KULEUVEN
Faculty of Bioscience Engineering

IMPACT OF HIGH-SALT DIET ON TYPE 1 DIABETES

Erasmus Dissertation

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Faculty of Medicine
CHROMETA
Clinical and Experimental Endocrinology

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SUMMARY

Today, autoimmune diseases are a serious problem for humanity and constitute severe, sometimes debilitating health complications. Approximately 6% of the population has an autoimmune disease and the incidence is increasing worldwide. One of the reasons for their development is the improper regulation of the immune system, in which regulatory T cells (Tregs) play an important role. Genetic and environment factors have a major impact on the initiation and progression of these diseases. Developed countries create a milieu in which cardiovascular, metabolic, and autoimmune diseases flourish. In particular, Western diet, including high-fat, high-sugar, high-protein, and high-salt consumption, along with regular consumption of processed and 'fast foods', encourages the development of obesity, metabolic syndrome, and cardiovascular morbidity and mortality. In some recent studies, high-salt diet has also been reported to influence the symptoms of autoimmune diseases and decrease Treg functionality. Therefore, the aim of the current project was to study whether a high-salt diet could trigger the development of autoimmune diseases in the non-obese diabetic (NOD) mouse model, focusing in this dissertation on the development of type 1 diabetes.

Here, we found that a high-salt diet did not elicit type 1 diabetes development in 16-week-old NOD mice with ongoing islet inflammation. These results were unexpected as mice fed with a similar high-salt regimen developed a more severe type of experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis. Moreover, high-salt intake aggravated the symptoms of systemic lupus erythematosus, collagen-induced arthritis and experimental colitis. We assume however that the composition of the HSD and especially the origin of its proteins being 24% casein might have impeded the observations. When maintained on a standard chow, which normally contains natural non-purified ingredients, like wheat middlings, wheat germ, and soybeans, NOD mice have the greatest diabetes incidence. In contrast, the introduction of semi-purified casein- or hydrolyzed casein-based diets are the least diabetogenic when mice are maintained on these diets from a very young age. Although our mice were kept until 16 week of age on a natural ingredient diet, the switch to a diet with casein as the major protein source might have prevented the further development of type 1 diabetes.

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LIST OF ABBREVIATIONS

AID	Autoimmune disease
APC	Allophycocyanin
CD25/IL2Rα	Interleukin-2 receptor α chain
CD	Celiac disease
CIA	Collagen-induced arthritis
CTLA4	Cytotoxic T-lymphocyte antigen 4
DM	Diabetes mellitus
DPBS	Dulbecco's phosphate-buffered saline
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FMO	Fluorescence Minus One
GD	Graves' disease
HLA	Human leukocyte antigen
HSD	High-salt diet
IDDM	Insulin-dependent diabetes mellitus
IPGTT	Intraperitoneal glucose tolerance test
MS	Multiple Sclerosis
NOD	Non-obese diabetic
NIDDM	Non-insulin-dependent diabetes mellitus
NSD	Normal salt diet
PE	Phycoerythrin
PLN	Pancreatic lymph node
RA	Rheumatoid arthritis
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SPL	Spleen
SLE	Systemic lupus erythematosus

SS	Sjögren’s syndrome
T1D	Type 1 diabetes
TH CELL	T helper cell

1. INTRODUCTION

1.1 AUTOIMMUNE DISEASES

Normally, the immune system protects from invading bacteria, viruses, fungi or parasites. Autoimmune diseases (AID) are caused by immune system' dysfunction [(1)] as an overactive and/or erroneous immune response is seen against substances and tissues present in the body. In general, the immune system can differentiate between self and non-self, but in an AID it recognises a part of the body as non-self [(2)]. As a result, proteins called autoantigens and autoantibodies are formed and further drive a chronic inflammatory process that disrupts the normal function of the tissue [(3)]. AID can affect different organs and vary significantly among people who are affected, in terms of severity and therapy responsiveness. There are over 80 different types of AID, such as rheumatoid arthritis (RA), Graves' disease (GD), celiac disease (CD), systemic lupus erythematosus (SLE), type 1 diabetes (T1D), multiple sclerosis (MS), or Sjögren's syndrome (SS) [(4)]. AID strike 1 in 15 individuals and seem to affect more women than men. In this dissertation, I will mainly focus on T1D (see section 1.4).

The hallmark of AID generally involves the presence of autoreactive T cells, autoantibodies and inflammation. During the process of autoimmunity, autoreactive $CD4^+$ and $CD8^+$ T cells appear [(5)]. Normally, these cells are deleted or inactivated in the thymus but in AID significant numbers of autoreactive T cells still escape to the periphery. It is largely unknown how these T cells escape clonal deletion. There are different populations of $CD4^+$ T cells, as depicted in Figure 1 [(6)]. Upon antigenic stimulation, naïve $CD4^+$ T cells are activated, expand, and differentiate into diverse effector subsets called T helper – (Th) Th1, Th2, Th9, and Th17 cells. Their differentiation into diverse lineages depends on specific cytokine signalling and transcription factors, as well as epigenetic modifications [(7)]. Interferon-γ (IFN-γ)-producing $CD4^+$ Th1 and interleukin-17 (IL-17)-producing $CD4^+$ Th17 cells have been identified as key pathogenic Th populations triggering the development of many AID. In this dissertation, specific attention is paid to a small group of $CD4^+$ T cells termed regulatory T cells (Tregs), which modulate the immune system, maintain tolerance to autoantigens and prevent AID [(8)] [(9)]. These cells co-express several markers including CD25 (IL-2Rα), CTLA4 (cytotoxic T-lymphocyte antigen 4), and the transcription factor FoxP3 [(10)]. There are natural or adaptive (induced) Tregs; natural Tregs develop and travel from thymus to periphery, while adaptive Tregs are non-regulatory T cells that acquire Treg assets outside of the thymus.

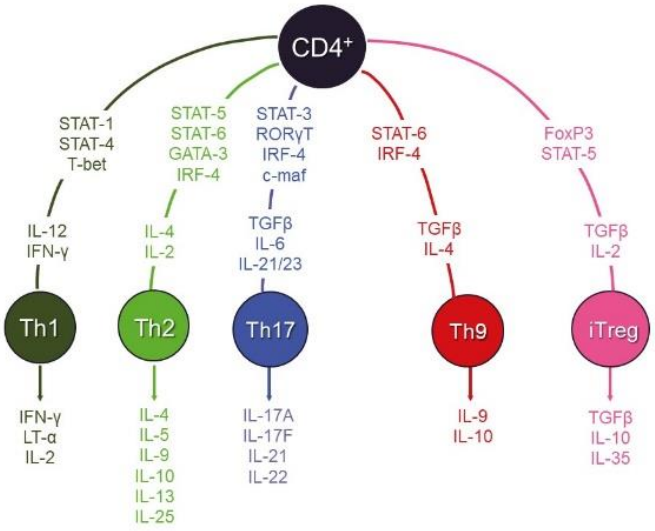

Figure 1. Schematic classification of CD4⁺ T cells [11]*. The scheme describes the corresponding transcription factors and the cytokines responsible for differentiation into the different T-cell subsets.*

1.2 GENETICS

Multiple genetic and environmental factors operate together in the etiology of AID [12]. Since many causes of AID are shared, people with an AID are more likely to suffer from more than one of these devastating diseases. Genes in the human leukocyte antigen (HLA) complex are the strongest predisposing genetic factors [13] [14]. The components of HLA class II, e.g. the HLA-DQ2/DQ8 genotype, have been linked to several AID including CD and T1D. These molecules can play an important role in the presentation of antigens to $CD4^+$ T cells, which indicates the importance of this pathway in the initiation and progression of AID [13] [17]. But also other genes in the HLA complex may contribute [15] [16].

1.3 ENVIRONMENTAL FACTORS – HIGH-SALT DIET

Although genetics is important, multiple environmental factors are implicated in the development of AID, such as toxic chemicals, dietary components, gut dysbiosis and infections (Figure 2), which can cover almost 70% of the causes of AID [18].

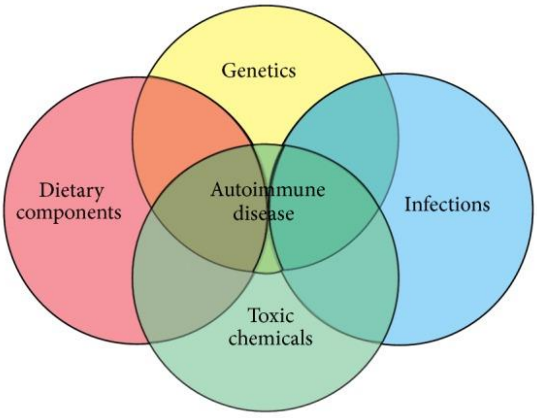

Figure 2. Representation of the factors that contribute to AID [18]*. Each factor is shown in a different colour.*

Diet has been postulated as a potential environmental risk factor for the growing incidence of AID in developed countries over recent decades. Salt (or sodium chloride; NaCl) is one dietary factor that is increasing due to the rising consumption of processed foods or 'fast foods' [19]. In these types of diet the sodium (Na) content can be more than 100 times higher compared to similar home-made meals [20] [21]. Although Na is an indispensable nutrient and is crucial for cellular function in appropriate amounts, the high intake of salt has far-reaching effects on cardiovascular, renal, and endocrine diseases. A Western lifestyle with a high-salt intake can induce hypertension, which is a leading contributor to cardiovascular morbidity and mortality worldwide. Moreover, a high-salt diet is linked to an increased risk of cerebrovascular diseases and dementia.

Interestingly, recent studies have demonstrated that a high-salt diet can promote the onset of MS and other related AID, which implicates high-salt consumption as an environmental trigger in autoimmunity [22]. The high-salt environment has also been shown to dramatically reduce the functionality of thymus-derived Tregs, which is needed to preserve self-tolerance, and to promote the differentiation of Th17 cells [22] [23]. However, new data demonstrated that induced Tregs were completely stable and fully functional under high salt conditions [22]. Furthermore, excessive salt intake reduced the lactobacilli in the gut, while the number of Th17 cells increased [24]. When the animals were given probiotic lactobacilli along with a high-salt diet, however, the frequency of Th17 helper cells decreased. The probiotics also alleviated the clinical symptoms of experimental autoimmune encephalomyelitis (EAE), a disease model for MS. In an animal model of SLE, a high-salt diet was associated with reduced survival [25] [26]. In RA research, for example, joint inflammation has been found to be more severe in mice on a high-salt diet compared to animals on a normal chow [23].

Clinical and epidemiological studies indicated that a high-salt intake positively correlated with increases in disease activity in patients with RA and MS [22].

1.4 TYPE 1 DIABETES

Diabetes mellitus (DM), commonly known as diabetes, describes a group of metabolic disorders of multiple etiology characterized by chronic hyperglycemia [27]. The classic symptoms are frequent urination, increased thirst and hunger and weight loss, whereas additional symptomes may include blurry vision, feeling tired and poor wound healing [28] [29].

There are three main types of DM. T1D is caused by the selective destruction of the insulin-producing beta cells in the pancreas, which usually leads to an absolute insulin deficiency [30]. T1D patients must inject themselves with insulin in order to stay alive. This form was previously denoted as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes" [31]. T1D typically occurs in children or young adults and is more prevalent in Northern Europeans and their descendants. Type 2 diabetes begins with insulin resistance, a condition in which cells fail to properly respond to insulin and, with the progression of the disease, a lack of insulin may also develop [32]. This form was previously referred as "non-insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". T2D comprises 90% of people with diabetes worldwide [33] and is largely the result of excess body weight and physical inactivity [31]. Gestational diabetes is the third major form, and occurs when pregnant women without a previous history of diabetes develop high blood glucose values [30]. All diabetic patients must balance their food intake and exercise and carefully monitor their blood glucose values.

Although the percentage of individuals with T1D is very small compared to the total number of patients with diabetes, the global prevalence of this type is estimated at 20 million patients [30]. Moreover, the worldwide incidence of T1D is growing with the strongest increase in children under 5 years of age. In this case, symptoms typically develop in a short period of time [34]. T1D is a T-cell-mediated AID. As explained above, genetic predisposition, but also environmental factors, are important for the development of T1D. T1D begins as a misdirected inflammatory response against the pancreatic insulin-producing beta cells that results in immune infiltration in the islets of Langerhans also known as insulitis [31].

The non-obese diabetic (NOD) mouse is an important model of T1D but also of other AID such as SS [35], thyroiditis [36] , prostatitis in male mice [37], peripheral polyneuropathy [38], and a few aspects of non-organ-specific AID such as late-onset anti-nuclear antibodies, a SLE-like disease after exposure to death mycobacteria [39]. The mouse develops a chronic lymphocytic infiltration of endocrine and exocrine glands. The strength of this inbred mouse strain for this project is that it spontaneously develops T1D, and shares many similarities with the human T1D situation. NOD mice have a generalized defect in their ability to generate effective numbers of Tregs. The incidence of spontaneous diabetes in the NOD mouse is between 50 and 70% in females and between 20 and 40% in males at 30 weeks of age and, when mice are maintained in a relatively germ-free environment, the incidence is higher than in mice in conventional “dirty” housing

facilities. Moreover, autoimmune diabetes is a food-influenced disease and interestingly, the diabetogenic agents are not carbohydrates but come from the protein fraction of natural food like wheat flour and soybean proteins. Diabetes onset typically occurs between 12 and 14 weeks of age in female mice and slightly later in male mice, which is early when compared to the onset of SS, which occurs at a later age (around 16 weeks of age). T1D and SS develop independently of each other.

Immune cell infiltrates are observed in the islets of Langerhans from approximately 3-4 weeks of age and most mice exhibit severe insulitis by 10 weeks of age [40] as depicted in Figure 3. Focal inflammation in the submandibular salivary glands and the lacrimal glands starts at approximately 12-16 weeks of age and increases with age, while simultaneously salivary function declines until complete secretory dysfunction by 16-18 weeks of age.

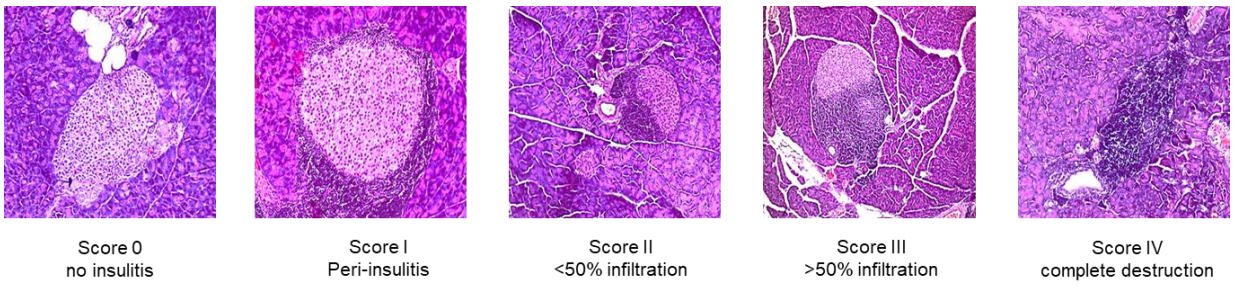

***Figure 3**. For the evaluation of insulitis the following score can be used: 0 (white - intact islets), 1 (vertical lines - peri-insulitis), 2 (grey - <50% infiltration), 3 (horizontal lines - >50% infiltration), IV (black - complete destruction).*

2. AIMS

2.1 GLOBAL OBJECTIVE

Prior studies have indicated that excessive salt consumption affects the immune system by shifting the immune balance towards a proinflammatory state which can cause the aggravation of AID such as SLE, MS, colitis and RA. They also noticed that a high-salt environment dramatically reduced Treg functionality. Therefore, the **overall objective** of the project was to study whether a high-salt diet can trigger the development of AID, focusing primarily on SS and later on T1D, in the NOD mouse model.

2.2 SPECIFIC AIMS

In this dissertation, **I concentrated on T1D** and investigated whether a high-salt intake can **elicit the development of T1D in NOD mice with ongoing islet inflammation**. Therefore, we studied different symptoms of the disease (i.e. blood glucose values, glucose tolerance, weight, food and water consumption) in 16-week-old mice fed with either a normal-salt or high-salt diet during 8 weeks. In a second aim, we wanted to study whether a high-salt environment had a negative impact on the frequencies of $CD4^+$ T cells, in particular Tregs, and $CD8^+$ T cells.

3. MATERIALS AND METHODS

3.1 SCHEMATIC OVERVIEW OF THE PROJECT

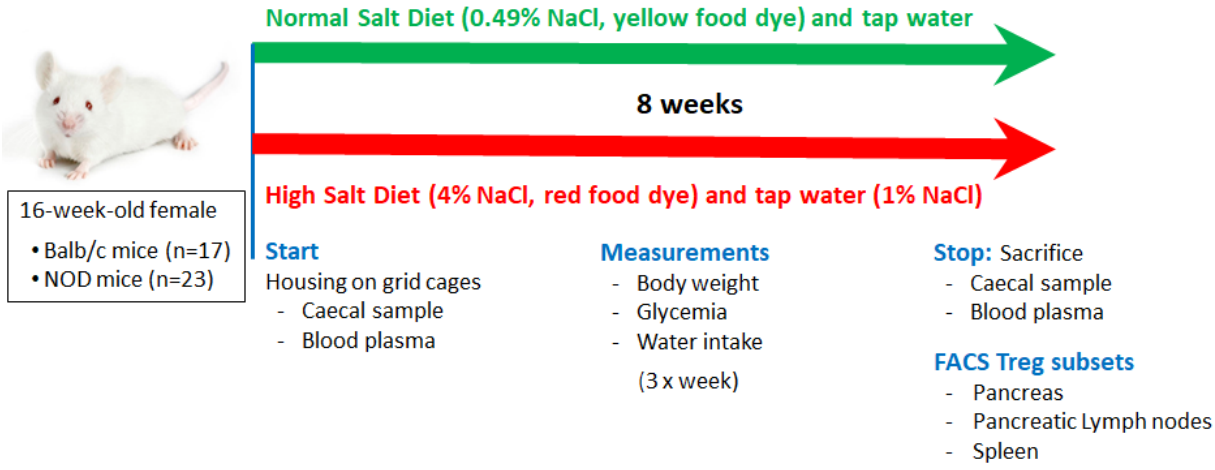

Figure 4: Experimental design to study the impact of high-salt diet on T1D development in NOD mouse model. *The normal salt diet (NSD) is shown in green and the high-salt diet (HSD) in red. All the information about the figure is explained below.*

3.2 INFORMATION ABOUT THE MICE

Salivary and lacrimal gland infiltration occurs later than in the pancreas at approximately 12-16 weeks of age, followed by secretory dysfunction by 16-18 weeks of age. Therefore, 16-week-old female mice, either diabetes-prone NOD mice, inbred at KULeuven since 1989, or commonly used Balb/c mice, purchased from Envigo (Horst, The Netherlands) and inbred at KULeuven, were randomly allocated into four experimental groups (see section 3.3 diets) and monitored during 8 weeks (see **Figure 4**). NOD mice were non-diabetic when starting the experiment. Mice were housed in grid cages at the animal facilities of the KULeuven. All the experimental protocols were reviewed and approved by the Ethics Committee of the KULeuven (Project no. 154-2018). During my training at KULeuven, I obtained my FELASA B certificate.

3.3 DIETS

Two types of diets were purchased from SSNIFF (normal-salt diet (NSD; E15430-047) and high-salt diet (HSD; E15431-347); Soest, Germany). Diets were gamma-irradiated (25 kGy) and were identical in composition (21.1% crude protein, 5.1% crude fat, 5.0% crude fibre, 9.2% crude ash, 26.9% starch, 11% sugar, different vitamins and minerals) except for NaCl content (NSD: 0.4% NaCl, HSD: 4% NaCl) [41]. Mice were maintained on the respective diet during the course of the experiment. Drinking water for HSD animals was supplemented with 1% NaCl.

3.4 MEASUREMENTS

Weight and glycemia

During the 8-week follow-up, mice were weighed twice weekly. Blood glucose values were measured with glucometer (Accu-Check Aviva glucometer, F. Hoffmann-La Roche Ltd., Basel, Switzerland) with the same schedule.

Intraperitoneal glucose tolerance test (IPGTT)

After 7 weeks of feeding, at the start of the 12-h light/dark cycle, food was removed but leaving mice unrestricted access to water. Following a 6 h starvation, mice were administered glucose by gavage (2 g/kg of body weight) and glucose concentrations were measured on blood drops taken from the tip of the tail at 0, 15, 30, 60, 90 and 120 min after glucose injection using a glucometer. Once the experiment was finished, mice were fed again.

Indirect calorimetry

Calocages are special cages for metabolic and behavioural investigations. A series of sensors are combined to integrate a vast amount of information for each animal [42]. For these experiments, mice already on the respective diet for 6 weeks were allowed to adapt for 7 days to single housing and specific drinking/feeding baskets. Thereafter, animals were individually housed in automated cages for indirect calorimetry (TSE Phenomaster Calocages, Bad Homburg, Germany) in a room at 22 °C ambient temperature and a 12-h light/dark cycle with *ad libitum* access to food and water. Food and water intake, O_2 consumption, CO_2 production, respiratory exchange ratio (RER, calculated as VCO_2/VO_2), heat production, mean temperature, and ambulatory activity were recorded over a 72 h period.

Urine collection

After 7 weeks of feeding, mice of all experimental groups were housed individually in metabolic cages and provided with drinking water freely to collected 24 h urine. Then, the 24 h urine samples were sent to the hospital laboratory for sodium ion testing.

Fecal sampling

For fecal microbiome analyses, mice of each strain maintained on NSD until 16-weeks of age were randomly distributed over the NSD and HSD groups. To avoid coprophagia, mice were housed in grid cages. Fresh fecal pellets were collected, both at start and stop of the experiment, directly from the anal orifices, immediately flash-frozen in liquid nitrogen and stored at -80°C for later analyses. These analyses are not included in this dissertation.

3.5 DISSECTION

After 8 weeks of feeding, mice were sacrificed, blood was taken by heart puncture and the following organs were collected: spleen, pancreas and pancreatic draining lymph nodes. We also collected salivary glands (e.g. submandibular and parotid) for SS project. Before the dissection, weight and glycemia were measured. The organs were placed in washing medium (RPMI 1640 medium, GlutaMAX™ supplement, HEPES cat# 72400054 Thermofisher, 2% fetal bovine serum (FCS; cat# 10270-106 Gibco, 5% penicillin-streptomycin (PEN/STREP; cat# 15140122 Thermofisher) until processing.

3.6 SINGLE CELL ISOLATION AND FACS (FLUORESCENCE ACTIVATED CELL SORTER) STAINING

Pancreas samples were minced and placed in pre-warmed digestion cocktail containing collagenase in Gentle MACS C tubes (Miltenyi Biotec cat# 130-093-237) in a Gentle MACS dissociator. Samples were incubated for 30 min in a warm water bath at 37℃ and vortexed every 10 minutes. The samples were re-suspended and passed through a cell strainer (70 µm Greiner Bio-One cat# 542070) into a 50 mL tube (Greiner Cellstar 50 mL cat# 227261). Thereafter, samples were washed and rinsed with 10 mL of washing medium. The cell suspension obtained was centrifuged at 300g (Rotina 420R, Hettich Zentrifugen), 5 min, washed with 5 mL of FACS buffer (DPBS cat# 14190144 Thermofisher, 2 mM EDTA Ambion cat# AM9260G, 0.1% bovine serum albumin (BSA)) and counted (Bürker chamber Marienfeld). Pancreatic lymph nodes and spleen were processed separately and transferred to cell strainers in 50 mL tubes, crushed with the tip of a 2.5 mL syringe and rinsed with 10 mL of washing medium. The pancreatic lymph nodes were transferred to 15 mL conical tubes (Corning 15 mL PP cat# COR430766 Elscolab) before centrifugation. All samples were spun down at 400g, 5 min and the supernatant was poured off. The pancreatic lymph nodes were resuspended in 250 µL of FACS buffer. For the spleen, red blood cell lysis was performed by mixing with 5 mL NH_4Cl, incubated in a warm water

bath at 37℃ for 3 min and 20 mL of washing medium was added to neutralize the NH_4Cl. The samples were spun down, the supernatant poured off and resuspended in 10 mL of washing medium for counting.

For the Treg staining, the following fluorochrome-conjugated antibodies were used: PerCP-Cy5.5 anti-CD3 (clone 145-2C11, eBioscience), PE-Cy7 anti-CD25 (clone PC61.5, eBioscience), APC-H7 anti-CD4 (clone GK1.5, BD Pharmingen), eFlour450 anti-CD8a (clone 53-6,7, eBioscience) and APC-anti FoxP3 (clone FJK-16s, eBioscience). To block Fc receptors, the CD16-CD32 (clone 93, eBioscience) mouse antibody was used. Zombie Yellow Fixable Viability dye (Biolegend, cat# 423104) was used to discriminate live/death cells. In the cell preparation, 10^6 cells/well were placed in a 96-well V-bottom plate (ThermoFischer Sterilin plates, cat# 634-009 VWR). For the blanc sample, 100 µL of spleen suspension was used and for the Zombie sample, 50 µL of live spleen cells and 50 µL of dead spleen cells were introduced. For the FMO´s samples, 100 µL of spleen suspension was used. In addition to the cells, single colour CompBeads (Ultracomp eBeads cat# 01-2222-42 Thermofisher) were placed in the 96-well V bottom plate (one drop of beads in each well). For the surface staining, plates were spun down at 400g, 3 min and supernatant was poured off. The samples were washed with 200 µL PBS (cat# 14190144 Thermofisher) to remove the excess protein, spun down and the supernatant was poured off. Cellular and Zombie samples were resuspended in 50 µL diluted Zombie Yellow Fixable Viability dye. All samples were incubated for 20 min at room temperature (RT) in the dark. The cells were washed twice with 200 µL of FACS buffer. The cells were incubated with antibodies against extracellular epitopes in the dark for 30 min. Cells were washed twice with 200 µL of FACS buffer. For the intracellular staining, fresh eBio Fix/Perm buffer and Perm/Wash (cat# 00-5523-00 Thermofisher) were prepared. 100 µL of fresh Fix/Perm was added per well (in cellular samples, Zombie, FMO´s and blanc samples) and pipetted up and down. The samples were incubated at RT for 30 min in the dark. 150 µL of Perm/Wash was added, spun down and flicked off supernatant. The samples were washed again with 200 µL of Perm/Wash. The cells were incubated with antibodies against intracellular epitopes in the dark for 30 min at RT. The samples were washed again twice and resuspended in 100 to 200 µL FACS buffer. The plate was kept at 4°C in the dark until acquisition. No extra fixation was needed.

3.7 FACS ACQUISITION AND ANALYSIS

The samples were acquired using Canto II flow cytometer and FACSDiva software. The results were analysed with FlowJo software (TreeStar, Ashland, OR). CompBeads were used to compensate the settings and FMO´s samples to better gate the populations. The gating strategy was as depicted in **Figure 5**. Live cells were gated using LIVE/DEAD staining for dead cells. Only the samples with a viability equal to or greater than 80% were further analysed. Within this group, single cells were obtained by forward-scatter height (FSC-H) versus forward-scatter area (FSC-A). The lymphocytes were gated within the single cell subset. The $CD3^+$ population (T cells) was obtained from the lymphocyte gate. Within the $CD3^+$ T cell subset, two subpopulations were gated: $CD4^+$ and $CD8^+$. The expression of CD25, FoxP3 was analysed in the total population of $CD4^+$: $CD4^+$ $CD25^+$, $CD4^+$ $FoxP3^+$.

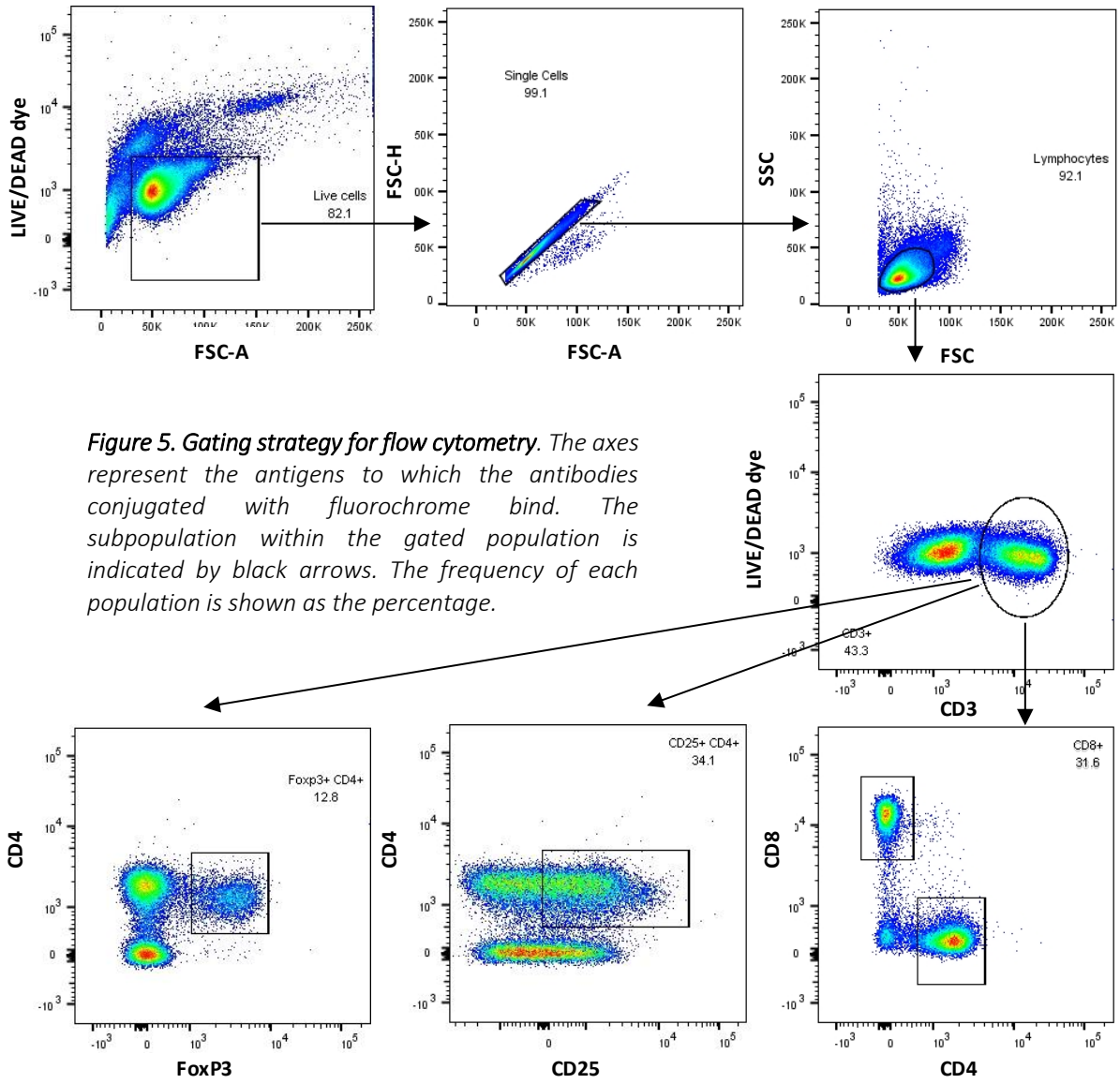

Figure 5. Gating strategy for flow cytometry. The axes represent the antigens to which the antibodies conjugated with fluorochrome bind. The subpopulation within the gated population is indicated by black arrows. The frequency of each population is shown as the percentage.

3.8 STATISTICS

All data were analysed using GraphPad Prism 8.1.1 (GraphPad Software, La Jolla, CA, USA). The results are expressed as means with error bars representing standard errors of the mean (SEM).

4. RESULTS

4.1 HIGH-SALT DIET DOES NOT TRIGGER T1D DEVELOPMENT IN NOD MICE, WHEN INITIATED AT 16 WEEKS OF AGE.

NOD mice were chosen for our study because this mouse strain is known to develop several autoimmune pathologies including T1D at an increasing incidence and severity with age. In our colony, the first signs of spontaneous diabetes appear in 12- to 14-week-old female mice. At 26 weeks of age, 50-60% of female mice normally exhibit obvious hyperglycemia. While T1D incidence and severity may differ among various colonies, comparable age-dependent effects of T1D have been reported. Here, our aim was to test whether a HSD (4% NaCl chow and 1% saline water to drink), initiated at 16 weeks of age when islet inflammation is ongoing, would trigger the development of T1D in female NOD mice. We applied a regimen previously used in models of hypertension and autoimmunity [18].

Surprisingly, as shown in **Figure 6**, placement of 16-week-old NOD mice on a HSD for 8 weeks did not increase mean blood glucose values nor did it elicit the development of T1D versus those on a NSD (0 out of 11 versus 0 out of 12 mice, respectively). As expected, mean blood glucose values recorded in naive Balb/c mice remained perfectly in normal range, irrespective of the diet. When analyzing the NOD data, although none of the mice developed overt T1D during the 8 week follow-up period, some mice on a HSD had short episodes of hyperglycemia in the initial weeks of the experiment.

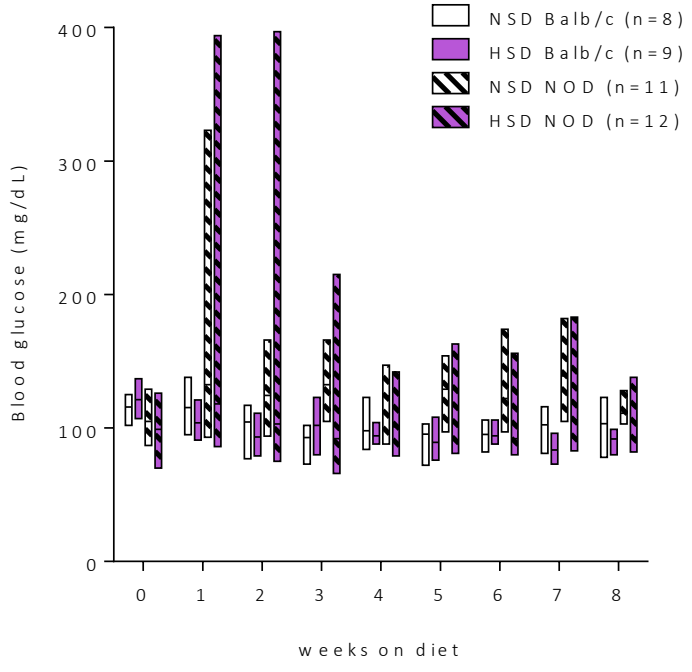

***Figure 6. Blood glucose measurements**. The time (in weeks on diet) is represented on the X-axis and the blood glucose values (in mg/dL) is represented on the Y-axis. NSD Balb/c (shown in a white box), HSD Balb/c (shown in a purple box), NSD NOD (shown in a white box with an angled striped pattern) and HSD (shown in a purple box with an angled striped pattern). Data are displayed in floating bar plots (displaying min-max and mean values).*

We also determined the degree of glycemic control by IPGTTs. Normoglycemic NOD mice already 7 weeks on a NSD or HSD showed a comparable although impaired glucose ultilization, with blood glucose values peaking after 30 min at more than 263 mg/dL and gradually lowering to 150 mg/dL at 120 min however not reaching initial blood glucose values (Figure 7). Peak blood glucose values were seen at 30 min rather than at 15 min, as exhibited by the naive Balb/c mice on both a NSD and HSD.

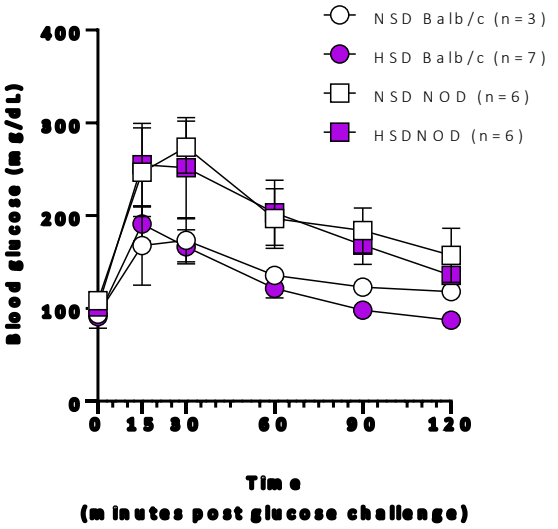

Figure 7. Intraperitoneal glucose tolerance test. Time (in minutes post glucose challenge) is plotted on the X-axis and the mean blood glucose values (in mg/dL) are plotted on the Y-axis. The four groups of mice are as follows: NSD Balb/c (shown in a white circle), HSD Balb/c (shown in a purple circle), NSD NOD (shown in a white square) and HSD NOD (shown in a purple square). Data are mean ± SEM.

4.2 BODY WEIGHT, FOOD AND WATER INTAKE, URINE, INDIRECT CALORIMETRY IN DIABETES-PRONE NOD MICE VERSUS NAÏVE BALB/C MICE ON A HSD

Diabetes-prone NOD mice and naïve Balb/c mice of 16 weeks of age were *ad libitum* fed with a NSD or HSD for 8 weeks. Although the Balb/c mice have lower mean body weights than the NOD mice at the start of the observation period, both groups of animals displayed similar weight distribution on a NSD and HSD (Figure 8).

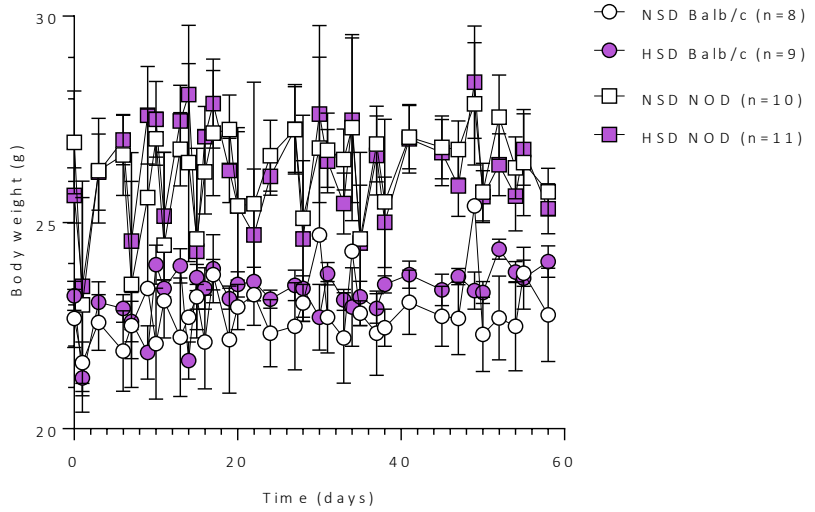

Figure 8. Weight measurements. The time (in days on diet) is plotted on the X-axis and the body weight (in g) is plotted on the Y-axis. The four groups of mice are as follows: NSD Balb/c (shown in a white circle), HSD Balb/c (shown in a purple circle), NSD NOD (shown in a white square) and HSD NOD (shown in a purple square). Data are mean ± SEM.

We chose to evaluate mouse behavior during the 12-h light/dark cycle using automated in-cage analysis tools. First, we observed that the food intake was not different between the two mouse strains, irrespective of the diet, but exhibited a nocturnal increase and then declined during the day (Figure 9A). Second, the NOD mice had a greater water intake than the naïve Balb/c mice on a NSD (3.34 ± 0.33 and 2.00 ± 0.57 mL/mouse/24 h, respectively), again with a distinct diurnal and nocturnal pattern. As expected, the HSD mice drank more than the NSD mice, but this behavior was more apparent in the NOD mice (3.61 ± 0.53 and 6.84 ± 2.72 mL/mouse/24 h in HSD Balb/c mice and HSD NOD mice, respectively)(Figure 9B). Although the data is still preliminary, we observed that NOD mice had a 33 % lower urine volume after a 24 h observation in metabolic cages compared to Balb/c mice on a NSD (1.75 ± 0.35 and 5.40 ± 1.27 mL/mouse/24 h, respectively). Balb/c mice on a HSD urinated less than their counterparts on a NSD, but this was less obvious in NOD mice (Figure 9C). Interestingly, the sodium ion concentration in urine of the HSD mice was significantly higher than that in NSD mice but did not differ between the two mouse strains, (Figure 9D). Finally, using indirect calorimetry, maximal O_2 consumption (VO_2) and CO_2 production (VCO_2), RER, mean temperature, heat production, and ambulatory activity were calculated for the entire light and dark cycles during a period of 5 days. Both Balb/c and NOD mice were more active during the night cycle. However, we observed that a HSD had a negative impact on the ambulatory activity compared to a NSD, especially in the NOD mice (8743 ± 1233 and 10640 ± 4074 counts in HSD NOD and NSD NOD mice, respectively)(Figure 9E). There were no apparent differences between the diets during the light cycle. Moreover, O_2 utilization and CO_2 production, RER, the mean temperature and heat production were similar in the Balb/c mice compared to the NOD mice on either a NSD or a HSD.

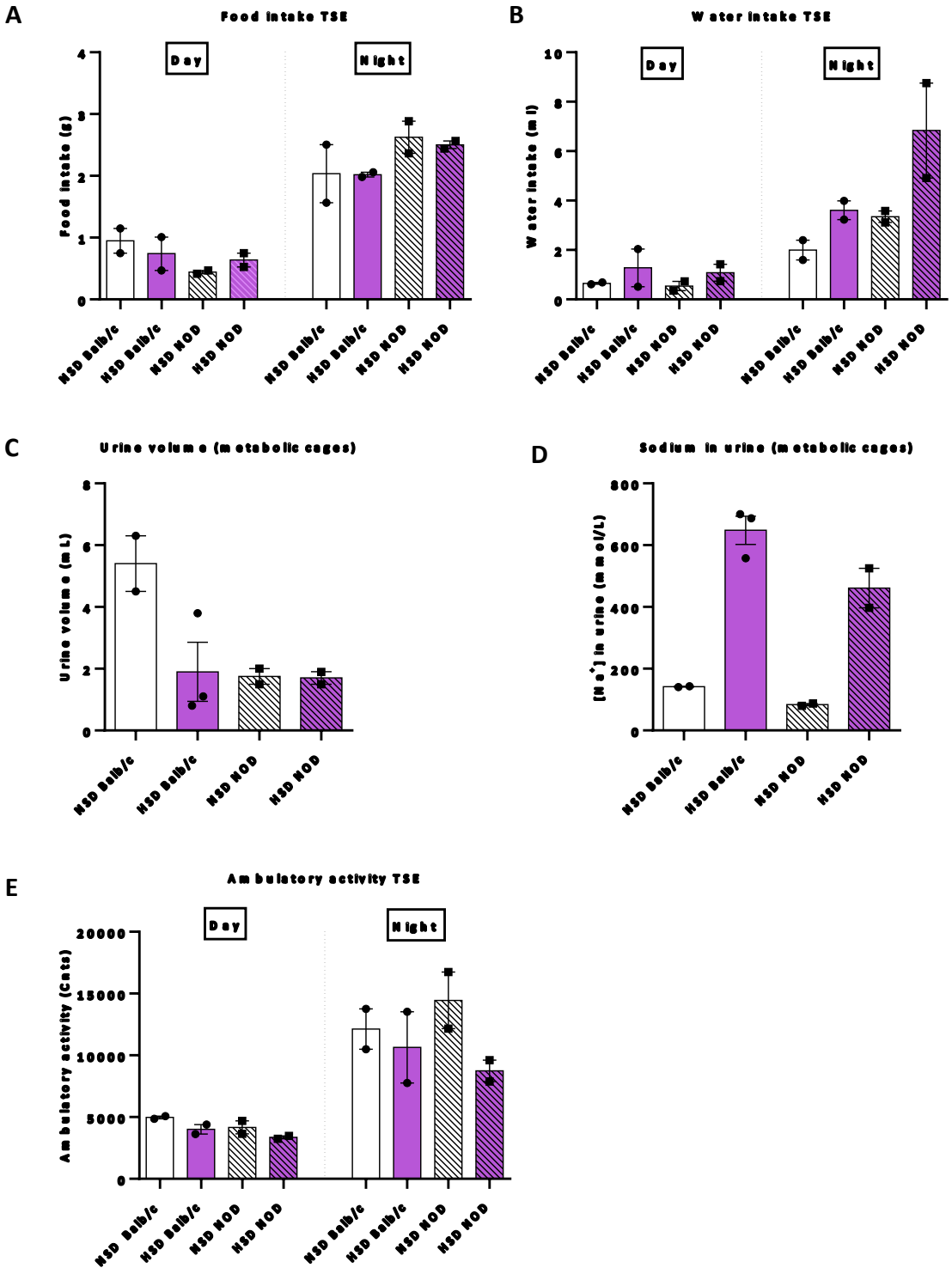

Figure 9. Parameters measured by indirect calorimetry (TSE system) or in metabolic cages. (A) Food intake (in g), (B) water intake (in mL), (C) urine volume (in mL for 24 h in metabolic cages), (D) sodium ion in urine (in mmol/L in urine collected in metabolic cages), and (E) ambulatory activity (in counts). NSD Balb/c (shown in a white box), HSD Balb/c (shown in a purple box), NSD NOD (shown in a white box with an angled stripped pattern) and HSD NOD (shown in a purple box with an angled stripped pattern). Data are presented In scatter dot plots with mean ± SEM. Each dot represents a different animal.

4.3 FLOW CYTOMETER ANALYSIS MAINLY FOCUSING ON THE TREG SUBSET

As a HSD can profoundly impact on the immune system via numerous mechanisms [22] [23], we first measured the abundance of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in different tissues (e.g. spleen and pancreatic draining lymph nodes) in diabetes-prone NOD mice versus naive Balb/c mice, receiving a HSD compared to those on a NSD. We also studied pancreas samples but unfortunately encountered technical problems and had too few live cells to reliable analyze the acquired data. Although the data are still preliminary (n=2 mice per experimental group), FACS analysis of spleens and pancreatic draining lymph nodes did not reveal major changes in these T-cell populations between the NOD and Balb/c mice, irrespective of the diet fed (Figure 10). However, compared with Balb/c mice, NOD mice on a NSD diet tended to have a higher proportion of $CD4^+$ T cells and a lower proportion of $CD8^+$ T cells in their pancreatic draining lymph nodes, which was more pronounced in NOD mice on a HSD.

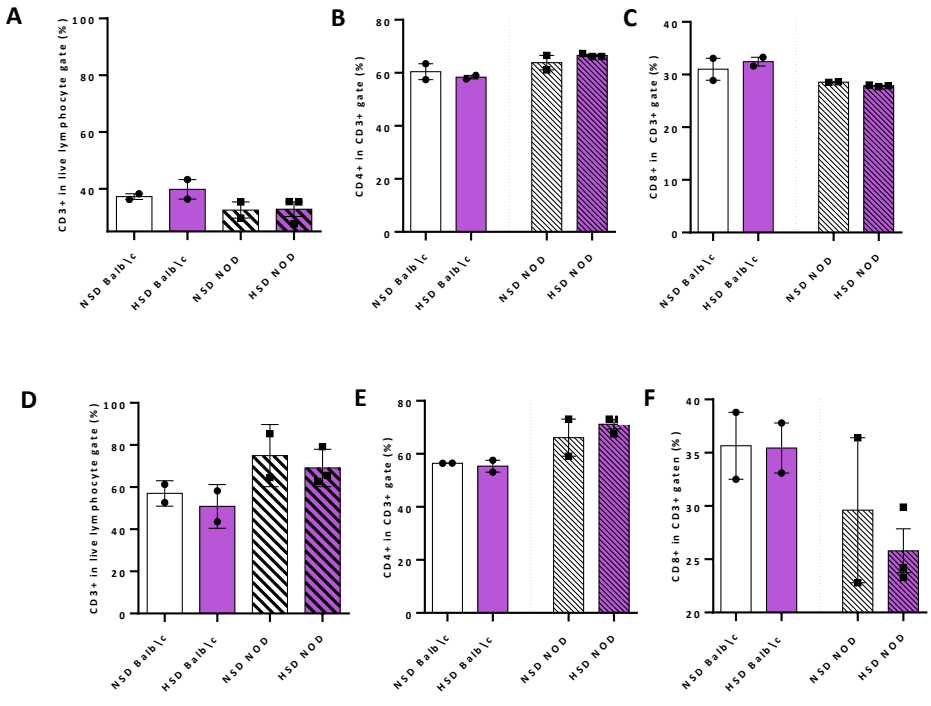

Figure 10. Flow cytometry analysis of spleen (A-C) and pancreatic draining lymph nodes (D-F) of diabetes-prone NOD mice on a HSD. *The abundance of different T-cell subsets is expressed: (A) $CD3^+$ T cells, (B) $CD4^+$ T cells, (C) $CD8^+$ T cells in spleen and (D) $CD3^+$T cells, (E) $CD4^+$ T cells, (F) $CD8^+$ T cells in pancreatic draining lymph nodes. NSD Balb/c (shown in a white box), HSD Balb/c (shown in a purple box), NSD NOD (shown in a white box with an angled striped pattern) and HSD NOD (shown in a purple box with an angled striped pattern). Data are presented In scatter dot plots with mean ± SEM. Each dot represents a different animal.*

The impact of excessive salt consumption on Treg frequencies and functionality is still under debate. In SLE and experimental colitis models, a HSD had little effect on the percentage of Tregs, although it significantly inhibited the suppressive function of Tregs [43]. Due to the limited sample size, it is difficult to make bold hypotheses. At present, we observed that a HSD did not change the percentage of $CD4^+Foxp3^+$ Tregs in spleen nor in pancreatic draining lymph nodes of NOD mice on a HSD compared to those on a NSD.

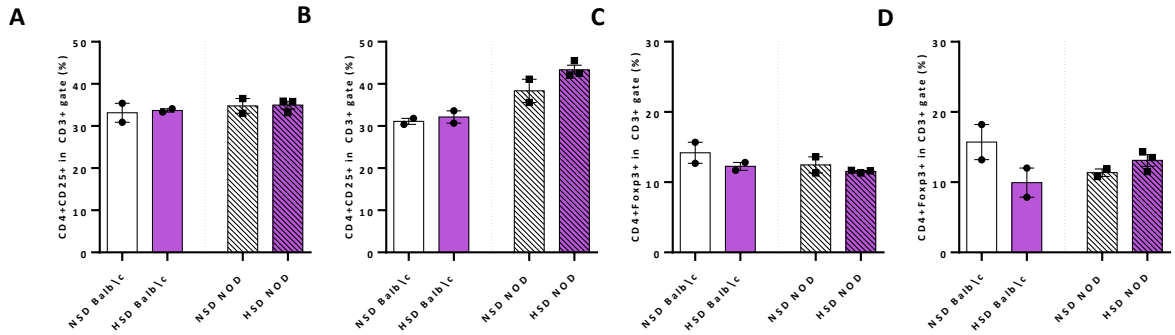

Figure 11. Flow cytometry analysis of spleen (A-C) and pancreatic draining lymph nodes (D-F) of diabetes-prone NOD mice on a HSD. *The abundance of Treg subsets is expressed: (A) $CD4^+CD25^+$ T cells in spleen (B) $CD4^+CD25^+$ T cells in pancreatic draining lymph nodes (C) $CD4^+$ $Foxp3^+$ T cells in spleen and (D) $CD4^+$ $Foxp3^+$ T cells in pancreatic draining lymph nodes. NSD Balb/c (shown in a white box), HSD Balb/c (shown in a purple box), NSD NOD (shown in a white box with an angled striped pattern) and HSD NOD (shown in a purple box with an angled striped pattern). Data are presented In scatter dot plots with mean ± SEM. Each dot represents a different animal.*

5. DISCUSSION

The overall objective of the project was to investigate whether a HSD, initiated at the early stages of SS around 16 weeks of age, would exacerbate the symptoms of the disease in the NOD mouse model. As this animal model also spontaneously develops T1D, I focused in this dissertation on whether placement of 16-week-old normoglycemic NOD mice on a HSD for 8 weeks would also elicit the development of T1D.

Here, we demonstrated that a high-salt regimen, which was previously shown to aggravate MS [22], SLE [26], colitis [44] and RA [45] in disease models, did not provoke the onset nor did it increase te severity of T1D in the NOD mice, even though the physiological impact of excessive salt intake was confirmed by enhanced water consumption and elevated concentrations of sodium ion in urine.

According to a recognized concept, high-salt consumption increases thirst and fluid intake. Efficacious excretion of excessive salt by the kidneys conversely increases the urine volume by osmotic diuresis and thereby corrects the extracellular volume [46]. Recent studies indicated that increasing salt intake indeed increased sodium excretion, but also unexpectedly caused the kidney to conserve water [47].

Although the mean blood glucose values of the HSD NOD mice were not different from the NSD NOD mice, we noticed especially in the initial weeks of the experiment that some HSD NOD mice developed transient hyperglycemia. It is remarkable that even in the instrumental EAE studies, a HSD aggravated the disease symptoms only during the first weeks of the dietary protocol [18] [49]. The HSD effects seemed to be temporary as no significant differences were found between the HSD and NSD animals at the end of the study around 25 days [48]. In the present work, it could also be that a HSD had only transient, non-sustained effects on T1D development in the NOD mice after the 8-week dieteray protocol. Furthermore, glucose utilization in the HSD NOD mice 7 weeks after feeding was not different from the NSD NOD mice but clearly impaired compared to naïve Balb/c mice on either a NSD or HSD. Similar transient observations were noted in a study performed by Kolypetri, E Randell, et al. who demonstrated that a HSD did not exacerbate murine autoimmune thyroiditis in the NOD.H2^{h4} mice after a 5-week feeding regimen [49]. Even more striking are the observations in CD86 knockout NOD mice, a model of chronic inflammatory demyelinating polyradiculoneuropathy, in which a HSD initiated at 7 weeks

of age and continued until 30 weeks of age ameliorated the clinical symptoms and the disease course with a reduced decline of locomotor function [50]. Additionally, they observed a reduced immune cell infiltration of sciatic nerves in CD86 knockout NOD mice which had received the HSD. Fascinatingly, several of these HSD studies have been conducted in AID models on NOD genetic background.

Still, significant effects on immune homeostasis and especially on peripherally-induced antigen-specific Th17 cells have been shown after high-salt intake [18]. After 8 weeks on a HSD, we only observed slightly higher frequecies of $CD4^+$ and lower frequencies of $CD8^+$ T cells in the pancreatic draining lymph nodes in NOD mice compared to those on a NSD. We did not yet investigated the frequencies of Th17 cells in the pancreas and its draining lymph nodes. However, although Th17 cells are strongly implicated in the development of EAE [51], lupus nephritis [52], collagen-induced arthritis [53], and experimental-induced colitis [54], their role in the initiation and progression of T1D is still under debate. Treatment with anti-IL-17 had no effect on T1D development in 5-week-old NOD mice, but prevented disease when treatment was started at 10 weeks of age [55]. Moreover, a HSD feeding has been shown to reverse the suppressive effects of Tregs [43]. In our study, we did not observe differences in the frequency of $CD4^+FoxP3^+$ Tregs in spleen nor in the pancreatic draining lymph nodes of mice on a HSD diet compared to those on a NSD. At present, we did not study the suppressive potential of the Tregs. Taking into account that we did not see an aggravation of T1D progression in the HSD NOD mice, we hypothesize that we will probably not see differences in the immune compartement of the HSD NOD mice.

Our data are puzzling since none of the 16-week-old NOD mice did develop T1D during the 8-week observation period, even not on the NSD. Both diets were purchased from SSNIFF and were purified diets with similar composition except for the NaCl content (0.4% versus 4% in NSD and HSD respectively). We had little information on the origin of the proteins, fat and fiber. After contacting the company, we discovered that the 21% protein was derived from casein, 5% fat from soybean oil and 5% fiber from refined cellulose. Although the effects of casein on T1D development are still controversial [56], many investigators demonstrated that casein-based diets significantly reduced the incidence of hyperglycemia [57] [58]. Therefore, we hypothesize that the high casein concentration in the HSD overruled the effects of the high-salt intake and prevented the progression of T1D in the HSD NOD mice. Moreover, the importance of early introduction of

a diet has been recognized in the NOD mice [59]. In addition, the maternal diet during pregnancy is already said to influence diabetes development in NOD progenies [60]. In future experiments, a HSD would have to be a natural ingredient diet supplemented with high NaCl content. Moreover, a HSD intervention might have more impact on T1D development when administered at a young age when the gut microbiome is still under development.

6. CONCLUSION

Excessive salt intake did not trigger the development of T1D in 16-week-old NOD mice with ongoing islet inflammation. Moreover, flow cytometry did not reveal important differences in major T-cell subsets in spleen and pancreatic draining lymph nodes of NOD mice on a HSD.

Our study had several shortcomings. First of all, we studied female diabetes-prone NOD mice of 16 weeks of age, the stage at which SS starts to develop but when islet inflammation is already fully established. An early dietary exposure around 3-5 weeks of age might be better to induce long-lasting effects on β-cell autoimmunity and T1D. Second, the composition of the diet is another point that should be taken into account as T1D development has been shown to be positively correlated with particular food ingredients. Purified diets containing casein and other products might negatively impact on the progression of the disease. Finally, the genetic background of the mice, being NOD, may have influenced the results. Other studies in which animals on the NOD background were used did not observe exacerbating effects of a HSD on autoimmunity, such as in autoimmune thyroiditis (NOD.H2^{h4} mouse model) [49] or did report improving effects in chronic inflammatory demyelinating polyradiculoneuropathy (CD86 knockout NOD mice) [50].

T1D is well known to be mediated by Th1 cells, but a role for Th17 cells in pathogenesis remains possible. My experiment is embedded in a broader project in which the number of mice will be increased and in which not only the abundance of Tregs, but also of Th17 cells will be studied.

In the future, I would propose to use young mice (<5 weeks of age) and a natural ingredient diet supplemented with 4% NaCl to study whether a high-salt intake would influence the development of AID, in particular T1D, in NOD mice at a later age.

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