coated magnetic nanoparticles using bispecific TAC. These complexes recognised both dextran and the cell surface antigen expressed on the unwanted cells (Figure 1). The small size of the magnetic dextran iron particles permitted efficient binding to the TAC-labeled cells. Next, the magnetically labelled cells were separated from the unlabelled target cells using the EasySep® procedure.

2.5f.1 Component description and principle

The EasySep® Negative Selection Human Neutrophil Enrichment Cocktail contained a combination of murine monoclonal antibodies (IgG1) purified from hybridoma culture supernatant by affinity chromatography using Protein A or Protein G Sepharose. These antibodies were bound in bispecific TAC which were directed against cell surface antigens on human blood cells, namely CD2, CD3, CD9, CD19, CD36, CD56, glycophorin A and dextran. The EasySep® Magnetic Nanoparticle was supplied as a suspension of magnetic dextran iron particles in water.

2.5f.2 Experimental protocol

The PBMC pellet was dissolved in 5 ml of PBS containing 2% FBS and 1 mM EDTA in a 5 ml polystyrene tube and centrifuged at 350 g for 10 min. The supernatant was discarded. The nucleated cell suspension was prepared by dissolving the pellet in 500 µl of PBS containing 2% FBS and 1 mM EDTA. To the cell suspension was added 25 µl of EasySep® Human Neutrophil Enrichment Cocktail followed by mixing and incubation for 10 min at RT. Next, 50 µl of the EasySep® magnetic nanoparticles were added followed by mixing and vigorously pipetting more than 5 times as
vortexing was not recommended. The solution was incubated for 10 min at RT. Next, the volume of the solution was increased to a total volume of 2.5 ml using PBS containing 2% FBS and 1 mM EDTA. The cells were then mixed by pipetting up and down to disrupt the red blood cell aggregates. The tube (without cap) was then placed into the magnet and set aside for 5 min. Next, the EasySep® Magnet was picked up, and in one continuous motion the magnet and tube were inverted, pouring off the desired fraction into a new 5 ml polystyrene tube. The magnetically labeled unwanted cells remained bound inside the original tube, held by the magnetic field of the magnet. The magnet and the tube were retained in the inverted position for 2-3 s, and then returned to upright position. The tube was removed from the EasySep® magnet and the new tube containing the supernatant fraction was placed into the magnet for 5 min and the inversion and collection step was repeated. The negatively selected enriched cells in the new tube were used for RNA extraction.

2.5g RNA extraction

To isolate RNA from the monocytes and neutrophils, the RNeasy isolation kit was used (catalog no. #74104; Qiagen).

2.5g.1 Component description and principle

The RNeasy Micro technology combined the selective binding properties of a silica-based membrane with the speed of microspin technology. The guanidine-thiocyanate-containing lysis buffer and ethanol were added to the sample to create conditions that promoted the selective binding of RNA to the RNeasy MinElute membrane. The sample was then applied to the RNeasy MinElute spin column whereby the RNA bound to the silica membrane. Next, traces of DNA that copuriﬁed were removed by DNase treatment on the RNeasy MinElute spin column. DNase and any contaminants were washed away and high-quality total RNA was eluted in RNase-free water. RNA molecules longer than 200 nucleotides were purified. The procedure enriched for mRNA, since most RNAs <200
nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together make up 15–20% of total RNA) were selectively excluded. The size distribution of the purified RNA was comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently. The kit utilised RLT buffer, which contained guanidine thiocyanate, buffer RW1 containing ethanol and RPE buffer.

2.5g.2 Experimental protocol

The cells were disrupted by adding 350 µl of buffer RLT followed by thorough mixing using a vortex. The lysate was directly pipetted into a QIAshredder 2 ml collection tube. Next, 350 µl of 70% ethanol was added to the lysate followed by pipetting up and down to mix. The 700 µl of sample including any precipitate that may have formed was transferred to an RNeasy MinElute spin column, which had been placed in a 2 ml collection tube. The lid was closed gently and the sample centrifuged for 30 s at 14,000 rpm. The flow through was discarded. Next, 700 µl of buffer RW1 was added to the RNeasy MinElute spin column. The lid was closed gently and the sample was centrifuged for 15 s at 14,000 rpm to wash the spin column membrane. The flow-through was then discarded. Next, 500 µl of buffer RPE was added to the spin column. The lid of the tube was closed gently and the sample was centrifuged for 15 s at 14,000 rpm. Thereafter, 500 µl of buffer RPE was added to the spin column followed by centrifugation for 2 min at 14,000 rpm to wash the sample. The RNeasy MinElute spin column was then placed in a new 1.5 ml collection tube and 30 µl RNase-free water was added directly to the center of the spin column membrane followed by centrifugation for 1 min at 14000 rpm to elute the RNA. The concentration of RNA was measured using a Nanodrop spectrophotometer, which was pre-blanked using 1 µl of RNAsse free water. A260/280 nm was determined to assess purity where a ratio of A260/A280 > 1.8 indicates minimal protein contamination in the RNA sample. RNA was stored at -80°C until further use.
2.5h Reverse transcription and first strand cDNA synthesis

To 1 µg of RNA in a total volume of 11 µl, was added 1 µl of random hexamers (100 pM/µl). This solution was mixed well and placed at 70°C for 5 min, followed by 5 min incubation on ice. To this solution, the other components/reagents were added in the following order, 4 µl of 5 × RT buffer (Promega), 2 µl of dNTP (10 mM), 1 µl of RNasin (Promega) and 1 µl of MMLV Reverse transcriptase (Promega) and mixed well. The samples were placed at 37°C for 40 min, followed by 42°C for 40 min and finally at 80°C for 5 min. The first strand cDNA was stored at -20°C until further use.

2.5i Quantitative real time PCR

Total first strand cDNA was used as a template for quantitative polymerase chain reaction (qPCR or real-time (RT)-PCR) quantification using a DyNAmoHS SYBR Green kit (Finnzymes) and a RT-PCR system (DNA Engine OPTICON® system; MJ Research). For the amplification of TLRs, cytokines, MMPs the respective primers were used as described in Table 2.6. For each TLR mRNA quantification, the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) was used as a reference point using the respective primers.
Table 2.6 Human real time PCR Oligonucleotides used for the amplification of human genes (TLR, cytokine and chemokine) and HPRT-housekeeping gene.

<table>
<thead>
<tr>
<th>Human Gene</th>
<th>Gene accession number</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
<th>Fragment size (bp)</th>
<th>Annealing Temp ($T_m$, °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>NM_000194.2</td>
<td>AGCTTGCTGGTGAAAAGGAC</td>
<td>TTATAGTCAAGGCGCATATCC</td>
<td>104</td>
<td>60</td>
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<tr>
<td>TLR1</td>
<td>NM_003263.3</td>
<td>TATCCCTCCTGTGGATATTGCTGCT</td>
<td>TAAATGGTGAACCTGCGACCCGAAG</td>
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<td>60</td>
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<tr>
<td>TLR2</td>
<td>NM_003264.3</td>
<td>ACCTGTCAACACAGGGTGACCT</td>
<td>TGTCAAGAATGGCGACCCGAAG</td>
<td>139</td>
<td>60</td>
</tr>
<tr>
<td>TLR3</td>
<td>NM_003265.2</td>
<td>AAGAAGCAGGCGAGAGAAGG</td>
<td>AAGAAGCAGGCGAGAGAAGG</td>
<td>182</td>
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</tr>
<tr>
<td>TLR4</td>
<td>NM_138554.3</td>
<td>GCCGAAAGGTGATTGGTGTTG</td>
<td>TACCCACAGCAGCTGGCTGAAAT</td>
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<td>60</td>
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<tr>
<td>TLR5</td>
<td>NM_003268</td>
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<td>AGCCCTGTTGGAGTGGCGTCTG</td>
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<td>TLR6</td>
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<tr>
<td>TLR7</td>
<td>NM_016562</td>
<td>TATTTTCAGCAACCAAGCAACACCA</td>
<td>GCAGCCTCTGGATGACATGTTG</td>
<td>164</td>
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</tr>
<tr>
<td>TLR8</td>
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<td>AGGCCTCGCTGGCTTACATGG</td>
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<tr>
<td>TLR9</td>
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<td>TGGAGAAGTCCCGGAGAGCAGTA</td>
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<td>TLR10</td>
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<td>IL-6</td>
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<td>CAGCCTCAAATGCTGTTGCCCCTCACA</td>
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<td>TNF-α</td>
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<td>IFN-β</td>
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<td>TGTCAGCCTACCTCAGTGGTGC</td>
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<td>RANTES</td>
<td>NM_002985.2</td>
<td>TGCCCTGTTCAGGCTGCTGCTG</td>
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<td>92</td>
<td>60</td>
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</tbody>
</table>
2.5j Cytokine / chemokines measurements

Analysis of cytokines was carried out at the ISL at the National Ireland University Maynooth. Fresh blood sample was transported in ice cold packs from MRH, Mullingar to ISL, Maynooth and processed on the same day. Serum was used for measuring TNFα, IL-6, IL-β, IL-10, IL-8, IL-12p70 and IFN-γ with a 96-Well MULTI-ARRAY 7-Multiplex Assay (Meso Scale Discovery, 9238 Gaither Road, Gaithersburg, Maryland 20877, USA). Serum was used for measuring IFN-β and Rantes using Meso Scale Discovery (MSD) single-plex 96-well plates. MSD was a sector 2400 analyser and operated through electrochemiluminescence technology. The instrument measured the intensity of emitted light to afford a quantitative measure of TNFα, IL-6, IL-β, IL-10, IL-8, IL-12p70, IFN-γ and Rantes present in the sample.

2.5j.1 Meso Scale Human Pro-inflammatory 7-Plex Assay

Serum from participants was used for measuring IL-1β, TNFα, IL-6, IL-8, IFNγ, IL-12p70, and IL-10 levels using a Meso Scale 96-Well Human Pro-inflammatory 7-plex ultra sensitive assay kit (MSD). The Human 7-Plex assay detected IL-1β, TNFα, IL-6, IL-8, IFNγ, IL-12p70, and IL-10 in a sandwich immunoassay format. The 7-plex assay was supplied as a 96 well ultra sensitive plate pre-coated with IL-1β, TNFα, IL-6, IL-8, IFNγ, IL-12p70, and IL-10 capture antibodies on spatially distinct spots on the plate. First, the pre-coated plate was incubated with 25 µl/well of Diluent 2 (R51BB-4) supplied with the kit for 30 min at RT with vigorous shaking at 1000 rpm. Next, the samples (cell free supernatants) or appropriate dilution of stock calibrator blend (highest calibrator point was obtained by diluting the stock by 100 fold in Diluent 2 and from this an 8 point standard curve with 4-fold serial dilution was prepared-assay range 2500 to 0 pg/ml) was added to separate wells of the MSD plate in duplicates. The plate was sealed with an adhesive seal and incubated for 2 h with vigorous shaking.
(1000 rpm) at RT. Next, the plate was washed three times with wash buffer (PBS-T) and was pat dry. The SULFO-TAG Detection Antibody Blend (D2008-2) provided as 50 × stock solution was diluted to a final working concentration of 1 ×, by mixing 60 µl of stock antibody blend with 2.94 ml of Diluent 3 (R51BA-4) supplied with the kit. Further, 25 µl/well of the 1 × detection antibody solution was added to each well of the MSD plate and the plate was sealed and incubated for 2 h with vigorous shaking (1000 rpm) at RT. Next, the wash step was repeated and 150 µl/well of 2× diluted Read Buffer T (R92TC-3) was added in to each well of the MSD plate. The plate was immediately read on the SECTOR Imager and the data was analysed using the MSD Discovery Workbench analysis software.

2.5j.2 Meso Scale Human RANTES and IFNβ Assay

Serum from participants was used for measuring RANTES and IFNβ levels using a meso scale 96-Well Human RANTES assay kit and Human IFNβ assay kit respectively (MSD). The Human RANTES assay plate (L451BFA-1) detected RANTES and Human IFNβ assay avidin plate (L15AA-1) detected IFNβ in a sandwich immunoassay format. The 96 well ultra sensitive plate was pre-coated with human RANTES capture antibody on the RANTES plate. Regarding the IFNβ plate, a 50× anti-hIFNβ biotinylated capture antibody was diluted to 1× working concentration in Diluent 100 (R50AA-4), and 20 µl/well of this 1 × capture antibody was manually pipetted onto the MSD plate and incubated at RT with gentle shaking for 1 h. Subsequently, the RANTES and IFNβ plates were incubated with 25 µl/well of Diluent 2 (R51BB-4) supplied with the kit for 30 min at RT with vigorous shaking at 1000 rpm. Next, the samples (cell free supernatants) or appropriate dilution of stock calibrators (highest calibrator point for RANTES was obtained by diluting the stock (C01BF-2)
by 100 fold in Diluent 2 and from this, an 8 point standard curve with 4-fold serial dilution was prepared-assay range 2500 to 0 pg/ml, and highest calibrator point for IFNβ was obtained by diluting the stock (C01AD-2) by 25 fold in Diluent 1 (R50CK-4) and from this, an 8 point standard curve with 4-fold serial dilution was prepared-assay range 25,000 to 1.5 pg/ml) were added to separate wells of the respective MSD plates in duplicates. The plates were sealed with an adhesive seal and incubated for 2 h with vigorous shaking (1000 rpm) at RT. Next; the plates were washed three times with wash buffer (PBS-T) and were pat dry. The SULFO-TAG Detection Antibody Blend (D21BF-2) for RANTES and (D21AD-2) for IFNβ detection, provided as 50 × stock solution were diluted to a final working concentration of 1 ×, by mixing 60 µl of stock antibody blend with 2.94 ml of Diluent 3 (R51BA-4). Next, 25 µl/well of the 1 × detection antibody solution was added to each well of the respective MSD plates and the plates were sealed and incubated for 2 h with vigorous shaking (1000 rpm) at RT. Next, the plates were washed three times with wash buffer (PBS-T) and were pat dry. Then, 150 µl/well of 2× diluted Read Buffer T (R92TC-3) was added to each well of the MSD plates. The plates were immediately read on the SECTOR Imager 2400 and the data was analysed using the MSD Discovery Workbench analysis software.
2.6 Statistical analysis

Data are expressed as median (interquartile range) and represented as box-and-whiskers plots. The dark midline in the box represents the distribution's median value. The top and bottom edges of the box respectively represent the 75th and 25th percentile values. The top and bottom of the vertical lines, the whisker respectively represent the upper and lower maximum value. χ² test or Fisher exact test (as appropriate) were used to compare proportions. The Mann–Whitney test was used in case of non-normally distributed parameters to compare median between two groups. Kruskal-Wallis test was used for multiple inter-group comparisons in case of parameters that did not show normal distribution. If a significant difference was found in inter-group comparisons, post hoc multiple comparison analysis with the Dunns multiple comparison test was performed. Data were transformed to lognormal format and multifactorial ANOVA was applied to evaluate the effect of variables (age, sex, BMI, WHR and duration of diabetes) on different inflammatory markers. Correlations between values were examined by calculating Spearman correlation coefficients. All the statistical analysis was done using the Prism 6.0 computer program (GraphPad, La Jolla, CA) and p value less than 0.05 was considered significant.
Chapter 3

Analysis of metabolic parameters in T2D subjects
A total of 146 subjects participated for this T2D inflammation study. Thirty-four healthy volunteers without diabetes acted as controls (NGT) and the rest (T2D, n=112) were classified into four groups based on glycaemic control and complications as mentioned previously: GC: n=27, GCC: n=32, PC: n=21 and PCC: n=32.

The role of various risk factors for the development and progression of diabetes have been demonstrated by several epidemiologic studies (Klein et al., 2002; Cai et al., 2006). These factors include type and duration of diabetes, age, gender, glycaemic control, hypertension, body mass index, smoking, serum lipids and presence of microalbuminuria. We have evaluated all these risk factors in the current study.

The females were 67.6% in NGT and 42.9% in T2D group. The median age was higher in T2D subjects compared to NGT controls (p<0.001).

The duration of diabetes was calculated for all groups and as it reflects the course of disease, the groups with complications and poor glycaemic control group had longer duration of diabetes compared to good glycaemic control group (p<0.001).

The profile for smoking was similar in both NGT and T2D groups: 20.5% were smokers in NGT compared to 20.5% in T2D groups, and 30.7% were ex-smoker in NGT as compared to 43.5% in T2D (Table 3.1).

There was significant difference in the BMI and Waist-hip-ratio among different groups (p<0.01). The T2D subjects had higher BMI and Waist-hip-ratio compared to NGT controls. The median systolic BP was seen
significantly raised in GC, GCC and PCC (p<0.05). However, no significant difference was seen in diastolic BP among different groups (Table 3.1).

The fasting glucose was higher in all T2D subjects compared to NGT controls (p<0.05). All T2D subjects had raised total cholesterol and triglycerides, and low HDL compared to NGT controls (p<0.05). LDL was low in T2D subjects compared to NGT likely secondary to lipid lowering agents.

Previous epidemiological studies have demonstrated increased concentrations of CRP in patients with T2D. However, patients with raised CRP, who had evidence of infection, were excluded from this study. This study was designed to explore the role of TLRs, cytokines/chemokines and adipokines in the pathophysiology of T2D. Knowing that these are highly sensitive biomarkers and get influenced by infection; the raised CRP was kept an exclusion criteria so as to avoid recruitment of subjects with infection, which would have otherwise confounded our results. Unsurprisingly, therefore, we did not find any significant difference in CRP between different groups (Table 3.2).

A detailed drug history was obtained for all subjects and it was observed that a higher percentage of subjects in PC (52.4%) and PCC (71.9%) were on insulin compared to GC (7.4%) and GCC (28.1%) groups. In all groups most T2D subjects were on lipid lowering agents: GC (85.2%), GCC (93.8%), PC (61.9%) and PCC (87.5%) and also on angiotensin converting enzyme (ACE) inhibitor and angiotensin receptor blocker (ARB) in view of HTN or micro/macro albuminuria: GC (74.1%), GCC (87.5%), PC (76.2%), and PCC (81.3%) (Table 3.3).
All PCC subjects had one or more microvascular complications (retinopathy, neuropathy and nephropathy). Additionally, 53.1% (17 subjects) of these had one or more macrovascular complications. In GCC group, 56.3% (18 subjects) had one or more microvascular complications, 34.4% (11 subjects) had both macro & microvascular complications and 9.4% (3 subjects) had only macrovascular complications (Table 3.4). Among microvascular complications, retinopathy was higher in PCC subjects (40.6%) compared to GCC subjects (18.6%). No significant difference was observed in neuropathy in between two subgroups. 15.6% PCC subjects had diabetic foot disease compared to 6.3% GCC subjects (Table 3.4).

In PCC, 68.8% subjects had nephropathy compared to 56.3% in GCC subgroup. Of these, 18.2% and 22.2% subjects had macroalbuminuria respectively. The proportion of T2D subjects having CKD was higher in PCC subgroup than GCC subgroup (CKD stage 3; 45.5% vs. 27.7%, CKD stage 4; 9% vs. 0%) (Table 3.5).
Table 3.1 Baseline subject characteristics: demographics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NGT</th>
<th>GC</th>
<th>GCC</th>
<th>PC</th>
<th>PCC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
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<td>27</td>
<td>32</td>
<td>21</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>11/23</td>
<td>12/15</td>
<td>20/12</td>
<td>13/8</td>
<td>19/13</td>
<td>0.068</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 [47-59]</td>
<td>62 [51-71]</td>
<td>65 [57-73]</td>
<td>56 [50-60]</td>
<td>60 [55-68]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker Y/N</td>
<td>7/24</td>
<td>3/21</td>
<td>6/26</td>
<td>5/16</td>
<td>9/22</td>
<td>0.659</td>
</tr>
<tr>
<td>Ex smoker Y/N</td>
<td>8/26</td>
<td>8/19</td>
<td>14/18</td>
<td>4/17</td>
<td>8/24</td>
<td>0.271</td>
</tr>
<tr>
<td>WHR</td>
<td>0.92 [0.84-0.96]</td>
<td>0.96 [0.90-1.04]</td>
<td>1.03 [0.95-1.06]</td>
<td>1.04 [0.93-1.18]</td>
<td>1.02 [0.97-1.08]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>129 [120-137]</td>
<td>145 [131-156]</td>
<td>142 [131-154]</td>
<td>130 [120-145]</td>
<td>147 [124-158]</td>
<td>0.005</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80 [77-85]</td>
<td>80 [71-85]</td>
<td>78 [73-84]</td>
<td>78 [73-88]</td>
<td>77 [68-86]</td>
<td>0.808</td>
</tr>
</tbody>
</table>

BMI, Body Mass Index, SBP, Systolic Blood Pressure, DBP, Diastolic Blood Pressure, WHR, Waist-to-hip ratio Data are expressed as median [Inter quartile range]. p value corresponds to the differences between groups.
Table 3.2 Baseline subject characteristics: biochemical parameters

<table>
<thead>
<tr>
<th>Characteristics</th>
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<th>GC</th>
<th>GCC</th>
<th>PC</th>
<th>PCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
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<td>27</td>
<td>32</td>
<td>21</td>
<td>32</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>4.8 [4.7-5.1]</td>
<td>6.5 [5.8-7.2]</td>
<td>7.6 [6.1-8.2]</td>
<td>10.9 [8.3-12.9]</td>
<td>10.6 [7.9-14.6]</td>
</tr>
<tr>
<td>CRP</td>
<td>0.10 [0.06-0.25]</td>
<td>0.20 [0.09-0.30]</td>
<td>0.18 [0.06-0.50]</td>
<td>0.15 [0.06-0.60]</td>
<td>0.20 [0.10-0.40]</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>5.9 [5.1-6.6]</td>
<td>3.9 [3.5-4.7]</td>
<td>3.7 [3.1-4.3]</td>
<td>4.2 [3.7-4.8]</td>
<td>3.9 [3.5-4.7]</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>3.6 [2.8-4.3]</td>
<td>2.2 [1.9-2.9]</td>
<td>1.8 [1.5-2.4]</td>
<td>2.3 [1.8-2.9]</td>
<td>2.0 [1.5-2.7]</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.6 [1.3-1.9]</td>
<td>1.3 [1.0-1.5]</td>
<td>1.1 [0.9-1.3]</td>
<td>1.1 [1.0-1.3]</td>
<td>1.1 [0.9-1.2]</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.9 [0.7-1.2]</td>
<td>1.5 [1.1-2.0]</td>
<td>1.4 [0.8-2.0]</td>
<td>1.5 [0.8-1.9]</td>
<td>1.8 [1.2-2.5]</td>
</tr>
</tbody>
</table>

HbA1c, glycosylated haemoglobin, HDL, high-density lipoprotein, LDL, low-density lipoprotein, CRP, C-reactive protein. Data are expressed as median [Inter quartile range].
Table 3.3 Baseline characteristics: medications and HTN in different subgroups

<table>
<thead>
<tr>
<th>Characteristics</th>
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<th>GC</th>
<th>GCC</th>
<th>PC</th>
<th>PCC</th>
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<tbody>
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<td>34</td>
<td>27</td>
<td>32</td>
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<td>32</td>
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<tr>
<td>Lipid lowering Agents</td>
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<td>Aspirin</td>
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<td>PPAR γ agonists</td>
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<td>3</td>
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<td>1</td>
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<tr>
<td>GLP 1 analogs</td>
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<td>2</td>
<td>1</td>
</tr>
<tr>
<td>DPP IV inhibitors</td>
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<td>3</td>
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<td>Insulin</td>
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<td>9</td>
<td>11</td>
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<td>ACE inhibitors</td>
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<td>20</td>
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<tr>
<td>HTN</td>
<td>-</td>
<td>17</td>
<td>6</td>
<td>7</td>
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PPAR γ agonists, Peroxisome proliferator-activated receptor gamma agonists, GLP-1 analogs, Glucagon-like peptide-1 analogs, DPP IV inhibitors, Inhibitors of dipeptidyl peptidase 4, ACE inhibitor, Angiotensin-converting-enzyme inhibitor, ARB, angiotensin receptor blockers, HTN, Hypertension.
Table 3.4 Baseline characteristics: micro/macro vascular complications in different subgroups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NGT</th>
<th>GC</th>
<th>GCC</th>
<th>PC</th>
<th>PCC</th>
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<td>Cerebrovascular disease</td>
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<td>4</td>
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<td>2</td>
</tr>
<tr>
<td>Diabetic foot</td>
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<td>0</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

CKD, Chronic kidney disease, IHD, Ischemic heart disease, PAD, Peripheral arterial disease
Table 3.5. Baseline characteristics - CKD staging and diabetic nephropathy in GCC and PCC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>GCC</th>
<th>PCC</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>MicroAlb</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>MacroAlb</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>CKD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stage 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stage 2</td>
<td>8</td>
<td>7</td>
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<td>Stage 3</td>
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<td>Stage 4</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Stage 5</td>
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</table>

CKD, Chronic Kidney Disease, MicroAlb, Microalbuminuria, MacroAlb, Macroalbuminuria
Chapter 4

Analysis of cytokines in T2D subjects
Analysis of cytokines mRNA expression in T2D subjects

4.1 Results

The cytokines were measured in NGT and T2D subjects in both monocytes and neutrophils. Both neutrophils and monocytes expressed all 5 cytokines, IL-6, TNFα, IL-1β, IFNβ and Rantes at the transcriptional level. The 2^(-ΔΔCt) method, commonly known as the "Delta Delta CT was used to analyse the relative changes in gene expression obtained using real-time quantitative PCR. Results of the real-time PCR data were represented as CT values, where CT was defined as the threshold cycle number of PCRs at which amplified product was first detected. There is an inverse correlation between CT and amount of target: lower amounts of target correspond to a higher CT value, and higher amounts of target have lower CT values. The average CT was calculated for both the target genes and GAPDH (glyceraldehyde-3-phosphate dehydrogenase is a protein-coding gene) and the ΔCT was determined as (CT values for the target gene) minus (the mean of the CT values for GAPDH). The ∆∆CT was calculated by the formula ∆∆CT = (ΔCT of target - ΔCT of normal). The N-fold differential expression in the target gene compared to the normal sample counterpart was expressed as 2-∆∆CT (Livak KJ and Schmittgen TD, 2001).

4.1a Cytokines expression in monocytes in T2D subjects with good and poor glucose control

We can see that the PC and PCC subjects exhibited significantly increased TNFα mRNA expression in the monocytes when compared to
NGT (Fig. 4.1A). The highest level of IL-6 mRNA was found in monocytes from GC subjects (Median value: 12.3-fold), followed by PC subjects (10.7-fold) when compared to NGT (Table 4.1). In contrast, complication groups, GCC (3.9-fold) and PCC subjects (4.2-fold) showed lesser overexpression, compared to NGT subjects (Fig. 4.1B).

IL-1β mRNA was suppressed in all T2D groups, lowest in GCC and PCC, compared with NGT (Fig. 4.1C). Interestingly, highest level of IFNβ mRNA was seen in monocytes from PC subjects (Median value: 27-fold), followed by PCC (12.4-fold), GC (11.1-fold) and GCC (5.4-fold) when compared to NGT (Fig. 4.1D). Similarly, highest level of Rantes mRNA was noticed in monocytes from PC subjects (16-fold) and PCC subjects (12.4-fold) (Fig. 4.2A).

The IL-6 (p<0.05), IL-1β (p<0.05), IFNβ (p<0.001) and Rantes (p<0.001) expression in monocytes remained significant among different groups, with multifactorial ANOVA after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) (Table 4.4). Additionally a statistically significant correlation was found between IL-1β mRNA in monocytes and BMI in T2D subjects (Spearman’s r = 0.41, p<0.05).

In summary, the highest level of IL-6 mRNA was observed in GC group, whereas highest level of IFNβ mRNA and Rantes mRNA were noticed in PC group, when compared to NGT. On the contrary, IL-1β mRNA remained suppressed in T2D groups, compared to NGT.
4.1b Cytokines expression in neutrophils in T2D subjects with good and poor glucose control

The highest level of TNFα mRNA was seen in neutrophils from GC subjects (Median value: 6.4-fold) and PC subjects (3.3-fold), compared to NGT. In contrast, TNFα mRNA expressions remain suppressed in PCC subjects (0.5-fold), compared to NGT (Fig. 4.3A). GC and PC neutrophils over expressed IL-6 mRNA by about 58-fold and 9-fold respectively, compared to NGT (Table 4.2). In contrast, complication group subjects, GCC (1.3-fold) and PCC subjects (1.2-fold) showed comparable expression, when compared to NGT control individuals (Fig 4.3B).

Similarly, IL-1β mRNA was overexpressed in GC (1.8-fold) and suppressed in GCC, PC and PCC groups, compared with NGT (Fig. 4.3C).

We can see that GC and PC neutrophils over expressed IFNβ mRNA by 35.4-fold and 4.8-fold respectively, compared to NGT (Table 4.3D). In contrast, neutrophils in the complication group, GCC (0.7-fold) and PCC subjects (1.2-fold) showed comparable expression, compared with NGT. Similarly, highest level of Rantes mRNA was seen in neutrophils from GC subjects (Median value: 28.7-fold), followed by PC subjects (4.1-fold), compared to NGT (Fig. 4.2B). In contrast expression was comparable in GCC and PCC subjects.

The TNFα (p<0.001), IL-6 (p<0.001), IFNβ (p<0.001) and Rantes (p<0.001) expression remained significant among different groups, using multifactorial ANOVA after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) (Table 4.4). Additionally, we did not find a correlation between cytokines mRNA and BMI in neutrophils.
Collectively, our data showed that TNFα, IL-6, IFNβ and Rantes mRNA expression were highest in GC neutrophils, when compared to NGT controls, whereas comparable expression was evident in GCC and PCC groups.

In summary, the study showed that TNFα, IL-6, IFNβ & Rantes mRNA expression were highest in GC neutrophils, and IL-6 & IFNβ mRNA were overexpressed in GC monocytes compared to NGT. In contrast, IL-1β mRNA remained suppressed in monocytes in PC, GCC & PCC, compared to NGT. These results suggest an enhanced anti-inflammatory mechanism in T2D subjects with good glycaemic control with no complications, compared to T2D subjects with poor glycaemic control, and subjects with complications.
Analysis of serum cytokines in T2D subjects

4.2 Results

4.2a Pro-inflammatory cytokine levels in T2D subjects with good and poor glucose control

In this study, we did not find statistical significant difference in serum IL-6, TNF-α and IL-1β among different groups (Fig. 4.4A, B). The median values of serum IL-6 and TNF-α were comparable in GC, GCC and PCC subjects compared to NGT (Table 4.3). Similarly, comparable levels of serum IL-1β were noticed in GC and GCC, suppressed levels in PC and PCC, when compared with NGT (Fig. 4.4C). In contrast, serum IFN-β was significantly increased in PC and comparable in GC group, compared to NGT control (Table 4.4D), decreased in GCC and PCC group (p<0.05). However, after adjustment for covariates the differences in serum IFN-β levels became statistically insignificant.

4.2b Anti-inflammatory cytokine and chemokine levels in T2D subjects with good and poor glucose control

IL-10 is an anti-inflammatory cytokine, which serves to suppress the production of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages and to enhance humoral immunity. Herein, serum IL-10 was increased in GC and PC subjects, supressed in GCC and PCC subjects, compared with NGT, but statistically insignificant (Fig. 4.5A). Next we assessed the serum levels of the early response chemokine Rantes and the inflammatory chemokine IL-8. Rantes was increased in the PC and PCC groups, comparable in GC and GCC groups, when compared with NGT control group (Table 4.3). Interestingly, Serum IL-8 was comparable in GC group, decreased in PC, GCC and
PCC, when compared with NGT. However, no statistically significant difference in serum IL-8 and Rantes levels were observed among different groups (Fig. 4.5C).

4.2c Levels of Th1 cytokines, IL-12 and IFN-γ in T2D subjects with good and poor glucose control
Interleukin 12 (IL-12) activates natural killer and T cells with the secondary synthesis and release of IFN-γ thus promoting a Th1- or cell-mediated immune response (Fig. 4.6). Decreased levels of IL-12 was detected in the GC, markedly decreased in GCC, PC and PCC patient groups compared to the NGT control group (Table 4.3). However, no significant difference in serum IL-12 was observed among different groups (Fig. 4.6A). IFNγ was lower in GC, markedly supressed in the GCC, PC and PCC subjects compared to NGT subjects. Again, we did not find any significant difference in serum IL-10 amongst different groups (Fig. 4.5B).

We applied multifactorial ANOVA using lognormal data after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) and applied, but did not notice any change in significance (Table 4.5). Additionally, we did not find correlation between serum cytokines and BMI in T2D subjects.

4.2d Cytokines mRNA expression in monocytes & neutrophil and serum levels of cytokine for the same individuals
We analysed the cytokines mRNA expression in monocytes and neutrophils, and the serum levels for the same individual in T2D cohort and NGT subjects. A scatterplot of this analysis is depicted in Fig. 4.7 (A-J)
IL-6 mRNA was significantly overexpressed in both monocytes and neutrophils from GC group, compared to monocytes from NGT (Fig. 4.7A). Similarly, IL-6 mRNA was significantly overexpressed in neutrophils from GC compared to IL-6 mRNA in neutrophils from NGT. The serum IL-6 levels remained statistically insignificant for the same individuals (Fig. 4.7B). The IFNβ mRNA was significantly overexpressed in neutrophils from GC group, compared to NGT neutrophils & monocytes (Fig. 4.7E). We did not find significant difference in serum IFNβ levels among different groups for the same individuals (Fig. 4.7F).

Rantes mRNA was significantly overexpressed in neutrophils from GC group, compared to Rantes mRNA levels in both monocytes and neutrophils from NGT (Fig. 4.7I). Similarly, Rantes was significantly overexpressed in monocytes from PC compared to monocytes from NGT. The serum Rantes levels did not show significant difference among different groups for the same individuals (Fig. 4.7J).
Table 4.1 Cytokines mRNA expressions in monocytes from type 2 diabetic subjects & healthy controls. Cytokines mRNA were measured in subjects with normal glucose targets (NGT), good glucose control (GC), good glucose control with micro/macroversacular complications (GCC), poor glucose control (PC), poor glucose control with micro/macroversacular complications (PCC) in monocytes at the transcriptional level using 2-(ΔΔCt) method and results are expressed as median [inter quartile range]. Statistical significance was determined by non-parametric Kruskal–Wallis one-way analysis of variance and p<0.05 was considered significant. p value corresponds to the differences between groups.

<table>
<thead>
<tr>
<th></th>
<th>NGT</th>
<th>GC</th>
<th>GCC</th>
<th>PC</th>
<th>PCC</th>
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<tr>
<td>TNFα</td>
<td>1.0 [0.9-1.2]</td>
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<td>1.2 [1.1-2.4]</td>
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<td>2.2 [1.9-2.3]</td>
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<td>IL-6</td>
<td>0.9 [0.9-1.0]</td>
<td>11.1 [10.4-12.6]</td>
<td>3.5 [2.3-5.5]</td>
<td>9.6 [7.4-12.2]</td>
<td>3.8 [3.6-3.9]</td>
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<td>IL-1β</td>
<td>0.9 [0.7-1.3]</td>
<td>0.1 [0.1-0.1]</td>
<td>0.04 [0.03-0.04]</td>
<td>0.1 [0.1-0.1]</td>
<td>.07 [.05-0.12]</td>
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<td>IFNβ</td>
<td>0.9 [0.8-1.2]</td>
<td>10.0 [9.5-11.2]</td>
<td>4.9 [4.8-5.2]</td>
<td>24.3 [20.9-25.2]</td>
<td>11.2 [10.1-12.5]</td>
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<td>Rantes</td>
<td>1.2 [0.7-1.6]</td>
<td>1.8 [1.3-2.8]</td>
<td>7.6 [7.2-8.0]</td>
<td>19.2 [14.6-21.5]</td>
<td>14.9 [13.5-16.6]</td>
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Table 4.2 Cytokines mRNA expressions in neutrophils from type 2 diabetic subjects & healthy controls. Cytokines mRNA were measured in neutrophils from subjects with normal glucose targets (NGT), good glucose control (GC), good glucose control with micro/macrovascular complications (GCC), poor glucose control (PC), poor glucose control with micro/macrovascular complications (PCC) at the transcriptional level using 2-(ΔΔCt) method and results are expressed as median [inter quartile range]. Statistical significance was determined by non-parametric Kruskal–Wallis one-way analysis of variance and p<0.05 was considered significant. p value corresponds to the differences between groups.

<table>
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<tr>
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<th>NGT</th>
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<th>PC</th>
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<td>TNFα</td>
<td>1.0[0.8-1.1]</td>
<td>6.4[5.4-8.0]</td>
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<td>IL-6</td>
<td>0.9[0.7-1.3]</td>
<td>52.1[44.4-56.0]</td>
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<td>IL-1β</td>
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<td>0.5[0.4-1.6]</td>
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<tr>
<td>IFNβ</td>
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<td>35.4[35.0-36.1]</td>
<td>0.7[0.4-1.1]</td>
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<td>Rantes</td>
<td>0.9[0.8-1.1]</td>
<td>25.8[25.5-26.1]</td>
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<td>3.7[3.6-3.8]</td>
<td>1.3[1.1-1.5]</td>
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Table 4.3  Fasting levels of cytokines in the serum in subjects with type 2 diabetes & controls. Serum levels of cytokines were measured in subjects with normal glucose targets (NGT), good glucose control (GC), good glucose control with micro/macrovascular complications (GCC), poor glucose control (PC), poor glucose control with micro/macrovascular complications (PCC) using multiplex cytokine profiling and results are expressed as median [IQR]. Statistical significance was determined by Kruskal–Wallis test and p<0.05 was considered significant. p value corresponds to the differences between groups.

<table>
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<th>NGT</th>
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<th>PC</th>
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<td>21</td>
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<tr>
<td>IL-6</td>
<td>12.3 [0.9-16.3]</td>
<td>13.2 [1.0-19.1]</td>
<td>12.4 [1.5-19.3]</td>
<td>3.8 [1.4-18.0]</td>
<td>13.4 [1.9-18.5]</td>
<td>0.36</td>
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<tr>
<td>IL-1β</td>
<td>6.0 [0.9-8.4]</td>
<td>5.9 [0.8-8.5]</td>
<td>6.1 [2.5-7.6]</td>
<td>4.6 [1.6-7.7]</td>
<td>3.9 [1.0-5.6]</td>
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<td>IFNβ</td>
<td>357.2 [8.5-685.4]</td>
<td>339.8 [126.3-1263]</td>
<td>85.8 [19.8-315.0]</td>
<td>1082 [736.6-1427]</td>
<td>156.0 [33.2-810.1]</td>
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<td>IL-8</td>
<td>63.5 [25.2-89.8]</td>
<td>57.3 [26.7-85.3]</td>
<td>39.3 [20.6-68.9]</td>
<td>41.7 [24.2-89.1]</td>
<td>49.4 [26.6-68.3]</td>
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<td>IL-12p70</td>
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<td>6.7 [2.2-9.9]</td>
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<tr>
<td>IFNγ</td>
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<td>5.2 [2.1-7.1]</td>
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Table 4.4. The multifactorial ANOVA analysis of cytokines mRNA in monocytes and neutrophils after adjustment for covariates. Statistical significance was determined after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins). p value corresponds to the differences between groups.

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<td>IL-6</td>
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<tr>
<td>IL-1β</td>
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<td>IL-1β</td>
<td>0.079</td>
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<tr>
<td>IFNβ</td>
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<td>IFNβ</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rantes</td>
<td>&lt;0.001</td>
<td>Rantes</td>
<td>&lt;0.001</td>
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Table 4.5. The multifactorial ANOVA analysis of serum cytokines after adjustment for covariates. Statistical significance was determined after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins). p value corresponds to the differences between groups.

<table>
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<th>Serum cytokines</th>
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<tr>
<td>TNFα</td>
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<td>IL-12p70</td>
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<tr>
<td>IFNβ</td>
<td>0.257</td>
<td>IFNγ</td>
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<td>Rantes</td>
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Figure 4.1 TNFα, IL-6, IL-1β and IFNβ mRNA expressions in monocytes in subjects with type 2 diabetes & controls. Top and bottom horizontal lines of the boxplots indicate 25th and 75th percentiles respectively; lines within the box indicate median values. TNFα (A), IL-6 (B) and IFNβ (D) were overexpressed in all T2D groups, compared with NGT. In contrast, IL-1β (C) was suppressed in all T2D groups (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs NGT.
Figure 4.2 Rantes mRNA expressions in monocytes & neutrophils in subjects with type 2 diabetes & controls. A: Rantes mRNA was overexpressed in monocytes from PC & PCC, compared with NGT. B: In neutrophils, Rantes was overexpressed in GC and PC group. NGT, Normal glycaemic targets, GC, good glucose control, GCC, good glucose control with micro/macrovascular complications, PC, poor glucose control, poor glucose control with micro/macrovascular complications (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs NGT.
Figure 4.3 TNFα, IL-6, IL-1β & IFNβ mRNA expressions in neutrophils in subjects with type 2 diabetes & controls. The TNFα (A), IL-6 (B), IL-1β (C) & IFNβ mRNAs (D) were overexpressed in neutrophils in T2D subjects, compared to NGT. The highest level of TNFα, IL-6 & IFNβ were seen in GC group (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs NGT
Figure 4.4 Serum levels of pro-inflammatory cytokines in subjects with type 2 diabetes & controls. Serum TNF-α (A), IL-6 (B) and IL-1β (C) levels were comparable among different groups. IFN-β (D) levels were significantly increased in PC group, compared to NGT. Values are expressed as pg/ml (n = 34 vs 27 vs 32 vs 21 vs 32). *p<0.05, **p<0.01, ***p<0.001 vs NGT.
Figure 4.5 Anti-inflammatory cytokine and chemokine serum levels in subjects with type 2 diabetes & healthy controls.

Serum IL-10 (A), Rantes (B) and IL-8 (C) were comparable in different T2D groups and NGT. NGT, Normal glycaemic targets (n = 34), GC, good glucose control (n = 27), GCC, good glucose control with micro/macrovacular complications (n = 32), PC, poor glucose control (n = 21), PCC, poor glucose control with micro/macrovacular complications (n = 32). Values are expressed as pg/ml. *p<0.05, **p<0.01, ***p<0.001 vs NGT.
Figure 4.6 Serum levels of IL-12 and IFN-γ in subjects with type 2 diabetes & healthy controls. Serum IL-12 (A) and IFN-γ (B) were comparable in different T2D groups and NGT. NGT, Normal glycaemic targets (n = 34), GC, good glucose control (n = 27), GCC, good glucose control with micro/macrovascular complications (n = 32), PC, poor glucose control (n = 21), PCC, poor glucose control with micro/macrovascular complications (n = 32). Values are expressed as pg/ml. *p<0.05, **p<0.01, ***p<0.001 vs NGT.
Figure 4.7 page 116 – 118. Scatter diagram plotting cytokines mRNA expression in monocytes/neutrophil and the serum levels for the same individuals. (A) IL-6 mRNA was overexpressed in both monocytes and neutrophils from GC group, compared to NGT neutrophils. Serum IL-6 (B), TNFα mRNA (C) and serum TNFα (D) remained statistically insignificant for the same individuals.
Figure 4.7 Contd. IFNβ mRNA was overexpressed in neutrophils from GC group, compared to NGT neutrophils & monocytes. Serum IFNβ (B), IL-1β mRNA (C) and serum IL-1β (D) remained statistically insignificant for the same individuals.
Figure 4.7 Contd. Rantes mRNA (I) was overexpressed in neutrophils from GC group, compared to NGT neutrophils & monocytes. Serum Rantes (J) remained statistically insignificant for the same individuals. NGT, Normal glycaemic targets, GC, good glucose control, GCC, good glucose control with micro/macrovascular complications, PC, poor glucose control, PCC, poor glucose control with micro/macrovascular complications, M, monocytes, N, neutrophils. Values are expressed as pg/ml. *p<0.05, **p<0.01, ***p<0.001 vs NGT.
4.3 Discussion

To the best of our knowledge, this is the first study that has sought to evaluate serum cytokine levels and cytokines mRNA expressions among diabetic subjects with good glycaemic control with/without complications and poor glycaemic with/without complications. Herein, we investigated the cytokines mRNA expression in the monocytes and neutrophils and also characterised the cytokines profile in serum of subjects with T2D and NGT and evaluated whether perturbations in cytokines occur as a consequence of micro- and macrovascular complications.

Previous cross-sectional and prospective studies have described an elevation in levels of circulating acute-phase proteins such as CRP, cytokines such as IL-1β, IL-6 and TNF-α, and chemokines in subjects with T2D (Donath & Shoelson, 2011). We determined serum levels of the Th1 cytokines (TNF-α, IL-1β, IL-6, IL-12p70 and IFN-γ) which stimulate cell-mediated immunity, and promote inflammation and cytotoxicity. We also assessed serum levels of the Th2 cytokine, IL-10, which is purported to mediate a protective function during diabetes. We also evaluated TNF-α mRNA, IL-1β mRNA, IL-6 mRNA, Rantes mRNA and IFN-γ mRNA expressions in both monocytes and neutrophils in T2D and NGT cohort. In the present study, strict exclusion criteria were applied to the study to include exclusion of subjects with intercurrent illnesses and infections.
Pro-inflammatory cytokine levels in T2D subjects with good and poor glucose control

Given the current hypothesis that T2D is an inflammatory condition (Donath & Shoelson, 2011), we initially evaluated levels of serum IL-1β, IL-6 and TNF-α. In relation to previous studies showing that elevated TNF-α, IL-6 and IL-1β are risk factors for T2D (Donath & Shoelson, 2011; Spranger et al., 2003; Masters et al., 2010), we did not find any significant difference in serum levels of IL-6 and TNF-α in subjects with T2D, compared with NGT. However, IL-6 mRNA was significantly overexpressed in both monocytes and neutrophil from GC and PC, compared with NGT. Similarly, TNF-α mRNA was significantly overexpressed in GC neutrophils, compared with NGT. TNF-α mRNA was significantly overexpressed in PC and PCC monocytes, but after adjustment for covariates, it became statistically insignificant (Table 4.4). Our results coincide with previous studies, which showed overexpressed TNF-α mRNA and IL-6 TNF-α in T2D subjects (Tsiotra et al., 2007; Gonzalez et al., 2012).

A study by Huseynova et al. (2013) found that subjects with T2D had increased levels of TNF-α and IL-8 in the stage of decompensation. In this study T2D subjects were classified according to disease duration into 3 groups: under one year- stage of compensation (n = 28), from six to ten years - stage of subcompensation (n = 28), over ten years- stage of decompensation (n =28). HbA1c was high in stage of decompensation (11.4 ± 0.32%) compared to compensation stage (6.1 ± 0.05). We think that elevated TNF-α and IL-8 in the T2D subjects in this study were related poor glycaemic status rather than the duration of disease i.e. decompensation. We did not find staging based on duration of diabetes. In our study, highest levels of TNF-α mRNA were present in GC compared to NGT.
We think that discrepancy among these two studies can be attributed to (I) duration of the diseases; the majority of subjects included in our study have a longer disease duration (mean duration 9 years) (II) small sample size, (III) different T2D study cohort; in our study subjects were classified as per glycemic status and presence/absence of complications. We think that duration of disease, glycaemic status and presence or absence of complications might explain the apparent discrepant results between these two studies.

Interestingly, our monocyte data showed significantly decreased expressions of IL-1β mRNA in GCC, PC and PCC subjects, compared with healthy controls (p<0.05). In parallel to our study, Mooradian et al. (1991) has described undetectable levels of IL-1α and IL-1β in a group of 137 patients with long-standing hyperglycaemia. The mean duration of diabetes in his group was 14.1 ±6.7 y and complications were not specified. In an another prospective study, cytokines were analysed in 53 T2D subjects with and without DR, and compared with healthy controls, and it was found that serum levels of IL-1β and IL-6 were undetectable in all diabetic subjects (Doganay et al., 2002). It has been shown that metformin (Isoda et al., 2006; Bestermann et al., 2011), PPAR-γ agonists (Jiang et al., 1998b) and GLP-1 analogues (Chaudhuri et al., 2012) lower the IL-1β concentration. In our study, IL-1β mRNA was significantly suppressed in neutrophils in PC subjects, compared with healthy controls (p<0.05), however after adjustment for various covariates it became statistically insignificant (Table 4.5). Furthermore, IFN-β has been shown to reduce the maturation of IL-1β by reducing the activation of nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3) (Guarda et al., 2011). The T2D subjects in our study have elevated IFN-β and this might have affected IL-1β as well.
IFN-β is associated with anti-viral immunity but has also shown to immunomodulate the Th1/Th2 cytokine profile by attenuating the secretion of IFN-γ and IL-12, while augmenting IL-4 and IL-10 secretion (Sellner et al., 2008). It has been shown that Interferon therapy can trigger induction of several autoimmune diseases, including T1D (Nakamura et al., 2011). A study on mice has suggested role of IFN-β in the onset of T1D (Pelegrin et al., 1998). In our study, serum levels of IFN-β were significantly raised in PC compared to NGT control (Table 4.4D), however, it became statistically insignificant after adjustment for covariates (Table 4.5D). We showed that IFN-β mRNA was significantly overexpressed in both monocytes and neutrophils from GC and PC group, compared to NGT. However, in the absence of human studies exploring the role of IFN-β in T2D, the significance of this finding is not known, and we think that larger studies are required to understand the role of IFN-β in T2D pathophysiology.

Collectively, our data showed that mRNA expressions of proinflammatory cytokines IL-6, TNF-α and IFN-β were increased in neutrophils, and IL-6 and IFN-β were increased in monocytes in GC group, compared to NGT. In contrast, IL-1β mRNA expression was significantly decreased in GCC, PC and PCC subjects, compared to NGT. It suggests an upregulated anti-inflammatory mechanism in T2D subjects with good glycaemic control with no complications, compared to T2D subjects with poor glycaemic control, and subjects with complications.
Anti-inflammatory cytokine and chemokine levels in T2D subjects with good and poor glucose control

Chemokines such as IL-8 and Rantes are cardinal in the pathogenesis of all inflammation since they mediate the arrival of inflammatory cells to the site of both acute and chronic inflammation (Maier et al., 2008). Regarding diabetes, three studies found that in mouse models and in humans, obesity was associated with infiltration of macrophages into adipose tissue and adipose tissue from obese mice exhibit a significant upregulation of chemokines (Herder et al., 2005). Few studies have demonstrated an elevated level of Rantes in patients with T2D (Maier et al., 2008; Herder et al., 2008). In our study, serum Rantes levels were comparable among different groups. However, we showed that Rantes mRNA was overexpressed in monocytes from PC subjects (16-fold) (p<0.001), followed by PCC subjects (12.4-fold) (p<0.001), compared to NGT. Similarly, in neutrophils, Rantes mRNA was overexpressed in GC (p<0.001) and PC (p<0.01) subjects, compared with NGT. Though the Rantes mRNA was overexpressed in our study, it was not possible to say whether it was a consequence of hyperglycaemia or had a causal role in it. However, Augsburg Cohort Study suggested that raised Rantes was a consequence rather than cause of hyperglycaemia. In this study, DNA samples were analysed from 502 individuals with and 1632 individuals without incident T2D, CCL5 genotypes and RANTES serum concentrations were determined, and associations between genotypes, haplotypes, serum levels, and T2D were assessed. It was noticed that RANTES/CCL5 gene variants and serum levels were not causally related with T2D.
The serum IL-8 was comparable among different groups in our study, which is in disagreement with previous studies (Fig. 4.5C). Esposito et al. (2003) has shown increased plasma levels of IL-8 in newly diagnosed T2D subjects (n = 30), compared with healthy subjects. In another similar study, Zozuliaska et al. (1999) showed elevated plasma levels of IL-8 in T2D subjects (n =20). The medications and diabetes associated micro/macrovasular complications status were not described in these studies. We think that our study cohort was different from these studies as our T2D subjects were on multiple oral agents and/or insulin, and subjects had longer duration of diabetes, which might have suppressed the IL-8 levels. Furthermore, both studies had small sample size, which could have affected their results. Elner et al. (1995) has suggested that IL-8 participates in the pathogenesis of diabetic retinopathy. He reported an increase in IL-8 concentrations in the vitreous of patients with diabetic retinopathy. This study involved 30 patients with proliferative diabetic retinopathy (PDR), 13 patients with proliferative vitreoretinopathy (PVR), and 26 control individuals, and IL-8 was measured in the vitreous. We did not analyse serum cytokines based on DR stages in this study, which was a limitation. We think that studies with larger sample size are required to draw a conclusion regarding the role of IL-8 in T2D.

In this study serum IL-10 levels were statistically insignificant among different groups. However, Yaghini et al. (2011) showed that the serum levels of IL-10 were decreased in T2D subjects (n = 131) compared to controls (n = 120). Similarly, the Leiden 85-Plus Study, which included a cohort of 553 subjects showed that low IL-10 production capacity was associated with high plasma glucose, and high HbA1c, (Exel et al., 2012). In this study blood samples were collected under nonfasting conditions, which was a limitation. In our study, we did not find a correlation between Hba1c & IL-10. The difference regarding IL-10 in our study and these studies is possibly due to smaller sample size in our study.
In summary, we observed that Rantes mRNA expression was markedly overexpressed in neutrophils from GC group, however, variable expression was seen in monocytes. Collectively, it indicates an activated inflammatory mechanism in subjects with T2D.

**Levels of Th1 cytokines, IL-12 and IFN-γ in T2D subjects with good and poor glucose control**

In this study, we observed low serum levels of IL-12 and IFN-γ in T2D subjects, which was statistically insignificant. Previous studies have shown conflicting results. Tsiavou et al. (2004) has demonstrated low serum levels of Th1 cytokines, IFN-γ and IL-12 in T2D subjects. In contrast, Wegner et al. (2008) demonstrated elevated IL-12 serum levels in T2D subjects. His study included 109 subjects and T2D subjects were treated with sulfonylurea. In another human study, which included 80 subjects, Winkler et al. (1998) demonstrated elevated serum IL-12 in T2D subjects. The patients medication were not specified in this study. Our study differ from these studies as our T2D study cohort was different and most of our T2D subjects were on multiple OHA’s and/or insulin, and this altogether might have suppressed serum IL-12 in the our T2D cohort. We think that larger studies are required to ascertain the role of cytokines in T2D.

An appropriate balance between proinflammatory (Th1) and anti-inflammatory (Th2) cytokines is critical to maintain homeostasis thus avoiding unwanted chronic inflammatory pathology. While the Th1 cytokines stimulate cell-mediated immunity, and promote inflammation and cytotoxicity, the Th2 cytokines assure regulatory function, thus mediate protective function during diabetes. Collectively, our data showed that mRNA expressions of proinflammatory cytokines IL-6, TNFα and IFN-β were significantly increased in neutrophils, and IL-6 and IFN-β were significantly increased in monocytes in GC group, compared to NGT.
Chemokine, Rantes mRNA was also found overexpressed in GC in neutrophils. In contrast, IL-1β mRNA expression was significantly decreased in GCC, PC and PCC subjects, compared to NGT. It suggests that T2D subjects with good glycaemic control with no complications have upregulated inflammatory mechanisms, whereas T2D subjects with poor glycaemic control, and subjects with complications have decreased inflammation.
Chapter 5

Analysis of adipokines in T2D subjects
5.1 Results

The adipokines were measured in NGT and T2D subjects in both monocytes and neutrophils. Both neutrophils and monocytes expressed all three adipokines, RBP-4, LCN-2 and adiponectin at the transcriptional level. The 2\(^{(\Delta\Delta\text{Ct})}\) method was used to analyse the relative changes in gene expression obtained using real-time quantitative PCR.

5.1a Adipokines expression in monocytes in T2D subjects with good and poor glucose control

The highest level of RBP4 mRNA was seen in monocytes from GC subjects (6.2-fold), followed by increased expression in GCC (2.8-fold) when compared to NGT (Fig. 5.1A). In contrast, PC and PCC subjects showed comparable RBP4 expression when compared to NGT control individuals (Table 5.1).

The highest level of LCN2 mRNA was observed in monocytes from GC subjects (2-fold) (Fig. 5.1B). In contrast, significantly decreased LCN2 expression was evident in PC and PCC subjects compared to NGT. Similarly, adiponectin mRNA was overexpressed in monocytes from GC subjects (1.4-fold) and significantly decreased levels were seen in PC and PCC subjects, compared with NGT (Fig. 5.1C). The RBP4, LCN2 and adiponectin mRNA expressions remained significant among different groups with multifactorial ANOVA using lognormal data after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) (p<0.001) (Table 5.3).
In summary, the RBP4, LCN2 and adiponectin were overexpressed in monocytes from GC subjects when compared to NGT controls. On the contrary, decreased expression of the LCN2 and adiponectin was seen in PC and PCC subjects compared to NGT individuals.

5.1b Adipokines expression in neutrophils in T2D subjects with good and poor glucose control

GC and PC neutrophils over expressed RBP4 mRNA, about 40-fold and 6-fold respectively, compared to NGT (Fig. 5.2A) (Table 5.2). Similarly, LCN2 mRNA expression was significantly upregulated in GC (25.2-fold) and PC (3.6-fold) compared with NGT (Fig. 5.2B). We can see that adiponectin mRNA expression was also significantly enhanced in GC (30.7-fold) compared to NGT (Fig. 5.2C). The RBP4, LCN2 and adiponectin mRNA expressions remained significant among different groups with multifactorial ANOVA using lognormal data after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) (p<0.001) (Table 5.3). Additionally, we did not find correlation between adipokines and BMI in both monocytes and neutrophils in T2D subjects.

In summary, our data showed that all three adipokines, RBP4, LCN2 and adiponectin were significantly overexpressed in GC neutrophils compared to NGT controls. Moreover, neutrophils from PC subjects also displayed the enhanced expression of LCN2 mRNA compared to NGT. In contrast, comparable expression of adipokines was evident in GCC and PCC subjects compared to NGT.
Collectively, the study showed that RBP4, LCN2 and adiponectin were overexpressed in both monocytes and neutrophils in GC subjects. In contrast, variable expression was evident in PC, GCC and PCC subjects compared to NGT controls.
Table 5.1 Adipokines mRNA expressions in monocytes from type 2 diabetic subjects & healthy controls. Adipokines mRNA were measured in subjects with normal glucose targets (NGT), good glucose control (GC), good glucose control with micro/macrovascular complications (GCC), poor glucose control (PC), poor glucose control with micro/macrovascular complications (PCC) in monocytes at the transcriptional level using 2-(ΔΔCt) method and results are expressed as median [inter quartile range]. Statistical significance was determined by Kruskal–Wallis test and p<0.05 was considered significant. p value corresponds to the differences between groups.

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<th>NGT</th>
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<tr>
<td>RBP4</td>
<td>1.0 [0.9-1.0]</td>
<td>6.2 [4.3-7.0]</td>
<td>2.8 [2.6-3.0]</td>
<td>1.3 [1.0-1.5]</td>
<td>1.0 [0.8-1.3]</td>
<td>&lt;0.001</td>
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<tr>
<td>LCN2</td>
<td>1.0 [0.9-1.1]</td>
<td>2.5 [2.3-2.6]</td>
<td>1.0 [0.9-1.0]</td>
<td>0.5 [0.5-0.6]</td>
<td>0.6 [0.5-0.7]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1.1 [0.7-1.5]</td>
<td>1.6 [1.4-1.7]</td>
<td>0.8 [0.7-0.8]</td>
<td>0.3 [0.3-0.4]</td>
<td>0.3 [0.2-0.3]</td>
<td>&lt;0.001</td>
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Table 5.2 Adipokines mRNA expression in neutrophils from type 2 diabetic subjects & healthy controls. Statistical significance was determined by Kruskal–Wallis test and p<0.05 was considered significant. p value corresponds to the differences between groups.

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<td>RBP4</td>
<td>1.0 [0.8-1.1]</td>
<td>39.6 [36.5-41.8]</td>
<td>1.1 [0.7-1.9]</td>
<td>5.4 [4.8-6.8]</td>
<td>0.4 [0.2-0.7]</td>
<td>&lt;0.001</td>
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<tr>
<td>LCN2</td>
<td>1.0 [0.9-1.1]</td>
<td>25.2 [18.8-32.9]</td>
<td>1.5 [1.3-1.7]</td>
<td>3.6 [3.2-4.3]</td>
<td>0.9 [0.3-1.6]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1.0 [0.9-1.0]</td>
<td>30.7 [29.5-31.6]</td>
<td>0.7 [0.5-1.0]</td>
<td>4.0 [2.7-6.7]</td>
<td>0.6 [0.3-1.5]</td>
<td>&lt;0.001</td>
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Table 5.3. The multifactorial ANOVA analysis of adipokines mRNA in monocytes and neutrophils after adjustment of covariates. Statistical significance was determined after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins). p value corresponds to the differences between groups.

<table>
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<th>Neutrophils</th>
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<td>LCN2</td>
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<td>Adiponectin</td>
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Figure 5.1 RBP4, LCN-2 & adiponectin mRNA expressions in monocytes in subjects with type 2 diabetes & controls. The RBP4 mRNA (A) was overexpressed in monocytes from GC and GCC, compared to NGT. LCN2 (B) and adiponectin (C) mRNAs showed decreased expression in PC and PCC monocytes, compared to NGT. RBP-4, Retinol binding protein 4, LCN-2, Lipocalin-2 (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs. NGT.
Figure 5.2 RBP4, LCN 2 & adiponectin mRNA expressions in neutrophils in subjects with type 2 diabetes & controls. The RBP4 (A), LCN2 (B) and adiponectin (C) mRNAs were significantly overexpressed in GC neutrophils, compared to NGT. LCN2 mRNA was also overexpressed in PC compared to NGT. RBP-4, Retinol binding protein 4, LCN-2, Lipocalin-2 (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs. NGT.
5.2 Discussion

In the present study, we have investigated three adipokines, i.e., adiponectin, LCN2 and RBP4 mRNA expression in monocytes and neutrophils in T2D subjects and compared them with the healthy controls.

Only few studies have evaluated the expression of human adiponectin at mRNA level in the monocytes. The adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) receptors are ubiquitously expressed in most organs as well as in human peripheral monocytes, and in monocyte-derived macrophages (Pang & Narendran, 2008). Most of the data published so far indicate that circulating adiponectin and the mRNA expression of AdipoR1 and AdipoR2 are reduced in IR postulating a state of adiponectin resistance in glucose intolerant animals and humans. (Weigert et al., 2008). One study has revealed a positive correlation between monocytic AdipoR1 mRNA and systemic adiponectin concentration in obesity (Weigert et al., 2008). Whereas, another study has shown a positive correlation between Lymphocytic AdipoR1 mRNA and serum adiponectin level (Alberti et al., 2007). In our study we evaluated adiponectin mRNA expression on monocytes and neutrophils but did not investigate AdipoR1 and AdipoR2 mRNA separately.

LCN2 is expressed in neutrophils and interestingly, neutrophil gelatinase-associated lipocalin was originally isolated from the supernatant of activated human neutrophils (Kjeldsen et al., 1993). There might be a correlation between LCN2 expression on leucocytes and serum LCN2 concentration, but we did not find any study, which evaluated LCN2 mRNA expression on leucocytes. Similarly, RBP4 expression has been studied in adipocytes and macrophages (Brochi et al., 2010) but the studies on RBP4 expression in monocytes or neutrophils are lacking.

Adiponectin has been identified as insulin sensitizing adipocyte-derived protein (Esteve et al., 2009), which is paradoxically decreased in obesity
(Bruun et al., 2003a). In a human study, Brun et al. (2003) showed that plasma levels of adiponectin were higher in lean subjects compared with obese subjects, and adiponectin levels inversely correlated with measures of adiposity [BMI (P 0.05)]. He further showed that weight loss resulted in increase in plasma adiponectin and an increase in adipose tissue mRNA levels. In our study, we did not find correlation between adipokines and BMI in both monocytes and neutrophils in T2D subjects. Previous studies have shown that the circulation levels of adiponectin are lower in T2D (Hotta et al., 2000; Weyer et al., 2001) and in IR states (Chang et al., 2009; Yang et al., 2002c). Similarly our data also showed suppressed adiponectin in monocytes from PC and PCC, compared to NGT. However, adiponectin mRNA was overexpressed in neutrophils from GC compared to NGT. Whether increased adiponectin in GC is a protective response to overcome IR or a reflection of excellent glycaemic control, median Hba1c of 6.4 [6.0-6.9] (median [IQR]), remains questionable. The lifestyle modification and weight reduction also enhances plasma adiponectin concentration in T2D subjects (Hotta et al., 2000; Balgopal et al., 2005), however, the contribution of these measures were not measured in our study. The subjects were not asked about weight loss and exercise programme they might be involved in, at the time of recruitment and IR was also not calculated. However, absence of micro or macrovascular complications and shorter duration of diabetes in GC as compared to other T2D groups, argues against IR in the GC group.

It has been shown that adiponectin plasma levels deteriorate early in the development of obesity and rapidly achieve trough levels with further weight gain and thus, the significance of low adiponectin levels for the metabolic and cardiovascular risk is not apparent in patients beyond a certain BMI cutoff or in patients with already visible end-organ damage (Engeli et al., 2003). Hotta et al., (2000) showed that serum adiponectin levels does not correlate with existing retinopathy in diabetic patients. Our
data also showed that adiponectin expression was significantly reduced in PCC in monocytes, compared to NGT.

In our study, highest levels of adiponectin mRNA & IL-6 mRNA were noticed in neutrophils in GC group compared to healthy controls and decreased levels were seen in PCC in monocytes (p<0.05). However, it has been shown that serum adiponectin concentrations are inversely associated with the degree of inflammatory response in both cardiovascular disease and critical illness (Ouedraogo et al., 2007). Adiponectin has been shown to have protective metabolic and anti-inflammatory properties (Engeli et al., 2003). A human study has demonstrated an independent inverse correlation between low adiponectin (plasma levels and adiponectin gene expression from adipose tissue) and high plasma levels of hs-CRP and IL-6 (Engeli et al., 2003). In a study on porcine macrophages adiponectin was shown to suppress LPS-stimulated release of IL-6 (Wulster-Radcliffe et al., 2004). We think that our results are at variance with previous findings because of different methodologies i.e, we measured adipokines mRNA expressions in monocytes and neutrophils, whereas other studies had measured adipokines in serum and adipocytes. Though, it is plausible to speculate that higher levels of adiponectin observed in GC is a protective measure for counteracting IR and inflammation, whereas decreased adiponectin in PCC is a reflection of downregulation of receptor as a result of sustained activation, resulting in complications, the interpretation is limited by the small sample size in this study.

The RBP4 is an adipokine associated with obesity and comorbidities, which correlates inversely with insulin sensitivity and higher levels have been seen in subjects with IGT and T2D (Yang et al., 2005; Graham et al., 2006; Cho et al., 2006). Interestingly, highest levels of RBP4 mRNA were seen in GC in both monocytes and neutrophils and a decreasing trend was evident in other T2D cohorts. The weight loss and exercise have been
shown to decrease RBP4 levels in subjects with IR and T2D (Graham et al., 2006; Ku at al., 2010), whereas insulin-sensitizing agent pioglitazone has been shown to increase RBP4 gene expression (Yao-Borengasser et al., 2007; Sell et al., 2007). However, in our study, only 5 T2D subjects were on PPAR-γ agonists out of 112 (Table 3.3). Interestingly, in line with our study, few other studies also did not find any correlation between circulating RBP4 levels and IR (Ulgen et al., 2010; Promintzer et al., 2007). We think that poor glycaemic control and the complications might have suppressed the RBP4 expression in PC, GCC and PCC, but the data is limited because of small sample size.

The serum RBP4 levels correlates with the degree of IR (Graham et al., 2006), but the mechanism by which RBP4 induces IR is not well known (Esteve et al., 2009). IR is associated with increased risk of both micro- and macrovascular complications (Kilpatrick et al., 2007). We tried to explore the role of RBP4 in diabetes associated chronic micro/macro vascular complication. One study has suggested role of RBP4 in chronic inflammatory DR; the serum RBP4 level were significantly elevated in individuals with proflerative DR compared with those with no DR or simple DR and (Li et al., 2010). In contrast, another study in DN suggested it is impaired kidney function that determine RBP4 serum concentration in T2D nephropathy patients rather than T2D per se; the RBP4 concentration was found elevated equally in T2D and non diabetic subjects with impaired renal function (Andrea et al., 2008). In our T2D subjects, retinopathy was seen in 40.6% PCC subjects, compared with 18.6% GCC subjects, neuropathy was evident in 41% GCC subjects and 44% PCC subjects, and nephropathy was seen in 68.8% PCC compared to 56.3% GCC subjects. However, we demonstrated suppressed RBP4 expression in our PCC group. It suggests that sustained activation of receptors, resulting in diabetic complication, lead to downregulation of receptor for limitation and termination of systemic inflammatory response.
Our study clearly showed that the LCN2 mRNA levels were highest in GC in both neutrophils (p<0.05) and monocytes, and a decreasing trend was evident in other T2D cohorts. Few studies have also demonstrated increased LCN2 expression in obese animals and human subjects with T2D (Wang et al., 2007; Yan et al., 2007). It has been shown that hyperglycaemia increases the expression of LCN2 by inducing IR, and the thiazolidinediones, an insulin-sensitizing agent reduces it (Wang et al., 2007; Yan et al., 2007). Given the anti-inflammatory role of LCN2, increased LCN2 in obesity and IR appears to be a protective mechanism against inflammation.

Collectively, we found that all three adipokines, adiponectin, LCN2 and RBP4 were overexpressed in the T2D subjects and indicate an inflammatory/anti-inflammatory and IR/insulin sensitizing milieu in early diabetes. We think that higher levels of adipokines observed in early diabetes is a reflection of protective measure to prevent complication, whereas suppressed expression observed in late diabetes is a reflection of downregulation of receptors to curtail the inflammation.

No study that we are aware of has compared adipokine expression among diabetic subjects with good glycaemic control with/without complications and poor glycaemic with/without complications. In presence of the limited data from this relatively small study it is difficult to interpret the exact role of adipokines in the pathophysiology of T2D, and further studies are needed in a similar but larger cohort and with an expanded profile including measurement of IR and serum adipokine levels.
Chapter 6

Analysis of TLR expression in T2D subjects
6.1 Results

6.1a TLR expression in monocytes in T2D subjects with good and poor glucose control

Toll like receptor gene expression was evaluated in the monocytes and neutrophils of subjects with T2D and NGT individuals. All TLRs 1-10 were expressed on both monocytes and neutrophils. The $2^{\Delta\Delta Ct}$ method was used to analyse the relative changes in gene expression obtained using real-time quantitative PCR.

The expression of TLR 1-10 mRNA in monocytes obtained from NGT and T2D are depicted in Fig 6.1 (Table 6.1). It was found that TLR1 mRNA was overexpressed by 3-fold and 2-fold in the monocytes of GC and GCC subjects, respectively, when compared with NGT ($p<0.001$). In contrast, comparable expression was evident in PC and PCC subjects (Fig. 6.1A).

TLR2 mRNA was overexpressed in the monocytes from GC subjects (1.6-fold), whereas decreased expression was evident in the PC (0.7-fold) and PCC subjects (0.5-fold), compared to NGT (Fig. 6.1B). Similarly, TLR3 mRNA was upregulated by 4.7-fold and 2.3-fold in the monocytes of GC and GCC subjects, respectively compared to NGT, whereas comparable expression was observed in the PC and PCC subjects (Fig. 6.1C).

Interestingly, T2D subjects in all groups exhibited a comparable TLR4 mRNA expression in the monocytes when compared to NGT (Fig. 6.1D). The highest level of TLR5 mRNA was seen in the monocytes from GC subjects (3.3-fold), followed by GCC (1.5-fold) compared to NGT (Fig. 6.1E). In contrast, comparable expression was noticed in PC and PCC subjects. TLR6 mRNA was overexpressed in the monocytes from GC
subjects (2.5-fold) compared to NGT, whereas, comparable expression was evident in GCC, PC and PCC subjects (Fig. 6.1F).

We can see that TLR7 mRNA expression was upregulated in the monocytes from PC (12-fold), GCC (9.3-fold) and PCC (3.9-fold), markedly upregulated in GC subjects (24.4-fold), when compared to NGT (Fig. 6.1G). The monocytes of GC overexpressed TLR8 mRNA by 2.1-fold, compared to NGT, whereas comparable expression was noticed in GCC (1.5-fold), PC (1.2-fold) and PCC subjects (1.3-fold) (Fig. 6.1H).

TLR9 mRNA was overexpressed by 6.3-fold and 2-fold in the monocytes of GC and GCC subjects compared to NGT (Fig. 6.1I). In contrast, PCC subjects (0.8-fold) exhibited decreased TLR9 mRNA expression when compared to NGT controls. Similarly, the monocytes of GC and GCC overexpressed TLR10 mRNA by 5.8-fold and 2.2-fold respectively compared to NGT, whereas suppressed expression was observed in the PCC subjects (Fig. 6.1J). The TLR mRNA 1, 3, 5, 6, 7, 9 & 10 remained statistically significant among different groups after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) using lognormal data with multifactorial ANOVA (Table 6.2). Additionally, We did not find correlation between TLRs mRNA in monocytes and BMI in T2D subjects.

In summary, monocytes from GC group overexpressed all TLRs mRNA 1-10 and mRNA levels of TLR1, 3, 5, 7, 9 & 10 were significantly highest compared to NGT. TLR 1, 5, 7 &-10 were overexpressed in the monocytes from GCC subjects compared to NGT (p<0.05).
Table 6.1 TLR mRNA expressions in monocytes from subjects with type 2 diabetes & healthy controls. TLRs mRNA were measured in subjects with normal glucose targets (NGT), good glucose control (GC), good glucose control with micro/macrovascular complications (GCC), poor glucose control (PC), poor glucose control with micro/macrovascular complications (PCC) in monocytes at the transcriptional level using 2-(ΔΔCt) method and results are expressed as median [inter quartile range]. Statistical significance was determined by Kruskal–Wallis test and p<0.05 was considered significant. p value corresponds to differences between groups.

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</tr>
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Table 6.2. The multifactorial ANOVA analysis of TLRs mRNA in monocytes and neutrophils after adjustment of covariates. Statistical significance was determined after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins). p value corresponds to the differences between groups.

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<td>TLR 6</td>
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<td>TLR 9</td>
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<td>TLR 9</td>
<td>&lt;0.001</td>
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<td>TLR 10</td>
<td>&lt;0.001</td>
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Figure 6.1 (Pages 146-147)

TLR mRNAs expression in monocytes of subjects with type 2 diabetes & controls. All TLRs mRNA 1-10 were overexpressed in GC group, and mRNA levels of TLR1, 3, 5, 7, 8, 9 & 10 were highest compared to NGT.
TLR 1, 5, 7 & 10 were overexpressed in the monocytes from GCC subjects compared to NGT. TLR7 mRNA was markedly overexpressed in monocytes from GC (22-fold) compared to NGT. TLR, Toll Like Receptor (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs NGT.

Figure 6.1 contd. from page 146
6.1b TLR expression in neutrophils in T2D subjects with good and poor glucose control

The expression of TLRs 1-10 mRNA in neutrophils obtained from NGT and T2D are depicted in Fig 6.2 (Table 6.3). It was found that TLR1 mRNA was overexpressed by 2.4-fold in the neutrophils of GC subjects compared to NGT (Fig. 6.2A). In contrast, comparable expression was evidenced in other groups. Similarly, TLR2 mRNA was overexpressed by 1.6-fold and 1.9-fold in the neutrophils of GC and GCC subjects respectively, compared to NGT, whereas comparable expression was evidenced in PC and PCC subjects (Fig. 6.2B).

Interestingly, TLR3 mRNA expression was markedly upregulated by about 28-fold and 4-fold in the neutrophils of GC and PC subjects, respectively compared to NGT (Fig. 6.2C). In contrast, suppressed expression was observed in the GCC and PCC subjects. We can see that highest level of TLR4 mRNA was seen in neutrophils from GC subjects (5.4-fold), followed by PCC (3.5-fold) and GCC (3.3-fold), compared to NGT. In contrast, TLR4 expression in PC (2.3-fold) group was comparable (Fig. 6.2D).

The TLR5 mRNA, similar to TLR3 mRNA, was markedly overexpressed by 27.5-fold and 5.9-fold in the neutrophils of GC and PC subjects compared to NGT, whereas GCC and PCC subjects overexpressed the TLR5 mRNA by 3.6-fold and 2.4-fold, respectively compared to NGT control individual (Fig. 6.2E).

TLR6 mRNA expression was upregulated by 9.7-fold in the neutrophils of GC subjects compared to NGT. In contrast, comparable expression was evidenced in GCC subjects (0.9-fold), and suppressed expression in PCC subjects (0.5-fold) (Fig. 6.2F). Interestingly, the neutrophils of GC overexpressed TLR7 mRNA by 61.5-fold, followed by increased
expression in PC by 4.9-fold compared to NGT. In contrast, GCC and PCC exhibited comparable expression compared to NGT (Fig. 6.2G).

The neutrophils of GC and GCC overexpressed TLR8 mRNA by 4.5-fold and 2.1-fold respectively, when compared with NGT, whereas PCC (0.7-fold) exhibited decreased expression compared to NGT (Fig. 6.2H).

The TLR9 mRNA expression was markedly enhanced in the neutrophils of GC (25.5-fold) and PC (3.6-fold), comparable in GCC (1.9-fold), when compared with NGT. In contrast, PCC subjects (0.5-fold) showed decreased expression, compared to NGT (Fig. 6.2I). Similarly, highest level of TLR10 mRNA was seen in neutrophils from GC subjects (12.8-fold) compared to NGT. In contrast GCC (1.3 fold) and PC group (2.3-fold) exhibited comparable expression, and PCC group (0.8-fold) showed decreased expression, compared to NGT (Fig. 6.2J). All TLRs 1-10 except TLR 2 remained statistically significant among different groups after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) using lognormal data with multifactorial ANOVA (Table 6.2). Additionally, we did not find correlation between TLRs mRNA in neutrophils and BMI in T2D subjects.

In summary, the highest expression of TLRs mRNA 1-10, except TLR 2 were noticed in the neutrophils from GC subjects compared to NGT. Similarly, neutrophils from GCC subjects overexpressed TLRs 4 & 5 compared to NGT. In contrast, the neutrophils from PCC significantly overexpressed only TLR4 compared with NGT.

Collectively, these data show that TLRs are overexpressed in type 2 diabetes subjects in both monocytes and neutrophils and suggest the role of TLRs in the pathophysiology of T2D and associated complications.
Table 6.3 TLR mRNA expressions in neutrophils from subjects with type 2 diabetes & healthy controls. TLRs mRNA were measured in subjects with normal glucose targets (NGT), good glucose control (GC), good glucose control with micro/macrovascular complications (GCC), poor glucose control (PC), poor glucose control with micro/macrovascular complications (PCC) in neutrophils at the transcriptional level using 2-(ΔΔCt) method and results are expressed as median [inter quartile range]. Statistical significance was determined by Kruskal–Wallis test and p < 0.05 was considered significant. p value corresponds to differences between groups.

<table>
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<td>TLR 4</td>
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<td>3.6 [2.8-4.8]</td>
<td>5.9 [5.2-6.9]</td>
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Figure 6.2 (pages 151-152)
TLRs mRNA expressions in neutrophils of subjects with type 2 diabetes & healthy controls:
Figure 6.2 (cont. from page 151)

TLRs mRNA 1-10, except TLR 2 were significantly overexpressed in GC, and TLRs 2, 4 & 5 were overexpressed in GCC in neutrophils, compared to NGT. The neutrophils from PCC significantly overexpressed only TLR4 compared with NGT. TLR, Toll Like Receptor (n = 8 in each group). *p<0.05, **p<0.01, ***p<0.001 vs. NGT.
6.2 Discussion

In the present study, we have demonstrated that in monocytes, all TLRs mRNA 1-10 were overexpressed in GC group, and mRNA levels of TLR1, 3, 5, 7 9 & 10 were highest, compared to NGT. Similarly, in neutrophils, all TLRs mRNA, except TLR 2 were significantly overexpressed in GC group, when compared to NGT. Similar to our results, previous studies have demonstrated increased expression of TLR2 and TLR4 in the monocytes derived from patients with type 2 diabetes (Dasu et al., 2010). Previous studies did not explore other TLRs in diabetes, but were mainly focussed on TLR2 and TLR4, so data on role of other TLRs in T2D is lacking. Our novel study suggest that other TLRs are also overexpressed in T2D and warrant further investigation to explore the role of different TLRs in the pathophysiology of T2D.

We have demonstrated differential expression of TLRs in monocyte and neutrophils. The monocytes from GC subjects markedly overexpressed TLR7 mRNA (24.4-fold), whereas neutrophils from GC subjects markedly overexpressed TLR3 mRNA (28.6-fold), TLR5 mRNA (27.5-fold), TLR7 mRNA (61.5-fold), TLR9 mRNA (25.5-fold) and TLR10 mRNA (12.8-fold) compared to NGT. The differential expression on monocyte and neutrophil was also seen in other groups of diabetes. It has been demonstrated previously that monocytes and neutrophils differ in the surface expression of TLR mRNAs and this might have attributed to differential expression. The Neutrophils constitutively express all TLRs except TLR5, while monocytes lack expression of TLR3, TLR6, TLR7, and TLR10 (Muzio et al., 2000). Furthermore, it was shown that constitutive expression of TLR5 was less in neutrophils compared to monocytes and expression of TLR4 was greater and that of TLR9 lower in monocyte-derived macrophages compared to monocytes (O’Mahony et al., 2008). It has also been shown that IFN-γ up-regulates TLR2 and TLR4 expression in neutrophils and
monocytes, and inflammatory cytokines down-regulate TLR5 expression in monocytes (O’Mahony et al., 2008).

The expression patterns of TLRs in different cell types may be an important regulatory mechanism of the innate immune response to various pathogens. The neutrophils are the hallmark of acute inflammation, get recruited to the site of injury within minutes and have shorter half life in circulation of about 6-8 hours (Christian et al., 2011). In contrast, the blood monocytes differentiate into tissue macrophages and survive for long period and produces various cytokines like IL-1, IL-12 and TNFα (Heitbrock, 2007). The increased monocytic activity and elevated biomarkers have been shown in previous studies in diabetic subjects (Devaraj et al., 2006). Most of the studies have used monocytes for TLR expression in various diseases but the studies using neutrophils in TLR expression in T2D inflammation are lacking. Our study demonstrates that neutrophils and monocytes expresses different TLR mRNA in T2D subjects and thus suggest the significance of neutrophils in TLR evaluation.

In our study, T2D subjects with poor glucose control, and subjects with complications showed a trend towards suppressed TLR mRNA expression. In monocytes, GCC moderately overexpressed TLR 1, 5, 7 & 10, whereas PC overexpressed only TLR 7, compared to NGT. Similarly, in neutrophils, GCC moderately overexpressed TLR 4 & 5, whereas PC overexpressed TLR 5, 7 & 9 compared to NGT. PCC subjects overexpressed only TLR 4 in neutrophils (p<0.05), and a statistically insignificant decreased expression of TLR 3, 6, 8, 9 & 10 was noticed in PCC compared to NGT. It is plausible to speculate that downregulation of TLRs might have occurred to curtail their sustained activation and to block the concomitant increase in proinflammatory cytokines. Whether decreased TLR expression leads to compromised innate immune
signalling mechanisms and a concomitant predisposition to infections, needs to be investigated.

We have shown that neutrophils as well as monocytes markedly overexpressed TLR7 mRNA, compared to NGT. We couldn't not find any association of TLR7 with T2D in the literature. However, recently it has been shown that TLR7 promotes autoimmune diabetes in animal model by activating and producing proinflammatory cytokines, IF-α (Lee et al., 2011). The significance of raised TLR7 in T2D, as demonstrated in our study, needs to be investigated.

It has been argued previously that TLR4 expression and signalling correlates with IR (Reyna et al., 2008; Dasu et al., 2010). In contrast, in our study the highest level of TLR4 was seen in GC subjects in neutrophils (p<0.05) compared with NGT. The HbA1c and duration of diabetes was significantly less in GC compared to other T2D groups, which argue against IR in this group. Knowing that most TLRs were markedly overexpressed in both monocytes and neutrophils in GC subjects, we think that TLRs overexpression in early diabetes indicates low grade inflammation and plays a role in the pathogenesis of disease.

It has been shown that statins, PPAR-γ agonists (Dasu et al., 2009a), metformin (Andrews et al., 2012) and angiotensin receptor blockers (Dasu et al., 2009b) decrease TLR2 and TLR4 expression and signaling. In our T2D cohort, comparable number of subjects in each group were on lipid lowering agents, GC (85.2%), GCC (93.8%), PC (61.9%) and PCC (87.5%) , and ACE inhibitor/ARB, GC (74.1%), GCC (87.5%), PC (76.2%), and PCC (81.3%). A higher percentage of subjects in GC (81%) and GCC (81%) were on metformin, compared to PC (62%) and PCC (59%), despite that highest level of TLR4 mRNA was observed in neutrophil in GC group compared to NGT (p<0.05). It has been shown that insulin infusion significantly suppressed TLRs 1, 2, 4, 7, and 9 mRNA expressions in mononuclear cells (Ghanim et al., 2008). In our study a higher percentage
of subjects in PC (52.4%) and PCC (71.9%) were on insulin compared to GC (7.4%) and GCC (28.1%) groups. Interestingly, TLR 4 was significantly overexpressed in neutrophils in PCC, TLR 7 was overexpressed in monocytes from PC, and TLR 5, 7 & 9 were overexpressed in neutrophils from PC group compared to NGT (p<0.05). We think that glycaemic control and the complications affect the expressions of TLRs to a great extent.

In summary, our study demonstrated that TLRs are overexpressed in type 2 diabetes subjects in both monocytes and neutrophils. The surface expression of TLR was different in monocytes and neutrophils and also in different groups of T2D. The increased expression of TLRs is suggestive of an inflammatory milieu in T2D, however, to understand the significance of raised TLRs in T2D pathophysiology and micro/macrovascular complication, further investigations are required.
Chapter 7

Cytokine profiling of Pre-diabetic subjects
7.1 Introduction

Several studies have investigated and reported that proinflammatory cytokines are elevated in patients with IGT (Muller et al., 2002; Ruotsalainen et al., 2006), and predict the conversion to T2D (Pradhan et al., 2001). However, there is lack of data regarding the association between IFN-β and pre-diabetes. There is also limited information about association between TLRs and pre-diabetes. With this in mind, we analysed a cohort of 42 healthy Irish subjects to evaluate relationship between IGT and circulating cytokine levels, including IFN-β and TLRs. However, due to the limited number of pre-diabetes subjects in this group thus limiting statistical significance, we opted to preclude the analysis of adipokine and TLR expression levels in pre-diabetes.

Our study comprised 34 NGT and 9 pre-diabetes subjects (8 had IFG and 1 had both IFG and impaired 2h post prandial glucose). Pre-diabetic participants were healthy volunteers and were not on any medications. They were 18 years or older and met the exclusion criteria as described before. They were diagnosed as pre-diabetes based on ADA criteria after an OGTT test (Table 2.3).

7.2 Analysis of metabolic parameters in pre-diabetic subjects

The median age and smoking status was comparable in the NGT and pre-diabetes group. In NGT group, 22.5% were smokers, 51.6% non-smoker and 25.8% ex-smoker, whereas in pre-diabetes group 25% were smokers, 50% non-smoker and 25 % ex-smoker (Table 7.1). Pre-diabetes subjects had significantly higher BMI compared to NGT controls (p<0.05). However, no significant difference was seen in the WHR and blood pressure in between the two groups.
The fasting glucose and HbA1c were significantly higher in pre-diabetes subjects compared to NGT controls (p<0.05). Interestingly, CRP was significantly raised in pre-diabetes subjects compared to NGT (p<0.05). However, no significant difference was seen in lipid profile in between these two groups (Table 7.2). SBP and DBP were taken after a rest of 5 min and two readings were taken one minute apart. There was no significant difference in BP in between the two groups.

7.3 Results
Serum levels of cytokines were measured in subjects with NGT and pre-diabetes.

7.3a Pro-inflammatory cytokine levels in subjects with pre-diabetes and NGT
Our data showed that serum levels of IL-6, TNFα and IFNβ were increased in pre-diabetes compared with healthy controls (Table 7.3). However, there was statistically insignificant difference between the two groups (Fig. 7.1A &B).

7.3b Anti-inflammatory cytokine and chemokine levels in subjects with pre-diabetes and NGT
The serum levels of anti-inflammatory cytokine, IL-10 were raised in pre-diabetic subjects compared to NGT (Table 7.3) (Fig. 7.2E). However, the difference remained statistically insignificant between the two groups. The serum levels of chemokines, Rantes and IL-8 were elevated in pre-diabetes group compared with healthy controls (Fig. 7.2D, F). Here again, we did not find any statistically significant difference between the two groups.
7.3c Levels of Th1 cytokines, IL-12 and IFN-γ in subjects with good and poor glucose control

The serum IL-12 and FN-γ levels were comparable in pre-diabetes and NGT groups (Table 7.3), and statistically no significant difference was observed.

The multifactorial ANOVA was applied on all cytokines using lognormal values and data were adjusted for age, sex, BMI and WHR, it did not reveal any significant difference between pre-diabetes group and healthy controls. Additionally, we did not find correlation between serum cytokines and BMI in pre-diabetes subjects.
Table 7.1 Baseline characteristics of pre-diabetes and healthy controls

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<td>Sex (M/F)</td>
<td>11/23</td>
<td>2/7</td>
<td>0.249</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.5 [46.7-59.0]</td>
<td>49 [42.5-58.5]</td>
<td>0.439</td>
</tr>
<tr>
<td>Smokers Y/N</td>
<td>7/16</td>
<td>2/4</td>
<td>1.00</td>
</tr>
<tr>
<td>Ex smoker Y/N</td>
<td>8/16</td>
<td>2/4</td>
<td>1.00</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 [24.4-30.0]</td>
<td>35.4 [25.1-44.1]</td>
<td>0.047</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>91.0 [82.0-100.8]</td>
<td>111.0 [86.0-119.0]</td>
<td>0.062</td>
</tr>
<tr>
<td>WHR</td>
<td>0.9 [0.8-0.9]</td>
<td>0.9 [0.8-1.0]</td>
<td>0.683</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>129.0 [120.0-137.5]</td>
<td>127.5 [113.8-141.8]</td>
<td>0.921</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.0 [75.0-85.5]</td>
<td>74.0 [71.2-82.5]</td>
<td>0.172</td>
</tr>
</tbody>
</table>

BMI, Body Mass Index, WHR, Waist-Hip ratio, SBP, Systolic Blood Pressure, DBP, Diastolic Blood Pressure, data are expressed as median [IQR] (*p<0.05 vs NGT)
Table 7.2 Baseline biochemical parameters of pre-diabetes and healthy control

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NGT</th>
<th>Pre-diabetes</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>34</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fasting Glucose (mM)</td>
<td>4.8 [4.7-5.1]</td>
<td>5.9 [5.7-6.5]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5 [5.4-5.7]</td>
<td>6.0 [5.7-6.2]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>0.1 [0.1-0.3]</td>
<td>0.6 [0.1-1.1]</td>
<td>0.011</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>5.9 [5.1-6.6]</td>
<td>5.5 [4.5-5.9]</td>
<td>0.176</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>3.6 [2.8-4.3]</td>
<td>3.5 [2.2-4.1]</td>
<td>0.431</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.6 [1.3-1.9]</td>
<td>1.2 [1.2-1.8]</td>
<td>0.206</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.9 [0.7-1.2]</td>
<td>1.4 [0.9-1.8]</td>
<td>0.078</td>
</tr>
</tbody>
</table>

HbA1c, glycosylated haemoglobin, HDL, high-density lipoprotein; LDL, low-density lipoprotein, CRP, C-reactive protein. Data are expressed as median [IQR] (*p<0.05 vs NGT)
Table 7.3 Fasting levels of serum cytokines in subjects with pre-diabetes and healthy controls

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Normal</th>
<th>Pre-diabetes</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>34</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>12.3 [0.8-16.3]</td>
<td>17.3 [2.0-20.6]</td>
<td>0.086</td>
</tr>
<tr>
<td>TNFα</td>
<td>18.6 [14.5-22.5]</td>
<td>19.7 [17.1-25.7]</td>
<td>0.348</td>
</tr>
<tr>
<td>IL-1β</td>
<td>6.0 [0.9-8.4]</td>
<td>3.3 [0.9-8.9]</td>
<td>0.948</td>
</tr>
<tr>
<td>IFN-β</td>
<td>357.2 [8.5-685.4]</td>
<td>532.1 [202.7-1315]</td>
<td>0.305</td>
</tr>
<tr>
<td>Rantes (ng/ml)</td>
<td>54703 [41015-77823]</td>
<td>57587 [38739-620256]</td>
<td>0.510</td>
</tr>
<tr>
<td>IL-8</td>
<td>63.5 [25.2-89.8]</td>
<td>80.6 [20.0-153.4]</td>
<td>0.487</td>
</tr>
<tr>
<td>IL-10</td>
<td>6.8 [5.1-8.6]</td>
<td>8.4 [5.6-10.8]</td>
<td>0.213</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>9.3 [3.9-12.9]</td>
<td>9.8 [3.1-13.2]</td>
<td>0.956</td>
</tr>
<tr>
<td>IFNγ</td>
<td>8.7 [2.3-11.9]</td>
<td>5.2 [1.9-12.5]</td>
<td>0.838</td>
</tr>
</tbody>
</table>

IL-6, interleukin 6, TNFα, Tumour Necrosis Factor α, IL-1β, interleukin 1β, IFN-β interferon β Data are expressed as median [IQR] (p<0.05 vs. NGT)
Figure 7.1 Serum levels of pro-inflammatory cytokines and Th1 cytokines, IL-12 and IFN-γ in normal and pre-diabetic subjects. A: IL-6 and TNF-α levels comparable in pre-diabetic subjects and healthy controls. B: IFN-β levels comparable in pre-diabetic subjects and NGT. C: IL-12 and IFN-γ levels comparable in pre-diabetic subjects and NGT. Values are expressed as pg/ml (Rantes as ng/ml) (n = 34 vs 9). *p<0.05, **p<0.01, ***p<0.001 vs NGT.
Figure 7.2 Serum levels of anti-inflammatory cytokine and chemokine in normal and pre-diabetic subjects.

Serum levels of cytokines/chemokines were measured using Multiplex assays as described in the research design and methods. Values are expressed as pg/ml (Rantes as ng/ml). Rantes (D), IL-10 (E) and IL-8 (F) were comparable in pre-diabetic subjects and healthy controls. NGT, Normal Glucose Tolerant (n = 34 vs 9). *p<0.05, **p<0.01, ***p<0.001 vs NGT.
7.4 Discussion

In this study we have evaluated cytokine profiling in pre-diabetic subjects and compared them with healthy controls. We observed elevated serum levels of IL-6 and TNFα, and suppressed levels of IL-1β in pre-diabetes subjects, but the differences were not statistically significant. A prospective, case-control study has shown elevated serum levels of pro-inflammatory cytokines, IL-6 and TNFα, but not IL-1β, in subjects with pre-diabetes (Spranger et al., 2003). We did not find any human study that has evaluated role of IFN-β in relation to pre-diabetes. IFN-β attenuates the secretion of pro-inflammatory cytokines while it augments the anti-inflammatory cytokines (Sellner et al., 2008). In our study, we did not find significant difference in serum levels of IFN-β between the two groups. Similarly, Th1 cytokines, IL-12 and IFN-γ levels remained statistically insignificant.

A population-based KORA Survey S4 study, which included 1653 subjects, has shown that elevated levels of Rantes were present in pre-diabetes subjects (Herder et al., 2005). However, our data did not show statistically significant differences in the serum levels of anti-inflammatory cytokines/chemokines, Rantes, IL-8, and Th2 cytokine IL-12 between the two groups. We think that the small sample size of our pre-diabetes cohort affected the results.

Few prospective studies have shown an independent positive association of CRP with the risk of developing T2D in pre-diabetes subjects (Pradhan et al., 2001; Spranger et al., 2003; Hu et al., 2004; Doi et al., 2005). Our study also revealed significantly elevated CRP in pre-diabetes compared to healthy controls.

In summary, in this study we did not find significant differences between Th1 and Th2 cytokines in the pre-diabetes and NGT cohort. The raised
CRP alone, in absence of significant levels of other biomarkers, is not enough for us to comment on the inflammatory state in pre-diabetic subjects. One of the major limitations in this study is the small number of pre-diabetes subjects (n=9). Furthermore, among pre-diabetes subjects, 8 had IFG and 1 had IGT. The pre-diabetes subjects were heavier than the NGT subjects, with a mean BMI 35.4 Kg/m² and 25.8 Kg/m² respectively, and this difference in BMI could have affected the cytokine profile between the two cohorts. However, findings were not altered after adjustment for age, sex, BMI and WHR. In this study, we did not analyse cytokines mRNA expressions in pre-diabetes cohort, because of the small number of pre-diabetes subjects, but it would have been interesting to see mRNA expressions in pre-diabetes subjects in a larger study. Overall, in presence of these limitations, it is difficult to draw definitive conclusions from this pre-diabetes study. However, we can suggest a potential role of these cytokines as biomarkers for the detection of early/subclinical disease and a larger study should be carried out to explore this possibility.
Chapter 8

General discussion
Correlation between cytokines, chemokines, adipokines, TLRs, T2D and adiposity

The main objective of this study was to investigate the role of inflammatory biomarkers in the pathophysiology of T2D. We evaluated cytokines and TLRs in T2D subjects with good glycaemic control with/without complications and poor glycaemic control with/without complications in both monocytes and neutrophils, and compared with the NGT controls. In addition, given that a number of studies have indicated an association between adipokines and T2D, we also investigated the adipokine expression in both monocytes and neutrophils in our T2D subjects and compared with the NGT controls. It has been shown in previous studies that inflammation in T2D subjects starts years before the development of T2D. Hence, we also investigated the serum cytokines in pre-diabetes subjects to explore the inflammatory cascade at the onset of diabetes. However, this was not the part of research protocol.

Pre-diabetes represents an elevation of plasma glucose above the normal range but below that of clinical diabetes and is associated with a high risk for T2D, subclinical inflammation, early atherosclerosis, and CVD (Bardini et al., 2010). It has been shown that pre-diabetes was associated with retinopathy in 2% to 4% of affected subjects in Pima Indians (Gabir et al., 2000). A prospective, nested case-control study has shown that elevated levels of CRP and IL-6 predict the development of T2D (Pradhan et al., 2001). In contrast, another prospective, case-control study showed that a combined elevation of IL-6 and IL-1β, rather than isolated elevation of IL-6 alone, independently increased the risk of T2D (Spranger et al., 2003). Our study showed that subjects with pre-diabetes have elevated serum levels of pro-inflammatory cytokines, IL-6, TNFα, and Th1 cytokine, IL-12, when compared with NGT controls, but statistically insignificant. Serum IL-1β and Th1 cytokine, IFN-γ levels were comparable in pre-diabetes
subjects. Our study has revealed a significantly elevated CRP in prediabetes cohort compared to healthy controls. However, the elevated CRP alone does not suffice to comment on inflammatory status in the pre-diabetes in a small cohort.

In this study we showed that in neutrophils, the highest levels of pro-inflammatory cytokines IL-6 mRNA, TNFα mRNA and IFNβ mRNA including chemokine, Rantes were present in GC group, when compared to NGT control. Similarly, in monocytes, IL-6 mRNA, and IFN-β were overexpressed from GC subjects, when compared to NGT. Collectively, these data indicate that an inflammatory milieu exists in T2D, and that subjects with good glycaemic control with no complications have upregulated inflammatory mechanism.

We demonstrated that IL-1β mRNA expression was significantly decreased in monocytes from GCC, PC and PCC subjects, compared to NGT. Similarly, in monocytes a variable overexpression of IL-6 mRNA, TNFα mRNA, IFN-β mRNA and Rantes mRNA was observed in PC, GCC and PCC groups, compared with NGT. The IL-6 mRNA, IFN-β mRNA and Rantes mRNA were overexpressed in neutrophils from PC group (less than GC), compared with NGT. These data further suggest that an inflammatory milieu exists in T2D, and subjects with poor glycaemic control, and subjects with complications have compromised Th1 cells.

We also investigated adipokines expression in both monocytes and neutrophils in different T2D groups and compared with the NGT controls. Our study showed that all three adipokines: adiponectin, RBP4 and LCN2 were overexpressed in both monocytes and neutrophils in T2D subjects, compared to NGT. It has been shown that LCN2 exhibits anti-inflammatory
properties; antagonises the detrimental effect of TNFα on adipocytes and macrophages, upregulates PPAR-γ, adiponectin and leptin and protects adipocytes from TNF-α–induced production of IL-6 and MCP-1 (Zhang et al., 2008). This suggests that overexpressed LCN2 in T2D subjects is a measure to counteract ongoing inflammation in T2D, which is evidenced by raised pro-inflammatory cytokines in these subjects. It has been shown that circulating RBP4 levels correlate with the degree of IR, and increased levels are present in IGT and T2D subjects (Graham et al., 2006). In line with previous studies, our study also showed overexpressed RBP4 in both monocytes and neutrophils in T2D subjects. In addition, our study also showed overexpressed adiponectin in both monocytes and neutrophils in T2D subjects. Adiponectin has been shown to exert an insulin-sensitizing effect through binding to its receptors AdipoR1 and AdipoR2, and activation of AMPK, PPAR-α, and some other unknown signaling pathways (Yadav et al., 2013). It is plausible to speculate that overexpressed RBP4 is indicating development of IR in the T2D cohort, whereas overexpressed adiponectin is reflecting genesis of insulin sensitivity, a measure to counteract the IR. Collectively, we found that all three adipokines, adiponectin, lipocalin and RBP4 were overexpressed in the T2D subjects indicating that there is an inflammatory and IR milieu, which is linked with the pathophysiology of T2D and associated complications.

Furthermore, adiponectin has been shown to suppress the LPS-stimulated release of IL-6 and induce IL-10 in human monocytes and macrophages (Weigert et al., 2008; Kumada et al., 2004; Wulster-Radcliffe et al., 2004). In our study, we have demonstrated that IL-6 mRNA was significantly overexpressed in GC and PC group, compared with NGT, and serum IL-10 levels were comparable. Whether the elevated IL-6 has enhanced the adiponectin expression or increased adiponectin has suppressed IL-6 to a certain extent, remains questionable.
Our study clearly showed that the adipokines adiponectin, RBP4 and LCN2 mRNA were markedly overexpressed in both neutrophils and monocytes in GC subjects, compared with other groups. In contrast, all three adipokines were suppressed in both neutrophils and monocytes in PCC subjects, and variable expression was evident in GCC and PC subjects. It indicates that GC subjects have enhanced inflammatory and IR milieu, whereas, PCC subjects have less inflammatory and IR milieu. It is plausible to speculate that downregulation of adipokine receptors occurs following sustained activation to terminate the inflammation and IR. It is also evident that GC subjects have enhanced anti-inflammatory and insulin sensitizing milieu, whereas PCC subjects have down-regulated anti-inflammatory and insulin sensitizing milieu. It reflects that T2D subjects with complications and poor glycaemic control have impaired defensive mechanism to combat the inflammation and IR. Collectively, this suggests that in T2D subjects with good glycaemic control with no complications, there is a release of excess of adipokines and cytokines.

To further investigate the inflammatory cascade in T2D we explored the TLRs in this study. Our data clearly showed in monocytes, highest level of all TLRs 1-10 [TLR1, 3, 5, 7, 9 & 10 (p<0.05)] were observed in GC group, compared with NGT. Similarly, in neutrophils, highest level of all TLRs, except TLR 2 were observed in GC, when compared with NGT controls (p<0.05). The overexpressed TLRs in both monocytes and neutrophils suggest ongoing inflammation in T2D subjects. In contrast, both monocytes and neutrophils from GCC, PC and PCC subjects showed a trend towards decreased TLR mRNA expression. Whether the decreased TLR expression in poor glycaemic state is an indication of compromised innate immune signalling mechanisms or a reflection of protective mechanism to curtail further increase in pro-inflammatory cytokines, remains questionable.
The immune response is determined by the actual TLRs that are activated. The TLR3, TLR7, TLR8 and TLR9 upon activation induce type I IFNs (IFN-β), whereas stimulation of human monocyte-derived macrophages and dendritic cells with the TLR8 ligand together with the TLR3 or TLR4 ligand lead to synergistic IL-6, IL-10, IL-12, and TNF-α mRNA expression and cytokine production (Makela et al., 2009). In parallel to TLRs, our data demonstrated overexpressed IL-6 mRNA and IFN-β mRNA in monocytes and neutrophils from GC and PC, and TNF-α mRNA and in neutrophils from GC group, compared with NGT. However, our study failed to show elevated serum IL-10 and IL-12 levels in T2D subjects despite overexpressed TLRs in these subjects. The expression of IL-10 and IL-12 is dependent upon TLR2/4 activation and in our study TLR 2 and TLR 4 in monocyte, and TLR2 in neutrophil was minimally overexpressed, compared to other TLRs. It has been shown that metformin (Andrews et al., 2012), statins, PPAR-γ agonists (Dasu et al., 2009a) and angiotensin receptor blockers (Dasu et al., 2009b) lower the expression of TLR 2 /4 in T2D patients. Most of our T2D subjects were on these medications and this likely explains the decreased expression of TLR 2 and TLR 4 in our T2D cohort.

Recent studies have shown that adipocytes play an important role in the physiological regulation of immune responses in fat deposits via TLR signaling cascades. Adipose tissue produces and releases a variety of pro-inflammatory and anti-inflammatory factors, including the adipokines leptin, adiponectin, resistin, RBP4 and visfatin, as well as cytokines and chemokines, such as TNF-α, IL-6, monocyte chemo-attractant protein 1, and others (Lago et al., 2007). In an animal model, the obesity led to upregulated expression of TLR1–9 and TLR11–13 in adipose tissue (Kima et al., 2012). The multifactorial ANOVA using lognormal data of cytokines, adipokines and TLRs after adjustment for age, sex, WHR, BMI, duration of
diabetes, creatinine and medications (insulin, sulfonylurea, metformin, GLP-1 analogues, DPP IV inhibitors, aspirin and statins) did not alter the results of most inflammatory markers, except of TNF-α, TLR2 & 8 in monocytes, IL-1β mRNA and TLR2 in neutrophils. This suggests that there is an inflammatory milieu, independent of obesity, which is linked with development of T2D and associated micro/macrovascular complications.

Studies have shown that low-grade chronic inflammation and activated innate immune mechanisms are involved in the pathogenesis of T2D (Pickup et al., 2004). It has been shown that TNF-α signaling impairs insulin signaling through serine phosphorylation of IRS-1, reduces GLUT4 gene expression, and mediates IR (Hotamisligil et al., 1994). Furthermore studies revealed that TLR4 contributes to the development of insulin resistance and inflammation through activation of pro-inflammatory kinases and ROS, and indirectly, via activation of cytokine signaling cascades and systemic release of pro-inflammatory, insulin-desensitizing factors (Kim et al., 2010). We have also shown that our subjects with T2D displayed subclinical, low-grade systemic inflammation including increases in cytokines, chemokines, adipokines and TLRs, although the degree of immune activation was far below that is seen in acute infections. We think, that this perturbations in the normal homeostatic balance between cytokines, chemokines, adipokines and TLRs serve to promote onset and progression of late diabetic complications.

Previous studies have shown that inflammation, and more specifically pro-inflammatory cytokines, play a determinant role in the development of microvascular complications and diabetic cardiovascular diseases. Studies have suggested a role of pro-inflammatory cytokines IL-1, IL-6, IL-18, TNF-α and TGF-β1 in diabetes microvascular complications (Navale & Paranjape, 2013). Our study also demonstrated variable overexpression of
IL-6 mRNA, TNF-α mRNA including IFNβ mRNA and Rantes mRNA in different T2D groups. In relation to role of TLRs in diabetes complication, our PCC subjects overexpressed TLR 4 in neutrophils, and GCC subjects overexpressed TLR 1, 5, 7 & 10 in monocytes and TLR 4 & 5 in neutrophils, when compared with NGT. It has been shown that TLR4, due to its additional roles in immunostimulation, modulation of inflammation, angiogenesis, and tissue repair and regeneration plays a vital molecule in wound healing (Seki et al., 2005; Vink et al., 2002; Grote et al., 2011; Mollen et al, 2006). TLR4 is also shown to be responsible for the expression and regulation of Vascular Endothelial Growth Factor (Pei et al., 2008). Wounds devoid of TLR4 or its downstream targets like MyD88, take longer time to heal and often develop into chronic non-healing ulcers (Macedo et al., 2007). Kanhaiya et al. (2013) has clearly shown that any deregulation in the TLR4 mediated downstream signaling lead to chronic non-healing ulcers in humans. In line with these studies, TLR4 mRNA was overexpressed in both neutrophils and monocytes in our study and none of the T2D subjects in our study exhibited active foot ulcer. TLR4 has also been shown to promote tubulointerstitial inflammation in DN by upregulating IL-6 (Lin et al., 2012). In our T2D cohort, nephropathy was seen in 68.8% and 56.3% in PCC and GCC group, respectively. We did not correlate diabetes associated micro/macrovascular complications separately with different TLRs. The importance of these overexpressed TLRs in diabetes associated complication needs to be investigated.

In our study, a trend toward decreased TLR expression was noticed in PC, GCC and PCC monocytes and neutrophils. TLR 2, 3, 9 and 10 were relatively suppressed in the PCC monocytes, and TLRs 3, 6, 8, 9 and 10 were relatively supressed in the PCC neutrophils, compared to NGT. Similarly, all three adipokines were relatively suppressed in both neutrophils and monocytes in PCC subjects (LCN2 & adiponectin in monocytes p<0.05), compared with NGT, and variable expression was evident in GCC and PC subjects. We also observed suppressed IL-
1b mRNA expressions in monocytes in GCC, PC and PCC groups, compared to NGT. To determine that whether decline in immune function in PC, GCC and PCC group is due to poor glycaemic control and diabetes complications or due to prolonged duration of diabetes, we applied multifactorial ANOVA model and adjusted duration of diabetes as a covariant, but the difference among inflammatory markers among different groups remained significant. This suggests that decline in immunity in T2D is related to poor glycaemic control and diabetes complications rather than the duration of disease. Whether decline in immunity is the result of disease process itself or whether patients with suppressed inflammatory markers are more susceptible to develop diabetes and associated microvascular and macrovascular complications, remains to be investigated.
Chapter 9

Conclusion, Limitations and Further Studies
Conclusion, Limitations and Further Studies

To the best of our knowledge, this is the first study that has sought to evaluate cytokine levels, adipokines and TLRs expressions among type 2 diabetic subjects with good glycaemic control with/without complications and poor glycaemic with/without complications and compared them with healthy controls. The key points are summarized as below-

1. In T2D with good glycaemic control with no complications, the inflammatory and anti-inflammatory mechanisms are overactive; and Th1 cells are overactive.

2. In advanced diabetes i.e., poorly controlled T2D and diabetes with complications, inflammatory and anti-inflammatory mechanisms gets impaired, and Th1 and Th2 response gets suppressed.

3. In T2D subjects with good glycaemic control with no complications, RBP4, an adipokine that induces IR, was overexpressed indicating an inflammatory and IR milieu. In contrast, adiponectin, an insulin sensitizing adipokine, and lipocalin, an anti-inflammatory adipokine were also overexpressed indicating a compensatory mechanism to counteract inflammation and protecting from IR.

4. All three adipokines were suppressed in both neutrophils and monocytes in PCC subjects, and variable expressed in GCC and PC subjects. This indicates that downregulation of adipokine receptors occurs in poorly controlled diabetic subjects and in subjects with complications.

5. TLRs were overexpressed in monocytes and neutrophils from GC, and a trend toward decreased TLR expression was noticed in PC, GCC and PCC monocytes and neutrophils. It suggests that downregulation of TLR receptors occurs in poorly controlled diabetic subjects and in subjects with complications.
6. The multifactorial ANOVA using lognormal data of cytokines, adipokines and TLRs after adjustment for age, sex, WHR, BMI, duration of diabetes, creatinine and medications did not alter the results of most inflammatory markers, except of TNF-α, TLR2 & 8 in monocytes, IL-1β mRNA and TLR2 in neutrophils.

7. No correlation was found between serum cytokines and BMI, adipokines mRNA and BMI, and TLRs mRNA and BMI. Among cytokines mRNA expression a correlation statistically significant was found between IL-1β mRNA in monocytes and BMI in T2D subjects (Spearman’s r = 0.41, p<0.05). Our data suggests that the inflammatory milieu in diabetes was linked with diabetes itself, rather than obesity.

8. Few TLRs have been linked with complications in diabetes. Our PCC subjects overexpressed TLR 4 in neutrophils, and GCC subjects overexpressed TLR 1, 5, 7 & 10 in monocytes and TLR 2, 4 & 5 in neutrophils, when compared with NGT. The relevance of these overexpressed TLRs in diabetes associated complication need to be investigated.

9. Our study demonstrated that neutrophils and monocytes express different TLR mRNA in T2D subjects. This indicates the significance of neutrophils in TLR evaluation, in addition to the more commonly evaluated monocytes.

In summary, our study demonstrates that subjects with T2D exhibit perturbations in cytokines, chemokines and adipokines, and TLRs, supporting their role in T2D progression and complications. Whether this perturbation is the result of disease process itself or whether patients with perturbed inflammatory markers are more susceptible to develop diabetes and associated microvascular and macrovascular complications, remains to be investigated. However the consistently and significantly suppressed cytokines, chemokines and adipokines, and TLRs, in with poor glycaemic
control and with complications suggests a “burnt out” disease state re-emphasizing the role of early management to achieve good glycaemic control and avoiding complications. These findings along with other previous and ongoing studies might pave the way in the future in designing certain therapeutic measures that are aimed at suppressing the low grade inflammatory state in early diabetes through anti-inflammatory cytokines and targeted TLR therapy.

A major limitation of the present work is the sample size; we think that more control subjects and T2D subjects should be involved. Another limitation is insulin level, which was not measured; hence IR could not be calculated in T2D subjects. Further, during recruitment the T2D subjects were not asked about exercise programs that they may be involved in, recent weight loss and change of medications. These factors impact insulin sensitivity and thus affect inflammatory markers in diabetes. The T2D subjects were on anti-diabetic medications, insulin, statins and ARBs, which would have affected the inflammatory markers, but it would have been unethical to discontinue the medications to continue the research. Finally, we think that serum adipokine levels, which were not measured, would have given more information about the role of adipocytes in the inflammatory cascade in T2D.

Though this small but exhaustive study has thrown up many interesting trends regarding the role of inflammation in diabetes and the notion of burnt out diabetes, more robust prospective studies with larger sample size are needed to arrive at more conclusive results. Therefore, a prospective study should be designed with a larger sample size in a similar cohort with expanded profile including measurement of IR and serum adipokine levels. The subjects with poorly controlled T2D and with complications should be treated intensively and inflammatory markers
should be measured at baseline and after one year to assess the change in inflammatory markers with change in glycaemic state, to support or refute the concept of burnt out diabetes. Additionally, a study should be carried out on pre-diabetes subjects with expanded profile to explore that whether these inflammatory markers can be used as biomarker to predict the development of T2D. This will help in our understanding of the role of inflammation in pathophysiology of T2D and the occurrence of diabetic complications and designing new therapies in the future.
Chapter 10

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Appendix 1

A. Oral presentation

S Gupta, A Maratha1, A Natarajan, T Gajanayake, J Siednienko1, S Hoashi and S Miggin. Burnt Out Diabetes: Cytokines, Adipokines and TLRs Modulation in Type 2 diabetes. The Endocrine society (Chicago) 2014, (Late Breaking Abstract)

S Gupta, A Maratha1, A Natarajan, T Gajanayake, J Siednienko1, S Hoashi and S Miggin. Association between Type 2 Diabetes & Inflammation. Irish Endocrine Society, Kilkenny, November 2013
Irish Journal Medical Sciences, 2013; 182 (9): 400

B. Poster presentations

S Gupta, A Maratha, A Natarajan, T Gajanayake, J Siednienko1, S Hoashi and S Miggin. The Endocrine society (Boston) 2011. Cytokine profiling in Type 2 Diabetes
Endocr Rev. 2011: 32; P2-91

Endocrine Abstracts 2011: 25;P119

Irish J Med Scien 2010: 179 (13); S513
Siednienko J, Gupta S, Mangan B, Miggins S, Hoashi S. Modulation of Toll-Like Receptors in Type 2 Diabetes. American Diabetes Association 2009 57-LB (Late Breaking Abstract) *Diabetes* 2009: 58 (1A); LB15

**C. Bursary award**

MD work proposal was awarded a bursary by the Irish Endocrine Society in 2009.