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CHARACTERISATION AND MODULATION

BY
TINA OKDAHL

DISSERTATION SUBMITTED 2023

AALBORG UNIVERSITY
DENMARK

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Publications

1. **Okdahl T**, Emmanuel A, Morlion B, Farmer A, Varrassi G, Drewes AM. Recommendations for the management of opioid-induced constipation - how to improve usability in clinical practice. 2023;17(10):975–84.

2. **Okdahl T**, Mark EB, Nedergaard RB, Knoph CS, Cook ME, Krogh K, et al. Effects of opium tincture on the enteric and central nervous systems: A randomized controlled trial. 2023 Feb 27;

3. Bertoli D, Mark EB, Liao D, **Okdahl T**, Nauser S, Daugberg LH, et al. MRI-Based Quantification of Pan-Alimentary Function and Motility in Subjects with Diabetes and Gastrointestinal Symptoms. 2023;12(18):5968.

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6. **Okdahl T**, Wegeberg A, Birthe A, Jensen H, Jensen ST, Riis H, et al. Systemic Cytokine Expression in Diabetes Is Associated with Prolonged Gastrointestinal Transit Times and Cardinal Gastroparesis Symptoms. 2023;

7. **Okdahl T**, Wegeberg A-M, Pociot F, Brock B, Størling J, Brock C. Low-grade inflammation in type 2 diabetes: a cross-sectional study from a Danish diabetes outpatient clinic. 2022 Dec 14;12(12):e062188.

8. Wegeberg AM, **Okdahl T**, Riahi S, Ejskjaer N, Pociot F, Størling J, et al. Elevated levels of interleukin-12/23p40 may serve as a potential indicator of dysfunctional heart rate variability in type 2 diabetes. 2022;21(1):1–10.

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11. Wegeberg AML, **Okdahl T**, Fløyel T, Brock C, Ejskjaer N, Riahi S, et al. Circulating Inflammatory Markers Are Inversely Associated with Heart Rate Variability Measures in Type 1 Diabetes. 2020;2020.

12. **Okdahl T**, Brock C, Fløyel T, Wegeberg AL, Jakobsen PE, Ejskjaer N, et al. Increased levels of inflammatory factors are associated with severity of polyneuropathy in type 1 diabetes. 2020 Oct 24;93(4):419–28.

LIST OF PAPERS

This PhD thesis is based on the following papers:

1. **Okdahl T**, Wegeberg A-M, Pociot F, et al. Low-grade inflammation in type 2 diabetes: a cross-sectional study from a Danish diabetes outpatient clinic. BMJ Open. 2022 Dec 14;12(12):e062188.

2. **Okdahl T**, Brock C, Fløyel T, et al. Increased levels of inflammatory factors are associated with severity of polyneuropathy in type 1 diabetes. Clin Endocrinol (Oxf). 2020 Oct 24;93(4):419–28.

3. **Okdahl T**, Wegeberg A, Birthe A, et al. Systemic Cytokine Expression in Diabetes Is Associated with Prolonged Gastrointestinal Transit Times and Cardinal Gastroparesis Symptoms. 2023;

4. **Okdahl T**, Kufaishi H, Kornum D, et al. Transcutaneous vagus nerve stimulation has no anti-inflammatory effect in diabetes: a secondary analysis of a randomized double-blind sham-controlled trial (*to be submitted when the primary outcome has been published*)

ABBREVIATIONS

AGE	Advanced glycation end product
ATP	Adenosine 5'-triphosphate
BMI	Body mass index
CAM	Cell adhesion molecule
CCL	C-C motif chemokine
CD	Cluster of differentiation
COVID	Coronavirus disease
CRP	C-reactive protein
CXCL	C-X-C motif chemokine
DPP	Dipeptidyl peptidase
DSPN	Distal symmetrical polyneuropathy
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
GCSI	Gastroparesis Cardinal Symptom Index
GFAT	Glucosamine-fructose amidotransferase
GLP-1	Glucagon-like-peptide-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
iCAM	Intracellular cell adhesion molecule
IFN	Interferon
IL	Interleukin
IP	IFN-γ-induced protein
iVNS	Invasive vagus nerve stimulation
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MIP	Macrophage inflammatory protein
MSD	Mesoscale Discovery
nAChR	Nicotinic-acetylcholine-receptor
NK	Natural killer
RAGE	AGE receptor

ROS	Reactive oxygen species
SGLT	Sodium-glucose cotransporter
sICAM	Soluble intracellular cell adhesion molecule
suPAR	Soluble urokinase plasminogen activator receptor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TARC	Thymus and activation regulated chemokine
TNF	Tumour necrosis factor
tVNS	Transcutaneous vagus nerve stimulation
VNS	Vagus nerve stimulation
β2AR	β2-adrenergic-receptor

ENGLISH SUMMARY

The global incidence of diabetes is on the rise. Although treatment options and their efficacy have improved, the persistence of long-term macro- and microvascular complications remains a significant obstacle to achieving optimal patient care. Inflammation is an underlying component in the development of both type 1 and type 2 diabetes and in addition, chronic hyperglycaemia and hypoglycaemic events in diabetes cause metabolic alterations, which facilitate an overall low-grade inflammatory state of the organism. Though not fully understood, this low-grade inflammatory status is believed to be involved in the pathogenesis of several diabetic complications. Consequently, there is a clear need for deeper insight into this field and for exploration of potential anti-inflammatory treatment options for diabetes.

This PhD aimed to characterize and investigate circulating inflammatory biomarkers in diabetes and diabetic complications. For this purpose, cross-sectional data from three clinical studies were analysed and published in three peer-reviewed original research papers (Papers 1, 2, and 3). Moreover, the aim was to investigate the potential anti-inflammatory effect of transcutaneous vagus nerve stimulation (tVNS) in diabetes. Data from a randomised controlled trial was utilized for this purpose, and the results are presented in paper 4 (to be submitted to a peer-reviewed journal).

In paper 1, the presence of inflammation in type 2 diabetes was explored. The results showed correlations between several inflammatory biomarkers and various clinical characteristics such as obesity, glycaemic control, treatment, and sex. Moreover, increased levels of inflammatory biomarkers were associated with the presence of multiple diabetic complications. These findings emphasise the need for addressing the inflammatory status in the management of type 2 diabetes.

Paper 2 focused on the possible connections between inflammatory biomarkers and the presence of distal symmetrical polyneuropathy (DSPN) in type 1 diabetes. A connection between increased levels of inflammatory biomarkers and the presence of DSPN was found. Moreover, concentrations of certain inflammatory biomarkers correlated with measures of vibration and tactile perception. These observations indicate that inflammation is associated with the progression of DSPN.

Paper 3 explored associations between inflammation and gastrointestinal dysfunction in diabetes. Elevated levels of specific inflammatory biomarkers and increased transit time of the stomach and colon were found, while an inverse relationship with gastrointestinal symptoms was seen. These results indicate a relationship between inflammation and gastrointestinal function in diabetes, which needs further elucidation.

In paper 4, tVNS was explored as a strategy for lowering the inflammatory status in diabetes. However, no such effect was seen after both short-term and long-term treatment. It can be speculated that autonomic dysfunction in diabetes may have hindered an anti-inflammatory response through the vagus nerve. This hypothesis should be further tested by including individuals with diabetes but without signs of autonomic dysfunction.

In conclusion, the results from this PhD thesis support an association between circulating inflammatory factors and diabetic complications in both type 1 and type 2 diabetes but failed to show an anti-inflammatory effect of tVNS in diabetes. Going forward, assessment of the inflammatory status and possible application of anti-inflammatory treatment in the management of diabetes should be explored as strategies for preventing the development of long-term complications and improving patient outcomes.

DANSK RESUME

Den globale forekomst af diabetes er stigende. Selvom behandlingsmulighederne og deres effektivitet er forbedret, udgør makro- og mikrovaskulære komplikationer stadig en betydelig hindring for at opnå optimal patientpleje. Inflammation er en underliggende komponent i udviklingen af både type 1 og type 2 diabetes, og desuden forårsager kronisk hyperglykæmi og hypoglykæmiske episoder i diabetes metaboliske forandringer, der faciliterer en systemisk inflammatorisk tilstand i organismen. Selvom det ikke er fuldt forstået, tyder det på, at denne inflammatoriske tilstand er involveret i udviklingen af flere diabetiske komplikationer. Der er derfor et klart behov for dybere indblik i dette felt og for udforskning af potentielle antiinflammatoriske behandlingsmuligheder i diabetes.

Dette ph.d.-projekt havde til formål at karakterisere og undersøge systemiske inflammatoriske biomarkører i diabetes og diabetiske komplikationer. Til dette formål blev tværsnitsdata fra tre kliniske studier analyseret og offentliggjort i tre fagfællebedømte originale forskningsartikler (Artikel 1, 2 og 3). Derudover var målet at undersøge den potentielle antiinflammatoriske effekt af transkutan vagusnervestimulation (tVNS) i diabetes. Data fra et randomiseret kontrolleret studie blev anvendt til dette formål, og resultaterne præsenteres i artikel 4 (vil blive indsendt til et fagfællebedømt tidsskrift).

I artikel 1 blev tilstedeværelsen af inflammation i type 2 diabetes udforsket. Resultaterne viste korrelationer mellem flere inflammatoriske biomarkører og forskellige kliniske karakteristika såsom fedme, glykæmisk kontrol, behandling og køn. Desuden var øgede niveauer af inflammatoriske biomarkører forbundet med tilstedeværelsen af flere diabetiske komplikationer. Disse fund understreger behovet for at håndtere inflammatorisk status i behandlingen af type 2 diabetes.

Artikel 2 fokuserede på mulige forbindelserne mellem inflammatoriske biomarkører og tilstedeværelsen af distal symmetrisk polyneuropati (DSPN) i type 1 diabetes. Der blev fundet en forbindelse mellem øgede niveauer af inflammatoriske biomarkører og tilstedeværelsen af DSPN. Desuden korrelerede koncentrationer af visse inflammatoriske biomarkører med målinger af vibrationssans og taktil perception. Disse observationer indikerer, at inflammation er associeret med udviklingen af DSPN.

Artikel 3 udforskede forbindelserne mellem inflammation og dysfunktion i mavetarmkanalen i diabetes. Der blev fundet forhøjede niveauer af specifikke inflammatoriske biomarkører og øget transittid i maven og tyktarmen, mens der blev observeret en omvendt sammenhæng med symptomer fra mavetarmkanalen. Disse resultater indikerer en forbindelse mellem inflammation og mavetarmfunktion i diabetes, som bør undersøges nærmere.

I artikel 4 blev tVNS udforsket som en strategi til at mindske den inflammatoriske status i diabetes. Dog blev der ikke observeret en sådan effekt efter hverken kort- eller langvarig behandling. Det kan tænkes, at autonom dysfunktion i diabetes muligvis har forhindret et antiinflammatorisk respons via vagusnerven. Denne hypotese bør testes ved at inkludere personer med diabetes, men uden tegn på autonom dysfunktion.

Samlet set understøtter resultaterne fra denne ph.d.-afhandling en forbindelse mellem systemiske inflammatoriske faktorer og diabetiske komplikationer i både type 1 og type 2 diabetes. Dog mislykkedes forsøget på at dæmpe inflammationen hos personer med diabetes ved hjælp af tVNS. Fremadrettet bør vurdering af inflammatorisk status og mulig anvendelse af antiinflammatorisk terapi i behandlingen af diabetes udforskes som strategier til at forhindre udviklingen af langvarige komplikationer og forbedre det enkelte patientforløb.

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Tina Okdahl, December 2023

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CHAPTER 1. BACKGROUND

1.1. INFLAMMATION

Inflammation is a double-edged sword in human physiology. It is vital for resolving infections and healing tissue injuries, but disruptions in the immune response can pose a considerable threat to the organism. An illustrative case of an excessively reactive response is the autoimmune destruction of pancreatic beta-cells, which are responsible for insulin production. This phenomenon leads to type 1 diabetes, which was fatal until the discovery of insulin in 1922 [1]. Other examples of conditions caused by an overreactive immune system include rheumatoid arthritis, multiple sclerosis, and psoriasis [2]. On the other hand, if the immune response is insufficient, e.g., due to immunodeficiencies, consequences may be frequent and severe infections or even the development of cancer [3]. Thus, the balance between pro-inflammatory and anti-inflammatory responses is a delicate dynamic process crucial for maintaining biological homeostasis (figure 1).

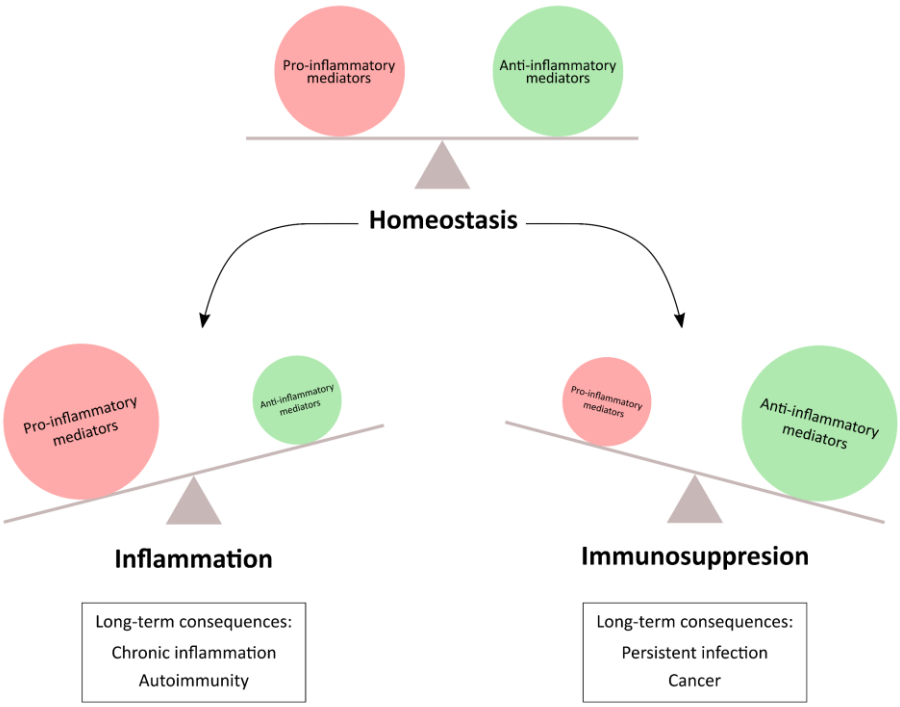


Figure 1 – Schematic representation of the balance between pro- and anti-inflammatory mediators in the regulation of the immune response.

1.1.1. INFLAMMATORY FACTORS

CYTOKINES

The orchestration of a suitable immunological response requires recruitment, activation, differentiation, and proliferation of specific immune cell types. Each of these steps is essential and relies on accurate and efficient communication between cells [4].

Cytokines, derived from the Greek words "cyto" (cell) and "kinos" (movement), constitute a diverse and pivotal group of small soluble proteins (<70 kDa) that play a central role in the intricate communication network between cells, particularly in the immune system [5]. Defined as soluble factors produced by one cell and causing functional changes in a target cell, cytokines share many similarities with hormones, but in terms of potency, cytokines far exceed that of hormones [1]. As such, cytokines are normally produced in very small amounts (picogram range) [6]. Moreover, unlike hormones, which are primarily produced by specialized endocrine cells, cytokines can be produced by all cell types in the organism except for erythrocytes [1]. Other important hallmarks of cytokines include their ability to act synergistically with one another and their exhibition of pleiotropism and redundancy, meaning that they can affect various cell types in different ways and that multiple cytokines may have similar effects. Most cytokines facilitate autocrine or paracrine communication between cells, but endocrine communication is also seen [7]. Upon binding of cytokines to their respective cell surface receptor, intracellular signalling pathways are initiated, causing amplification of the receptor signal. Thus, even very low concentrations of a specific cytokine (down to a few molecules) may induce a large effect in the target cell [6].

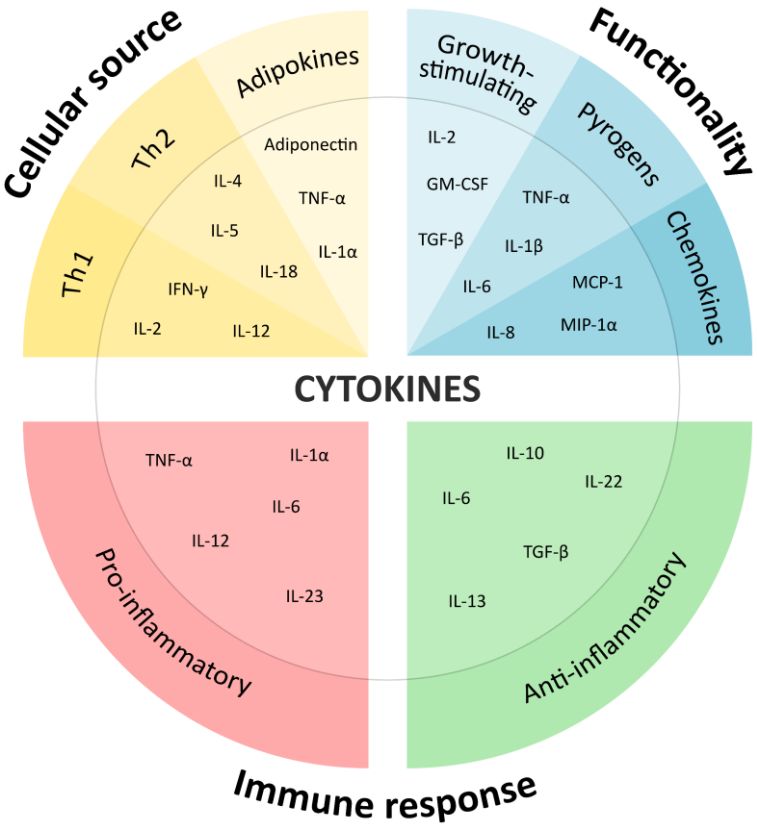


Figure 2 – Cytokines can be classified into several subgroups including, but not limited to, cellular source, functionality, and immune response.

The overall term *cytokine* covers many different subgroups (figure 2). A common classification system divides cytokines into those with pro-inflammatory or anti-inflammatory features. Some cytokines, however, may possess both pro- and anti-inflammatory properties under different circumstances. An example of this is interleukin (IL)-6, which is known to play a central role in acute inflammation but also has regenerative and regulatory functions [8]. Interleukins comprise a large group of cytokines, which primarily are produced by leucocytes and also act on leucocytes [7]. Other examples of cytokine families include interferons, which contain cytokines produced in response to the detection of pathogens, and tumour necrosis factors, which comprise cytokines capable of inducing cell death, among other features [1]. Other ways of categorising cytokines include allocation based on cellular source or functionality [7]. Table 1 provides an overview of a subset of some of the most common cytokines and their functions.

	Cellular source	Immune response	Biological activity	Half-life
IL-6	Antigen-presenting cells, activated Th2 cells	Pro- and anti-inflammatory	Initiation of acute-phase response, stimulation of B cell proliferation	15.5 hours
IL-8	Macrophages, endothelial cells, epithelial cells	Pro-inflammatory	Recruitment of leukocytes	24 minutes
IL-10	Macrophages, Th2 cells, B cells	Anti-inflammatory	Inhibition of cytokine production and Th1 immune response	-
TNF-α	Macrophages, mast cells, NK cells,	Pro-inflammatory	Activation of neutrophils, stimulation of adhesion molecules	18.2 minutes
IFN-γ	T- cells, NK-cells, macrophages	Pro-inflammatory	Promotion of Th1 immune response	-

Table 1 – Overview of a subset of the most common cytokines. IL: interleukin; TNF: tumour necrosis factor; IFN: interferon; NK: natural killer

CELL ADHESION MOLECULES

Cell adhesion molecules (CAMs) comprise a heterogeneous group of cell-cell and cell-matrix attachment mediators and play a crucial role in the inflammatory response, mainly in leucocyte activation and trafficking. During acute inflammation, tumour necrosis factor (TNF)-α activates endothelial cells to express different CAMs capable of tethering circulating immune cells, thereby facilitating extravasation. CAMs are also crucial for leucocyte activation, which requires cell-cell interactions, e.g., between a Th cell and an antigen-presenting cell [9].

1.1.2. INFLAMMATORY FACTORS AS BIOMARKERS OF DISEASE

Given the importance of inflammatory factors such as cytokines and CAMs in the immune response and biological homeostasis, exploring specific profiles in different diseases may be relevant for both prognostic, diagnostic, and therapeutic evaluation. A recent example of this is the identification of elevated IL-6 levels in coronavirus disease (COVID)-19-infected individuals as being prognostic for the development of cytokine storms [10].

Both cytokines and CAMs can be quantified from various biological materials, including plasma, serum, saliva, and urine, which can be easily collected in non- or minimally-invasive manners, further underlining their relevance as biomarkers of disease [5].

1.2. DIABETES

Diabetes mellitus is a condition in which the immune system and inflammation play major roles during both the development and progression of the disease and related complications [11]. Over the last decades, diabetes has become one of the most common chronic diseases in the world, with an estimated number of 537 million adults affected world-wide in 2021. The prevalence continues to rise partly due to longer life-expectancies, increased incidence rates, and rises in risk factors, leading to an expected number of 783 million cases in 2045 [12]. Several subtypes of diabetes exist, with type 1 and type 2 being the most prevalent by far, accounting for approximately 5-10% and 90% of all cases, respectively [13].

Type 1 diabetes is characterized by T cell-mediated autoimmune destruction of insulin-producing β-cells in the pancreas, resulting in chronic insulin deficiency. Consequently, the management of this condition requires lifelong exogenous insulin therapy. The trigger for the self-destruction of pancreatic cells is debated, but predisposing enteroviral infection of β-cells as a driver of autoimmune development has been proposed [14].

Type 2 diabetes is characterized by insulin resistance, which may or may not be accompanied by relative insulin deficiency. Risk factors for the condition include obesity and an inactive lifestyle [13]. Contrary to type 1 diabetes, insulin-replacement therapy is rarely required, and instead, management focuses on lifestyle modifications, such as increased physical activity, dietary changes, and weight control. These interventions are aimed at improving insulin sensitivity and reducing blood glucose levels. Furthermore, pharmacological interventions in the form of antidiabetic medications designed to enhance insulin sensitivity or stimulate endogenous insulin secretion can be used. Type 2 diabetes usually develops in adulthood, but an increased incidence rate in children is seen due to the rising prevalence of environmental risk factors such as obesity and a sedentary lifestyle in this age group [15].

1.2.1. DIABETIC COMPLICATIONS

With increased life-expectancies and a growing diabetes incidence rate, a substantial increase in long-term diabetic complications is seen [16]. However, new encouraging national data show declining incidence rates for neuropathy [17]. Nonetheless, a demand for better prevention and treatment of these conditions exists. Traditionally,

diabetic complications are subdivided into macrovascular and microvascular complications (figure 3).

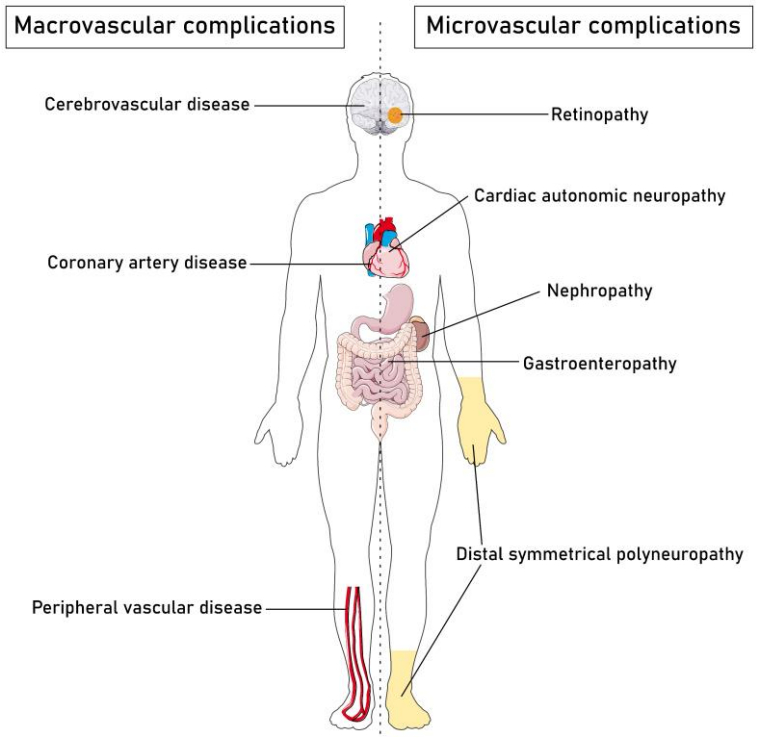


Figure 3 – Macro- and microvascular complications in diabetes.

Macrovascular complications are conditions in which blood flow through large blood vessels is compromised due to the development of atherosclerosis. Consequences may be cerebrovascular disease, coronary artery disease, and peripheral vascular disease. As such, macrovascular complications in diabetes are associated with severe morbidity and increased mortality rates [18].

Microvascular complications include diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. The pathogenesis of these conditions is complex, but inflammation and thickening of the basement membrane in capillaries and arterioles, causing inadequate diffusion of nutrients and gases from the bloodstream to the surrounding tissue, play key roles in the development [19]. Retinal nerves, nephrons, and neurons all share a vulnerability to fluctuations in blood glucose levels because their glucose uptake is independent of insulin. Consequently, intracellular glucose accumulation during hyperglycaemia is seen, leading to altered metabolic processes

[20]. Diabetic neuropathy is the most prevalent long-term complication of diabetes [21], and in the following, subtypes of this condition are described.

DISTAL SYMMETRICAL POLYNEUROPATHY

The most frequent type of diabetic neuropathy is distal symmetrical polyneuropathy (DSPN), in which neuronal damage to peripheral nerves causes a distinct pattern of symptoms. The condition usually begins distally in the lower extremities and progresses proximally over time. Involvement of hands may also occur gradually. The symptoms include numbness, tingling, and possibly pain, while objective signs comprise impaired nerve conduction and loss of peripheral tactile, vibration, and thermal sensation [21–23].

In type 1 diabetes, up to 20% of people with long-term disease duration suffer from DSPN, whereas in type 2 diabetes, the prevalence increases to 50% [21]. The condition is associated with the development of diabetic foot ulcers, which is the leading cause of lower extremity amputations in diabetes [24,25]. Moreover, a significant increase in all-cause mortality is associated with the DSPN [26]. While strict glucose control has a significant effect in reducing the risk of DSPN in type 1 diabetes, a more inconclusive pattern is seen in type 2 diabetes [21,27]. This underlines the complex pathophysiology of DSPN, which expands beyond hyperglycaemia and also includes oxidative stress, dyslipidaemia, mitochondrial dysfunction and even external factors such as environmental exposures [23,28–32].

DIABETIC AUTONOMIC NEUROPATHY

Neuropathy of the autonomic nerves (diabetic autonomic neuropathy) may have several different manifestations depending on the nerves involved and the target organ. The most severe is cardiac autonomic neuropathy, in which the autonomic regulation of the heart rate is compromised. This condition is associated with symptoms such as dizziness and fainting and may be the cause of dysrhythmia, stroke, and sudden cardiac arrest [33,34]. Cardiac autonomic neuropathy, therefore, contributes to the overrepresentation of death in diabetes [35,36]. Other manifestations of diabetic autonomic neuropathy include decreased sudomotor function, orthostatic hypotension, and urinary incontinence [37].

DIABETIC GASTROENTEROPATHY

Gastrointestinal symptoms, collectively referred to as gastroenteropathy, are common in diabetes and may be considered a subgroup of diabetic autonomic neuropathy [37,38]. The gastrointestinal tract is innervated by the submucosal and myenteric plexus of the enteric nervous system situated in the wall of the gastrointestinal tract. Located near the mucosa, the submucosal plexus regulates secretory function, while the myenteric plexus, being in close proximity to the smooth muscle layers, controls motility [38]. In addition to this local neuronal network, regulation of the gastrointestinal tract also happens in close collaboration with the autonomic nervous

system, involving both afferent and efferent projections [38]. The complex pathophysiology of diabetic gastroenteropathy is not yet fully understood but seems to involve histological alterations in the wall of the gastrointestinal tract, including myopathy of smooth muscle cells, loss of glial cells and deterioration of the enteric nervous system itself [38].

The most commonly described form of diabetic gastroenteropathy is gastroparesis, in which the passage of food from the stomach to the duodenum is delayed, leading to symptoms such as early satiety, nausea, and bloating [39]. However, disturbed motility patterns may be present in all segments of the gastrointestinal tract, and thus, symptoms such as abdominal pain, weight loss, diarrhoea, constipation, and faecal incontinence can also be seen [40–42].

Despite the increased risk of experiencing gastrointestinal symptoms in diabetes, diabetic gastroenteropathy seems to be somewhat overlooked both by clinicians and patients. This is despite the fact that these symptoms may have a substantial negative effect on health-related quality of life [43]. The condition may also be asymptomatic [44,45], but even in such cases, proper diagnosis and management are important due to the direct effect on glycaemic control and hypoglycaemic episodes caused by dyscoordinated peristalsis and nutrient uptake [46,47]. The tendency to overlook gastroenteropathy in clinical practice is probably, at least in part, linked to the fact that this type of diabetic neuropathy is not directly associated with severe morbidities or increased mortality rates, such as peripheral neuropathy or cardiac autonomic neuropathy.

1.2.2. INFLAMMATION IN DIABETES

The shared characteristic between type 1 and type 2 diabetes is the inability to effectively manage glucose transportation from the bloodstream to the cells, resulting in a persistent state of hyperglycaemia. Under normal circumstances, glucose metabolism results in the production of adenosine 5’-triphosphate (ATP) through glycolysis and oxidative phosphorylation, but when excess amounts of glucose are available, alternative metabolic pathways are initiated [48], some of which are outlined in the following (figure 4).

POLYOL PATHWAY

The polyol pathway is initiated as a consequence of excess glucose levels, and in diabetes, up to 30% of glucose is metabolized through this pathway. The consequences of the polyol pathway are accumulation of sorbitol and fructose and depletion of cofactors NADPH and NAD^+ [49]. This, in turn, causes osmotic imbalance, oxidative stress, and vascular impairment [48].

HEXOSAMINE PATHWAY

The hexosamine pathway is a metabolic pathway only active under hyperglycaemic conditions. In this pathway, fructose-6-phosphate, an intermediate of glycolysis, is converted into glucosamine-6-phosphate catalysed by the enzyme glucosamine-fructose amidotransferase (GFAT). Glucosamine-6-phosphate is then further modified and used in glycosylation of proteins and lipids, causing altered gene expression, vascular dysfunction and inflammation [48,50].

ADVANCED GLYCATION END PRODUCTS

Glucose accumulation triggers autooxidation, in which glucose is converted into glyoxal. Glyoxal can form irreversible bonds with lipids and proteins both inside and outside cells, thus forming so-called advanced glycation end products (AGEs). AGEs engage with AGE receptors (RAGEs), activating the nuclear factor-κB (NF-κB) transcription factor, leading to the production of pro-inflammatory cytokines. Moreover, AGE-RAGE interaction promotes the generation of reactive oxygen species (ROS) and impairs vascular function by reducing nitric oxide levels crucial for vasodilation [50,51].

ADDITIONAL MECHANISMS

In addition to the pathways mentioned above, other mechanisms contribute to the development of inflammation in diabetes. These include protein kinase C activation [48,50], altered lipid metabolism [30,31], and mitochondrial injury [28].

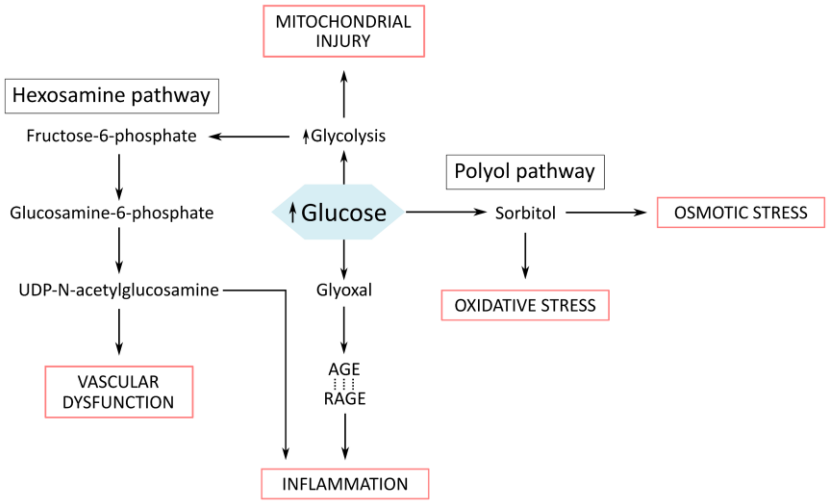


Figure 4 – Alternative metabolic pathways in diabetes brought on by hyperglycaemia lead to inflammation, oxidative stress, osmotic stress, mitochondrial injury, and vascular dysfunction.

1.3. THE INFLAMMATORY REFLEX

In 2002, the concept of a physiological inflammatory reflex mediated by the vagus nerve was introduced by Kevin Tracey and colleagues after observing that acetylcholine, the main parasympathetic neurotransmitter, inhibited the release of pro-inflammatory cytokines from human macrophage cultures [52]. This finding led to the hypothesis of parasympathetic involvement in the anti-inflammatory response, which they called the cholinergic anti-inflammatory pathway. Tracey et al. further discovered that electrical stimulation of the vagus nerve attenuated TNF release in a rat model of endotoxemia [53]. Additional insights regarding the components of the cholinergic anti-inflammatory response were provided later when it was discovered that cutting the splenic nerve or complete splenectomy prevented the anti-inflammatory effects of electrical vagus stimulation [54,55].

Currently, the understanding of the steps in the inflammatory reflex is as follows [56–58] (figure 5):

Afferent pathway:

1) Activation of the afferent vagus nerve by inflammatory factors

Central processing:

2) Signal relaying in the nucleus tractus solitarius

Efferent pathway (the cholinergic anti-inflammatory pathway):

3) Activation of the efferent vagus nerve
4) Release of acetylcholine by the efferent vagus nerve terminal
5) Binding of acetylcholine on the splenic nerve
6) Activation of the splenic nerve
7) Release of noradrenaline at the splenic nerve terminal
8) Binding of noradrenalin to β2-adrenergic-receptor-positive (β2AR+) T cells
9) Release of acetylcholine by (β2AR+) T cells
10) Binding of acetylcholine to nicotinic-acetylcholine-receptor-positive (nAChR+) macrophages in the spleen
11) Inhibition of production and release of pro-inflammatory factors from nAChR+ macrophages in the spleen

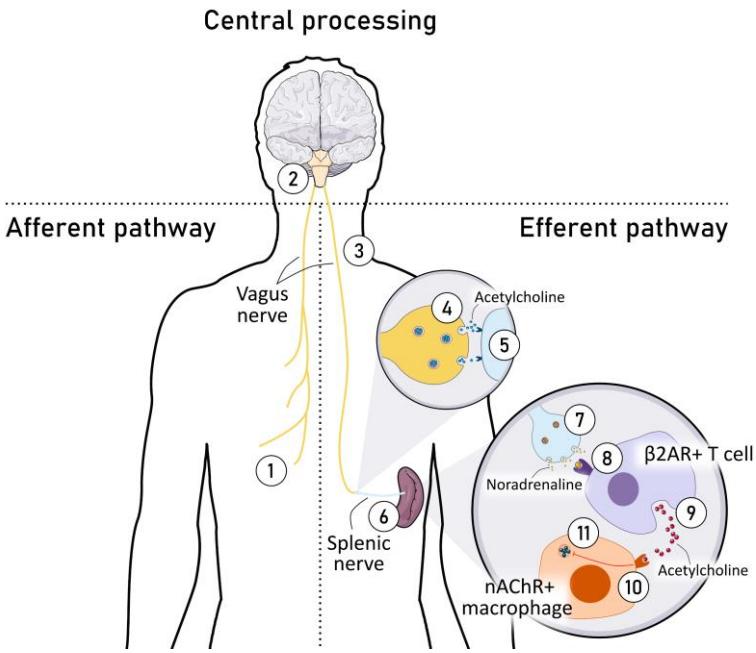


Figure 5 – The proposed mechanisms of the inflammatory reflex starting with the activation of the afferent vagus nerve. After signal relaying in the brain stem, the signal continues through the efferent pathway, also called the cholinergic anti-inflammatory pathway. Refer to the text for an explanation of numbers.

In addition to the efferent cholinergic anti-inflammatory pathway, an afferent pathway conveyed by the vagus nerve through the hypothalamic-pituitary-adrenal axis exists, causing the release of glucocorticoids from the adrenal gland, thus facilitating the suppression of systemic inflammation [59].

1.3.1. MANIPULATION OF THE INFLAMMATORY REFLEX

The identification of the inflammatory reflex has given rise to a new area of research at the intersection of immunology and neuromodulation, aiming at utilizing this physiological response in the management of various diseases with inflammatory aspects [57,60]. Activation of the cholinergic anti-inflammatory pathway has been achieved pharmacologically by selective α7nAChR-agonists in preclinical studies [61,62], and with the discovery of promising ligands for human use, clinical application may be feasible in the near feature [63].

Vagus nerve stimulation (VNS) may also be achieved electrically. In fact, the concept of electrical neuromodulation as a treatment of inflammatory disease dates back as far as 2000 years, when electric fish were used to treat arthritis in the Roman empire [64].

More recently, implantable vagus nerve stimulation (iVNS) has been developed, and in 1997, the first iVNS device was approved by the Food and Drug Administration (FDA) for use in epilepsy. In 2005, the indication for iVNS was expanded to include depression as well [65]. The use of iVNS requires invasive surgery in which an electrode is placed around the cervical part of the left vagus nerve. This procedure is generally well-tolerated, but a wish for non-invasive approaches in which VNS is achieved transcutaneously (tVNS) is desirable in many conditions due to the reduced risk of adverse events and reduced costs [57]. Furthermore, for acute conditions requiring only temporary neuromodulation, implantation of electrodes is unsuitable.

Transcutaneous stimulation of the auricular branch of the vagus nerve through an in-ear device is currently being used in epilepsy, depression, migraine, and pain [65]. Stimulation of the cervical part of the vagus nerve has also gained much attention due to its easy accessibility. Commercially available devices for this purpose have been developed and are currently approved for use in migraine, cluster headache, and, more recently, asthma exacerbations in COVID-19 [60,66,67].

In clinical trials, anti-inflammatory effects of tVNS have been shown both in healthy individuals [68,69] and in diseases such as IBD [70], rheumatoid arthritis [71], psoriatic arthritis [72], ankylosing spondylitis [72], and COVID-19 [73]. Thus, the application of VNS in conditions with known immune-mediated inflammatory pathways is intriguing. However, despite the well-known connection between chronic inflammation in diabetes and the development of diabetic complications, no clinical or preclinical studies have explored the potential anti-inflammatory effect of VNS in diabetes.

CHAPTER 2. AIMS AND HYPOTHESIS

The overall aim of this PhD thesis was to characterize and investigate circulating inflammatory biomarkers in diabetes and their connections with diabetic complications. Furthermore, the aim was to investigate the potential anti-inflammatory effect of transcutaneous vagus nerve stimulation in diabetes.

Consequently, this PhD thesis will explore the following four specific aims:

AIM 1

To investigate the presence of inflammatory biomarkers in type 2 diabetes and to identify clinical characteristics associated with the levels of these.

We hypothesised that people with diabetes have higher levels of systemic inflammatory biomarkers compared to healthy, and that clinical characteristics such as disease duration, glycaemic control, and therapeutical management are associated with the level of inflammatory biomarkers.

Aim 1 was investigated in **Paper 1**, which used data on people with type 2 diabetes from a cross-sectional study (**Study I**) and data from a control cohort of healthy participants (**Study III**)

AIM 2

To investigate associations between inflammatory biomarkers in type 1 diabetes and the presence and severity of DSPN.

We hypothesised that people with diabetes and DSPN have higher levels of systemic inflammatory biomarkers compared to healthy and to people with diabetes and no signs of DSPN. Moreover, we hypothesised that the severity of DSPN is associated with higher levels of inflammatory biomarkers.

Aim 2 was investigated in **Paper 2**, which used data on people with type 1 diabetes and no signs of DSPN from a cross-sectional study (**Study I**) and baseline data from a randomised controlled trial including people with type 1 diabetes and verified DSPN (**Study II**). Data from a control cohort of healthy participants (**Study III)** was also included.

AIM 3

To investigate associations between inflammatory biomarkers in diabetes and the presence of gastrointestinal problems.

We hypothesised that people with diabetes and increased segmental gastrointestinal transit times have higher levels of inflammatory biomarkers compared to people with diabetes and normal transit times. Moreover, we hypothesised that levels of inflammatory biomarkers were associated with gastrointestinal symptoms.

Aim 3 was investigated in **Paper 3**, which used data from a cross-sectional study including people with type 1 or type 2 diabetes (**Study I)** and data from a control cohort of healthy participants (**Study III)**.

AIM 4

To investigate the potential anti-inflammatory effect of transcutaneous vagus nerve stimulation in people with diabetes.

We hypothesised that transcutaneous vagus nerve stimulation reduces the level of inflammatory biomarkers in diabetes compared to sham stimulation.

Aim 4 was investigated in **Paper 4**, which used data from a randomised controlled trial (**Study IV**) exploring the effects of transcutaneous vagus nerve stimulation in people with type 1 or type 2 diabetes.

CHAPTER 3. MATERIALS & METHODS

This PhD thesis is based on three peer-reviewed original papers and a fourth paper draft, which will be submitted to a peer-reviewed journal. The data presented in these four papers originate from four clinical studies (two cross-sectional studies and two randomised controlled trials) (figure 6). In this chapter, studies and applied methods will be presented.

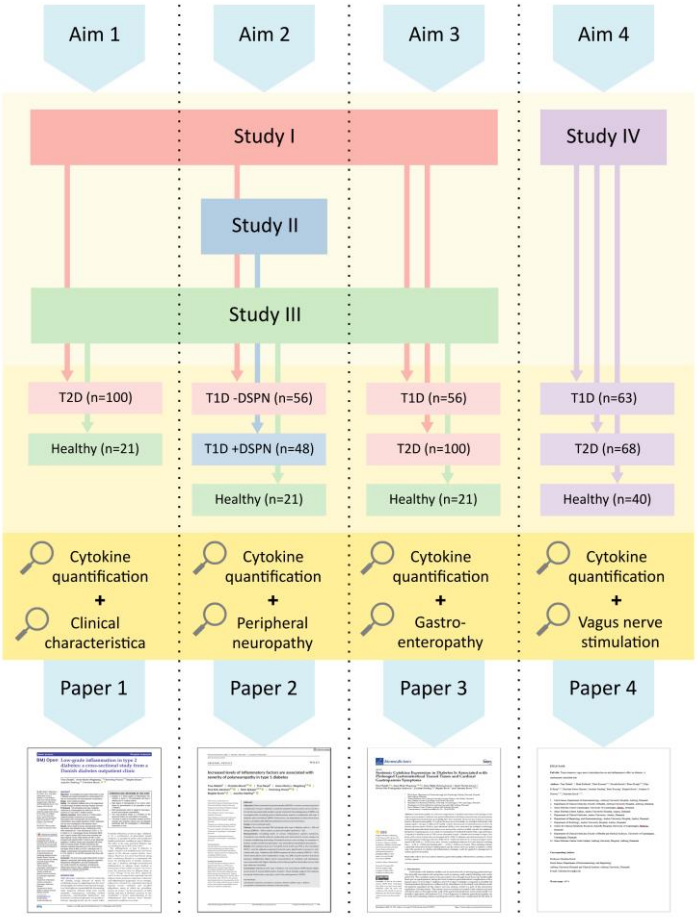


Figure 6 – Overview of aims, studies, cohorts, methods, and papers included in the thesis. T1D: type 1 diabetes; T2D: type 2 diabetes

3.1. INCLUDED STUDIES

3.1.1. STUDY I

STUDY OVERVIEW

Study I was a cross-sectional study with the primary objective of characterizing the prevalence and severity of cardiac autonomic neuropathy in people with type 1 or type 2 diabetes [74,75]. The study was approved by The North Denmark Region Committee on Health Research Ethics (N-20170045) and was conducted at Aalborg University Hospital, Denmark, from November 2017 to August 2020.

STUDY POPULATION

In total, 56 participants with verified type 1 diabetes and 100 participants with type 2 diabetes (both HbA1c $\geq$ 6.5% for a minimum of one year) were included in the study. Participants were on stable diabetes treatment for at least one month prior to inclusion in the study. To ensure a diverse cohort representative of the Danish diabetes population, all patients scheduled for annual diabetes visits at the outpatient diabetes clinic at the Department of Endocrinology at Aalborg University Hospital, Denmark, were invited consecutively to participate in the study. People with concomitant cardiac or neurological pathologies were excluded from the study.

3.1.2. STUDY II

STUDY OVERVIEW

Study II (NCT02138045) was a randomised, double-blinded, cross-over, placebo-controlled trial investigating the neuroprotective effects of glucagon-like-peptide-1 (GLP-1) agonist treatment in type 1 diabetes [76]. The study protocol was approved by The Danish Health and Medicines Authority (2013–004375-12) and The North Denmark Region Committee on Health Research Ethics (N-20130077). The trial was conducted at Aalborg University Hospital, Denmark, from June 2014 to January 2017. Only baseline data from study II was included in this PhD thesis.

STUDY POPULATION

In total, 48 participants with verified type 1 diabetes (HbA1c $\geq$ 7.0% for a minimum of two years) and on stable diabetes treatment for at least two months were included in the trial. Moreover, participants were included based on the presence of DSPN (verified by abnormal nerve conduction velocities). People with other neurological pathologies were excluded from the trial.

3.1.3. STUDY III

STUDY OVERVIEW

Study III was a cross-sectional study of healthy volunteers. The study aimed to provide an age- and sex-matched control cohort for the diabetes cohort in study II. Identical investigations of study II were thus carried out in study III. The study protocol was approved by The North Denmark Region Committee on Health Research Ethics (N-20090008) from June 2014 to January 2017.

STUDY POPULATION

In total, 21 healthy participants were included in study III.

3.1.4. STUDY IV

STUDY OVERVIEW

Study IV (NCT04143269) was a randomised, double-blinded, parallel-group, sham-controlled, multicentre trial aimed at investigating the effects of transcutaneous vagus nerve stimulation on the alleviation of gastrointestinal symptoms in people with type 1 or type 2 diabetes [77]. Study IV also included a control cohort of age- and sex-matched healthy participants. The study protocol was approved by The Danish Health and Medicines Authority (CIV-19-07-029105) and The North Denmark Region Committee on Health Research Ethics (N-20190020). The trial was conducted at Steno Diabetes Centre North Jutland, Steno Diabetes Centre Aarhus, and Steno Diabetes Centre Copenhagen (all in Denmark) from June 2020 to August 2022. Figure 7 provides an overview of the design of study IV relevant to paper 4.

STUDY POPULATION

In total, 131 participants with a verified diagnosis of type 1 (n=63) or type 2 diabetes (n=68) for a minimum of one year were included in the trial. Participants were included based on the presence of gastrointestinal symptoms (assessed by patient-reported outcome measures) and on the presence of diabetic autonomic neuropathy (assessed by objective (sudomotor function and cardiac reflex testing) and patient-reported outcome measures). People with gastrointestinal disorders were excluded from the trial. The control cohort included 40 participants in total.

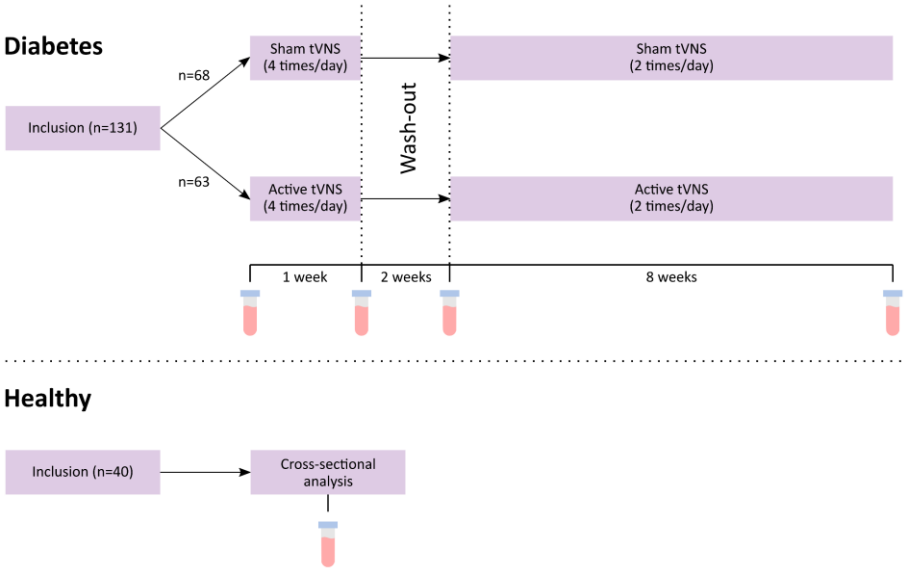


Figure 7 – Overview of the design of study IV. In total, 131 people with diabetes were included and randomised to either sham (n=68) or active tVNS (n=63). In study period 1, intervention was performed four times per day, while in study period 2, intervention was performed two times per day. The two study periods were separated by a wash-out period of at least two weeks. Blood was collected from participants at the beginning and the end of each study period. In addition, 40 healthy participants were included as a control cohort for cross-sectional analysis. A single blood sample was collected from this cohort.

3.2. COLLECTION OF BLOOD SAMPLES

In all four studies, blood was collected from participants during the morning (between 07:00 AM – 10:00 AM) and after at least six hours of fasting. In studies I, II, and III, blood was collected in clot activator tubes, while EDTA tubes were used in study IV. Hence, serum was used for analysis in study I, II, and III, while plasma was used in study IV. The blood samples were centrifuged for 10 minutes at 4°C at 1.000 g, and isolated serum or plasma was collected and stored at -80°C until analysis.

3.3. QUANTIFICATION OF CYTOKINES

Serum and plasma samples were analysed at Steno Diabetes Center Copenhagen by experienced personnel using two different multiplex technology platforms. To ensure the stability of cytokines, freeze/thaw cycles were kept to a minimum and did not exceed two cycles [78]. To avoid thawing during transportation from the site of collection to the site of analysis, samples were embedded in dry ice. For each specific aim, samples were analysed together to minimize the risk of interplate variability.

Table 2 provides an overview of investigated analytes and methods of investigation for each aim.

	Biological material	**Kit name**	**Platform**	**Analytes investigated**	
Aim 1	Serum	V-PLEX Neuro-inflammation Panel 1 Human Kit	Mesoscale Discovery	IL-1α IL-1β IL-2 IL-4 IL-5 **IL-6** **IL-7** **IL-8** **IL-10** **IL-12/ IL-23p40** IL-13 **IL-15** **IL-16** IL-17A	**IFN-γ** **TNF-α** TNF-β **Eotaxin** Eotaxin-3 **IP-10** **MCP-1** MCP-4 **MDC** MIP-1α **MIP-1β** **TARC** **CRP**
Aim 2	Serum	Inflammation 20-Plex Human ProcartaPlex ™ Panel	Luminex	**IL-1α** IL-1β **IL-4** IL-6 IL-8 IL-10 **IL-12p70** **IL-13** **IL-17A** IFN-α	IFN-γ **TNF-α** **CXCL10** **MCP-1** **CCL3** **CCL4** GM-CSF **ICAM-1** **E-selectin** **P-selectin**
Aim 3	Serum	V-PLEX Neuro-inflammation Panel 1 Human Kit	Mesoscale Discovery	**IL-6** **IL-8** **IL-10**	**TNF-α** **IFN-γ** **CRP**
Aim 4	Plasma	V-PLEX Pro-inflammatory Panel 1 Human Kit	Mesoscale Discovery	IL-1β IL-2 IL-4 **IL-6** **IL-8**	**IL-10** IL-12p70 IL-13 **TNF-α** **IFN-γ**

Table 2 – Overview of the cytokine quantifications for the four investigated aims. Analytes in bold: Included in the statistical analysis. Analytes in grey: Excluded from the statistical analysis due to being undetectable in the sample or having insufficient data quality. CCL: C-C motif chemokine; CRP: C-reactive protein; CXCL: C-X-C motif chemokine; GM-CSF: granulocyte-macrophage colony-stimulating factor; ICAM: intracellular cell adhesion molecule; IFN: interferon; IL: interleukin; IP: IFN-γ-induced protein; MCP: monocyte chemoattractant protein; MDC: macrophage-derived chemokine; MIP: macrophage inflammatory protein; TARC: thymus and activation regulated chemokine; TNF: tumour necrosis factor.

3.3.1. MESOSCALE DISCOVERY

Cytokine quantification for aims 1, 3 and 4 was conducted by multiplex technology using the Mesoscale Discovery (MSD) platform, represented in figure 8. The MSD platform uses a solid-phase immunoassay approach. In this technique, specific capture antibodies are immobilized onto ten separate spots on the surface of the solid phase, provided by a microtiter plate, allowing for simultaneous detection of ten analytes. These antibodies selectively bind to the target cytokines present in the sample. Following sample incubation, detection antibodies labelled with electrochemiluminescent tags are introduced. The electrochemiluminescent signal generated upon application of an electrical current allows for the precise quantification of the bound cytokines. The MSD instrument, a MESO QuickPlex SQ 120, then measures this electrochemiluminescent signal, providing a quantitative assessment of cytokine concentrations in the samples [5,79].

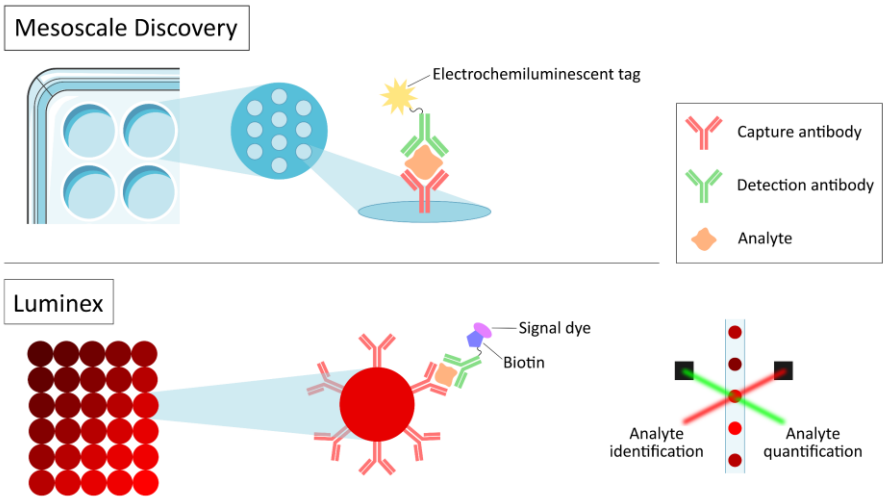


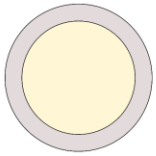
Figure 8 – Schematic presentation of the principles behind the multiplex assays provided by Mesoscale Discovery and Luminex.

3.3.2. LUMINEX

Cytokine quantification for aim 2 was carried out using the Luminex multiplex platform (figure 8). This is a liquid-based assay in which samples are mixed with beads coated with varying degrees of red and infrared dyes. The specific ratio of dyes is linked to a specific analyte of interest by conjugation of monoclonal antibodies. After mixing of beads and sample, biotinylated detection antibodies are added to the mixture, causing sandwich-like complexes to be formed if the specific analyte is present in the sample. Next, a signal dye able to bind to the biotinylated detecting antibody is added to the mixture. Finally, a flow-based detection instrument, a Luminex MAGPIX, is used to read beads one by one. Classification of the specific analyte is achieved by one laser measuring the unique infrared dye on beads, while quantification of analyte concentration is measured by a second laser able to detect the degree of signal dye present on each bead [5,79].

3.4. ASSESSMENT OF PERIPHERAL NEUROPATHY

For aim 2, associations between peripheral neuropathy and systemic cytokine levels were explored. Peripheral neurons can largely be divided into large and small fibres with distinct modes of function (figure 9). Thorough characterization of peripheral neuropathy requires assessment of multiple nerve types. In studies I, II, and III, this was achieved by quantitative sensory testing described below.



Classification	Aα	Aβ	Aδ	C
Type	Ia/Ib	II	III	IV
Diameter (µm)	13-20	6-12	1-5	0.2-1.5
Myelin	Yes	Yes	Thin	No
Conduction velocity (ms)	80-120	35-75	5-30	0.5-2.0

Figure 9 – Characteristics of different subtypes of peripheral sensory neurons.

3.4.1. VIBRATION PERCEPTION THRESHOLD

Vibration perception is mediated through large afferent myelinated Aβ-neurons [80]. Thresholds were determined by applying a vibration stimulus to the dorsum of the first phalanxes using a biothesiometer (Bio-Medical Instruments). This device allows for the application of increasing vibration stimuli up to a maximum of 50 volts. Participants reported to the study personnel when the vibration was first perceived.

3.4.2. TACTILE PERCEPTION THRESHOLD

Mediation of tactile perception is facilitated by large afferent myelinated Aα- and Aβ-neurons [80]. Tactile perception thresholds were determined using monofilaments (Optihair von Frey Filaments, Marstock nervetest). Increasing forces from 0.008 to 300 g were applied, and participants were told to indicate when the stimulus was first recognized. In the cohort included in study I, forces were applied to the planar side of the first phalanx, while forces were applied to the forearm in study II. Similar application sites in the studies would have been preferable, especially due to the uneven development of DSPN in the upper and lower extremities.

3.4.3. HEAT PERCEPTION THRESHOLD

Heat perception is conveyed by small afferent non-myelinated C-fibres [80]. Thresholds were assessed by thermal stimuli of the forearm with a thermode (Pathway, Medoc Ltd.). The heat tolerance threshold was determined by increasing the temperature of the thermode until participants reported a sensation of pain (or until a maximum of 52°C was reached).

3.5. ASSESSMENT OF GASTROENTEROPATHY

3.5.1. GASTROINTESTINAL TRANSIT TIME

For aim 3, associations between gastrointestinal function and systemic cytokine levels were explored. For this purpose, participants underwent investigations of gastrointestinal transit times by the wireless motility capsule (SmartPill, Given Imaging, Israel) as described in [81]. Once ingested, the wireless motility capsule records continuous data regarding temperature, pH, and pressure throughout the gastrointestinal tract. By analysing the recording according to falls and rises in temperature and pH, segmental transit times can be derived (figure 10).

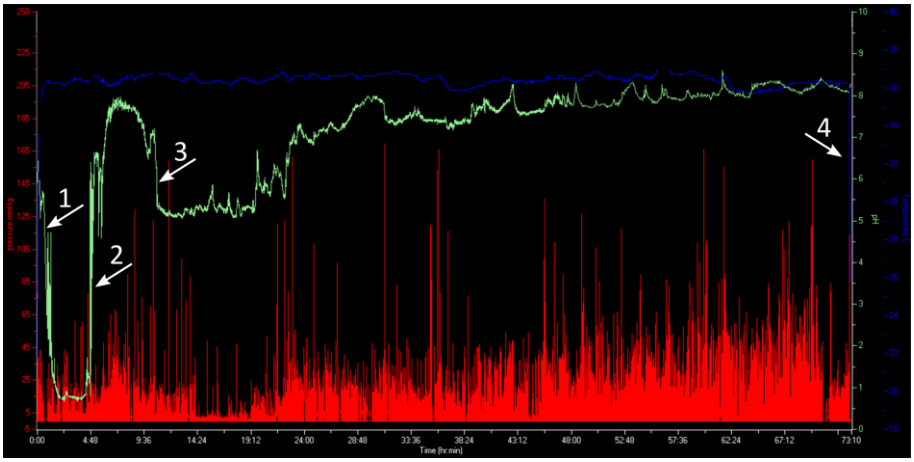


Figure 10 – Representative example of a wireless motility capsule recording. The blue and light green lines represent temperature and pH, respectively. The red bars represent pressure. A typical recording include a rapid temperature rise and a pH fall at the time of ingestion of the capsule (arrow 1). Passage through the pylorus is seen as a rapid and large increase in pH (arrow 2) followed by a plateau. The transition through the ileocecal junction is seen as a rapid but modest fall in pH (arrow 3). Lastly, the expulsion of the capsule is seen as a rapid temperature drop (arrow 4).

3.5.2. GASTROINTESTINAL SYMPTOMS

To account for the fact that gastrointestinal dysfunction is not always accompanied by prolonged gastrointestinal transit times [45], a patient-reported outcome measure was also included in the assessment of gastroenteropathy. Participants completed the Gastroparesis Cardinal Symptom Index (GCSI) questionnaire, which was developed specifically to evaluate symptoms of gastroparesis [82], which is among the most common gastrointestinal complications in diabetes [39]. The GCSI consists of nine items and covers three subdomains specific for gastroparesis (nausea/vomiting, bloating, and early satiety).

3.6. TRANSCUTANEOUS VAGUS NERVE STIMULATION

For aim 4, the anti-inflammatory effect of tVNS was explored. In study IV, participants self-administered tVNS according to a predefined protocol using a commercially available device known as GammaCore Sapphire (ElectroCore LLC, Basking Ridge, New Jersey, USA). This is a medical device able to generate a low-voltage electrical signal to the cervical part of the vagus nerve via two stainless steel electrodes. Participants were randomly assigned to receive either active or sham tVNS. Sham devices were similar in appearance to active devices but were unable to produce electrical signals. Due to possible visual ques during stimulation regarding

randomisation (e.g., muscle contractions of the oral commissure), unblinded personnel were in charge of device instructions, while blinded personnel collected data. To ensure blinding of participants, a parallel group design was chosen to avoid the ability to compare the different physical sensations associated with active and sham stimulation. Proper device placement was instructed to participants to be in the triangular area between the sternocleidomastoid muscle and the trachea, with stimulation intensity adjusted to the highest tolerable level (figure 11). Both short-term, high-intensity stimulation (four stimulations a day for one week) and long-term, medium-intensity stimulation (two stimulation per day for eight weeks) were investigated (figure 7).

Figure 11 – Participant self-administration of tVNS. By looking at a handheld mirror, correct positioning of the device is ensured. Written consent for the use of the picture was given.

CHAPTER 4. KEY RESULTS

AIM 1

To investigate the presence of inflammatory biomarkers in type 2 diabetes and to identify clinical characteristics associated with the levels of these.

Key results:

- People with type 2 diabetes had higher levels of TNF-α and eotaxin and lower levels of IL-7 compared to healthy individuals after adjustment for age and body mass index (BMI).
- People with long-term type 2 diabetes (over ten years) had higher levels of IL-10 compared to people with short-term type 2 diabetes after adjustment for age and BMI.
- Obesity, sex, glycaemic control, and therapeutical management were associated with increased levels of several inflammatory biomarkers.
- People with three or more diabetic complications displayed elevated levels of five inflammatory biomarkers (IL-6, IL-10, IL-12/IL-23p40, IL-15, and CRP) compared to people with fewer complications.

Interpretation

The extent of low-grade inflammation in type 2 diabetes correlates with factors such as obesity, glycaemic control, treatment, sex, and complications. These findings emphasize the significance of addressing inflammatory concerns in type 2 diabetes, as they may contribute to the predisposition for debilitating comorbidities.

AIM 2

To investigate associations between inflammatory biomarkers in type 1 diabetes and the presence and severity of DSPN.

Key results

- People with type 1 diabetes and distal symmetrical polyneuropathy had higher levels of several inflammatory biomarkers (IL-1α, IL-4, IL-12p70, IL-13, IL-17A, TNF-α, MCP-1, E-selectin) compared to people with type 1 diabetes but no signs of distal symmetrical polyneuropathy.
- These inflammatory biomarkers all had sensitivities and specificities above 75% for the detection of DSPN.
- Several of the measured inflammatory biomarkers correlated with measures of vibration, tactile, and heat perception thresholds.

Interpretation

Individuals diagnosed with type 1 diabetes and concurrent DSPN exhibit elevated serum levels of various inflammatory markers. Moreover, these inflammatory markers correlate with the severity of DSPN. These observations suggest that systemic low-grade inflammation may contribute to the pathogenesis of DSPN.

AIM 3

To investigate associations between inflammatory biomarkers in diabetes and the presence of gastrointestinal problems.

Key results

- People with diabetes and increased gastric emptying time had higher levels of IL-8 compared to people with diabetes and normal gastric emptying time.
- People with diabetes and increased colonic transit had higher levels of IL-10 compared to people with diabetes and normal colonic transit time.
- Symptoms of nausea and bloating were inversely correlated with levels of IL-6 in people with diabetes.

Interpretation

A connection between gastrointestinal transit times and systemic inflammatory biomarkers in diabetes may exist. However, the findings from this study present an inconclusive pattern.

AIM 4

To investigate the potential anti-inflammatory effect of transcutaneous vagus nerve stimulation in people with diabetes.

Key results

- No differences in the measured inflammatory biomarkers were seen after short-term or long-term tVNS or sham treatment.
- A tendency towards reduced TNF-α levels after active tVNS treatment was seen in those without signs of cardiac autonomic neuropathy.

Interpretation

tVNS was ineffective in lowering systemic inflammation in diabetes. This may be linked to vagal nerve dysfunction.

CHAPTER 5. DISCUSSION

The primary objective of this PhD thesis was to analyse and study inflammatory biomarkers in diabetes and their associations with diabetic complications. Additionally, the objective was to explore the potential anti-inflammatory effect of tVNS in diabetes as it has shown anti-inflammatory potential in other diseases.

In summary, we found several associations between diabetes, DSPN, and gastroenteropathy and various inflammatory biomarkers. We were unable to provide evidence for an immunomodulatory effect of tVNS in the cohort studied.

In the following chapter, inflammation in diabetes and the potential for modulation are discussed. This is followed by considerations regarding the applied methodology of the studies, including strengths and limitations. Lastly, future perspectives are discussed.

5.1. INFLAMMATION IN DIABETES AND DIABETIC COMPLICATIONS

When diving into the field of inflammation in diabetes and the connection with disease progression and the development of macro- and microvascular complications, one can easily feel overwhelmed. The literature is vast, and numerous potential inflammatory biomarkers have been proposed by several research groups worldwide. When adding the inherent nature of inflammatory mediators, such as the fact that many cytokines are pleiotropic with both pro- and anti-inflammatory properties depending on a given situation, the plot thickens even more. However, what we and several other researchers have shown is that a connection exists between increased biomarkers of inflammatory processes and the presence of diabetes and diabetic complications. Table 3 summarizes some of the current findings regarding biomarkers of inflammation in DSPN and CAN. Based on the increasing amount of evidence, it seems imperative that modulation of inflammation in diabetes should be explored as a new add-on treatment regime.

	Inflammatory biomarkers	Findings	Ref
Diabetic peripheral neuropathy	TNF-α, ICAM-1	Increased levels predicted the development of diabetic peripheral neuropathy over five years in T2D	[83]
	TNF-α, IL-6, sICAM-1, IL-1RA	Increased levels of TNF-α and IL-6 predicted DSPN development over 6,5 years, while associations between sICAM-1 and IL-1RA and DSPN were seen	[84]
	CCL7, CXCL9, CXCL10	Associated with incident DSPN	[85]
	IL-6, sICAM-1	Associated with the presence of painful DSPN	[86]
Cardiac autonomic neuropathy	IL-12/23p40	Associated with dysfunctional heart rate variability in T1D.	[75]
	IL-6, IL-18, CRP	Associated with dysfunctional heart rate variability in T2D.	[87]
	IL-1α, IL-4, CCL2, E-selectin	Increased in T2D+CAN compared to T2D-CAN.	[88]
	IL-18, adiponectin, ICAM, E-selectin	Associated with measures of cardiac autonomic neuropathy in recent-onset T2D, but not T1D.	[89]

Table 3 – Overview of some of the current findings regarding the link between inflammatory biomarkers and diabetic peripheral neuropathy and cardiac autonomic neuropathy. CAN: cardiac autonomic neuropathy; CCL: C-C motif chemokine; CRP: C-reactive protein; CXCL: C-X-C motif chemokine; DSPN: distal symmetrical polyneuropathy; ICAM: intracellular cell adhesion molecule; IL: interleukin; RA: receptor antagonist; sICAM: soluble intracellular cell adhesion molecule; T1D: type 1 diabetes; T2D: type 2 diabetes; TNF: tumour necrosis factor.

5.1.1. MODULATION OF INFLAMMATION IN DIABETES

As of now, specific anti-inflammatory therapies are not integrated with standard management of diabetes despite the well-established link between inflammation and disease progression and complications [90]. Even though glycaemic control has some effect in the prevention of microvascular complications, especially in type 1 diabetes, the persistent incidence of diabetic complications, despite advancements in antidiabetic medications, underscores the necessity for exploring therapeutic approaches that extend beyond glycaemic control [21,90].

Interestingly, many antidiabetic drugs also exhibit anti-inflammatory properties, though these may be derivative effects of their metabolic actions. However, it is also possible that these drugs have direct effects on the immune response. For instance, metformin has been shown to reduce the pro-inflammatory response in preclinical studies [91], although the anti-inflammatory effect is less convincing in clinical studies [92]. Other antidiabetic drug classes, including dipeptidyl peptidase-(DPP) 4 inhibitors, GLP-1 receptor agonists, and sodium-glucose cotransporter (SGLT) 2 inhibitors, are also known to exhibit anti-inflammatory properties in preclinical and proof-of-concept clinical studies [91]. Of note, insulin in itself is known to inhibit inflammation [93], but evidently, these actions are insufficient for the alleviation of inflammation in type 1 diabetes. Consequently, a need for targeted anti-inflammatory therapies in diabetes potent enough to challenge the ongoing hyperglycaemia-induced pro-inflammatory microenvironment is evident.

Preclinical studies have identified several promising anti-inflammatory compounds for lowering inflammation in diabetes and improvement of complication status. However, most fail when introduced in a clinical setting where comorbidities, genetic variations, and environmental factors influence the outcome. Nonetheless, the growing knowledge of inflammatory processes in diabetes and how they facilitate the development of diabetic complications is important for better treatment options in the future.

A milestone in the application of therapy with immunomodulatory properties in diabetes was reached in November 2022, when the FDA approved the use of teplizumab for the preservation of endogenous insulin secretion in stage 2 type 1 diabetes. Teplizumab is a monoclonal antibody specific for the cluster of differentiation (CD)3 molecule, which is essential in the activation of β-cell autoreactive T-cells in diabetes [94]. Thus, teplizumab preserves the function of pancreatic β-cells and reduces the need for exogenous insulin therapy in type 1 diabetes [95].

In terms of diabetic microvascular complications, the damages seem to be progressive and irreversible once they occur, and thus, a proactive approach utilizing preventive rather than curative treatment may prove more successful in achieving significant clinical benefits. Preventive treatment, however, must be suitable for prolonged usage, which calls for minimal side effects to ensure patient adherence and overall well-being. Evidence has been provided for the efficacy of low-dose aspirin as a prophylactic treatment of major cardiovascular events in diabetes due to its antithrombotic and anti-inflammatory properties. The integration into clinical practice, however, is complicated by an accompanying increased risk of major haemorrhage [96]. Accordingly, individual assessments regarding the risk-benefit ratio are required for the application of aspirin in diabetes.

Regarding DSPN, various vitamins and dietary supplements have shown potential in the prevention or even treatment of this microvascular complication [97]. An example of this is the utilization of the anti-inflammatory and antioxidant capacities of curcumin, the active component of the spice turmeric, which was shown to improve the severity of DSPN in a randomised controlled trial [98]. Another new and interesting approach for the modulation of DSPN is through manipulation of the gut microbiota. Recently, a randomised controlled trial (n=39) in people with DSPN showed improvements in the active group receiving faecal microbiota transplantation from healthy donors both in terms of clinical assessment of DSPN by the Toronto Clinical Scoring System and in relief of pain. This was accompanied by reductions in serum levels of IL-6 and TNF-α in the active group [99]. Further research in the area of faecal microbiota transplantation in diabetes is intriguing, especially because intestinal dysbiosis is present in this patient group [100].

In study IV, we explored the potential anti-inflammatory effects of tVNS as a candidate for an easy-to-use, non-invasive treatment option. Even though we were unable to show any cytokine-lowering effects of this treatment, further studies into the utilization of the physiological cholinergic anti-inflammatory pathway are intriguing. Possibly, inoperable VNS devices at the cervical level for continuous activating or at the efferent vagal projections towards the spleen for circumvention of central relying could prove more effective in the setting of diabetes. This is, however, highly speculative.

5.2. METHODOLOGICAL CONSIDERATIONS

A common limitation in all four papers included in this PhD thesis is the fact that the investigated aims were secondary analyses of larger studies. Therefore, the analyses must be considered experimental and may be underpowered. Consequently, risks of type II errors exist. Furthermore, aim 3 and 4 were investigated across both type 1 and type 2 diabetes. While these pathologies share several aspects, such as hyperglycaemia, they differ in many others. Stratification based on diabetes type could potentially have revealed additional insights on specific nuances within each subtype. Moreover, the cross-sectional data from studies I, II, and III hinders any conclusions regarding causality between the observed associations.

Limitations regarding the investigational approaches applied in the different studies are discussed in the following.

5.2.1. QUANTIFICATION OF INFLAMMATORY BIOMARKERS

Cytokines are secreted in a highly dynamic manner and often have short half-lives, and thus, interpretations of measured levels should always be made with caution [5]. However, with the growing knowledge of the roles of cytokines in various pathologies, the field investigating cytokines as biomarkers of disease has expanded.

Therefore, quantification methods with high throughput and high precision are of great interest. For a long time, the preferred method for cytokine quantification was through enzyme-linked immunosorbent assay (ELISA), which utilizes antibodies for specific identification of analytes [101]. However, this approach is time-consuming, typically requires relatively large volumes of samples, and is limited to assessing one analyte at a time [6]. Consequently, so-called multiplex assays have been developed by which several cytokines can by quantified simultaneously in a single sample in a rapid and efficient manner [79].

We used modern multiplex assays for the detection of inflammatory biomarkers. While such assays are efficient and fast, the possibility of cross-reactions and unspecific binding when simultaneously introducing several antibodies to the same sample must be considered. However, the platforms provided by Luminex and MSD have both been shown to be suitable for measuring cytokine profiles in a clinical setting, with the MSD platform being more sensitive and the Luminex providing better precision [79]. For cytokine quantification, high sensitivity is essential because these proteins are expressed in small amounts within the blood. In paper 2, we used the Luminex platform and found that levels of IL-6 and IFN-γ were below the levels of detection. Perhaps the enhanced sensitivity of the MSD platform would have been able to quantify these analytes, as was the case in papers 1, 3, and 4. Another discrepancy in cytokine quantification between the studies included was the collected biological material. In study I, II, and III, we collected serum, while plasma was collected in study IV. Both the coagulation process and the addition of anticoagulants may interfere with cytokine levels, and therefore, direct comparisons between measured cytokine levels in different blood specimens should be avoided [5].

5.2.2. ASSESSMENT OF DIABETIC NEUROPATHY

In paper 2, we looked at the associations between cytokine profiles and DSPN. As with most diabetic complications, DSPN is typically first recognized in the late stages, when irreversible damage to the nerves has occurred [102]. Consequently, there is great potential for improving patient outcomes if predictive biomarkers for DSPN susceptibility could be identified. Circulating cytokines may be relevant candidates in this regard because even though they are not specific, they are believed to reflect pathological processes within the endoneurium.

However, a substantial challenge in the diagnosis of DSPN is its detection in the early stages. While large fibre neuropathy is easily assessed with standard nerve fibre conduction testing, assessment of small fibre neuropathy still lacks a golden clinically applicable standard. To assess large fibre integrity, we used vibration detection threshold and tactile sensation, whereas small fibre involvement was assessed by heat perception thresholds for quantitative sensory testing in paper 2, but other approaches may be even better for detecting small fibre damage in the early stages. Among these are corneal confocal microscopy and assessment of intra-epidermal nerve fibre

density [103,104]. While these methods have shown potential both in research and in the clinical setting, a common consensus among the scientific community regarding the optimal method for early detecting of DSPN complicates the validation of robust and easily accessible biomarkers such as cytokine profiles.

5.2.3. ASSESSMENT OF DIABETIC GASTROENTEROPATHY

Assessment of gastroenteropathy is challenging, and no golden standard for diagnosis exists. Evaluation of neural integrity of submucosal biopsies from the gastrointestinal tract is a possible but often inappropriate method. Non-invasive approaches for assessing motility, such as scintigraphy and manometry, however, are often more suitable, especially in research, but they are also less accurate as they rely on proxy measures of gastrointestinal function [38]. For aim 3, we used the wireless motility system to assess regional and whole-gut gastrointestinal transit times, which are increased in people with diabetes compared to healthy [40,45].

The wireless motility capsule system is a minimally invasive and reliable investigational method for gastrointestinal transit time [105]. However, a major limitation of the system is the lack of information regarding the real-time position of the capsule. Therefore, the data provides no information on the degree of anterograde and retrograde motions. In regard to diabetic gastroenteropathy, this information would be important, however, because detailed phenotyping of the dysfunctional propulsion within the gastrointestinal tract is crucial for the development of focused treatment strategies. For example, the wireless motility system would not be able to distinguish between an overall decreased motility causing slow progression or dys-coordinated motility patterns with a mix of forward and backward motions. Evidently, the underlying mechanism and, thus, the appropriate treatment are different in these two cases.

The 3D-transit system is another available method for investigations of gastrointestinal transit times [106]. As opposed to the wireless motility system, the 3D-transit capsule records the rotation and specific position of the capsule in real-time. From these data, detailed information regarding motility patterns and segmental colonic transit times can be derived [106,107]. The 3D-transit system has been used previously for investigations in diabetes [108,109], but the major limitation of the system is the risk of technical problems leading to incomplete recordings. Thus, to ensure robust recordings of gastrointestinal transit times in studies I and III, the wireless motility system was chosen.

Another limitation of the wireless motility capsule system specific for the application in diabetes is the required ingestion of a rather comprehensive standardised meal. For individuals with gastroparesis, this can be difficult or even impossible to adhere to. Therefore, the calorie-intake together with the capsule was less in some subjects because they were unable to finish the standardised meal. What variation in gastric

emptying time this has introduced to the analysis is unknown, but one study suggests that the effect is limited [110]. However, for diabetic cohorts, a smaller meal could be considered in future studies to ensure equal calorie intake.

5.2.4. TRANSCUTANEOUS VAGUS NERVE STIMULATION

In study IV, the potential anti-inflammatory effect of tVNS applied to the cervical part of the vagus nerve was explored. No changes in the investigated inflammatory biomarkers were seen after short-term (7 days) or long-term (56 days) treatment.

In preclinical studies, it has been convincingly established that the vagus nerve regulates inflammation through the cholinergic anti-inflammatory pathway. In humans, similar results have been shown, but the majority of the studies investigated have been small, non-randomised and without control groups [57]. However, if we accept that the vagus nerve exhibits anti-inflammatory effects in humans through the cholinergic anti-inflammatory pathway, the negative findings in study IV could be attributed to several factors: 1) the characteristics of the studied *cohort*, 2) the specifications of the *medical device*, 3) the *stimulation protocol* utilized, and/or 4) the approach employed in our *investigation*.

Cohort
Regarding the cohort, which included people with diabetes and signs of autonomic neuropathy, it could be speculated that a dysfunctional vagus nerve due to diabetes-induced neuropathy was unable to convey the electrical signal sufficiently. To explore this hypothesis, the introduction of several control groups would have been intriguing. Firstly, the application of tVNS to the healthy control group included in study IV would have confirmed that the medical device and the stimulation protocol were able to induce anti-inflammatory effects in people with intact vagal nerve function. However, it is possible that tVNS would be ineffective in dampening inflammation if there is no systemic inflammation ongoing. Therefore, a cohort consisting of patients known to have a high degree of systemic inflammation but no autonomic neuropathy could have been included. Recently, a randomised controlled trial in systemic lupus erythematosus utilizing the GammaCore device and a similar stimulation protocol has been finalized [111]. Whether or not tVNS was able to induce an anti-inflammatory response in this highly inflamed cohort is interesting.

Moreover, a cohort consisting of people with newly diagnosed diabetes and no signs of autonomic neuropathy could have elucidated if tVNS exhibits anti-inflammatory effects in people with diabetes but intact vagal function. Lastly, in future trials, it would be reasonable to ensure the exclusion of participants who previously had undergone splenectomy, given that the spleen is crucial in the cholinergic anti-inflammatory pathway.

Medical device

As for the medical device used in study IV, a tVNS device designed for cervical application was chosen. The cervical part of the vagus nerve consists of both afferent (80%) and efferent fibres (20%) [112], and, in theory, electrical stimulation of this area may induce both anterograde and retrograde effects. Methodological limitations have complicated the thorough characterization of the electrical transduction in detail. Recently, however, optogenetic studies in animals have provided new insights into the specific circuits which are activated by vagus nerve stimulation [30]. From these studies, evidence has been provided for an anti-inflammatory effect of both anterograde afferent and efferent vagal nerve signalling. This is important in regard to study IV, given the fact that the anti-inflammatory effect of VNS traditionally has been attributed to the efferent cholinergic anti-inflammatory pathway established by Tracey et al. [52] and that the GammaCore used in study IV device has been designed to target the afferent fibres of the vagal nerve predominantly. However, as optogenetic studies have shown, afferent signalling should be able to induce anti-inflammatory effects.

Stimulation protocol

The most robust data regarding the anti-inflammatory effect of VNS comes from surgically implanted stimulators with, e.g., reductions in TNF-α levels have been shown [113]. Very little evidence exists regarding the optimal way of applying tVNS in regard to frequency, intensity, and duration. Therefore, it cannot be ruled out that the applied stimulations in study IV were insufficient in activating the anti-inflammatory cholinergic pathway sufficiently for a response in plasma cytokine levels to be measurable. During study period 1, participants used the device bilaterally four times a day, and maintaining high compliance might prove challenging if additional doses are needed to induce an anti-inflammatory response. Nevertheless, dose-response studies are warranted in order to elucidate and standardise the optimal tVNS stimulation protocol.

Investigations

In study IV, the anti-inflammatory effect of tVNS was investigated as changes in plasma levels of inflammatory biomarkers. However, local changes in inflammatory biomarkers, e.g., in the spleen, may have been introduced during the intervention, which our investigational approach was unable to detect. Another aspect to consider is the fact that we collected blood samples up to several hours after tVNS application. Most cytokines have very limited half-lives, [5] and it is, therefore, possible that any changes induced by tVNS conducted in the morning were normalized when collected blood samples were collected. Whether or not such a short-term dynamic response would have any clinically relevant effect on inflammation is unknown. Blood sampling just prior to tVNS and at regular time intervals after the stimulation could be an interesting exploratory approach to investigate the dynamics in the cytokine response. If a long-term response (up to several hours) were to be seen, a phenotypical switch of immune cells induced by the cholinergic anti-inflammatory pathway could

be the underlying mechanism. Therefore, it could also have been interesting to have explored changes in circulating immune cells, e.g., by flow cytometry. Indeed, anti-inflammatory effects of VNS beyond cytokine regulation have been shown preclinically [114].

Moreover, study IV could have explored the indirect anti-inflammatory effect of tVNS in diabetes as a result of improved glycaemic control. Beneficial antidiabetic effects of tVNS have been shown previously in preclinical studies, including stabilization of blood glucose levels [115,116]. Interestingly, rat models have shown that specific afferent VNS causes an increase in glycemia through suppression of insulin secretion, while specific efferent VNS leads to a decrease in glycemia through the secretion of glucagon. Selective efferent VNS may thus have potential as antidiabetic therapy, especially in type 2 diabetes [34,36]. In humans, a randomised controlled pilot study in people with pre-diabetes showed that auricular tVNS improved glucose tolerance and HbA1c levels [118]. However, as the findings from study IV indicate, tVNS in established diabetes may be inappropriate.

5.3. FUTURE PERSPECTIVES

Diabetes and diabetic complications have reached pandemic proportions during the last decades, and their influence on the quality of life of affected individuals, as well as on healthcare expenditures, cannot and must not be overlooked [119]. Therefore, research must be dedicated to exploring possible ways of improving these conditions.

5.3.1. A NEED FOR NEW BIOMARKERS?

Currently, most research regarding inflammatory biomarkers in diabetes focuses on classical cytokines such as interleukins, chemokines, and adipokines, which are often more indicative of acute rather than chronic inflammation. Therefore, other candidates may also have a role to play in the identification of individuals with a high degree of systemic inflammation due to chronic diseases such as diabetes [120].

Potential candidates for emerging inflammatory biomarkers include lipid mediators and microRNAs [121]. Another promising biomarker in this regard is the soluble urokinase plasminogen activator receptor (suPAR), which has been shown to be associated with various pathologies in which chronic inflammation is implicated. An important aspect of suPAR, which implicates this biomarker as relevant in identifying chronic inflammation, is the fact that it is highly unaffected by acute inflammation [122]. In addition, it seems to be associated with chronic inflammation per se rather than with the underlying condition. Associations between suPAR and diabetes morbidity and mortality have been shown [123,124]. Further evidence regarding the robustness of suPAR as a predictive biomarker of diabetic complications and possibly also as a relevant outcome measure in intervention studies is anticipated.

In addition to markers of chronic inflammation, disease-specific biomarkers may also hold promise in the future of diabetes research. By looking beyond inflammatory factors, which are unspecific in nature, soluble factors directly linked to the pathology in question may provide important insights into disease development. Disease-specific biomarkers in diabetic neuropathies [125,126] and nephropathy [127] have been reported but are still needed in complications such as gastroenteropathy. Promising approaches for the discovery of new disease-specific biomarkers in various diseases include lipidomics, proteomics, and metabolomics [128–130]. Technologies such as mass spectrometry enable large-scale profiling of lipids, proteins, and metabolites, and by investigating the expression of these molecules in both health and disease, potential pathogenetic mechanisms and drug targets can be identified [131]. Currently, a mass spectrometry study looking to identify novel biomarkers of DSPN by serum proteome phenotyping in patients with type 1 diabetes and verified DSPN is being conducted. This study is expected to provide new knowledge regarding the development of DSPN [132].

5.3.2. SUGGESTIONS FOR FUTURE STUDIES

Going forward, several aspects of the relationship between inflammation and diabetes and diabetic complications need further elucidation for optimal application in the clinic. Suggestions for future studies within the area are listed below.

First of all, large-scale longitudinal cohort studies are warranted in order to clarify the causal link between cytokine profiles and the development of diabetic complications. Though it has been established that low-grade inflammation and diabetes are intertwined, evidence regarding the temporal sequence is lacking. That is, does a specific cytokine profile predispose for the development of, e.g., DSPN, or does DSPN cause the display of a specific cytokine profile? In this regard, the inclusion of newly diagnosed people is crucial, as well as a follow-up period extending up to several decades since the development of these long-term complications is slow. The KORA F4/FF4 study is an example of a large-scale cohort study in which older adults with type 2 diabetes are followed over several years [133]. Through this study, evidence regarding the predictive nature of certain cytokines for the development of DSPN has been provided [84].

Secondly, case-control studies are warranted to examine the progression of complications in specific subgroups of individuals with diabetes exposed to certain factors over time. For example, investigating the distribution of DSPN development between those who have received anti-inflammatory treatment since diabetes diagnosis and those who have not would be intriguing. This treatment may include anti-inflammatory medications prescribed for other conditions or antidiabetic GLP-1-agonist treatment. One hypothesis could be that the group receiving anti-inflammatory treatment might exhibit a lower incidence of microvascular complications compared to the untreated group. Utilizing the Danish national health registry for such case-

control studies is advantageous, given its comprehensive data on diagnosis codes and medication linked through unique identification numbers [134].

Thirdly, intervention studies testing new potential candidates for lowering inflammation in diabetes are crucial. Both medical compounds, e.g., monoclonal antibodies, and bioelectronics such as VNS devices should be investigated. When designing intervention studies, care must be taken when choosing study populations and outcomes. When including people with long-term diabetes, a point-of-no-return in terms of microvascular complications may have been reached, and any intervention may thus prove inefficient. However, the inclusion of newly diagnosed people may also be challenging due to the slow progression of these complications and the relatively short intervention period applied in most clinical trials. Regarding outcomes, both patient-reported outcomes and objective measures should be included to ensure a multifaceted characterization of the investigated intervention.

Lastly, the possibility of utilizing artificial intelligence for the identification of specific patterns in diabetes and diabetic complications is intriguing. The integration of big data and machine learning techniques has and continues to revolutionize the healthcare industry [135]. By combining information regarding inflammatory profiles and clinical data such as age, gender, BMI, family history, and medication, a comprehensive dataset can be prepared. Machine learning algorithms can then process this data to create predictive models that estimate the likelihood of a diabetic patient experiencing complications related to chronic inflammation. These models may enable healthcare professionals to identify individuals who may benefit most from anti-inflammatory prophylactic treatment, thus providing personalized and more effective care. This approach has the potential not only to improve patient outcomes but also to optimize the use of resources within the healthcare systems by targeting interventions where they are most needed. Currently, these studies are already in progress [136–139], and undoubtedly, a substantial advancement in this field is anticipated in the years to come.

CHAPTER 6. CONCLUSION

This PhD study has explored inflammatory biomarkers in different diabetes contexts. Four specific aims were investigated for this purpose:

AIM 1

We found a link between specific inflammatory biomarkers and clinical characteristics in type 2 diabetes. Of note, an increased inflammatory profile was associated with the presence of multiple diabetic complications. These findings suggest that the inflammatory status should be considered when managing type 2 diabetes.

AIM 2

In type 1 diabetes, we found a connection between increased levels of inflammatory biomarkers and the presence of DSPN. Moreover, concentrations of certain inflammatory biomarkers correlated with measures of vibration and tactile perception. These observations indicate that inflammation is implicated in the progression of DSPN.

AIM 3

Specific inflammatory biomarkers were associated with increased transit time of the stomach and colon, which may be indicative of diabetic gastroenteropathy. An inverse correlation between gastrointestinal symptoms and IL-6 was also found, which may seem counterintuitive.

AIM 4

No observable differences in inflammatory status were seen between individuals receiving active tVNS or sham treatment. This was true both for short-term intervention and long-term intervention. We did, however, observe a tendency towards reduced TNF-α levels during active treatment in individuals with no signs of cardiac autonomic neuropathy compared to those without.

Taken together, our findings, along with a growing body of evidence in the scientific community, implicate chronic inflammation in the progression of diabetes and diabetic complications. It seems essential that this aspect should be explored as a management strategy for the prevention of diabetic complications. However, utilization of the physiological cholinergic anti-inflammatory pathway through tVNS may not be relevant in diabetes, possibly due to deterioration of autonomic function.

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