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Molecular Mechanisms of Graves' Ophthalmopathy. A focus on smoking and radioiodine.

Shahida, Bushra

2021

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Shahida, B. (2021). *Molecular Mechanisms of Graves' Ophthalmopathy. A focus on smoking and radioiodine.* [Doctoral Thesis (compilation), Department of Clinical Sciences, Malmö]. Lund University, Faculty of Medicine.

Total number of authors:
1

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Molecular Mechanisms of Graves' Ophthalmopathy

A focus on smoking and radioiodine

BUSHRA SHAHIDA
CLINICAL SCIENCES, MALMÖ | FACULTY OF MEDICINE | LUND UNIVERSITY

BUSHRA SHAHIDA is a graduate in Biomedical Science from the University College Copenhagen, Denmark. She moved to Sweden in 2010 to pursue a Master's in Molecular Biology at Lund University. After completion of her Master's degree in 2013 she started as a research assistant at the Clinical Research Center, Malmo. She began her Ph.D. studies in 2016 at the Department of Genomics, Diabetes and Endocrinology, focusing on Graves' disease. The main focus of the thesis has been on investigating the effects of smoking and radioiodine on the pathogenesis of Graves' opthalmopathy as well as possible novel treatments. Bushra believes resilience is a wonderful trait to have which leads to a plethora of positive outcomes. She believes in kindness and admires the advice by Mewlana;

In Generosity and Helping others be like a River
In Compassion and Grace be like Sun
In Concealing others' faults be like Night
In Anger and Fury be like Dead
In Modesty and Humility be like Earth
In Tolerance be like a Sea
Either exist as you Are or be as you Look

Mewlana Jalāl ad-Dīn Mohammad Rūmī

Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences, Malmö
Genomics, Diabetes and Endocrinology

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2021:59
ISBN 978-91-8021-065-2
ISSN 1652-8220

“As you start to walk on the way.
The way appears”

Mewlana Jalāl ad-Dīn Mohammad Rūmī

Molecular Mechanisms of Graves' Ophthalmopathy

A focus on smoking and radioiodine

Bushra Shahida

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DOCTORAL DISSERTATION

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To be defended at Agardh Lecture Hall, CRC, Jan Waldenströms Gata 35, Malmö
Date June 9, 2021 at 9:00

Faculty opponent
Toke Bek, MD, PhD
University of Århus, Denmark

Organization LUND UNIVERSITY Department of Clinical Sciences, Malmö Genomics, Diabetes and Endocrinology Faculty of Medicine	**Document name** **DOCTORAL DISSERTATION**
	Date of issue June 9th 2021
Author Bushra Shahida	Sponsoring organization

Title and subtitle

Molecular Mechanisms of Graves' opthalmopathy,
A focus on smoking and radioiodine

Abstract

Graves' disease (GD) is an autoimmune disease characterized by hyperthyroidism and is caused by an interplay of genetic and environmental factors. One-third of patients with GD develop Graves' ophthalmopathy (GO). Key processes in the pathogenesis of GO are inflammation and adipogenesis in orbital tissue. Strong risk factors associated with the development of GO are smoking and radioiodine treatment for hyperthyroidism. The aim of this thesis was to explore the molecular mechanisms behind the effects of smoking and radioiodine treatment on the pathogenesis of GO.

In **Study I,** microarray results showed upregulation of *IL-1ß*, *IL-6* and adipocyte-related immediate early genes (IEGs), *CYR61* and *PTGS2,* in intraorbital adipose/connective tissue from smokers compared with nonsmokers with active GO. We confirmed the results with Reverse transcription polymerase chain reaction (RT-PCR). Using bioinformatic tools, we investigated whether the overexpressed genes were associated with pathways upregulated in adipogenesis. We found the overexpressed genes to be significantly associated with pathways involving inflammation, adipogenesis, and immune responses.

In **Study II,** we studied the direct effect of cigarette smoke extract (CSE) on the gene expression of *IL-1ß*, *IL-6*, *CYR61*, and *PTGS2* and on the adipogenesis process using an in vitro model. Orbital fibroblasts (OFs) were isolated from intraorbital adipose/connective tissue from GO patients. The OFs were exposed to CSE, and gene expression was measured. We found that CSE alone could upregulate the gene expression of *IEGs*, *IL-1ß,* and *IL-6* but not the late adipogenic genes *SCD* and *PPARγ*. Moreover, we investigated whether simvastatin could downregulate *IEGs*, *IL-6* and adipogenesis. Simvastatin downregulated *IEGs*, *IL-6,* and adipogenesis in OFs. Additionally, we found that IGF-1 treatment in 3T3-L1 preadipocytes led to mature adipocytes.

In **Study III,** we investigated the effect of CSE exposure and simvastatin and diclofenac treatment on peripheral blood mononuclear cells (PBMCs) isolated from newly diagnosed GD patients. We found that CSE exposure alone could upregulate the expression of *PTGS2*, *IL-1ß,* and *IL-6* and the release of PGE_2, IL-1ß, and IL-6. Furthermore, the proliferation of B lymphocytes and T lymphocytes was upregulated in response to CSE exposure. Simvastatin and diclofenac downregulated the gene expression of *PTGS2*, *IL-1ß,* and *IL-6* and the release of PGE_2, IL-1ß, and IL-6 by PBMCs. Furthermore, the proliferation of B- and T lymphocytes was downregulated. The effects of simvastatin and diclofenac were even stronger when the treatments were combined. Additionally, IGF-1-treated PBMCs upregulated the proliferation of B- and T lymphocytes.

In **Study IV,** GD patients were treated with radioiodine (RI). Thyrotropin receptor autoantibody (TRAb) levels were measured 3 months after treatment. DNA was collected using buccal swabs. We found that TRAb levels were significantly increased in 70% percent of the patients 3 months after RI treatment. TRAb levels were significantly elevated in the proportion of patients who developed GO after RI treatment compared to those GD patients that did not develop GO after RI treatment. A single nucleotide polymorphism (SNP) rs231775 in the *CTLA-4* gene was associated with TRAb levels in GD patients after RI treatment.

Key words Graves' disease. Graves' opthalmopathy, radioiodine, inflammation, adipogenesis, *IL-1ß, IL-6*. *PTGS2/COX2*, PGE_2, *CTLA-4*, B lymphocytes, T lymphocytes, Simvastatin, Diclofenac, SNP, gene expresion

Classification system and/or index terms (if any)		
Supplementary bibliographical information		**Language** English
ISSN and key title 1652-8220		**ISBN** 978-91-8021-065-2
Recipient's notes	**Number of pages** 109	Price
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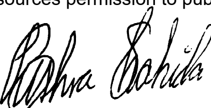
Signature  Date 2021-05-03

Molecular Mechanisms of Graves' Ophthalmopathy

A focus on smoking and radioiodine

Bushra Shahida

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Cover photo by the talented artist Alma Ahnlide. Cover concept by Bushra Shahida. The cover photo depicts a woman with bulging eyes shown in flowers and with a butterfly in the front of the neck, representing the thyroid gland.

Figures were created with Biorender.com, if not otherwise stated.

The quotes and poetry in the thesis, is written by Mewlana Jalāl ad-Dīn Mohammad Rūmī. He was a 13th-century Persian poet. His work generated a great following for its ability to bridge barriers, as it transcended cultures, ethnicities, languages and spirituality.

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Paper VI © Shahida et al. (Manuscript unpublished)

Faculty of Medicine
Department of Clinical Sciences, Malmö. Lund University

ISBN 978-91-8021-065-2
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2020

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se

To my dear parents. You are the reason behind everything I am today, thank you for your unconditional support.

“No one can take your place in existence or absence”

Mewlana Jalāl ad-Dīn Mohammad Rūmī

There is a lifeforce
Within your soul,
Seek that life.
There is a gem in the body,
seek that mine.
O traveller, if you are in search of that,
don’t look outside,
look inside yourself
And seek That.

Mewlana Jalāl ad-Dīn Mohammad Rūmī

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I. Planck T, **Shahida B**, Parikh H, Ström K, Åsman P, Brorson H, Hallengren B, Lantz M. 2014. Smoking Induces Overexpression of Immediate Early Genes in Active Graves' Ophthalmopathy. Thyroid 24, 1524–1532.

II. **Shahida B**, Johnson P.S, Jain R, Brorson H, Åsman P, Planck T, Lantz M. 2019. Simvastatin downregulates adipogenesis in 3T3-L1 preadipocytes and orbital fibroblasts from Graves´ ophthalmopathy patients. Endocrine Connections 8, 1230–1239.

III. **Shahida B**, Planck T, Singh T, Lantz M. 2021. Effects of smoking on inflammatory and proliferation markers in peripheral blood mononuclear cells from Graves' patients and Healthy individuals. *Manuscript not submitted yet*

IV. **Shahida, B**, Tsoumani K, Planck T, Modhukur V, Asp P, Sundlöv A, Tennvall J, Åsman P, Lindgren O, Lantz M. 2021. Increased risk of Graves´ophthalmopathy in patients with increasing TRAb after radioiodine treatment – The impact of prednisolone treatment and risk genes. *Manuscript submitted*

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Abbreviations

AITD	Autoimmune thyroid diseases
ANG II	Angiotensin II
Anti-TG	Antithyroglobulin (anti-TG)
Anti-TPO	Anti–thyroid peroxidase
APC	Antigen presenting cells
ATD	Antithyroid drugs
BCR	B-cell receptor
BTG2	B cell translocation gene 2
C/EBPα	CCAAT/enhancer binding protein α
cAMP	Cyclic adenosine monophosphate
CD40	B-cell surface antigen CD40
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CTLA-4	Cytotoxic T lymphocyte associated factor 4
CYR61	Cysteine-rich angiogenic inducer 61
DIO2	Deiodinase type 2
DUSP1	Dual specificity phosphatase 1
EGR1	Early growth response 1
FCRL3	Fc receptor-like 3 gene
FOXP3	Forkhead box P3
GAGs	Glycosaminoglycans
GPCR	G-protein coupled receptor
GWAS	Genome Wide Association Studies
HLA-DR	Human leukocyte antigen
HMG-CoA	Reductase 3-Hydroxy-3-methylglutaryl coenzyme A reductase
ICAM-1	Intracellular adhesion molecule 1
IEGs	Immediate early genes
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor 1
IgG	Circulating immunoglobulin G
IL-10	Interleukin-10
IL-1ß	Interlekin-1ß
IL-2	Interleukin-2

IL-4	Interleukin-4
IL-6	Interleukin-6
LDL-C	Low-density lipoprotein cholesterol
MHC	Major Histocompatibility Complex
NGS	Next-generation sequencing
NSAID	Nonsteroidal anti-inflammatory drug
OFs	Orbital fibroblasts
PGE_2	Prostaglandin E_2
PPARγ	Peroxisome proliferator-activated receptor γ
PTPN22	Protein thyrosine phosphatase-22
RA	Rheumathoid arthritis
RTX	Rituximab
SCD	Stearoyl-coenzyme A desaturase
SNPs	Single nucleotide polymorphisms
T3	Trijodthyronine
T4	Thyroxine
TAO	Thyroid-associated ophthalmopathy
TCR	T-cell receptor
Tg	Thyroglobulin
TGF-ß	Transforming growth factor-beta
Th1	T-helper 1 lymphocytes
Th2	T-helper 2 lymphocytes
TNF-α	Tumor necrosis factor- α
TRAb	Antibodies against TSHR
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
XCI	Skewed inactivation of X chromosome

Introduction

Graves’ disease

Historical notes

Graves’ disease (GD) was named after the Irish physician Robert James Graves, who described a case of goiter, palpitations and exophthalmos in 1835(1), and the German physician Karl Adolph von Basedow independently reported the same form of symptoms in 1840 and 1848(2). However, the condition was already mentioned as early as the fifth century BC, in writings by Aristotle and Xenophon. Later, in 1110, the Persian physician Abu-l-Fadail Ismail ibn al-Hussain al Jurjani, in his work dedicated to the Shah of Khwarazm, a Treasure of Medicine, clearly described the combination of exophthalmos with goiter. Centuries later, a few reports about patients with exophthalmos and goiter are described, but none of the authors could see the significance of the connection between these two conditions. Without knowing these connections, physicians such as Charles de Saint-Yves mentioned the combination of exophthalmos and goiter in 1722, and Guiseppe Flajani reported in 1802 that he had successfully treated two patients with exophthalmic goiter and palpitations. Finally, Caleb Hillier Perry described the first truly classic case in 1786(1, 3). Many researchers before Robert James Graves and Karl Adolph von Basedow contributed to the understanding and knowledge about GD, but Graves’ disease and Basedow’s disease is the most widely used terms for the disease.

Epidemiology

The most common cause of hyperthyroidism is Graves’ disease (GD), which accounts for 60-80% of hyperthyroid cases(4). During the period of 2003-2005, the incidence of GD in Sweden was 21/100 000 individuals per year(5). GD is a complex disease caused by an interplay of genetic, environmental and endogenous factors, such as age, female sex, fetal microchimerism and pregnancy(6). While this illness can occur at any age, it is most commonly seen between 40 and 60 years(7), and the incidence is 5-10 times higher in women(4, 6, 8).

In Malmo, Sweden the peak incidence of GD in women is at 50-60 years.(7). The reasons for the higher incidence in women are not fully understood, but one suggestion is that hormonal changes at menopause may be an explanation(7). Another explanation may be the skewed inactivation of the X chromosome (XCI) in women. Females inherit one X chromosome by their father and one by their mother, and one of these X chromosomes is inactivated by random methylation in early embryonic life(9). Hence, females are mosaics for the genetic activity of the maternal and paternal X chromosomes, where approximately 50% of genetic activity accounts for each(10). Skewing is defined as ≥80% activation of either the paternal or maternal X chromosome in the same tissue, and the associations of XCI with GD and with autoimmune thyroid diseases (AITD) suggest that epigenetic phenomena may contribute to this relationship(9, 10).

Environmental factors such as smoking, dietary iodine and emotional stress are some of the frequently cited factors(11) (Figure 1). Endogenous factors such as fetal microchimerism and the passage of cells from fetus to mother at the early stage of pregnancy have been reported at the onset of thyroid immune disease(12). In populations with severe iodine deficiency, goiter and hypothyroidism are common, while mild to moderate deficiency may result in hyperthyroidism in some individuals, despite increased activity of the thyroid to maintain euthyroidism(13). In Sweden, the iodization of table salt was introduced in 1936, and according to World Health Organization (WHO), the Swedish population is iodine-sufficient(14). Other environmental factors, such as infection with *Yersinia enterolitica, Coxsackie B, retrovirus, Helicobacter pylori and hepatitis C virus,* have been associated with GD(12, 15, 16), as well as drugs such as immunotherapy or antiretroviral medications(12, 17). Reduced vitamin D levels have been associated with new-onset GD(18-20) and with increased TRAb titers in GD patients(21). However, the underlying mechanism remains unsolved, and further studies are needed to define the role of vitamin D treatment in GD(19).

Dysbiosis, which is an alteration in bacterial function and diversity in the gut, may contribute to autoimmune diseases such as rheumatoid arthritis, diabetes mellitus type I, and, now, GD(22). Next-generation sequencing has been applied to study microbiota in GD patients with and without GO, and the accumulated data from these studies suggest that the microbial composition in GD patients with and without GD is altered compared to controls and may be associated with the development of GD and/or GO(23-25). Cigarette smoking is a strong risk factor for developing GD with an odds ratio (OR) of 3.3, and the risk increases with the number of cigarettes smoked daily(26, 27). Studies have shown that smoking decreases the levels of thyroid stimulating hormone (TSH), increases thyroid hormones, and may also reduce the efficiency of treatment, thus having a significant effect on thyroid function(28).

Genetics in GD

Based on twin studies that showed a concordance of 35-36% in monozygotic twins compared to 3-7% in dizygotic twins, it is well known that genetic factors contribute to the onset of GD (29). More recently, a study including two large twin cohorts attributed a 75-80% role to genetic factors versus 20-25% to environmental factors(30). Since the early 1970s, there has been a race to explain the genetic contribution to GD, and it seems that the predisposition is polygenic. Several different approaches have been applied to investigate genetic predisposition. First, linkage analysis was used, involving a series of microsatellite markers spread across the genome, looking for cosegregation in family-based data sets. Later, the candidate gene approach was used, investigating single nucleotide polymorphisms (SNPs). More recently, genome-wide association studies (GWAS), next-generation sequencing (NGS) and bioinformatics have identified many predisposing genes involved in GD(12, 31). However, only a few susceptibility genes from the studies have been replicated, including, cytotoxic T lymphocyte-associated factor 4 (*CTLA-4*), B-cell surface antigen CD40 (*CD40*), protein thyrosine phosphatase-22 (*PTPN22*), thyroglobulin (*Tg*), thyroid-stimulating hormone receptor (*TSHR*), forkhead box P3 (*FOXP3*), Fc receptor-like 3 gene (*FCRL3*)(32-34) and human leukocyte antigen (*HLA-DR*) in which four class II HLAs have been associated in Caucasian populations (Table 1)(35). Other genes have been associated with GD, such as, deiodinase type 2 (*DIO2*), interleukin-6 (*IL-6*), interleukin-10 (*IL-10*), interlukin-2 (*IL-2*)(32, 34, 36-38). GD susceptibility genes include both target-tissue-regulatory and immune-regulatory genes. In recent years, studies have shown that the interactions between environmental and genetic factors are due to epigenetic-genetic interactions. Genetic risk alone contributes to a moderately increased risk, whereas the risk is magnified when combined with synergistic epigenetic modifications of the regulatory regions controlling gene expression. Epigenetic effects include RNA interference through microRNAs, DNA methylation and histone modification, which can all increase the risk associated with an inherited polymorphism. These combined effects lead to an elevated risk of disease. It is believed that understanding these synergistic effects will lead to promising therapeutic targets(36).

Table 1: Association with GD. Adapted from (35, 36, 39, 40)

Gene	Associated Variants	Populations
CTLA-4	A/G 49 SNP (rs231775) 3'UTR (AT) microsatellite	Caucasian
HLA-DR	*DR3* *DQB1*03:01* *DQA1*05* *DQB1*02*	Caucasian
CD40	CT (Kozak) SNP	Caucasian Japanese Koreans
TSHR	Intron 1 and 7 SNPs	Caucasian Japanese
PTPN22	R620W SNP	Caucasian
Tg	S374A SNP M1028V SNP T2334C SNP	Japanese Caucasian
FOXP3	rs3761548 rs3761549	Asians
FCRL3	rs3761959 rs11264798	Caucasian

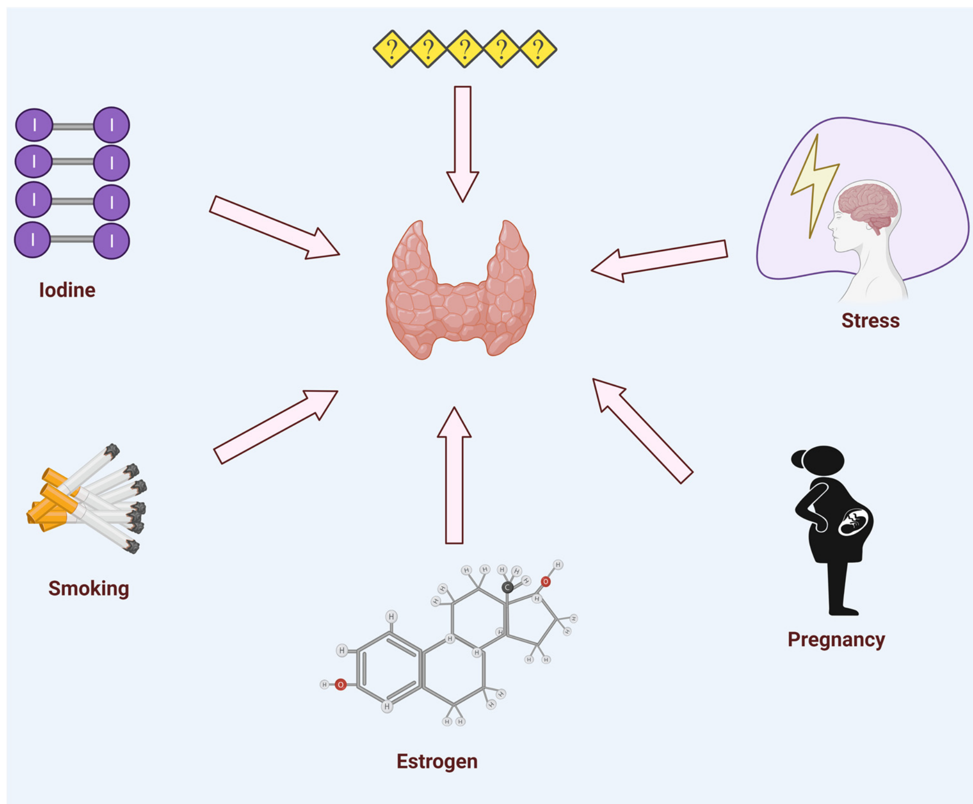

Figure 1: Risk factors associated with the development of Graves' disease.

Pathogenesis

The active phase of GD is associated with the T-helper 1 (Th1) lymphocyte response, while the later phases are associated with T-helper 2 (Th2) lymphocytes. This relationship is interesting since 70% of genes hitherto associated with the risk of GD are involved in T-cell function, implying the importance of T-cell function in the pathogenesis of GO. The autoimmune reaction in GD originates from circulating immunoglobulin G (IgG) antibodies against TSHR (TRAb), and these antibodies, produced by B-cell clones, stimulate thyroid hormone production. TRAb binds to TSHR, which leads to activation of the G-protein coupled receptor (GPCR) pathway with downstream activation of adenylate cyclase, leading to the production of cyclic adenosine monophosphate (cAMP)(41). Elevated levels of cAMP result in induced proliferation of thyrocytes, thyroid growth and secretion of thyroid hormones T3 and T4(42, 43). Inflammation in the thyroid gland in GD is characterized by the infiltration of mononuclear cells, including T lymphocytes and antigen-presenting cells, such as dendritic cells, monocytes, and B lymphocytes. T-lymphocytes proliferate in response to antigen-presenting cells, some proliferate into effector T lymphocyte phenotypes, and others develop into regulatory T-lymphocytes, which can attenuate immune reactivity(44). The duration and magnitude of autoimmune inflammation in GD depends on the balance between proinflammatory factors and factors that decrease immunoreactivity. B-lymphocytes require two signals to differentiate into plasma cells. The first signal comes from antigen binding to the B-lymphocyte receptor, and the second comes from the interaction between CD40 on the B-cell surface and the CD40 ligand on T lymphocytes(45). These interactions lead to the production of cytokines that are critical, as they promote antibody secretion and support T-lymphocyte class switching. First, B lymphocytes produce IgM, which can be switched into IgG or IgE. Intrathyroidal B lymphocytes spontaneously produce anti-thyroid receptor antibodies and are assumed to be the primary source of autoantibodies in GD. The role of thyroid epithelial cells is not completely understood, but it is known that they release several chemokines and express MHC class II molecules. Despite not being antigen-presenting cells, these cells are able to present thyroid antigens to T-lymphocytes. Additionally, they express CD40, suggesting their potential to directly interact with antigen-specific T lymphocytes in GD. The initiation of inflammation in GD involves B lymphocytes and dendritic cells, and later, the thyroid tissue infiltrates by T lymphocytes in response to cytokines, sustaining the autoimmune inflammation process in the thyroid gland(11).

Clinical features and manifestations

Elevated levels of thyroid hormone affect several different body systems and therefore lead to symptoms that can vary strongly between and within GD patients. Common symptoms and physical findings in GD patients include heat sensitivity, tremor, anxiety, mood changes, weight loss, fatigue, frequent bowel movements, palpitations, sleep disturbances, difficulty concentrating, and enlargement of the thyroid gland (goiter). Men may suffer from erectile dysfunction or decreased libido, whereas women suffer from alterations in their menstrual cycles. Less common are symptoms related to Graves' ophthalmopathy (GO), with an incidence rate of 25-40% depending on the definition of GO(43, 46, 47); pretibial myxedema (PTM), also known as Graves' dermopathy, with an incidence rate of approximately 1-5%; and acropachy (clubbing of the fingers or toes), which is present only in patients with Graves' dermopathy(11, 48).

The diagnosis of GD is based on clinical features and biochemical abnormalities, including suppressed TSH levels, elevated free T4 and/or free T3 levels, and elevated TRAb levels. TRAb assays have become more reliable, as the sensitivity and specificity have increased in recent years(8). If the clinical diagnosis is unclear, thyroid peroxidase antibodies can be measured as they are present in 75% of GD patients(6), or thyroid nuclide scans can be performed, which in GD, show diffusely enhanced uptake in an enlarged thyroid. The presence of pretibial myxedema or GO is sufficient for the diagnosis of GD(6).

Treatment

Treatments for GD include antithyroid drugs (ATDs), radioiodine, and thyroidectomy. There are disadvantages and advantages of these treatments. The favoured treatment in Europe is ATD for uncomplicated cases, whereas in the United States, radioiodine treatment is preferred. For ATD treatment, the remission of GD in a Swedish study was reported to be 45%(49). Some patients may become hypothyroid after ATD treatment and may require lifelong L-thyroxine treatment. Radioiodine treatment (RI) is associated with a larger decrease in relapse rate than ATD but a higher risk of developing hypothyroidism(50). RI is also a strong risk factor for developing GO. This factor might also be associated with elevated levels of TRAb, which increase to a maximum within three months(51). In patients treated with ATD or thyroidectomy, TRAb decline slowly(51, 52). Thyroidectomy, total or near total removal of the thyroid, decreases the risk of relapse but does not decrease the risk of developing GO. The intervention carries the risks associated with surgery and leads to permanent hypothyroidism, requiring lifelong treatment with levothyroxine medications to maintain a euthyroid state(50).

Graves´ ophthalmopathy (GO)

Epidemiology

GO, also known as Graves' orbitopathy or thyroid-associated ophthalmopathy (TAO), is mostly associated with GD but can occur in patients with other autoimmune thyroid diseases(53). Approximately 25-40% develop clinically significant GO, and of those, 5-6% are patients suffering from moderate to severe GO(47, 54). GO is more common in women than in men, with a ratio of 4.2:1(5), and is more severe in men and older patients(27, 54). The ethnic prevalence of GO is unclear, as there have been studies reporting the incidence of GO in Caucasians to be higher than in Asians, possibly in relation to smoking. On the other hand, a recent meta-analysis and systematic review reported a slightly lower prevalence among Caucasians than among Asians(46, 55). Studies have examined the association between GO and major histocompatibility complex (*MHC*), adipogenesis-related genes, *CTLA-4, PTPN22,* interleukins, and TSH receptor or thyroglobulin, but the results of these studies have been conflicting(56, 57).

Several risk factors have been identified to be associated with the occurrence and progression of GO (Figure 2). Cigarette smoking is the strongest modifiable risk factor for GO. Graves' patients who are smokers have a higher risk of developing GO than nonsmokers and a higher risk of developing severe GO(27, 58). The mechanisms behind the effect of smoking on the progression of GO are not fully understood but might involve hypoxia in the orbit, elevated production of cytokines, stimulation of adipogenesis and oxygen free radical generation(59). The development of GO occurs more frequently after radioiodine treatment (RI)(52). TRAb correlate with the activity and severity of GO and is the only specific biomarker for GD and GO(60, 61). It has been suggested as an independent risk factor and predictor for the severity of GO(54, 62). Other risk factors, such as selenium deficiency (63) and glitazones (64-66), have been associated with an increased risk of GO.

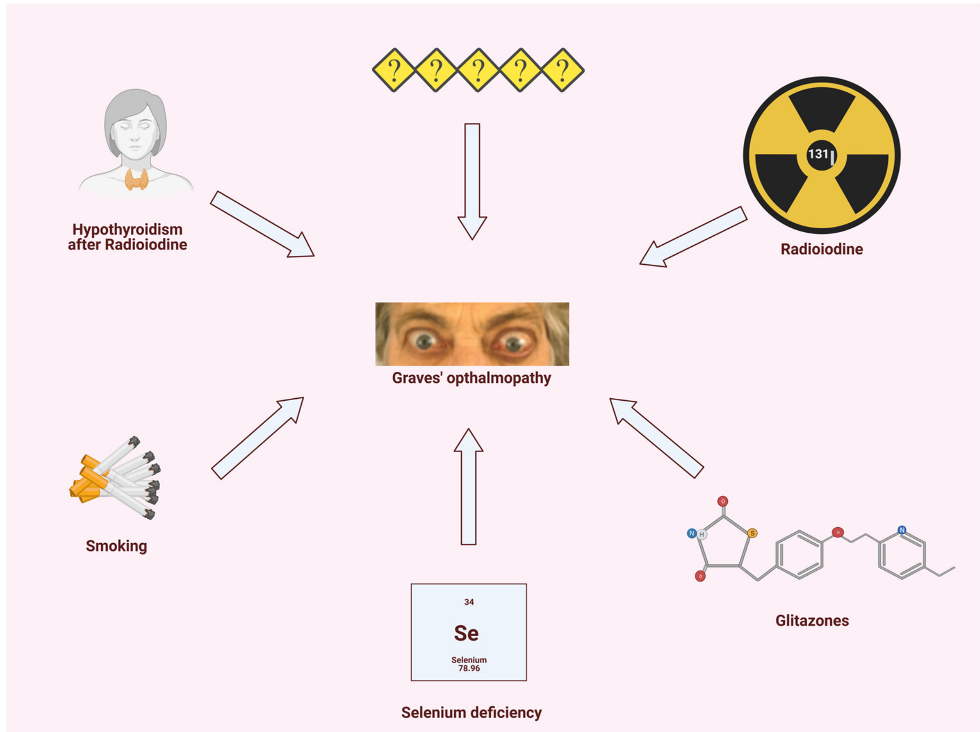

Figure 2: Risk factors associated with Graves' opthalmopathy

Pathogenesis

The pathogenesis of GO can be categorized in contributions from the cells in the inflamed orbital tissue, including extraocular myocytes, orbital fibroblasts (OFs), adipocytes, and mononuclear cells, which all have their own role in the main processes of GO. The pathogenesis of GO includes inflammation, adipogenesis, excess production of hydrophilic glycosaminoglycans leading to edema, and later-stage fibrosis. The early active phase of GO is characterized by infiltration of extraocular muscles and connective/adipose tissue with mononuclear cells, mainly CD4+ T lymphocytes, CD8+ T lymphocytes, B lymphocytes, macrophages, mast cells, and plasma cells. The early active phase of GO is primarily dominated by Th1 lymphocytes and the subsequent production of cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-α (TNF-α). The later phase, where tissue remodeling and fibrosis are seen, may be dominated by Th2 lymphocytes and other cytokines, including interleukin-4 (IL-4) and interleukin-10 (IL-10)(67-69).

The inflammatory activity of OFs in GO includes the production of chemokines and chemoattractions of T lymphocytes, which then stimulate OFs to produce cytokines and chemokines that recruit and activate monocytes, T lymphocytes, B lymphocytes

and mast cells (Figure 3)(70). Moreover, the production of interlekin-1β (IL-1β) stimulates OFs to produce prostaglandin E2 (PGE_2), which in turn stimulates IL-6 production and B-lymphocyte maturation, activates mast cells and induces Th2 skewing(71, 72). Activation of leukocytes requires not only local chemokine gradients but also the expression of adhesion and costimulatory molecules on leukocytes, tissue resident cells and endothelial cells. The expression of intracellular adhesion molecule 1 (ICAM-1) on orbital fibroblasts is upregulated by IL-1β, TNF-α and IFN-γ and promotes the migration of leukocytes to inflammatory sites(73-75).

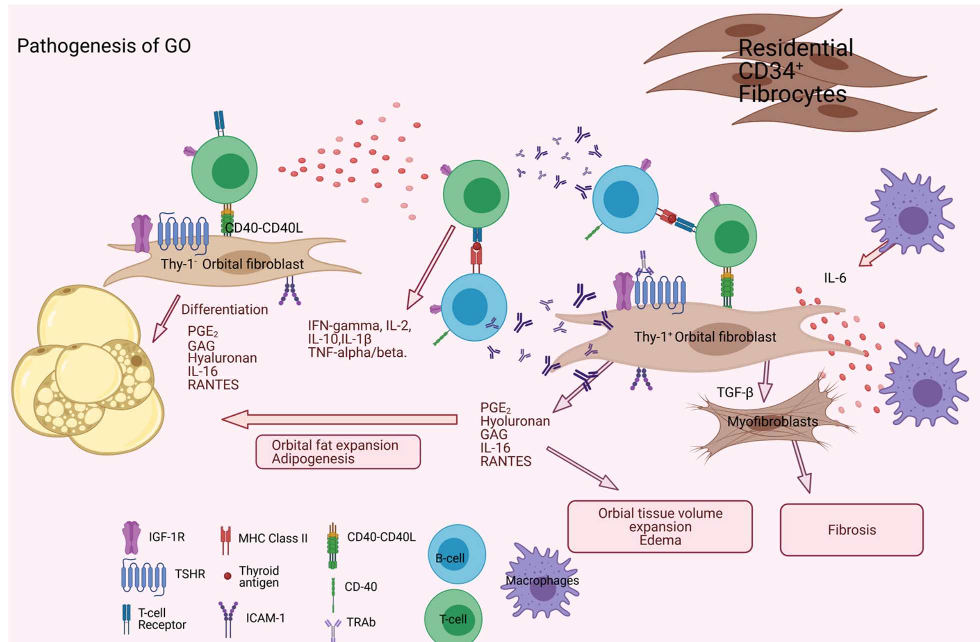

Figure 3: Pathogenesis of Graves' ophthalmopathy. The orbit becomes infiltrated with mononuclear cells. Residential CD34+ fibrocytes can differentiate into myofibroblasts (Thy-1+) or adipocytes (Thy-1-). Th1 activation produces cytokines, including IFN-γ, IL-2, IL-10, IL-1β, TNF-alpha/beta and glycosaminoglycan synthesis, leading to inflammation. Macrophages produce IL-6, which augments B-cell maturation. Activated orbital fibroblasts produce prostglandin E_2,. Thy-1+ orbital fibroblasts produce hyaluronan and other glycosaminoglycans, leading to edema and increased volume of the intraorbital tissue.

OFs express the costimulatory molecule CD40, which in general is also expressed on antigen-presenting cells (APCs) such as B lymphocytes and macrophages. T-lymphocytes express CD40 L, and the formation of CD40-CD40 L bridges between T lymphocytes and OFs leads to elevated ICAM-1 expression, production of IL-6 (which augments B lymphocyte maturation and antibody production), IL-1β, synthesis of hyaluronan, PGE_2 synthesis and proliferation of OFs. The proliferation and production of hyaluronan by OFs are important factors contributing to the process of orbital tissue expansion by attracting water, leading to edema and later

fibrosis in GO(68). Another important role of OFs in their contribution to GO is adipogenesis. OFs are classified into two subgroups according to their ability to differentiate into adipocytes or myofibroblasts. The OFs subtypes are Thy1(CD19)$^{+}$ and Thy1^{-}. Thy1^{+} OFs are capable of differentiating into myofibroblasts upon exposure to transforming growth factor-beta (TGF-ß) produced by OFs. Myofibroblasts participate in inflammation, repair and fibrosis(68, 76). Subtype Thy1^{-} can differentiate into adipocytes, contributing to the increased adipose tissue volume associated with GO(76, 77).

Adipogenesis, the differentiation of preadipocytes into adipocytes, is an important feature of the pathogenesis of GO. The formation of new adipocytes from precursor cells occurs in response to the upregulation of adipocyte-specific genes. This process includes the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (*PPARγ*), which is highly expressed in adipose tissue, leptin and adiponectin. All three are gene markers of adipocyte differentiation and are highly expressed in orbital tissue from GO patients compared to controls(78). Furthermore, the expression of these genes correlates positively with TSHR gene expression, and adipogenesis increases in parallel with TSHR expression(79).

The hypothesis of the shared autoantigen in GO and GD, TSHR, is supported by the positive correlation between TRAb titer, activity and severity of the disease(69).

Studies have also shown that TSHR is overexpressed in OFs from GO patients compared to controls. TSHR is generally not expressed on fibroblasts from other anatomical sites; hence, it seems to be a unique feature of OFs. TRAb stimulates OFs to differentiate and to increase the production of hyaluronan and IL-6(80, 81), suggesting that activation of OFs by ligation of TRAb to TSHR directly contributes to the pathogenesis of GO.

Another suggested autoantigen is insulin-like growth factor 1 receptor (IGF-1R), which has many roles in the maintenance and development of mammalian tissues and seems to diverge in individuals with autoimmune diseases. IGF-1R expression is increased in OFs from GO patients. Different hypotheses regarding how IGF-1 contributes to the pathogenesis of GO have been suggested. One hypothesis is that autoantibodies against IGF-1R and TSHR are present in GO patients and that these activate both receptors concomitantly (82). Another hypothesis is that only TRAb is present in GO patients and that there are no IGF-1R-stimulating antibodies. In this case, it is suggested that the activation of the IGF-1 pathway is stimulated by the binding of TRAb to TSHR, which activates IGF-1R by “cross talk” (82-84). Anti-IGF-1R antibodies have been detected in GD patients, but whether these antibodies can initiate signaling through IGF-1R is still unclear, with conflicting results. Some studies found that these antibodies could activate OFs through IGF-1R and stimulate the production of T-lymphocyte chemoattractants (e.g., RANTES and IL-16) and hyaluronan(85), others could not confirm these findings (86). Although the overall

understanding of the pathogenesis process involved in GO has increased, many questions, such as the triggering of the disease, still need to be answered.

Clinical features

Patients with severe or moderate GO are highly negatively impacted, not only physically but also mentally(87, 88). It has been reported that GO patients suffer from anxiety and depression(89). Clinical features in GO patients arise mainly from the increased volume of orbital tissue leading to increased pressure within the bony cavity. The common symptoms of GO include eye irritation, increased tearing, double vision, changes in appearance, light sensitivity and a sense of pressure or pain behind the eye, and in severe cases, incomplete closure of the eyes and occasionally but rarely blindness. Common signs include upper eyelid retraction, proptosis, eyelid swelling and erythema, conjunctival injection (redness) and chemosis (edema), exposure keratitis (corneal injury due to dryness) and extraocular eye muscle dysfunction(90-92).

Certain anatomical features, such as a wide angle of the lateral wall, have been associated with severe forms of GO, as orbits with these features may not be able to provide enough space for orbital tissue expansion(93).

Treatment

The standard treatment options for GO today are corticosteroids, Nonsteroidal anti-inflammatory drug (NSAIDs), orbital irradiation or surgery. The choice of treatment to use is often based on the clinical activity score (CAS). This system evaluates the activity of GO and helps in better management of the disease(94, 95). For patients with mild symptoms, treatment with immunosuppressive drugs or surgery is usually not justified, but instead supportive treatments such as artificial tears, sunglasses or head elevation during sleep are preferred. In the case of moderate to severe GO, immunosuppressive therapy, orbital irradiation or both are necessary. In cases with severe sight-threatening GO and no response to immunosuppressive drugs, orbital decompression may be required(94).

Rituximab (RTX) is a monoclonal CD20 antibody that in some cases has been used in clinical practice for the treatment of GO(96). RTX inhibits the proliferation and maturation of B lymphocytes, leading to inhibition of the autoimmune response(97). In a double-blinded randomized trial, RTX treatment was compared with methylprednisolone in GO patients with active to moderate severe GO. The activity score decreased more with RTX treatment at weeks 16 and 20. At week 24, RTX was effective in inactivating moderate to severe GO in 100% of the patients compared to 69% in the methylprednisolone-treated (98) In another randomized

double-masked placebo-controlled trial, they did not find any differences in clinical activity in the RTX-treated group compared to the placebo group(99). A novel drug has recently been approved by the U.S. Food & Drug Administration, teprotumumab. It is a fully human monoclonal IGF-1R antagonist that has proven to give promising response in GO patients(100) in randomized trials.

In a randomized trial from 2010-2013, 88 patients were included and treated with teprotumumab or placebo for 24 weeks. Eighteen patients treated with teprotumumab already showed a response in week 6 with a reduction in proptosis and clinical activity compared to the placebo group. In week 24, 69% of the patients showed a reduction of 4 mm proptosis and a decrease in activity score. There was no reduction in proptosis in the placebo group(101). In another randomized, double-masked, placebo-controlled phase 3 multicenter trial, it was seen that patients with proptosis responded at week 24 to the treatment with a reduction in exophthalmos of 2 mm, and an overall response was seen with a reduction of 2 CAS score units. The reported side effects of teprotumumab are muscle cramps, hyperglycemia, and weight loss. These side effects were seen in less than 5% of the patients(102), a safety profile that is promising.

Statins have recently been suggested as a treatment for GO, as they may protect against the development of GO in newly diagnosed GD patients. A register study from our department analyzed newly diagnosed Swedish GD patients between 2005 and 2018. The incidence of GO was compared between statin users and nonusers. The study reported that statin users were less likely to develop GO, and other lipid-lowering drugs did not exhibit a protective effect(103). Diclofenac is an anti-inflammatory drug that inhibits adipogenesis in 3T3-L1 preadipocytes(104). Adipogenesis is an important process in the pathogenesis of GO and targeting adipogenesis in GO patients may lead to a decrease in adipocytes in GO patients.

Other drugs have been suggested for the management of moderate to severe conditions of GO. One is tocilizumab, which selectively targets IL-6, a key inflammatory mediator in the pathogenesis of GO. It has a favorable side effect profile. However, the long-term effect of tocilizumab is unknown(105).

T-lymphocyte inhibitors that target CD3 receptors on T lymphocytes (teplizumab) leading to T-lymphocyte depletion might act as an effective treatment for GO. Inhibitors have shown some advantages in type 1 diabetes by preserving β-cells when given at the initial stage of the disease. Another T-lymphocyte inhibitor, abatacept, an analog of CTLA-4, acts by inhibiting the activation of T lymphocytes and has been used in rheumatoid arthritis patients(106). Cytokine antagonists (infliximab) are suggested to target TNF-α, which is a cytokine involved in the terminal event in enhancing the production of glycosaminoglycans (GAGs) and hyaluronan. It has been reported to improve color vision and CAS in dysthyroid optic neuropathy(107).

Adipogenesis

In the past two decades, knowledge of adipose tissue physiology has increased. Adipose tissue is an endocrine organ that secretes many active proteins and hormones, known as adipokines. Many of these adipokines are involved in the regulation of various cellular functions, including metabolism and immune homeostasis(108). The adipokines adiponectin and leptin reduce fibrosis and inflammation in other tissues. Leptin has been shown to be involved in the development of systemic hypertension, cardiovascular disease and renal dysfunction, whereas adiponectin is known to protect the heart and blood vessels against inflammation, atherosclerosis and oxidative stress. (109-112). Furthermore, adipose tissue contains immune cells from both innate and adaptive immunity systems, including eosinophils, neutrophils, mast cells, macrophages, T-lymphocytes and B lymphocytes(113-116).

Adipogenesis is the process by which fibroblast-like progenitor cells commit their fate to become mature adipocytes. In the first step, a mesenchymal precursor restricts itself to the adipocyte lineage, and without any morphological changes, it forms a preadipocyte. This commitment step is then followed by differentiation in which the specified preadipocytes undergo growth arrest, and in response to mitogens, they accumulate lipids and form mature adipocytes(117, 118).

In vitro studies have been crucial for understanding the factors required for adipogenesis, and they have made it possible to intensively study the process of adipogenesis. Knowledge about adipogenesis is based on studies on adipogenic cell lines, such as 3T3-L1 and 3T3-F442A(119). In the first stage of the differentiation of adipocytes, the cells go into growth arrest when confluent, followed by clonal expansion (Figure 4). Mitogens stimulate the expression of *IEGs* that subsequently induce the cell expansion phase, which includes one or two rounds of replication (days 1-2)(120). The function of IEGs is to trigger the transcriptional cascade leading to the adipocyte phenotype(121). The clonal expansion phase ceases coincidentally with the expression of the central regulators of adipogenesis, the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα). After clonal expansion, the preadipocytes enter a second final period of growth arrest (days 3-4). This stage is followed by the terminal phase when the cells differentiate into the mature adipocyte phenotype (days 4-10)(122), which expresses characteristic genes such as stearoyl-coenzyme A desaturase (*SCD*) and fatty acid synthase encoded by the *FASN* gene(123).

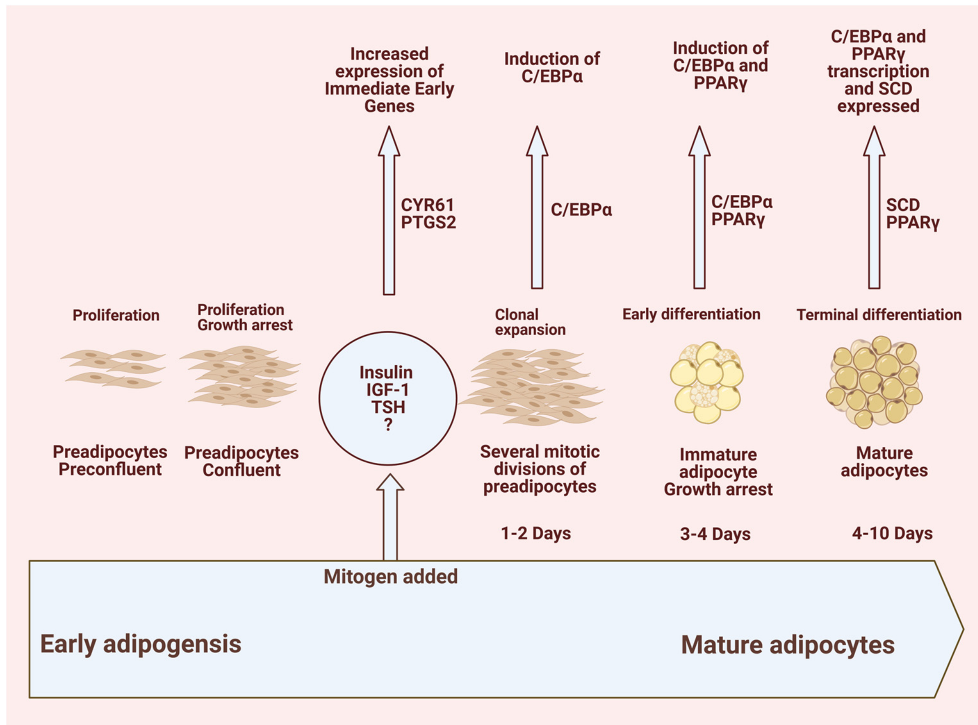

Figure 4: Illustration of the phases in adipogenesis in the 3T3-L1 preadipocyte cell line. De novo adipogenesis is enhanced in GO patients. Studies have found that adipocyte-specific genes leptin, adiponectin, *FASN*, and *PPARγ* are overexpressed in orbital tissue from GO patients compared to healthy controls(78, 124). Adipocyte-related immediate early genes (IEGs), including cysteine-rich angiogenic inducer 61 (*CYR61*) and *COX-2,* were found to be significantly overexpressed in patients with severe active GO compared to healthy controls(121). The elevated expression of adipocyte-specific genes, including CYR61 and *COX-2*, which both also have a role in inflammation, may suggest that several pathways involved in inflammation and adipogenesis are triggered, leading to enhanced adipogenesis in GO patients.

Peripheral Mononuclear Blood Cells

A peripheral mononuclear blood cell (PBMC) is any blood cell with a round nucleus, such as T lymphocytes, B lymphocytes, and monocytes. T- and B-lymphocytes are renowned by their site of maturation and receptors. T lymphocytes mature in the thymus and express the T cell receptor (TCR), whereas B-lymphocytes mature in the bone marrow and carry the B-cell receptor (BCR).

T lymphocytes are generally classified into two groups: helper T cells (T_{Hs}), which express CD4 receptors on the surface, and cytotoxic T cells (T_{Cs}), which express CD8 receptors on the surface. Both CD4+ and CD8+ T lymphocytes play an important role in the immune synapse between the TCR and antigens bound to the major histocompatibility complex (MHC) on antigen-presenting cells(125). T_H cells can be divided into subtypes, including Th1, Th2, Th17, T follicular helper (Tfh), regulatory

T (Treg), Th9, and Th22 cells; all these subtypes produce a large range of cytokines upon activation. Previous studies have shown that at least Th1 cells and Th2 cells are involved in the development of GO(126), where Th1 cells produce IL-1β, IL-2, IFN-y, and TNF-β(127) and Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13(128). CD8+ T lymphocytes mediate and suppress cell-mediated immune responses and can be divided into the following subgroups: Tc1 secreting IFN-γ, Tc2 secreting IL-5, Tc9 secreting IL-9, and Treg secreting TGF-ß(129). CD8+ T lymphocytes are involved in most autoimmune disorders, such as rheumatoid arthritis (RA) and GD. Under normal conditions, the balance and stability of the CD4+/CD8+ ratio is an important factor for the body's immune function(130). A reduction and dysfunction of CD8+ T-lymphocytes leads to impaired surveillance of the body's immune response, resulting in an increased number of T_h lymphocytes that respond to autoantigens, which in turn gives rise to an amplified immune response. It has been demonstrated that antibody-mediated depletion of CD8+ T lymphocytes in BALB/c mice immediately increases the incidence of hyperthyroidism, indicating that a reduction in CD8+ T lymphocytes leads to a weak monitoring of the immune response (131). In a study investigating the population of T lymphocytes in PBMCs isolated from GD patients with or without active GO, the authors found elevated levels of CD4+ T lymphocytes and decreased levels of CD8+ T lymphocytes in the patient group compared to the healthy control group(132).

B lymphocytes undergo a maturation process, where lymphoid progenitor cells develop into B lymphocytes and memory B lymphocytes, from which plasma cells are derived.

B lymphocytes provide surveillance to the body for signs of infection, and they express clonally diverse cell surface immunoglobulins (Igs) that recognize their target antigen. When B lymphocytes encounter the target antigen, they become plasma cells, which leads to the production and secretion of large amounts of antibodies that can bind to the target protein and neutralize it. Apart from producing antibodies, B lymphocytes have multiple roles in cascades of events that regulate the progression of an autoimmune disease and immune reaction(133). Upon an immune reaction, they interact with T lymphocytes and produce multiple cytokines, such as IL-10, IL-4, IL-6, TGF-β, and IFN-γ(134).

B lymphocytes have an important role in the autoimmune response of GD and GO. It has been reported that B lymphocytes from GD patients are skewed towards the IGF-1R+ phenotype. The IGF-1R+ phenotype leads to enhanced expansion of B-lymphocytes, which gives rise to increased Ab production. IGF-1+ B lymphocytes have been found in the peripheral circulation and orbital tissue from GO patients(135).

GO and GD are T-lymphocyte-dependent and B-lymphocyte-mediated autoimmune diseases. Targeting B lymphocyte depletion in GD and GO might be an effective

treatment, as B lymphocytes are important not only for antibody-producing cells but also for antigen-presenting cells in the early phase of GD and GO(133, 136).

The effect of smoking on T-and B lymphocytes

Cigarettes contain numerous harmful chemicals, such as carbon monoxide, nitrogen, oxides, nicotine, and cadmium, and affect the innate and adaptive immune systems of smokers(137, 138). Cigarette smoking has been implicated in the production of many immune or inflammatory mediators, including pro- and anti-inflammatory cytokines(139).

The adaptive immune system involves immune responses that are induced by pathogens and executed primarily by T- and B lymphocytes(140). The T-lymphocyte population in smokers is altered compared to that in nonsmokers(141, 142). Several studies have reported that the CD8+ T lymphocyte count is decreased in smokers, resulting in an altered CD4+/CD8+ T lymphocyte ratio compared to nonsmokers(143, 144). It has been reported that cigarette smoking may affect the immune system by dysregulating B lymphocytes and result in suppression of normal B lymphocyte function, leading to an increase in the inflammatory reaction(145). Furthermore, it has been reported that B lymphocytes in smokers produce larger amounts of IgE than those in nonsmokers, while the IgA and IgM concentrations did not differ significantly between smokers and nonsmokers(146, 147). It is now known that smoking is always harmful rather than beneficial for the development of autoimmune disorders, even though it suppresses the immune system. Paradoxically, smoking generally weakens immunity against infectious diseases but promotes autoimmunity(139, 148). This phenomenon is not fully understood. However, it has been speculated that weakened immunity with prolonged chronic infection may result in cross-reactive autoimmunity against pathogens and self-tissue(144, 149). Another explanation may be that smoking has different effects on immunity in different anatomical regions and diseases(144, 150).

Cigarette smoking has been linked to the development of multiple autoimmune diseases, including RA, GD and multiple sclerosis(151-154). In RA, it has been demonstrated that smoking is associated with increased RA severity as well as nodule formation, increased joint destruction, increased pulmonary disease, and decreased functional abilities(155-157). The severity of RA among smokers may be caused by the change in the ratio of TNF-α to soluble TNF-receptor, which may cause increased TNF-α activity(158, 159). Several studies have reported an increased risk of developing GD and GO in smokers. In a meta-analysis, it was reported that smokers were at higher risk of developing GD (OR 3.30) and of even higher risk of developing GO (OR 4.40) than nonsmokers(160). In another case-control study with age- and sex-matched control subjects, it was found that smoking

was associated with the development of severe GO(153). In a Swedish cohort, a case-control study found an interaction between smoking and a single nucleotide polymorphism (SNP) in *CYR61* to increase the risk of GO(161). It has been suggested that cigarette smoke may correlate with increased venous congestion in GO(162). Another study reported that proteins involved in tissue inflammation and adipose tissue differentiation from orbital tissue in GO patients were downregulated by steroids, but the effect of steroid treatment was reduced by smoking(163). Furthermore, an in vitro study reported that cigarette smoke extract induced adipogenesis in orbital fibroblasts from GO patients(164), and the expression of HLA-DR increased when nicotine combined with IFN-γ was added to cultured orbital fibroblasts from GO patients(165). Hence, there is much evidence that cigarette smoke increases the risk and severity of RA, GD, and GO, but the underlying mechanisms are still not fully explained.

Simvastatin

Statins have been used to prevent coronary artery diseases and stroke for three decades through their ability to reduce circulating low-density lipoprotein cholesterol (LDL-C)(166). Simvastatin acts by binding to 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), thereby blocking the conversion of HMG-CoA to mevalonate, leading to decreased biosynthesis of cholesterol(167). In addition to the effects on LDL-C, they also exhibit anti-inflammatory and antioxidant properties known as pleiotropic effects(168, 169). It has been known for a decade that simvastatin has antiproliferative effects on lymphocytes and other cell types(170, 171), but only recently have studies proven the immunomodulatory properties of statins that affect T-lymphocyte and APC function(172). It has been observed that statins inhibit cytokine-inducible expression of MHC class II molecules by APCs, thereby preventing antigen presentation to CD4+ T lymphocytes, leading to suppression of the Th1 response and increasing the anti-inflammatory Th2 lymphocyte response(173-177). Based on the anti-inflammatory abilities of statins, they have been suggested as a novel anti-inflammatory therapy for a number of conditions, including autoimmune diseases(178-180).

In RA, atorvastatin has been shown significantly to improve symptoms, such as joint swelling and tendon pressure points, and to decrease the levels of C reactive protein and blood sedimentation rate(181). In a double-blinded placebo-controlled trial in RA, those receiving atorvastatin had decreased significantly in disease activity score compared to those receiving placebo(181). In vitro studies have shown that simvastatin is able to downregulate the expression of *CYR61* in synovial fibroblasts(182). *CYR61* is an immediate early gene that is important in the

pathogenesis of RA and GO(182, 183). It has been described that B lymphocytes activated through CD-40 upregulate enzymes in the mevalonate pathway involved in cholesterol biosynthesis and hence are a target for simvastatin(184). A study by Vornhagen et al. investigated whether simvastatin could downregulate CD40-activated B lymphocytes. The authors found that B lymphocytes were downregulated in vitro after simvastatin treatment and that this effect was exclusive to CD40-activated B lymphocytes, as they did not find any effect of simvastatin on dendritic cells(184).

In GO, it has been suggested that statins may have an effect on the proinflammatory Th1 response by upregulating Th2 lymphocytes(185). Moreover, statins may be able to mobilize disease-specific proinflammatory T lymphocytes from the inflammation site into the blood, leading to clinical remission(185). A large cohort study including 8404 newly diagnosed GD patients investigated the risk factors associated with the development of GO. GD patients treated with statins for more than 60 days had a 40% decreased hazard of developing GO(186). As mentioned above, a study in our department reported that use of statins may reduce the risk of developing GO(103).

Diclofenac

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) and has anti-inflammatory, pain-relieving and antipyretic properties. It inhibits cyclooxygenase (*COX-2* (*PTGS2*)), the enzyme that catalyzes the first step in the conversion of arachidonic acid to prostaglandin (PGE_2)(187). However, NSAIDs have also been recognized to have cyclooxygenase-independent properties, such as antagonization of PPARγ, leading to inhibition of adipogenesis in the preadipocyte cell line 3T3-L1 (188-191). In an in vitro study, it was shown that diclofenac inhibited adipogenesis in the preadipocyte cell line 3T3-L1 in a dose-dependent manner(104).

NSAIDs have been more efficient in treating symptoms in patients with RA than placebo treatment(192). In a pilot study, oral sodium diclofenac was used to treat mild to moderate GO. The treatment resulted in relief of pain with a significant improvement in extraocular muscle restriction(193). A Swedish prospective multicenter study included newly diagnosed GD patients without clinical GO. The patients were treated with methimazole and diclofenac as an adjuvant treatment for 12 months. The study found that the number of GO patients was lower in the group treated with diclofenac than in those without diclofenac treatment. The finding did not meet significance, which might have been due to a low number of patients who developed GO. Moreover, the study reported that the anti-TPO titer was lower in the group treated with diclofenac, but no changes in TRAb titers were seen(191).

Aims

GO is a disease that currently is not preventable and in its severe form is sight-threatening. The treatment options for GO are limited and associated with side effects. Smoking and radioiodine treatment are strong risk factors for the development of GO. Knowledge about the mechanisms behind the triggering effects of smoking and radioiodine treatment is limited, and new treatment modalities are required.

Specific aims:

I. To investigate whether smoking has an effect on the expression of immediate early genes in severe active GO and to study pathways affected by smoking.

II. To develop an in vitro model to study the effects of smoking on human orbital fibroblasts from patients with severe GO and 3T3-L1 preadipocytes and to investigate whether simvastatin influenced adipogenesis.

III. To explore the effect of cigarette smoke on inflammatory mediators and proliferation in PBMCs from GD patients and whether the effects could be modulated by simvastatin and diclofenac.

IV. To investigate the relationship between TRAb levels in patients treated with radioiodine and the development of GO and to investigate the impact of known risk genes for GD

Subjects

The orbital adipose/connective tissue in **Study I** and **Study II**, blood samples in **Study III** and the DNA in **Study IV** were all collected after the patients provided informed consent and approval was granted from the ethical board of Lund University, Malmö/Lund Sweden.

Study I

The intraorbital adipose/connective tissue was obtained during orbital decompression surgery. All the patients included in the study were in the active phase of GO. The study included 15 smokers, 7 (47%) males and 8 (53%) females. Ten (67%) patients had been treated with steroids, and the mean time of disease was 10 months. In the nonsmoker group, 12 patients were included, 2 (17%) males and 10 females (83%), in whom 7 (58%) were treated with steroids and the duration of disease was 14 months.

Clinical characteristics are presented in the Tables 2 and 3.

Table 2. Clinical characteristics of smokers (n=15) with active Graves' opthalmopathy. Smokers 1-8 were analyzed both with microarray and real-time PCR. Smokers 9-15 were only included in the real-time PCR analyses

Patients	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Duration of active GO before operation (months)	9	8	14	7	6	22	6	7	12	5	18	13	10	7	13
Sex (male/female)	M	M	F	F	F	F	M	M	F	F	M	F	M	F	M
Age at operation (years)	47	68	46	49	55	70	68	56	55	68	61	71	57	58	39
Steroid treatment (months before operation)	9	8	10	5	3	22	6	7	12	3	4	13	4	6	4
Optic nerve dysfunction	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes

Table 3. Clinical characteristics of nonsmokers (n=12) with active Graves' opthalmopathy. Nonsmokers 16-23 were analyzed both with microarray and real-time PCR. Nonsmokers 24-27 were only included in the real-time PCR analyses.

Patients	16	17	18	19	20	21	22	23	24	25	26	27
Duration of active GO before operation (months)	10	16	24	22	10	8	14	5	5	24	24	7
Sex (male/female)	F	M	F	F	F	F	F	F	M	F	F	F
Age at operation (years)	62	57	44	41	65	86	63	76	59	73	52	51
Steroid treatment (months before operation)	10	3	3	1	8	3	14	5	5	22	23	3
Optic nerve dysfunction	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Study II

Adipose/connective tissue was obtained from 4 (1 male, 3 females) patients with GO who underwent decompression surgery. They had all been treated with steroids. Six controls were obtained from healthy individuals during blepharoplasty after cutting the 'orbital septum'. This tissue is part of the same adipose/connective tissue as the retrobulbar adipose/connective tissue.

The clinical data of the patients who underwent decompression surgery are presented in Table 4.

The adipose/connective tissue was cultured directly after the surgery.

Table 4. Clinical data of patients with Graves´ ophthalmopathy who underwent orbital decompression that were included in the study.

Sex		**M**	**F**	**F**	**F**
Age at operation		67	38	82	81
Smoking at diagnosis of ophthalmopathy		No	Yes	No	No
Treatment of ophthalmopathy	Steroid treatment	Yes	Yes	Yes	Yes
	Retrobulbar irradiation	No	No	No	No
Duration of ophthalmopathy before operation (months)		8	4	13	60

Study III

The blood samples used in this study were collected from 7 newly diagnosed Graves' patients at the Endocrinology Clinic, Skåne University Hospital Malmo (Table 5), and 9 healthy controls were obtained from personnel at the Endocrinology Clinic, Skåne University Hospital, Malmo.

Table 5. Clinical data on newly diagnosed Graves' disease patients

Sex	**F**	**F**	**F**	**M**	**F**	**F**	**M**
Smokers	Yes	No	Yes	No	No	Yes	Yes
Age	63	40	39	31	33	29	42
Treatment	No	No	No	No	No	No	No

Study IV

Graves´ patients included in this study (n=204) were admitted to the Department of Oncology for treatment with radioiodine. Patients (n=45) with risk factors for the development of GO after RI treatment, received prednisolone 30 mg per day for 1 month and after that the dose was slowly decreased during the following two months and stopped after 3 months. Clinical parameters are presented in Table 6

Table 6. Changes in TRAb levels after treatment with radioiodine and relation to clinical parameters.

Fold change TRAb	<1.1	≥1.1	P value*
Patients (%)	57 (31)	125 (69)	
Females (%)	45 (79)	97 (78)	0.84
Age males and females (years)	56 ± 17	54 ± 16	0.67
Age females (years)	54 ± 17	52 ± 16	0.49
Smokers (%)	8 (14)	31 (25)	0.10
Born outside of Sweden (%)	12 (21)	35 (28)	0.32
Treatment with steroids (%)	41 (73)	79 (63)	0.23
Treatment with >120 Gy	10 (18)	21 (16)	0.99
Duration of GD (months)	12 (5-25)	8 (3-33)	0.48
Primary treatment with ATD (%)	37 (65)	70 (56)	0.33
Duration of GO (months)	6 ± 6	5 ± 3	0.52
Treatment with corticosteroids in GO patients (%)	2 (22)	4 (17)	0.99
Smokers in GO patients (%)	0	2	0.99

For the genotyping analysis, 117 patients were included, where 57 patients had TRAb levels below the median and 60 patients had TRAb levels above the median. Detailed characteristics of the patients are given in Table 7.

Table 7. Characteristics of the genotyped individuals

	TRAb (IU/L) < median (15 IU/L)	TRAb (IU/L) ≥ median (15 IU/L)
N	57	60
Age (years)	55±18	54±15
Sex		
Male	13 (23)	15 (25)
Female	44 (77)	45 (75)
Ethnicity		
Born in Sweden	44 (77)	47 (78)
Born in Europe	7 (12)	7 (12)
Born outside Europe	6 (11)	6 (10)
Smokers	12 (21)	10 (17)
Non-Smoker	41(72)	39 (65)
Missing	4 (7)	11 (18)
TRAb IU/L	6 (3-9)	31 (24-39)

Values are expressed as the mean (±SD) and presented as n (%) unless otherwise stated.
Information was missing in ten patients.

Methods

RNA and DNA Extraction

All orbital tissue biopsies were treated with RNAlater (Sigma Aldrich, Germany) to minimize RNA degradation. RNA was extracted using the RNAeasy Plus Mini Kit (Qiagen, Germany) followed by quantification of RNA concentration and quality check on a NanoDrop ND-1000.

DNA was obtained from buccal swabs and extracted using a QiAamp UCP DNA microkit (Qiagen). The purity and concentration of DNA were checked on a NanoDrop ND-1000.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

PCR is a common laboratory technique used to amplify a particular region of DNA. In the first step of PCR, the RNA strand is reverse transcribed into its complementary DNA strand (cDNA) using reverse transcriptase followed by amplification of cDNA using PCR.

PCR was used to amplify DNA extracted from buccal swabs using the Repli-g Screening Kit (Qiagen) and for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). In S**tudy IV**, the TaqMan™ Allelic Discrimination Assay (Applied Biosystems, Sweden) was used to genotype the cohort.

Relative quantification by TaqMan quantitative polymerase chain reaction (qPCR) is a technique used to measure the expression of genes (mRNA) in a sample. This technique is based on amplification of a DNA sequence of interest. A fluorescent probe specific to the coding part of mRNA is cleaved by nuclease activity when TaqDNA polymerase reaches it, resulting in imitation of fluorescence. The intensity of the fluorescence is proportional to the amount of mRNA target that is present in the sample (Figure 5). Real-time PCR experiments were performed on an ABI-PRISM 7900 HT system (Applied Biosystems) (**Study I**) and QuantStudio 7 Flex system (Applied Biosystems) (**Studies II** and **III**) using TaqMan Gene Expression Assays (Applied Biosystems, Sweden).

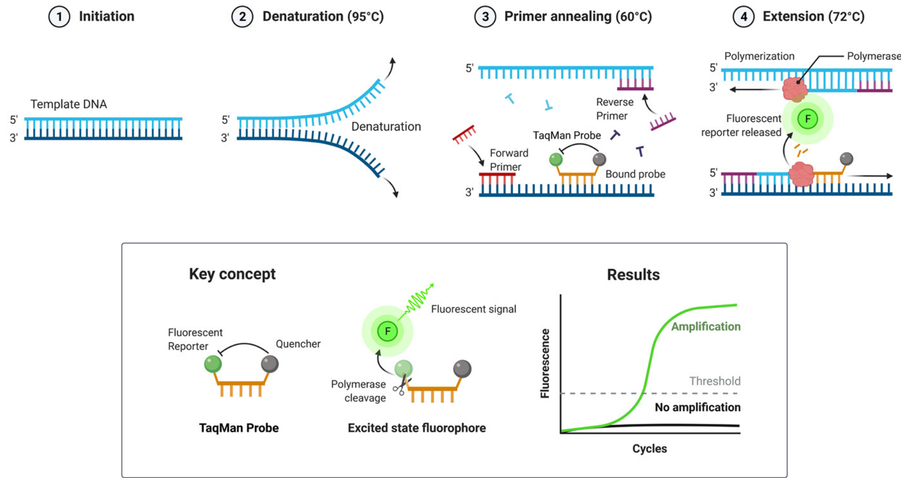

Figure 5: Schematic overview of the steps required for amplification of mRNA. The key concept is that nuclease activity of the DNA polymerase detaches the reporter dye from the quencher, and the reporter dye emits fluorescence

The standard curve approach was applied to analyze the mRNA expression data. The standard curve method is a relative quantification method that determines the mRNA expression signal of the target gene in relation to a standard curve. The advantage of using the standard curve method is that it gives highly accurate and quantitative results based on known quantities provided by the standard curve. The mRNA expression of the target gene was normalized to an endogenous control(194). In Studies I-III, cyclophilin A was used as an endogenous control for mRNA expression in human tissue and orbital fibroblasts. For the mouse 3T3-L1 preadipocyte cell line, beta-actin (ß-actin) was used as an endogenous control.

Microarray analysis

A microarray is a laboratory tool used to detect the expression of thousands of genes at the same time. The technique is used for gene expression profiling or SNP detection. Affymetrix microarrays are microscope slides printed with thousands of tiny spots in defined positions, with each spot containing known oligonucleotide probes specific for a certain transcript. The extracted RNA from the samples that need to be analyzed is labeled with a fluorescent dye and hybridized with its complimentary RNA followed by scanning with a laser that detects the emitted fluorescence in the hybridized samples (Figure 6).

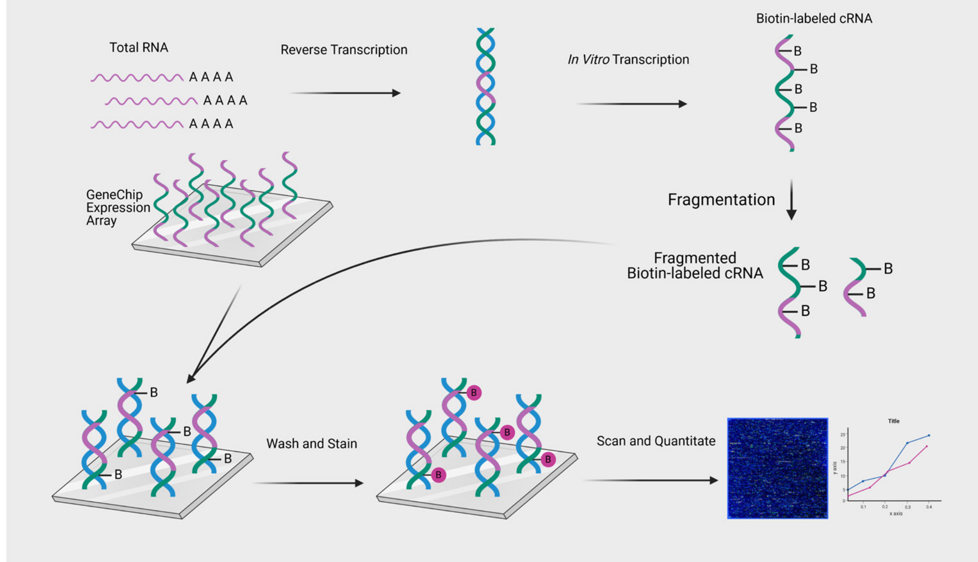

Figure 6: Schematic overview of the steps included in the Affymetrix microarray technique (reproduced from Thermo Fisher Scientific).

This measurement was used to analyze the differences in gene expression between groups of samples.

In **Study I**, the SCIBLU Microarray Resource Center at Lund University performed the gene expression analyses. To regroup the individual probes and to remap the correct sets of genes for the Affymetrix GeneChip Human Gene 1.0 ST Array, the software Entrez custom chip definition files (http://brainarray.mbni.med.umich.edu v17.0) were used. These analyses resulted in a total of 19,718 genes. It is important to choose the right method to calculate the gene expression data provided by the array, as it may have a major impact on the outcome(195, 196). Therefore, we used the approach of two methods to normalize and to summarize our gene expression data. We applied tools that combined the multiple probe intensities for each gene to produce an expression value using Affymetrix Power Tools (APT). The first tool applied was the probe logarithmic intensity error (PLIER) method (Affymetrix), and the second tool was the robust multiarray average (RMA) method(197, 198). After applying the methods, we were left with 12747 genes in GO. Genes were selected on the basis of their overexpression in smokers compared to nonsmokers, according to an expression ratio of >2-fold and ≤ 0.5-fold, respectively. Ranked differentially expressed genes (> 2 and < 0.5) were subjected to pathway analysis using the Ingenuity Pathways Analysis (IPA) library of canonical pathways (Ingenuity_

Systems; www.ingenuity.com) and the Web-based Gene Set Analysis Toolkit (WebGestalt)(199). WebGasalt is a suite of tools for functional enrichment analysis in various biological contexts. The tools make it possible to compare uploaded genes of interest with genes in predefined functional categories. The tools also integrate a number of pathways, such as KEGG Pathways, Gene Ontology and Pathway Commons.

Cell culture studies

Cell culture studies allow us to investigate and to understand the mechanism of interest better and are faster to perform than in vivo experiments. Another advantage of cell culture is that it allows one to perform experiments in a controlled environment, and the results are reproducible. One disadvantage of in vitro experiments is that when cells are isolated from their natural environment, it leads to an elimination or reduction of interactions of biological events in real life. In our studies, we used a cell line and primary cells to investigate the mechanisms behind the effect of cigarette smoke extract exposure (CSE), mitogens, and certain drugs. The advantages of using immortal cell lines are that they provide an unlimited supply of material and that there are no ethical concerns (200). Primary cells allow experiments using human tissue to retain the morphological and functional characteristics of their origin(201). These cells have limited potential in regard to their ability to differentiate and to keep the morphology in more than a certain number of passages, even when given appropriate conditions, and they take more time to grow. As the cells age in culture, they may change in morphology and function, which is why it is recommended to use them in early passages. However, these cells may provide more relevant results than cell lines(202).

The 3T3-L1 preadipocyte cell culture

In **Study II**, the mouse 3T3-L1 preadipocyte cell line and orbital fibroblasts (OFs) isolated from GO patients were used. The 3T3-L1 cell line is well established and has been used in studies on adipose tissue. The cells have a fibroblast-like morphology and are able to differentiate into mature adipocytes under appropriate conditions. We used this cell line to investigate the effects of CSE and simvastatin on the process of adipogenesis.

The 3T3-L1 preadipocyte cell line was grown to confluence. Two days postconfluent cells were treated with simvastatin for 24 hours, followed by the addition of a standard differentiation cocktail containing rosiglitazone (Sigma Aldrich), dexamethasone (Sigma Aldrich), and insulin (Sigma Aldrich) with or

without CSE for either 30-120 minutes or 6 days. In some experiments, insulin was replaced by IGF-1. On differentiation day 6, the lipid content in the differentiated adipocytes was measured after staining with Oil Red O (Sigma Aldrich). The mRNA of IEGs and late adipogenic genes was quantified.

Cell culture orbital fibroblasts (OFs)

In **Study II**, OFs were isolated from adipose/connective tissue that was obtained from GO patients who underwent decompression surgery and from healthy controls during blepharoplasty. The obtained tissue was cut into pieces of 1-3 mm, trypsinized and cultured.

The effect of CSE and simvastatin on *IEGs* and late adipogenic genes was investigated by gene expression. To study the effect of CSE on the expression of IEGs, OFs were exposed to CSE for 30-120 minutes.

To investigate whether late adipogenic genes could be downregulated by simvastatin, OFs were pretreated with simvastatin for 24 hours followed by the addition of a standard differentiation cocktail containing dexamethasone (Sigma Aldrich), insulin (Sigma Aldrich), rosiglitazone (Sigma Aldrich), and 3-isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich) and cultured for 12 days. Gene expression was measured using RT-PCR. To visualize the effects of simvastatin on adipogenesis, mature adipocytes were stained with Oil Red O and visualized by light microscopy.

To investigate whether IGF-1 could mimic the effects of insulin, insulin was replaced with IGF-1 in some experiments. Oil Red O staining was performed in differentiated adipocytes at differentiation day 12.

Cigarette smoke extract (CSE)

The CSE was generated by using a validated pump system (Figure 7)(203). Four cigarettes (Marlboro Red Class A) were smoked as follows: 35 ml in 2 seconds, 28 second pause, and 35 ml again in 2 seconds. Each cigarette was smoked with 10 puffs. The smoke was bubbled through 30 ml prewarmed (37°) DMEM (-FBS), giving a 100% concentration of CSE.

The media with the extracted CSE was sterile filtered through a 0.2 μM sterile filter, and the absorbance was measured at 380 nm.

A control was also made, where only air was bubbled through the media followed by sterile filtering and measurement of absorbance.

Cytotoxicity assay

To investigate if CSE exposure or simvastatin and diclofenac treatment effected the cell viability, the Cell Proliferation Kit I (MTT assay, Sigma Aldrich) was used. The kit is a colorimetric assay that quantifies the cellular viability and cytotoxicity in the cells. The water-soluble yellow dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted to an insoluble purple formazan dye by the action of mitochondrial reductase. This reaction only occurs in viable cells and is related to NAD(P)H production through glycolysis. Therefore, the formation of NAD(P)H directly reflects the number of viable cells in a solution. The solution is measured at 570 nm by a spectrophotometer(204).

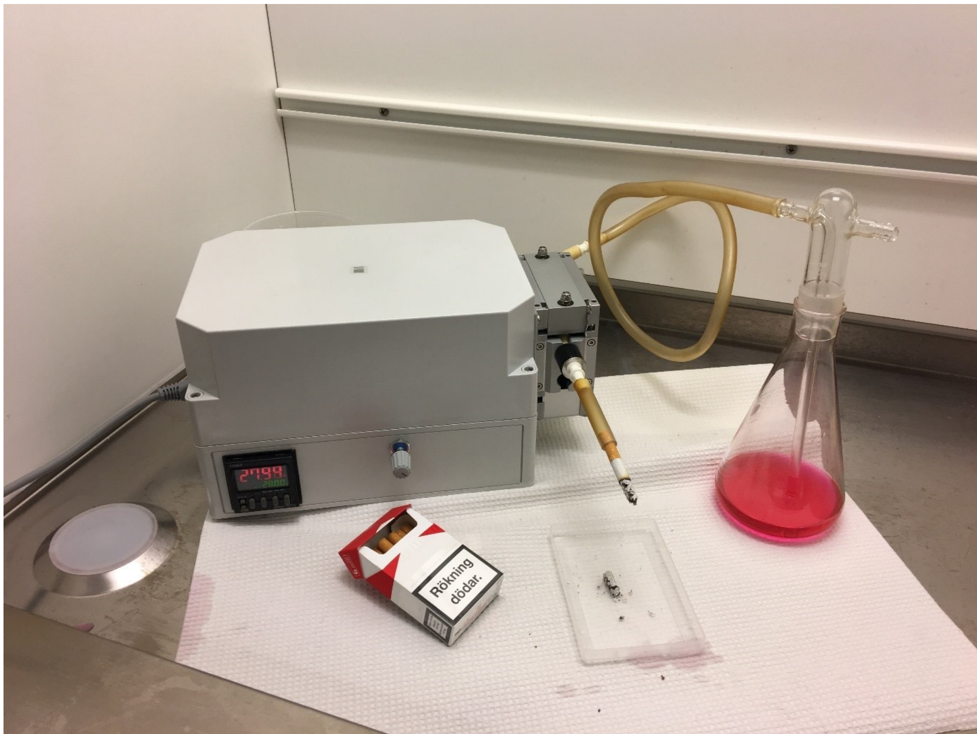

Figure 7: The validated pump system generating 100% cigarette smoke extract

Cell culture PBMCs

PBMCs were isolated from whole blood samples obtained from newly diagnosed GD patients and healthy controls using density centrifugation by Ficoll-Paque Plus (VWR/GE healthcare, Sweden).

To study the effects of CSE, simvastatin, and diclofenac, PBMCs were treated with phytohemagglutin (PHA) (Thermo Fisher Scientific, Sweden) and CSE with or without simvastatin and/or diclofenac for various time periods. The gene expression levels of *PTGS2*, *IL-6*, and *IL-1ß* were measured.

To analyze the PBMCs using flow cytometry, PBMCs were cultured for 5 days. Cells were activated using PHA and recombinant human CD-40-Ligand, cross linking antibody, and IL-4 (B-cell expansion kit, Miltenyi Biotec, Germany). After 72 hours of incubation, IL-2 was added and cultured for another 2 days.

Flow Cytometry

Flow cytometry is a powerful tool that can be applied in multiple fields, such as immunology, virology, and molecular biology. This technology allows us rapidly to analyze single cells suspended in a buffered salt solution. The technique distinguishes different cell types based on the expression of specific markers on the surface or inside the cell. The markers used for distinguishing between cell populations are antibodies that are labeled with fluorochromes. These labeled antibodies bind to markers on the cell. The cells are then led one by one through the analyzer, where they are hit by laser beams, leading to light imitation from the fluorochrome, which is then detected by the system. This tool allows us to quantify and to identify several different types of cells in the same sample or to sort specific populations(205). Flow cytometry was used in **Study III**. PMBCs were stained with the following markers: for pan T-lymphocyte population CD3 (PE-Vio 770, PC7), for monocytes CD14 (VioBlue, BV421) and for pan B-lymphocyte population CD19 (APC). Cell analysis was carried out on a CytoFLEX (Beckman Coulter, Sweden, Flow Cytometry Core Facility at LUDC, Malmö, Sweden).

ELISA

Enzyme-linked immunosorbent assay ELISA is a colorimetric technique that is widely used because of its ability to give fast and sensitive results at the same time and for its affordability. To determine the concentration of the target protein a standard curve is produced by using known concentrations of the protein. The

ELISA kits we used (R&D systems, Biotechne, Sweden), provides the standards, which are based on recombinant proteins. The data obtained from the standard curve is used to measure the unknown concentration of the protein in the samples by comparison of the linear region of the standard curve(206).

ELISA was used to measure IL-1ß, IL-6 and PGE_2 levels in supernatant obtained from PBMC cell cultures in study III.

The principle of analyzing IL-1ß and IL-6 was based on a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6/IL-1ß was precoated onto a microplate. Cell culture supernatant was pipetted into the well, and any IL-6/IL-1ß present would bind to the immobilized antibody. This step was followed by the addition of an enzyme-linked polyclonal antibody specific for human IL-6/IL1-ß. Colorimetric substrate solution was added, and the color developed in proportion to the amount of IL-6/IL-1ß bound. The intensity of color was measured at 540 nm using a microplate reader.

The analysis of PGE_2 was based on a competitive binding immunosorbent assay using antibody-antigen interactions. Here, the antigen binding sites were limited. In this assay, PGE_2 present in the sample competed with horseradish peroxidase (HRP)-labeled PGE_2 for a limited number of binding sites available on the monoclonal antibody. In the first incubation, PGE_2 bound to the antibody, followed by a second incubation in which HRP-labeled PGE_2 bound to the remaining antibody sites. Colorimetric substrate was then added, leading to color development, which increased proportionally with PGE_2 concentration in the sample. The intensity of color was measured at 540 nm using a microplate reader.

Genotyping

The DNA for genotyping was self-collected by the patients. A DNA swab was sent out to the patients, and they took a DNA sample from the buccal mucosa and returned it to our laboratory. The DNA was extracted and amplified, and the quality was checked as described above.

TaqMan™ Allelic discrimination assays provide optimized assays for SNP genotyping. Assays were labeled with VIC™ fluorescent dye, FAM™ fluorescent dye on the 5’ end, a minor groove binder (MGB) and a nonfluorescent quencher (NFQ). TaqMan probes hybridize to the target DNA between the two unlabeled PCR primers. During PCR, Taq polymerase extends the unlabeled primers guided by the template strand. When the polymerase reaches the TaqMan probe, it separates the dye from the quencher by cleaving the molecule, leading to a fluorescence signal from the unquenched VIC™ or FAM™ (Figure 8)(207, 208).

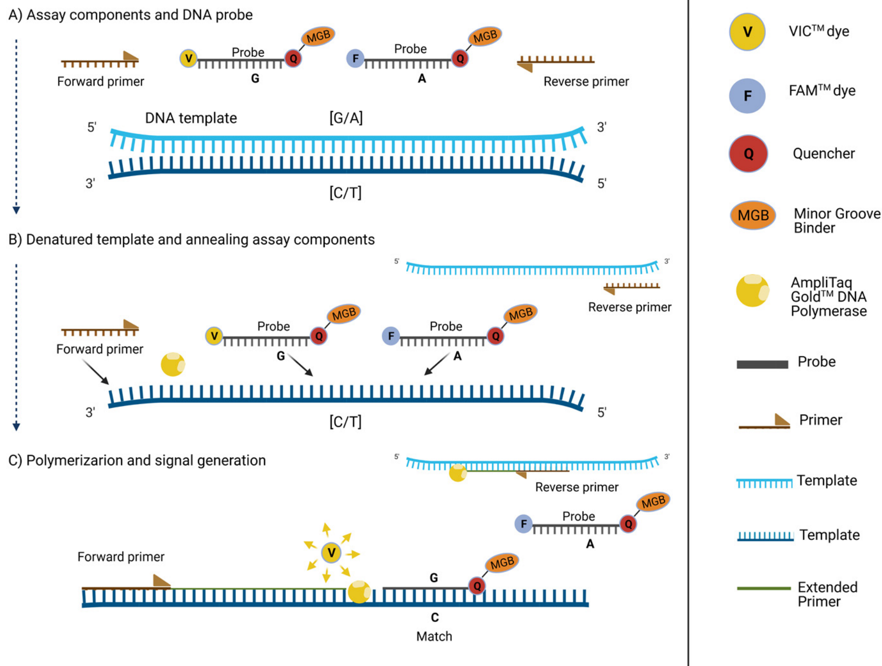

Figure 8: The complementary TaqMan probe fluoresces after it anneals to the template and after cleavage by AmpliTaq Gold DNA Polymerase. (Reproduced from Thermo Fisher Scientific)

The following SNPs were genotyped with TaqMan assays in 127 patients in **Study IV**: rs1378228 and rs12656618 in CYR61 and rs3087243 and rs231775 in CTLA4. These SNPs were chosen for analysis based on previous studies on GD and GO (161). Additionally, a tag SNP, rs6457617, HLA Class II, DR *Beta 1* (HLA-DR-DQQ), was analyzed, as this SNP has previously been reported to be associated with GD (35, 209). We also found HLA-DRB1 expression to be elevated in smokers compared with nonsmokers with active GO in **Study I**.

Statistical analysis

The Pearson Chi-square test is a non-parametric tool designed to analyse group differences when the dependent variables are nominal(210). We used the Chi-square test to assess differences in categorical variables in **Study I** and **Study IV**

The Mann-Whitney test is a non-parametric test, which compares the means between two independent groups with the assumptions that the data is not normally

distributed(211). The Mann-Whitney U rank sum test was applied for the small sample sizes (**Study I**, **Study II**).

The analysis of variance test (one-way ANOVA) is a parametric method and used for a comparison of means between more than two groups. The assumptions for the test are that, the dependent variable is continuous and normally distributed.

It gives an overall *P* value, that indicates, that mean values in at least one pair is significantly different from the other. To determine the specific significant pair, post hoc tests are used. In order to apply the post hoc test, the test for homogeneity of the variances among the groups is applied first(212). The ANOVA test was applied in **Study III** to assess the statistical difference between; CSE exposed and nonexposed PMBCs and simvastatin and/or diclofenac treated and nontreated PBMCs. When the overall test was found to be significant, the Brown-Forsythe test was applied, to test the homogeneity of the variances among the groups. If variances were found to be homogeneous ($P \geq 0.05$) the pairwise comparisons using Tukey's multiple comparisons test were applied to find the specific significant pairs.

T test is a parametric test used to compare the means of two groups. Assumption for t test are; data are normally distributed, and the dependent variable is continuous(213). In **Study IV** the independent t test was used to assess the statistical difference of TRAb values before and 3 months after RI treatment, the TRAb values in patients with GO compared to those without GO, and the difference between prednisolone treated and non-treated patients

The binomial exact test compares the observed distribution to the expected distribution. The assumptions for a binomial test are; two possible outcomes, samples are mutually independent, and the given probability of a given outcome is the same for all samples. In **Study IV** we applied a binomial test to assess if the observed proportion of patients differed from the expected proportion.

Correlation measures, an association between variables. The correlation coefficient "r" describes the strength of the linear correlation between the two random variables. The coefficient can be between -1 and 1, if r=0 there is no correlation between the variables. The assumptions for linear regression are; relationship between X and the mean of Y is linear, the observations are independent of each other, homoscedasticity (residuals have constant variance at every level of X) and residuals are normally distributed(214). In **Study IV** linear regression was applied to assess the correlation between anti-TPO and TRAb levels in RI treated patients.

Logistic regression is used to estimate the odds ratio (OR). OR is a prediction of the fold change in risk due to selected factors regarding the studied phenotype. The analysis allows for adjustment of factors such as gender and smoking. Assumptions for logistic regression are as follows; the dependent variable is binary, observations are independent of each other(215). In **Study IV** the frequency of SNP allele in the

two groups; patients that had TRAb < median(15IU/L)/ TRAb > median (15IU/L) was compared using logistic regression with sex and smoking as covariates. And the same was done for patients with GO and those without GO.

Data are presented as the mean ± standard deviation.

The genotyping data in **Study IV** are presented as odds ratios (ORs) with 95% confidence intervals (CIs). The p-values were based on additive models for the genetic variants.

The data were analyzed using GraphPad Prism, GraphPad Software 9.0 and IBM SPSS Statistics 22 software. Genetic analyses were performed using PLINK version 1.0. 0 (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml).

Significant results were defined as $p < 0.05$.

Results

Study I

Adipose/connective tissue from GO smokers and GO nonsmokers was collected and analyzed by microarray and RT-PCR to investigate the effect of smoking on the gene expression of *IEGs* in severe active ophthalmopathy.

The microarray results showed an upregulation of *IL-1ß* (2.3-fold), *IL-6* (2.4-fold), *HLA-DRB1* (2.1-fold), and the following *IEGs*, *CYR61* (2.18-fold) and *PTGS2* (1.86-fold), in smokers compared to nonsmokers with GO.

These results were confirmed with RT-PCR, where all 5 genes (*IL-1ß* (p=0.03), *IL-6* (p=0.004), *HLA-DRB1* (p=0.05), *CYR61* (p=0.009), and *PTGS2* (p=0.02) were significantly overexpressed in smokers compared to nonsmokers with GO (Figures 9 and 10).

The pathways affected by the genes we found to be upregulated in the microarray were “Humoral Immune Response” “Immune Cell Trafficking” and “Inflammatory Response”, as the networks, and “Immunological Disease” and “Inflammatory Disease” were the biological functions that were strongly associated with the upregulated genes in our data set. The significant canonical pathways were involved in inflammation, immune response, and adipogenesis.

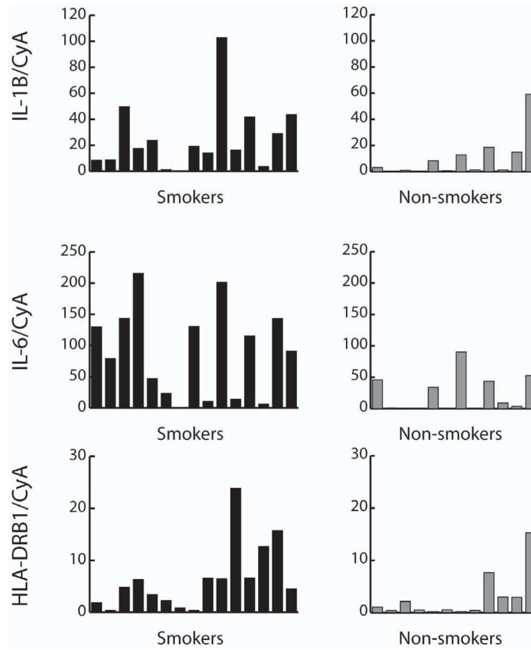

Figure 9. Gene expression in smokers (n=15) and nonsmokers (n=12) with GO analyzed with real-time PCR. *IL-1ß* (p=0.03), *IL-6* (p=0.004), and *HLA-DRB1* (p=0.05) (Mann-Whitney).

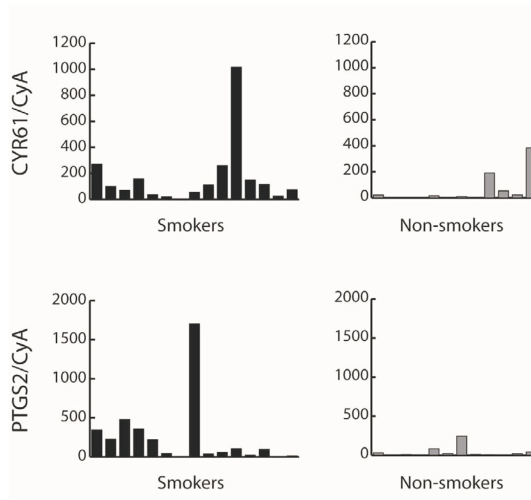

Figure 10. Gene expression of two *IEGs* in smokers (n=15) and nonsmokers (n=12) with GO analyzed with real-time PCR. Both genes selected for confirmation with real-time PCR were significantly overexpressed: *CYR61* (p=0.009), and *PTGS2* (p=0.02) (Mann-Whitney).

Study II

The effects of CSE and simvastatin on the expression of *IEGs*, late adipogenic genes and *IL-6* were investigated with in vitro studies using the 3T3-L1 preadipocyte cell line and OFs from GO patients.

We found that CSE exposure alone upregulated the expression of *IL-1ß* ($P < 0.05$) and *IL-6* ($P < 0.05$), and the following IEGs: *CYR61* ($P < 0.05$) and *PTGS2* ($P < 0.05$) in OFs (Figure 11).

The effect of simvastatin on the upregulated genes was investigated. We found that simvastatin downregulated the gene expression of *Cyr61* ($P < 0.05$), *Ptgs2* ($P < 0.05$) and *Il-6* ($P < 0.05$) in 3T3-L1 preadipocytes (Figures 12).

The effects of simvastatin on late adipogenic genes and adipogenesis were investigated, and simvastatin treatment of OFs downregulated the late adipogenic genes *PPARγ* ($P < 0.05$), and *SCD* ($P < 0.05$). Furthermore, adipogenesis was downregulated by simvastatin in OFs (Figure 13).

The effect of CSE exposure alone on the upregulation of the expression of *Scd1* and *Ppparγ* in 3T3-L1 preadipocytes was investigated. We did not find any upregulation of these genes by CSE exposure alone, indicating that the effect of CSE is adjuvant (Figure 14).

We investigated whether IGF-1 mimicked the effects of insulin on adipogenesis. and found that IGF-1 stimulated the differentiation process, resulting in mature adipocytes ($P < 0.05$) (Figure 15).

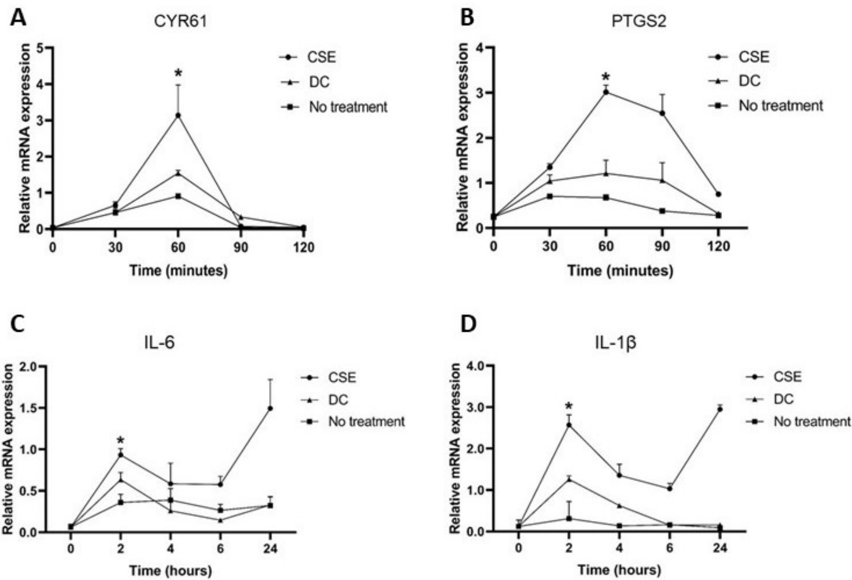

Figure 11: Effects of cigarette smoke extract on early adipogenic genes, *IL-6* and *IL-1β* in human primary orbital fibroblasts. Orbital fibroblasts from patients with GO were stimulated with cigarette smoke extract (CSE) or the differentiation cocktail (DC) for 30 to 120 minutes or 24 hours. Gene expression was quantified with RT-PCR of *CYR61* (A) and *PTGS2* (B), *IL-6* (C) and *IL-1β* (D) with cyclophilin A as an endogenous control. Values are the mean ± SD of 3 independent experiments. **P* < 0.05 (Mann-Whitney).

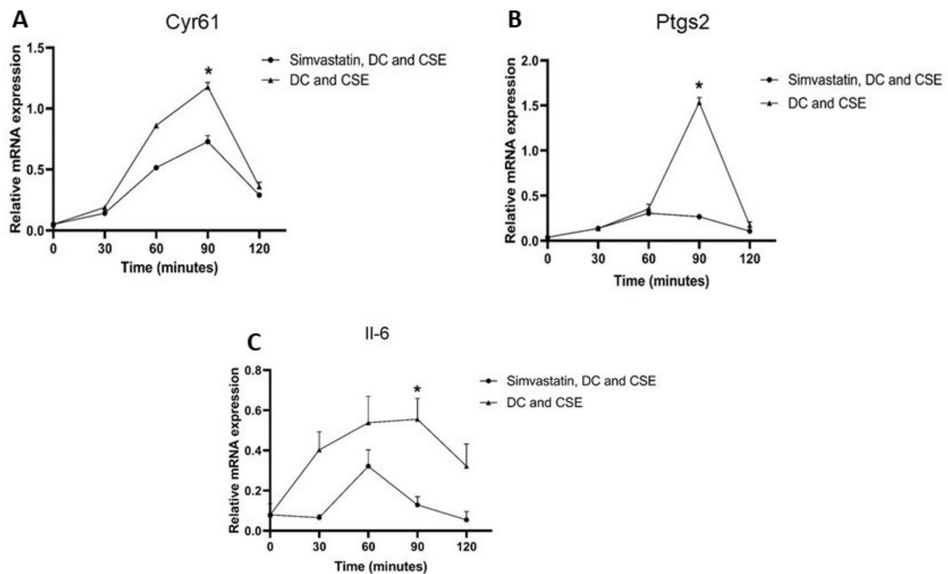

Figure 12: Effects of simvastatin on early adipogenic genes in 3T3-L1 preadipocytes. The 3T3-L1 preadipocytes were stimulated with the differentiation cocktail and cigarette smoke extract for 30-120 minutes in the presence or absence of simvastatin. Gene expression was quantified with RT-PCR. *Cyr61* (A), *Ptgs2* (B) and *Il-6* (C) with β-actin as an endogenous control. Values are the mean ± SD of 3 independent experiments. *P* < 0.05 (Mann-Whitney).

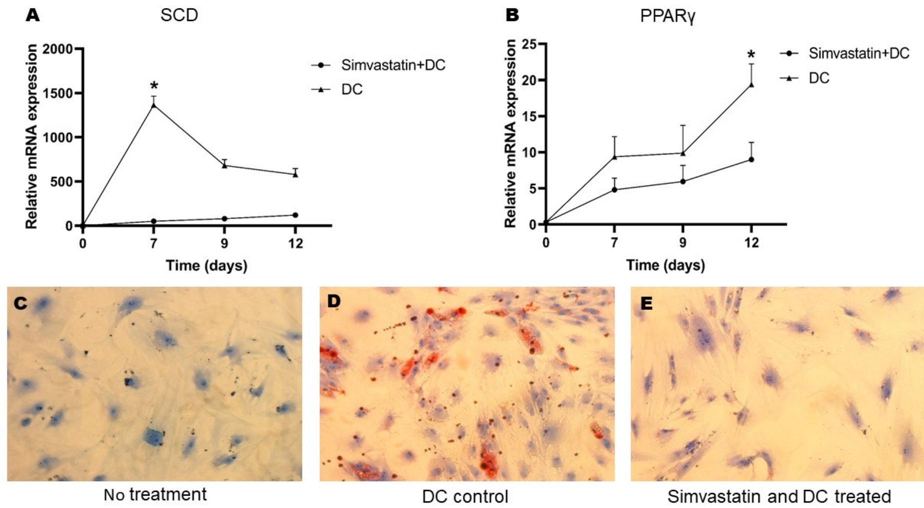

Figure 13: Effects of simvastatin on adipogenesis in human primary orbital fibroblasts. Orbital fibroblasts were treated with simvastatin and differentiation cocktail for 1 to 12 days. Gene expression was quantified with RT-PCR of the late adipogenic genes *SCD* (A) and *PPAR-γ* (B) with cyclophilin A as an endogenous control. Mature adipocytes at differentiation day 12 were stained with Oil Red O and visualized by light microscopy at x20. No treatment (C) treatment with the differentiation cocktail (D) treatment with simvastatin and the differentiation cocktail (E). Values are the mean ± SD of 3 independent experiments. $^{*}P < 0.05$ (Mann-Whitney).

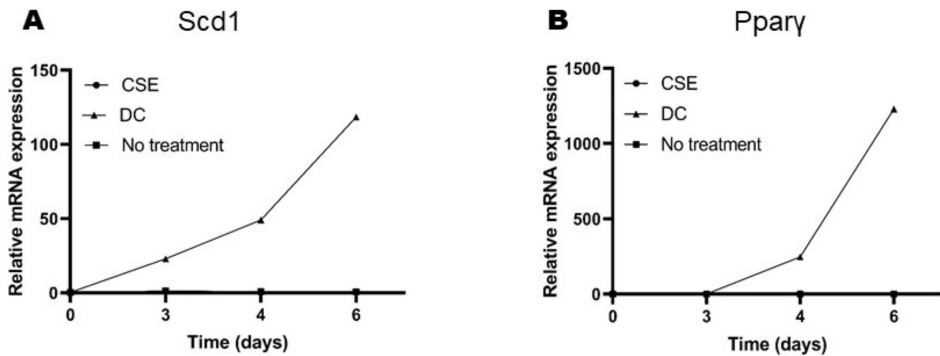

Figure 14: Effects of cigarette smoke extract on the expression of late adipogenic genes in 3T3-L1 preadipocytes. The 3T3-L1 preadipocytes were exposed to cigarette smoke extract. Gene expression was quantified with RT-PCR, *Scd1* (A) and *Ppar-γ* (B) after 3-6 days with β-actin as an endogenous control. Values are the mean ± SD of 3 independent experiments.

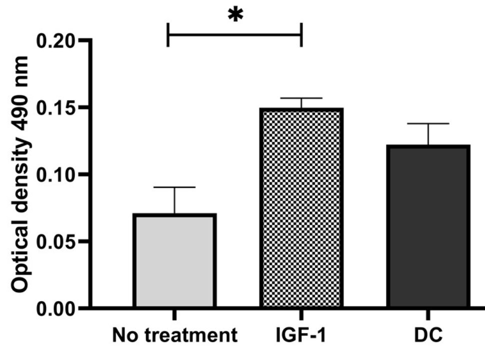

Figure 15. Human primary orbital fibroblasts stimulated to differentiate from IGF-1. Differentiation in orbital fibroblasts was stimulated by IGF-1. Matured adipocytes were stained with Oil Red O at differentiation day 12. Oil Red O was quantified from IGF-1-treated, insulin-treated and nontreated orbital fibroblasts after extraction at 490 nm. Values are the mean ± SD of 3 independent experiments. $^{*}P < 0.05$ (Mann-Whitney).

Study III

In this study, we used an in vitro model to investigate the effects of CSE and modulation of inflammation and proliferation of diclofenac and simvastatin on PBMCs isolated from GD patients and healthy individuals.

We exposed isolated PBMCs to CSE and found that CSE exposure alone could enhance the expression of *PTGS2* ($P \leq 0.0001$), *IL-1ß* ($P \leq 0.05$), and *IL-6* ($P \leq 0.05$). The expression increased even more when PHA treatment was combined with CSE (Figures 16 and 17).

PBMCs were pretreated with simvastatin and diclofenac separately and combined to investigate whether they could downregulate gene expression. We found that both simvastatin and diclofenac downregulated the expression levels of *IL-1ß*, *IL-6,* and *PTGS2* in PBMCs isolated from GD patients compared to untreated PBMCs. When both drugs were combined, the effect was enhanced on the downregulation of *IL-1ß*, *IL-6* and *PTGS2* ($P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively) (Figure 17).

The supernatants from the different conditions in the in vitro experiments were measured, and protein release was analyzed. We found that the effect of CSE exposure on PBMCs stimulated the release of PGE_2 ($P \leq 0.001$), IL-ß and IL-6 ($P \leq 0.001$, $P \leq 0.0001$) (Figure 18).

Furthermore, the effects of simvastatin and diclofenac on the release of PGE_2, IL-1ß and IL-6 were studied. We found that all decreased after treatment with the drugs separately, and when combined, the effect was enhanced (Figure 18).

The effects of CSE exposure on the proliferation of B- and T lymphocytes were studied. We found that CSE exposure significantly increased the proliferation index in B- and T lymphocytes compared to those that were not treated with CSE ($P \leq 0.01$ and $P \leq 0.0001$, respectively) (Figure 19).

The effect of simvastatin was investigated, and the proliferation index of the lymphocytes was defined. Simvastatin treatment significantly downregulated the proliferation index in both B- and T lymphocytes ($P \leq 0.01$) (Figure 19).

We investigated whether IGF-1 could affect the proliferation of B- and T-lymphocytes. Treatment with IGF-1 increased the proliferation of both lymphocyte populations ($P \leq 0.001$) (Figure 20).

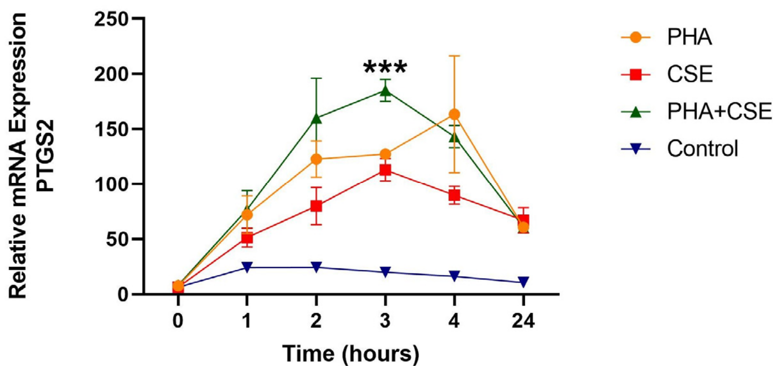

Figure 16: The effect of cigarette smoke extract on PBMC Graves' disease patients. PBMCs were isolated from Graves' disease patients followed by activation by 10% cigarette smoke extract (CSE) and/or phytohemagglutin (PHA). PBMCs were harvested after activation at 1 hour, 2 hours, 3 hours and 4 hours followed by mRNA expression of *PTGS2*. Values are the mean ± SD of three independent experiments. ***$P \leq 0.0001$ (One-way ANOVA).

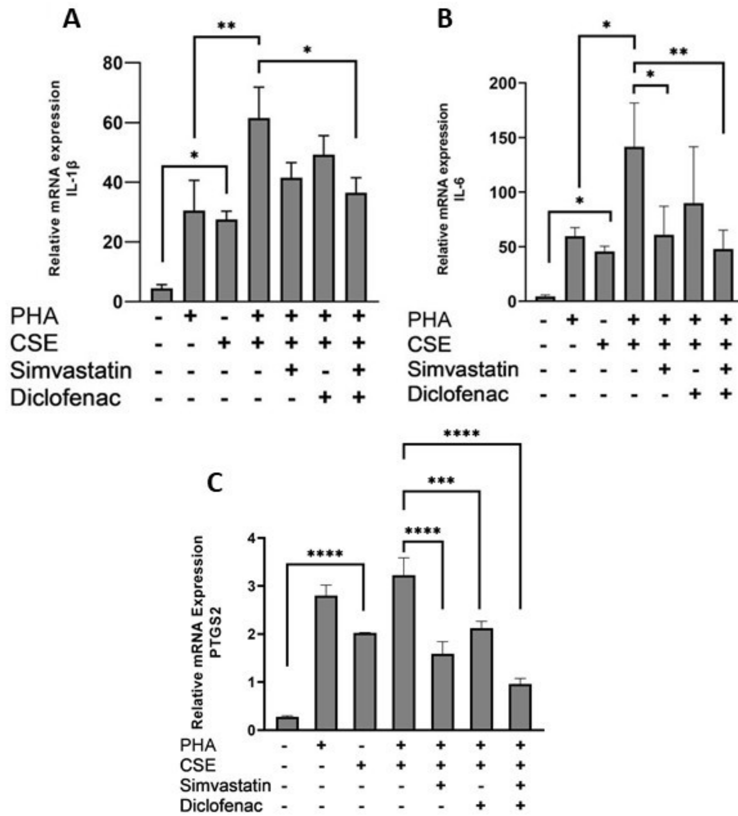

Figure 17: Effects of simvastatin and/or diclofenac on PBMCs from Graves' disease patients activated by cigarette smoke extract (CSE). PBMCs were isolated from Graves' disease patients and cultured with CSE and/or phytohemagglutin (PHA) with or without diclofenac and/or simvastatin and cultured for 72 hours. Cells were harvested, and the mRNA expressions of *IL-1ß* (A), *IL6* (B) and *PTGS2* (C) were quantified. Values are the mean ± SD of three independent experiments. *P ≤0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 (One-way ANOVA).

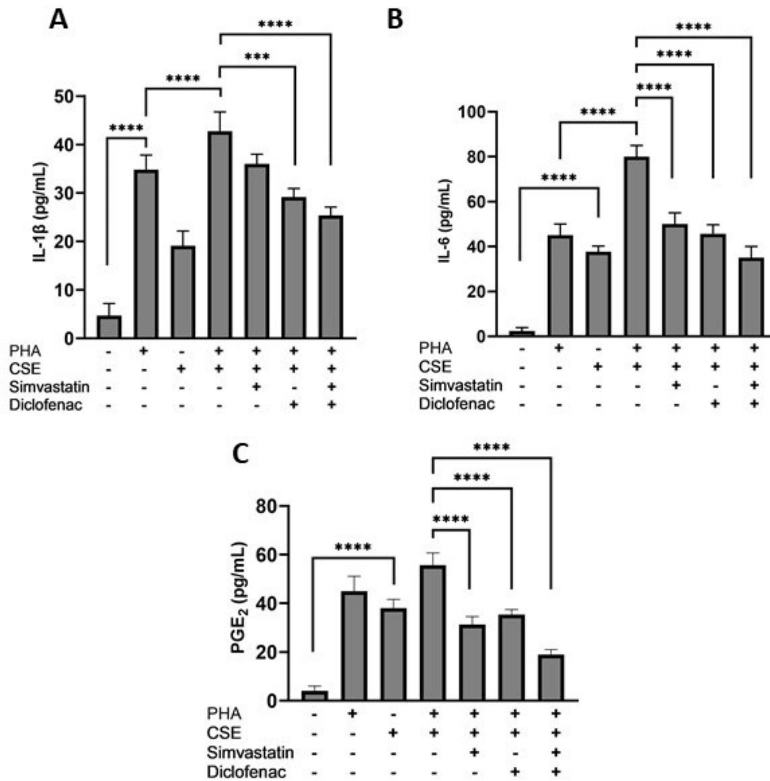

Figure 18: IL-1ß, IL-6 and PGE_2 levels in the supernatant from PBMCs isolated from Graves' patients after 72 hours of exposure to 10% CSE with or without simvastatin and/or diclofenac. Isolated PBMCs from Graves' disease patients were cultured followed by exposure to 10% cigarette smoke extract with or without simvastatin and/or diclofenac for 72 hours. Levels of IL-1ß (A), IL-6 (B) and PGE_2 were measured in the supernatant using ELISA. Values are the mean ± SD of three independent experiments. ***$P \leq 0.001$ and ****$P \leq 0.0001$ (One-way ANOVA).

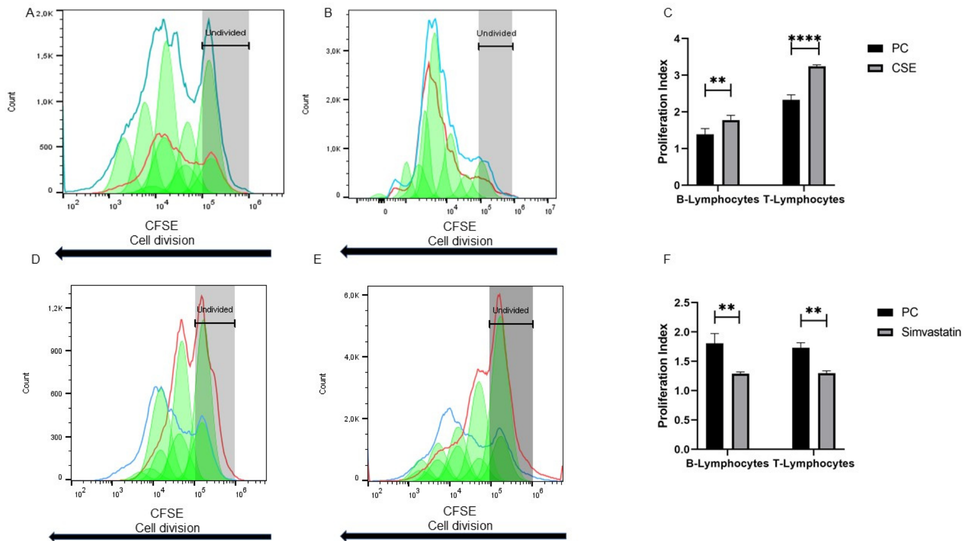

Figure 19: *Effects of Cigarette smoke extract exposure and simvastatin treatment on the proliferation of B-cells and T-cells isolated from Graves' disease patients.* Isolated PBMCs from Graves' disease patients were treated with phytohemagglutin (PHA), recombinant human CD40-Ligand, Cross-Linking Antibody and IL-4 (Proliferation Cocktail (PC)) with or without 10% cigarette smoke extract (CSE) or 10uM simvastatin for 5 days. The effects of CSE exposure and simvastatin treatment (marked with blue) on proliferation was measured in B lymphocytes (A and D), T lymphocytes (B and E) respectively compared with control (marked with red). Data on the proliferation index quantified in B-and T-lymphocytes exposed to CSE or simvastatin treated compared with PC treated (C and F) respectively are the mean ± SD from 3 independent experiments $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$. Proliferation index = total number of divisions divided by the number of cells that went into division calculated using FlowJo™ Version v10.7.

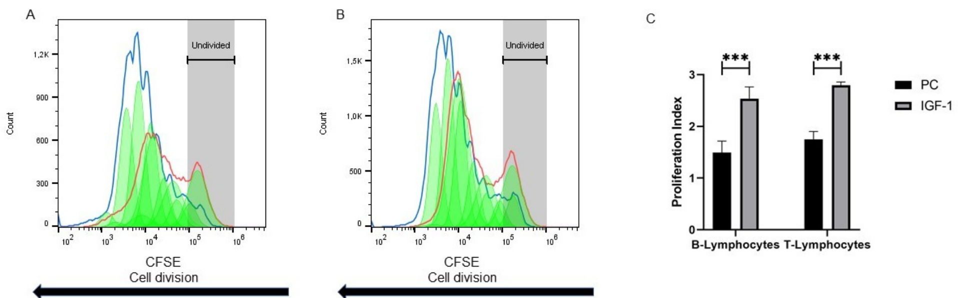

Figure 20: Effects of IGF-1 treatment on the proliferation of B- and T lymphocytes isolated from Graves' disease patients compared with controls. Isolated PBMCs from Graves' disease patients were treated with phytohemagglutin (PHA), recombinant human CD40 ligand, cross-linking antibody and IL-4 (proliferation cocktail (PC)) with or without IGF-1 for 5 days. The effects of IGF-1 treatment (marked in blue) on the proliferation of B lymphocytes (A) and T lymphocytes (B) compared with PC treatment (marked in red). Data on the proliferation index quantified in B- and T lymphocytes treated with IGF-1 compared with PC-treated cells (C) are the mean ± SD from 3 independent experiments $^{***}P \leq 0.0001$. Proliferation index = the total number of divisions divided by the number of cells that went into division calculated using FlowJo™ Version v10.7.

Study IV

In this follow-up study of GD patients without GO treated with radioiodine, we investigated the relationship of TRAb levels before and 3 months after radioiodine treatment. Furthermore, the effect of radioiodine treatment on the development of GO and if the the TRAb levels and GO development was associated with known risk genes for GD (specific risk genes for GO are not yet defined).

GD patients treated with RI had significantly increased TRAb ($P < 0.0001$) and anti-TPO ($P < 0.0001$) levels 3 months after treatment (Figure 21).

TRAb and anti-TPO levels in the patient group were evaluated, and, here, we found that an increased proportion of patients who later developed GO had elevated TRAb and anti-TPO levels ($P < 0.0001$ and $P \leq 0.01$, respectively) (Figure 22).

A correlation between an increase in TRAb and anti-TPO levels was found ($R=0.362$, $P < 0.0001$) (Figure 23).

The median TRAb titer measured 3 months after radioiodine treatment was significantly higher in GO patients than in patients without GO ($P< 0,0001$) (Figure 24).

The effect of prednisolone on TRAb and anti-TPO levels 3 months after RI treatment was investigated. We found that TRAb and anti-TPO levels were significantly lower in patients treated with prednisolone ($P< 0,0001$) (Figure 25).

We studied the association of risk genes with GD and TRAb levels. We found that one SNP (rs231775) in *CTLA-4* was associated with TRAb levels ($P = 0.005$). We did not find any significant association of risk genes with GD and GO ($P = 0.07$) (Table 8).

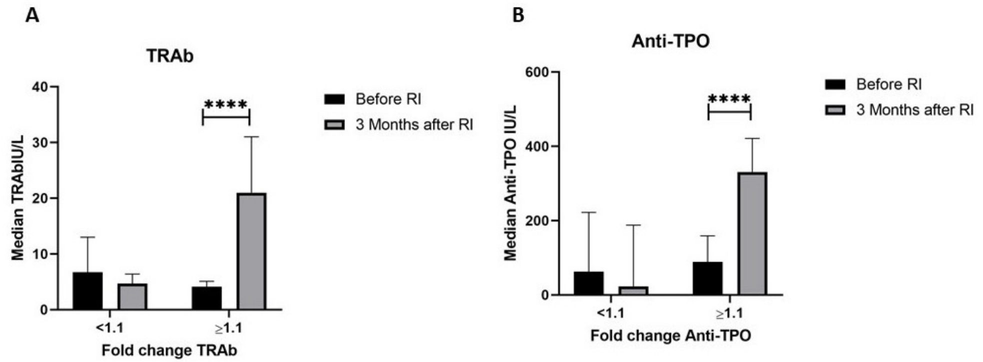

Figure 21: Fold change of TRAb and anti-TPO 3 months after treatment of Graves´ disease with radioiodine. The median level values of TRAb and anti-TPO before and after radioiodine in the group with fold change <1.1 and in the group with fold change ≥1.1 were all significant with p-values <0.0001 (t-test).

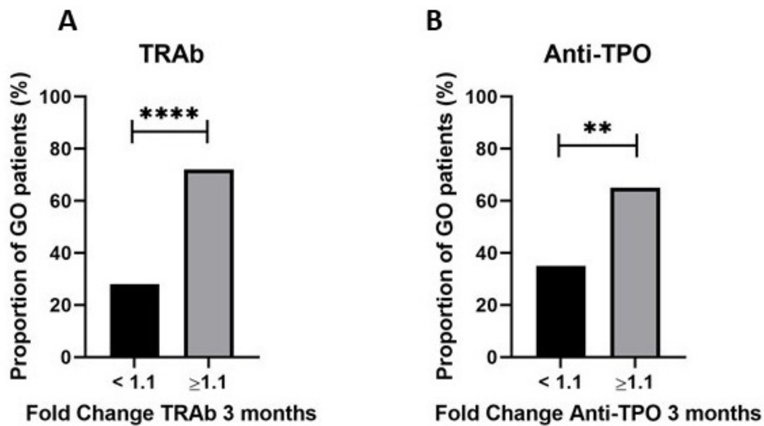

Figure 22: Proportion of TRAb and anti-TPO levels 3 months after treatment of Graves´ disease with radioiodine. Differences in proportions were calculated with a binomial test.

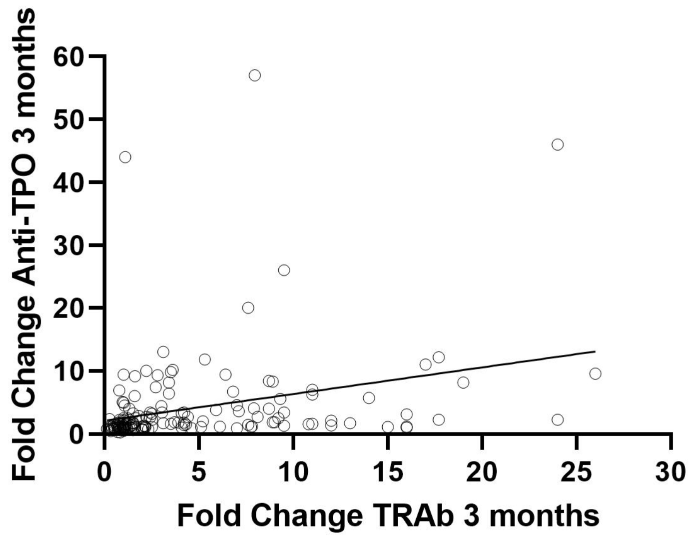

Figure 23: Correlation of fold change in TRAb and anti-TPO levels three months after treatment with RI (R=0.362, P < 0.0001)

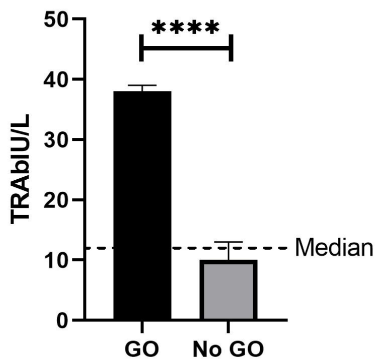

Figure 24: Development of Graves´ ophthalmopathy 1 year after RI in the whole group. Median values of TRAb in patients with and without GO with a dotted line that shows the median value of TRAb in all patients. To calculate the differences in median values, a t-test was used.

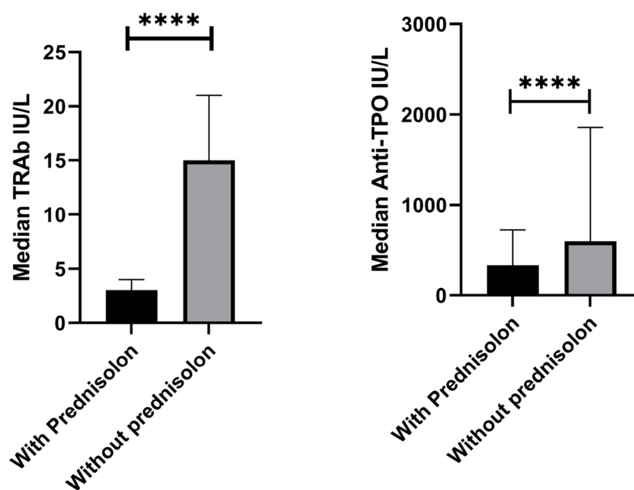

Figure 25: Development of TRAb (A), anti-TPO (B) levels, and response 3 months after treatment with radioiodine in Graves' patients pretreated with prednisolone compared with no treatment (n= 45). Significance between groups P < 0.0001 (t-test).

Table 8. SNPs in GD patients treated with radioiodine **A.** Association with TRAb below and above median 15 IU/L. **B**. Association with GO.

A

Gene	SNP	Allele Frequency TRAb (IU/L) < median (15 IU/L)	Allele Frequency TRAb (IU/L) ≥ median (15IU/L)	Associated Allele	OR for associated allele (95% CI)	p-value
CYR61	rs1378228	0.35	0.39	T	1.19 (0.72-1.99)	0.500
CTLA4	rs3087243	0.37	0.42	A	1.28 (0.77-2.12)	0.334
	rs231775	0.46	0.29	G	0.48 (0.28-0.80)	0.005
HLA-DRB1	rs6457617	0.42	0.42	C	1.30 (0.794-2.14)	0.295

B

Gene	SNP	Allele Frequency in patients without GO	Allele Frequency in patients with GO	Associated Allele	OR for associated allele (95% CI)	p-value
CYR61	rs1378228	0.36	0.4	T	1.181	0.58
CTLA4	rs3087243	0.37	0.48	A	1.62	0.10
	rs231775	0.41	0.28	G	0.56	0.07
HLA-DRB1	rs6457617	0.53	0.53	C	1.56	0.13

Discussion

The work in this thesis started with knowledge based on the expression of adipocyte-related IEGs in GO and their role in the adipogenesis and inflammation pathways in GO. The role of immune cells and adipogenesis in the pathogenesis of GO has been described in many studies. While it is well known that smoking is a strong risk factor for developing GD and GO, the mechanisms behind this effect are not well defined. Another well-known risk factor for developing GO is radioiodine treatment (RI); however, the exact underlying mechanism is not fully understood. Furthermore, no treatments specifically to treat GO are available today. Treatments have been suggested, but these are either not 100% effective or associated with many side effects. Therefore, we found it important to describe the mechanisms behind the development of GO in smokers, to identify risk genes associated with elevated levels of TRAb and the development of GO after RI treatment and to find possible treatments for GO.

Effects of cigarette smoke extract on *IEGs*, *IL-1β* and *IL-6* in orbital tissue

In a previous study, our group found that adipocyte-related *IEGs* were overexpressed in patients with severe active GO(121). We speculated that the effects of smoking may enhance gene expression in patients with active GO. In **Study I,** we compared orbital adipose/connective tissue from smokers and nonsmokers with active severe GO. Using a microarray, we found that adipocyte-related *IEGs*, *CYR61* and *PTGS2/COX-2,* and certain cytokines, such as *IL-1β* and *IL-6,* were overexpressed in smokers with active severe GO compared to nonsmokers with active severe GO, and the results were confirmed with real-time PCR. Furthermore, we found the genes to be involved in pathways associated with inflammation and adipogenesis. These results indicated that smoking enhanced the expression of IEGs in active severe GO.

We investigated whether smoking alone increased the expression of these genes or whether other mechanisms were involved in the pathogenesis of GO. To answer these questions, we developed an in vitro model in which we investigated the effect

of smoking on the expression of IEGs and the process of adipogenesis (**study II**). We exposed the 3T3-L1 preadipocyte cell line and OFs isolated from orbital adipose/connective tissue from GO patients to CSE. We found that *CYR61*, *PTGS2*, *IL-1ß,* and *IL-6* were all upregulated in response to CSE alone.

Previous studies have demonstrated that *Cyr61* participates in the regulation of the inflammatory microenvironment and is involved in the pathogenesis of GO and other autoimmune diseases. *Cyr61* is considered to be a novel proinflammatory factor(216-218) that is involved in many processes, such as extracellular matrix production, angiogenesis, inflammation, cell proliferation, adipogenesis, and fibrosis (217, 219, 220). Hence, there are many mechanisms by which *CYR61* could contribute to the pathogenesis of GO. In **Study II,** we found that CSE alone upregulated the expression of *CYR61* in OFs from GO patients and that the expression of *CYR61* was even higher than that in OFs treated with the standard differentiation cocktail.

IL-1ß is a *CYR61*-responsive gene, and it has been shown that *Cyr61* enhances *IL-1ß* -mediated inflammation (221). *IL-1ß* was overexpressed in smokers with active GO compared to nonsmokers (**Study I**). In OFs, CSE exposure enhanced the expression of *IL-1ß*, and the gene expression was even stronger than in the standard cocktail-treated OFs (**Study II**).

Effects of cigarette smoke extract on *PTGS2*, *IL-1ß* and *IL-6* in PBMCs

In Study III, we found that *IL-1ß* was more highly expressed in PBMCs exposed to CSE than in unexposed PBMCs, and the gene expression increased even more when the mitogens PHA and CSE were combined. The release of IL-1ß by PBMCs was also increased in response to CSE exposure (Study III). IL-1ß is a proinflammatory cytokine that exerts many inflammatory and immunomodulatory activities by stimulating the expression of genes associated with inflammation and autoimmune diseases(222, 223). GO pathogenesis includes connective tissue remodeling and fibrosis. It is believed that OFs are the source of GAG production leading to deposition in orbital tissue in patients with GO. IL-1ß has been shown to increase free radical production in OFs from GO patients, which leads to increased GAG production by OFs(224). It has also been reported that IL-1ß is elevated in tears from patients with active GO compared to patients in the inactive phase(225). Furthermore, *IL-1ß* has also been reported to upregulate *PTGS2* in OFs from GO patients(226). We found *PTGS2* to be upregulated in smokers with active GO (**Study I**) and in OFs exposed to CSE (**Study II**) and PBMCs exposed to CSE alone

or combined with PHA (**Study III**). Furthermore, the release of PGE_2 by PBMCs exposed to CSE was increased (**Study III**).

PGE_2 is a proinflammatory protein that is produced by the isoenzyme, that catalyzes the formation of prostaglandins from arachidonic acid and is activated by various stimuli, including cytokines and mitogens(227). PGE_2 has been reported to play a role in the maturation of B lymphocytes(11). In **Study III,** we found that B lymphocytes that were exposed to CSE had an increased proliferation index compared to those that were not exposed to CSE. This result means that cigarette smoke has an impact on inflammation by elevating the levels of B- and T-lymphocytes and subsequently the production of immunoglobulins. This finding may explain the severe state of GO and higher levels of TRAb in smokers Furthermore, PGE_2 has been shown to stimulate OFs from GO patients to produce IL-6(228). IL-6 is a proinflammatory cytokine and has been suggested to have many roles in the pathogenesis of autoimmunity(229). We found that *IL-6* was upregulated in smokers with active GO, OFs, and PBMCs exposed to CSE (**Studies I, II and III**). IL-6 release by PBMCs exposed to CSE was also found to be elevated compared to PBMCs that were not exposed to CSE (**Study III**).

Cigarettes contain numerous harmful chemicals that affect the innate and adaptive immune systems of smokers(137, 138). A study reported that smokers with GO had an elevated white blood cell and neutrophil count compared to former smokers(230).

In **Study III,** we found that the proliferation of B- and T lymphocytes was significantly increased when lymphocytes were exposed to CSE combined with PHA and CD40 activation.

In summary, adipocyte-related IEGs *CYR61* and *PTGS2* were upregulated in adipose/connective tissue from smokers with active GO compared to nonsmokers with active GO and in OFs exposed to CSE. *PTGS2* expression was upregulated in PBMCs isolated from GD patients in response to CSE. The expression was increased even more when PBMCs were exposed to CSE combined with PHA. Furthermore, the expression levels of *IL-6* and *IL-1ß* were upregulated in OFs and PBMCs in response to CSE, and the response was even stronger in PBMCs when CSE exposure was combined with the mitogen PHA. The release of PGE_2, IL-6 and IL-1β was increased by PBMCs exposed to CSE. The proliferation of B- and T-lymphocytes was significantly increased in response to CSE exposure. These findings suggest that cigarette smoke enhances the expression of adipocyte-related genes and proinflammatory cytokines and increases the proliferation of lymphocytes, all of which are mechanisms important in the pathogenesis of GO. This finding in part may explain why smokers suffer from more severe GO than nonsmokers. Other mechanisms not described here may also contribute to the

effects of cigarette smoke in orbital tissue such as demonstrated in a study on proteomics where the regulation of specific proteins was affected by smoking(163).

Cigarette smoke extract effects on late adipogenic genes

In **Study I**, we found that upregulated genes in the pathway analysis were associated with adipogenesis. We wanted to investigate the effect of CSE on late adipogenic genes stearoyl CoA desaturase (*SCD*) and *PPARγ* in our in vitro model.

SCD1 is an enzyme involved in lipid metabolism and is a marker of adipose tissue(104, 231). *Scd1* knockout mice have been shown to be protected against diet-induced weight gain, showing decreased body adiposity and increased insulin sensitivity(232, 233). Furthermore, it has been reported that genetic variants in the *SCD* gene were associated with insulin sensitivity and body fat distribution in Swedish men(234). A study by Planck et al. showed borderline association of one SNP in the *SCD* gene with GO, and it was speculated that the variant may promote adipogenesis, which is a key process in the pathogenesis of GO(161). In **Study II,** we exposed 3T3-L1 preadipocytes to CSE only to investigate whether CSE alone could induce adipogenesis. It was not possible for CSE alone to induce adipogenesis or upregulate the expression of *SCD. PPARγ* is a member of the nuclear receptor family and plays a central role in adipogenesis, as it serves as a key regulator of adipocyte differentiation(235). Novo adipogenesis is enhanced in GO patients and adipocyte-specific genes, such as *PPARγ,* are overexpressed in orbital tissue from GO patients compared to healthy controls(78, 124). The natural ligand of PPARγ is prostaglandin, PGJ2, which may be a link between inflammation and adipogenesis. We exposed 3T3-L1 preadiopocytes to CSE alone to investigate whether CSE exposure alone could induce adipogenesis. CSE alone could not induce adipogenesis or upregulate the expression of *PPARγ* in preadipocytes.

In summary, we did not find that CSE alone could induce adipogenesis or upregulation of late adipogenic genes in 3T3-L1 preadipocytes; therefore, CSE should be considered an enhancer of adipogenesis.

Effects of simvastatin and diclofenac on *IEGs*, *IL-1ß*, *IL-6* and lymphocytes

Apart from their cholesterol-lowering effect, statins are drugs known for their pleiotropic effects(168, 169). It has been known for a decade that simvastatin has antiproliferative effects on lymphocytes(170, 171), but only recently have studies proven the immunomodulatory properties of statins that affect the T-lymphocyte and APC function(172).

In vitro studies have shown that simvastatin downregulates the expression of *CYR61* in synovial fibroblasts(182). *CYR61* is an *IEG* that is important in the pathogenesis of RA and GO (182, 183). In **Study II,** we found that simvastatin downregulated the expression of *CYR61*.

PGE_2 is produced by orbital fibroblasts in GO patients(236). As mentioned earlier, PGE_2 plays a role in the maturation of B lymphocytes and stimulates the production of IL-6 by OFs. Moreover, it affects the differentiation of T lymphocytes and activates the degranulation of mast cells(71, 126). Hence, PGE_2 is involved in many processes leading to inflammation in GO. *PTGS2* is overexpressed in thyroiditis and in benign and malignant thyroid lesions but not in normal thyroid tissue(237), suggesting that the expression of *PTGS2* is associated with autoimmune disease and thyroid cancer. It has been shown that PGE_2 is decreased in response to NSAIDs(238). In addition, the NSAID diclofenac reduces the titers of anti-TPO in GD patients(191). A previous study showed that the expression of *PTGS2* in adipocytes was decreased to approximately 50% in response to diclofenac(104). In **Studies II** and **III**, the expression of *PTGS2* was downregulated by simvastatin. In **Study III,** PGE_2 release was decreased in response to simvastatin. Diclofenac alone could also downregulate the expression of *PTGS2* and PGE_2 release, but when both treatments were combined, the decrease was even stronger (**Study III**). Diclofenac not only decreases the gene expression of *PTGS2* but is also a well-defined inhibitor of the enzyme, which may also reduce the production of other prostaglandins.

IL-6 plays an important role in the activation of B lymphocytes and the development of antibody-producing plasma cells(239), which are both important in the pathogenesis of GO.

In **Studies II** and **III,** simvastatin downregulated the expression of *IL-6*. Furthermore, the release of IL-6 by PBMCs was also found to be downregulated as demonstrated in **Study III**. When simvastatin treatment was combined with diclofenac, the downregulation of *IL-6* and decrease in IL-6 release by PBMCs were even stronger (**Study III**).

B lymphocytes and T lymphocytes have important roles in the pathogenesis of GD and GO, as T lymphocytes provide the first signal to B lymphocytes and activate

them, leading to production of autoantibodies against TSHR. Targeting B- and T-lymphocytes may lead to a reduced production of autoantibodies against TSHR. In Study III, we found that the proliferation of B lymphocytes was downregulated by simvastatin. It has been reported that B lymphocytes activated through CD-40 upregulate enzymes in the mevalonate pathway involved in cholesterol biosynthesis and hence are a target for simvastatin(184). A study found that B lymphocytes were downregulated in vitro after simvastatin treatment(184). Simvastatin has been proven to have antiproliferative effects on T lymphocytes(170, 171). In **Study III,** T lymphocyte proliferation was downregulated by simvastatin.

IL-1ß is a proinflammatory cytokine that has been shown to be upregulated in active GO compared to controls(121). Simvastatin can downregulate IL-1ß expression in PBMCs isolated from hyperlipidemia patients(240). In **Study III,** we found that *IL-1ß* was downregulated by simvastatin and that the release of IL-1ß by PBMCs was decreased after simvastatin treatment. When simvastatin treatment was combined with diclofenac, the decrease in protein release and gene expression was even stronger (**Study III**).

In summary, we demonstrated that simvastatin downregulated the expression levels of *CYR61*, *PTGS2*, *IL-1ß,* and *IL-6* and the release of PGE_2, IL-1ß and IL-6. Diclofenac downregulated the expression levels and release of *PTGS2* (PGE_2), IL-1ß, and IL-6. The downregulation of gene expression and decrease in protein release were stronger when both treatments, diclofenac and simvastatin, were combined.

The proliferation of B- and T lymphocytes was downregulated in response to simvastatin treatment. These findings suggest that simvastatin and diclofenac may decrease the inflammatory process in the pathogenesis of GO and the production of immunoglobulins by downregulating the proliferation of B- and T lymphocytes. These findings suggest that the effects of simvastatin and diclofenac should be investigated in GO patients in a clinical trial.

Effects of IGF-1 on adipogenesis and proliferation in B- and T lymphocytes

Antibodies directed against IGF-1R have been found to circulate in GD patients compared to very few controls(241). Orbital fibroblasts isolated from GD patients were treated with IGF-1, which led to elevated levels of IL-16 and RANTES, which are both very strong T-lymphocyte chemoattractants. The same effect of IGF-1 was not seen in orbital fibroblasts from healthy subjects(242). A study reported that B lymphocytes from GD patients were more skewed towards the CD19 IGF-1R phenotype, which led to an enhanced expansion of B lymphocytes(135). In Study

II, we showed that IGF-1 led to the differentiation of preadipocytes into mature adipocytes, and in **Study III,** we showed that PBMCs activated with PHA and IGF-1 enhanced B- and T-lymphocyte expansion. These findings suggest that stimulation of IGF-1 leads to adipogenesis and expansion of lymphocytes, which both have important roles in the pathogenesis of GO.

Studies have suggested that the IGF-1 pathway has an important role in the pathogenesis of GO through autoantibody-activated signaling and autocrine/ paracrine mechanisms and therefore may be an important target for treating GO(243-245).

Effects of Radioiodine treatment

RI treatment is known to be a strong risk factor for the development of GO and might be mediated by the activation of TRAb (52). It has been shown that higher levels of TRAb increase the risk of GO at the time of GD diagnosis and after the diagnosis of GD (246, 247). Furthermore, it has been reported that TRAb increases within 3 months after GD patients are treated with RI (51). In **Study IV,** we showed that TRAb and anti-TPO levels significantly increased in 70% and 60% of the patients, respectively, 3 months after RI treatment. The increase of TRAb correlated with anti-TPO. Furthermore, we showed that the TRAb titer in the patients who developed GO was elevated compared to the group without GO. Patients who are at risk of developing GO are commonly treated with prednisolone during RI treatment. We found that prednisolone treatment decreased the TRAb and anti-TPO titers.

One group of patients who received RI treatment had decreased TRAb titers, and another group had increased TRAb titers. It is important to identify the group that increases in TRAb titers, as this group is more likely to develop GO. If patients with increases in TRAb titers can be identified, prednisolone can be selectively used only in these patients to prevent the development of GO. In **Study IV,** we investigated whether elevated levels of TRAb were associated with known risk genes for GD, as specific risk genes for GO have not yet been identified. We found that the *CTLA-4* SNP, +49A/G (rs231775), was associated with TRAb levels.

CTLA-4 is a negative immune regulator of T cell activation, with a principle function in maintaining immune homeostasis. It plays an important role in the prevention of autoimmune disease by inhibiting T cell activation (248, 249). CTLA-4 and CD28 are homologous receptors that are expressed on CD4+ and CD8+ T lymphocytes(250).

It has been shown that mice with a homozygous mutation in *Ctla-4* have a lethal lymphoproliferative disorder(251). Previous studies have shown that the *CTLA-4* SNP, +49A/G (rs231775), was associated with GD(252, 253). Furthermore, it has

been shown that the frequency of the G/G allele was higher in GD patients than in the frequency of the A/A allele, and the inhibitory effect of *CTLA-4* was found to be less potent in PBMCs from GD patients with the G/G allele(254). We found the G allele frequency to be significantly lower in RI-treated patients who had TRAb levels below the median. We found that rs231775 was almost significantly associated with GO, and the finding may not have been significant because of the small number of patients. However, the G allele frequency in patients without GO was lower than that in the group with GO. These results may indicate that the rs231775 variant in *CTLA-4* leads to decreased activation of T lymphocytes, which in turn affects TRAb levels and GO development. However, more detailed investigations are required to answer this question.

Limitations

I am aware that our studies have several limitations. In **study I,** the findings are based on a limited number of individuals who differ slightly in clinical parameters, such as age, sex, and duration of GD and GO. This factor may interact with the gene expression profile that we present in this study. Furthermore, all the smokers included in the microarray study had been treated with corticosteroids longer than nonsmokers. However, the pathways associated with inflammation were significantly upregulated in smokers compared to nonsmokers with GO. Conclusions from **Studies II** and **III** are drawn on the basis of the findings in, in vitro models, and in **Study II,** we did not investigate the protein expression of the IEGs. However, the studies were based on findings from **Study I**, where we show that the same group of genes was overexpressed in smokers with active GO compared to nonsmokers with active GO. **Study IV** is a follow-up study and includes a limited number of patients. However, we were still able to show the effect of RI treatment on the levels of TRAb and the development of GO; furthermore, we showed an SNP in *CTLA-4* to be associated with TRAb.

Future perspectives

GO is a complex disease that involves inflammation and adipogenesis. Smoking and radioiodine are strong risk factors. Future research should focus on investigating the effects of smoking on GO. Unfortunately, no in vivo model has been established at this point. An in vivo mouse model would have been a great tool to investigate the effects of smoking and radioiodine in less time. Patients that have GD or GO and undergo surgery for removal of orbital tissue or thyroid tissue are very few, which makes these studies very long, as there is a waiting time between available samples. With mouse models, we would be able to study many effects of smoking and radioiodine in less time. It would enable us to sort the cells located in the tissues and the effects of simvastatin and diclofenac on immune competent cells. Another focus in the future will be novel therapies for GO. The most promising drug that has passed phase III trials is teprotumumab; however, teprotumumab is not 100% effective. In the future, a combination of drugs such as teprotumumab, simvastatin, and diclofenac might achieve better outcomes. Investigating the effect of teprotumumab, simvastatin, and diclofenac, separately and combined, in randomized clinical trials will be of great interest.

Conclusions

I. *IEGs*, *IL-1ß*, and *IL-6,* were overexpressed in smokers with severe active GO compared to nonsmokers, suggesting that smoking in GO activates pathways associated with adipogenesis and inflammation.

II. Cigarette smoke extract exposure alone upregulated the gene expression of certain *IEGs*, *IL-1ß,* and *IL-6* in OFs and 3T3-L1 preadipocytes. Exposure to cigarette smoke extract enhanced adipogenesis but could not alone induce differentiation of preadipocytes or OFs into mature adipocytes. Simvastatin downregulated the gene expression of IEGs and late adipogenic genes and was able to downregulate adipogenesis both alone and in combination with diclofenac. IGF-1 was used as a mitogen that induced the differentiation of preadipocytes to mature adipocytes.

III. Cigarette smoke extract exposure significantly upregulated the gene expression and release of the inflammatory markers *PTGS2, IL-6 and IL-1ß* and the proliferation of B- and T lymphocytes. Simvastatin and diclofenac downregulated the expressions of *IEGs* and the proliferation of B- and T-lymphocytes, suggesting that these drugs might affect the treatment of GO. Furthermore, IGF-1 upregulated the proliferation of B- and T lymphocytes in our in vitro model.

IV. The increase in TRAb titers after radioidine treatment of GD patients without GO was associated with later development of GO. Prednisolone decreased the level of TRAb in the group that had increased TRAb titers 3 months after radioiodine treatment. The rs231775 genetic variant in *CTLA-4* was associated with higher levels of TRAb after radioiodine treatment, but the association with the development of GO did not reach significance because of the small number of patients developing GO after RI. Therefore, this study will be extended in the future.

Popular Science Summary

Graves' disease (GD) is a complex autoimmune disease that mostly affects women. Autoantibodies against the TSH receptor on the thyroid stimulate the thyroid to overproduce thyroid hormones, which leads to elevated levels of thyroid hormones in the body (hyperthyroidism). Patients who suffer from GD may develop associated eye disease, Graves' opthalmopathy (GO). Due to inflammation in GO, the eye muscles get swollen and the fat tissue behind the eye expands, leading to many symptoms such as protrusion of the eye, pain, eyelid swelling, eye redness, excess tearing, gritty sensation, light sensibility, double vision and impaired vision, among others.

Smokers with GD have a higher risk of developing GO and are more likely to suffer from severe GO. Another strong risk factor for developing GO is radioiodine treatment for hyperthyroidism. It is well known that smoking and radioiodine are strong risk factors associated with the development of GO, but the underlying mechanisms remain unclear. The therapies available for GO today are associated with many side effects; therefore, the development of novel therapies is of great importance.

The development of GO mainly includes two processes: inflammation and fat expansion. In this thesis, we studied the effects of smoking on genes important for the development of GO.

In **Study I,** GO patient smokers and nonsmokers underwent surgery to remove orbital tissue, and then, this tissue was investigated for gene expression. We found that genes with roles in the development of fat and inflammation were more highly expressed in smokers than in nonsmokers. The genes we found to be highly expressed, such as *CYR61* and *PTGS2*, both have roles in fat expansion and inflammation and are known as immediate early genes (*IEGs*). We found *IL-1ß* and *IL-6* to be more highly expressed in smokers than in nonsmokers. These genes are involved in inflammation. To investigate the pathways in which these genes were involved, we used bioinformatic tools. Bioinformatic tools are software programs that can help extract information about the role of genes in molecular pathways. By using the software, we found that the genes highly expressed in the group of smokers compared to nonsmokers were genes involved in the inflammatory, immune responsive and fat expansion pathways.

In **Study II**, we further studied the direct effect of cigarette smoke extract (CSE) on the process of fat expansion. We therefore established an in vitro model in which we isolated orbital fibroblasts (OFs) from orbital tissue removed from GO patients who underwent surgery. OFs can develop into fat cells under appropriate conditions. We exposed OFs to CSE. We found that CSE exposure alone upregulated the gene expression of both *IEGs*: *CYR61* and *PTGS2*. *IL1ß* and *IL-6* were also upregulated in response to CSE exposure alone. We did not find that CSE alone induced fat expansion and led to mature fat cells. Furthermore, we investigated whether simvastatin, a lipid-lowering drug, could downregulate the expression of *IEGs*, *IL-6* and fat expansion. Simvastatin has been shown to downregulate genes involved in inflammation, and as it is a lipid-lowering drug, we hypothesized that the drug might be able to decrease fat expansion in OFs. We found that simvastatin downregulated *IEGs*, *IL-6,* and fat expansion.

In the context of GO, the orbit is infiltrated with immunocompetent cells. In **Study III** we investigated the effect of CSE on peripheral blood mononuclear cells (PBMCs) isolated from GD patients. We found that the gene expression and release of *PTGS2* (PGE_2), *IL-1ß*, and *IL-6* were upregulated by CSE treatment alone. Furthermore, we found that the expansion of B- and T lymphocytes was upregulated when exposed to CSE. The effect of diclofenac, an anti-inflammatory drug, and simvastatin on PBMCs was also studied. We found that both drugs alone could downregulate the expression and protein release of *PTGS2*, *IL-1ß,* and *IL-6,* but when the drugs were combined, the downregulation was even stronger. Simvastatin could downregulate the proliferation of B- and T lymphocytes.

Radioiodine (RI) treatment in GD patients may in some patients lead to elevated levels of autoantibodies (TRAb). Patients with increased levels of TRAb may be at higher risk of developing GO. In **Study IV**, we found that RI treatment led to elevated levels of TRAb and that a significantly larger proportion of patients who developed GO after RI treatment had elevated levels of TRAb than those that did not develop GO. Furthermore, we studied whether those patients who had increased TRAb levels and developed GO had any gene variants (single nucleotide polymorphism [SNP]) in risk genes that was previously found to be associated with GD and/or GO. SNPs are being investigated in order to predict the development of a certain disease, the response to drugs or susceptibility to environmental factors in individuals. We found one SNP in the *CTLA-4* gene to be associated with TRAb levels. We did not find any significant association with GO; however, the same SNP was close to being significantly associated.

To summarize our findings,

IEGs with roles in fat expansion and inflammation were upregulated in smokers with active GO compared to nonsmokers. Furthermore, the upregulated genes were involved in pathways associated with fat expansion and inflammation.

CSE alone could upregulate gene expression in *IL-1ß*, *IL-6,* and *IEGs*, which have roles in fat expansion and inflammation. Simvastatin downregulated the gene expression of *IL-1ß*, *IL-6*, and *IEGs* and inhibited fat expansion in OFs isolated from GO patients.

PBMCs exposed to CSE had enhanced gene expression of *PTGS2*, *IL-1ß,* and *IL-6*. The protein release of PGE2, IL-1ß, and IL-6 was also elevated in response to CSE exposure alone. Simvastatin and diclofenac downregulated gene expression and protein release. Furthermore, CSE exposure resulted in increased expansion of B- and T lymphocytes, and proliferation was downregulated by simvastatin.

Radioiodine treatment led to elevated levels of TRAb and the development of GO. One SNP in the *CTLA-4* gene was associated with TRAb levels in patients treated with radioiodine.

Populärvetenskaplig sammanfattning

Graves sjukdom (GD) är en komplex autoimmun sjukdom som framförallt drabbar kvinnor. Antikroppar mot TSH-receptorn på sköldkörteln stimulerar sköldkörteln till ökad hormonsyntes vilket leder till ökade nivåer av sköldkörtelhormon i kroppen (hypertyreos). Patienter med Graves sjukdom kan även utveckla ögonsymtom, så kallad endokrin oftalmopati (GO). På grund av inflammation vid GO svullnar ögonmuskler upp och fettbildning i ögonhålan ökar vilket leder till en rad symtom hos patienten såsom utåtstående ögon, smärta, svullna ögonlock, röda ögon, ökat tårflöde, gruskänsla, ljuskänslighet, dubbelseende och nedsatt syn.

Rökare har större risk att utveckla GO än icke-rökare och drabbas oftare av svårare sjukdom. En annan riskfaktor för utveckling av GO är radiojodbehandling för hypertyreos. Det är välkänt att rökning och radiojod utgör starka riskfaktorer för utveckling av GO men mekanismer bakom detta är okända. De behandlingar som finns för GO är förknippade med många biverkningar och behovet att utveckla nya behandlingsalternativ är därför stort.

Två processer är viktiga vid utveckling av GO, inflammation och fettbildning. I denna avhandling studerades effekter av rökning på gener med funktioner i inflammation och fettbildning som bidrar till utvecklingen av GO.

I **Studie I** studerades genuttryck hos rökare och icke-rökare med GO i fettvävnad från ögonhålan som opererades bort vid ögonkirurgi. Gener med funktioner i fettbildning och inflammation var överuttryckta hos rökare jämfört med icke-rökare. En grupp av gener kända som,”immediately early genes” (*IEGs*), däribland *CYR61* och *PTGS2*, uttrycktes i högre grad hos rökare jämfört med icke-rökare. Dessa gener har funktioner i fettbildning och inflammation. Även *IL-1ß* och *IL-6* var överuttryckta hos rökare, dessa gener har funktioner i inflammation. Med hjälp av bioinformatiskt software har vi identifierat molekylära ”pathways” som gener överuttryckta hos rökare jämfört med icke-rökare är involverade i. Dessa gener var delar av ”pathways” med funktioner i inflammation, immunförsvar och fettbildning.

I S**tudie II** har vi vidare studerat effekt av rökextrakt (CSE) på fettbildning. I studie II har vi utvecklat en in vitro modell där orbitala fibroblaster (OFs) isolerades från fett från ögonhålan från opererade patienter med GO. OF är celler som har potential att utvecklas till fettceller efter stimulering. Vi exponerade OFs för CSE. Enbart CSE uppreglerade uttryck av både *IEGs*, *CYR61* och *PTGS2*. Även *IL-1ß* och *IL-6*

var överuttryckta efter CSE exponering. Enbart CSE kunde däremot inte trigga igång fettbildning med utveckling av mogna fettceller. Vi har även studerat huruvida simvastatin, en blodfettsänkande medicin, kunde nedreglera genuttryck av *IEGs* och *IL-6* samt fettbildning. Simvastatin har i andra studier visat sig kunna nedreglera gener med funktioner i inflammation och eftersom den har blodfettsänkande egenskaper, vi hypotiserade att medicinen även kunde minska fettbildning. I våra försök kunde simvastatin nedreglera *IEGs*, *IL-6* och fettbildning.

Vid GO infiltreras strukturer i ögonhålan med immunceller. I **Studie III** har vi undersökt effekt av CSE på perifera mononukleära blodceller (PBMCs) isolerade från patienter med GD. Genuttryck och proteinutsöndring av *PTGS2* (PGE_2), *IL-1ß* och *IL-6* var uppreglerad av enbart CSE. Dessutom uppreglerades proliferation av B- och T-celler av CSE. Vi har även studerat effekt av diklofenak, ett antiinflammatoriskt läkemedel, och simvastatin, på PMBCs. Båda läkemedel för sig kunde nedreglera genuttryck och proteinutsöndring av *PTGS2*, *IL-1ß* och *IL-6* men effekten var starkare när läkemedlen kombinerades. Simvastatin kunde även nedreglera proliferation av B- och T celler.

Behandling med radiojod (RI) för GD kan hos vissa patienter leda till stegrade nivåer av autoantikroppar (TRAK). Patienter som stiger i TRAK efter RI kan löpa större risk för utveckling av GO. I S**tudie IV** har vi påvisat att RI behandling ledde till ökade nivåer av TRAK och att större andel av patienter som utvecklade GO efter RI behandling hade stegrade TRAK nivåer jämfört med dem som inte utvecklade GO. Vi vidare undersökte huruvida individer som utvecklade höga nivåer av TRAK efter RI eller GO hade genvarianter (så kallade"single nucleotide polymorphisms [SNPs]") i riskgener som tidigare associerats med utveckling av GD och/eller GO. SNPs studeras med syftet att förutse utveckling av en viss sjukdom, svar på behandling eller individuell känslighet till miljöfaktorer. En SNP i *CTLA-4* genen var kopplad till TRAK nivåer. Samma SNP var nära statistiskt signifikant koppling även till GO.

Sammanfattningsvis har vi i denna avhandling visat att:

IEGs med funktioner i fettbildning och inflammation var uppreglerade i fettvävnad från ögonhålan hos rökare med aktiv GO jämfört med icke-rökare. Gener som var uppreglerade hos rökare var delar av "pathways" med funktioner i fettbildning och inflammation.

CSE uppreglerade genuttryck av *IL-1ß*, *IL-6* och *IEGs* med funktioner i fettbildning och inflammation i OF från patienter med GO. Simvastatin nedreglerade genuttryck av *IL-1ß*, *IL-6* och *IEGs* och minskade fettbildning i OFs isolerade från GO patienter.

PBMCs som exponerats för CSE hade ökat genuttryck av *PTGS2*, *IL-1ß* och *IL-6*. Proteinutsöndring av PGE2, IL-1ß och IL-6 ökade också efter CSE exponering. Simvastatin och

diklofenak nedreglerade detta genuttryck och proteinutsöndring. CSE ökade proliferation av B- och T lymfocyter och simvastatin minskade denna CSE effekt.

RI behandling resulterade i förhöjda nivåer av TRAK och utveckling av GO. En SNP i *CTLA4* var kopplad till TRAK nivåer efter RI behandling.

Acknowledgments

"Giving thanks for abundance is greater than abundance itself
Today, lets swim wildly, joyously in Gratitude"

Mewlana Jalāl ad-Dīn Mohammad Rūmī

Yes! And, finally!! The section of the whole thesis book that everyone reads. Even I think this section will be read by all before reading any other part of the thesis.

I think this part of the thesis was one of the most difficult parts to write. As I am not sure how I would be able to express my gratitude and appreciation to all the wonderful people who have been a part of this journey. As we all know, of course, no success is accomplished as an individual.

Success involves many people around you that have had some involvement in your achievements. It includes a multitude of sacrifices, their kindnesses, and the good work they contributed, seen and unseen, known and unknown.

First, I want to thank all the patients who agreed to participate in our studies. Without their consent, this thesis would never have been possible to finish.

Although there might exist many SUPERvisors in the world, I consider myself the luckiest and most fortunate PhD student to have been working under close supervison of **Mikael Lantz**, **Tereza Planck** and **Peter Åsman.**

My main SUPERvisor, **Mikael Lantz,** has been a true inspiration. Thank you for taking me in as a PhD student, for supporting conference attendance, for trusting in me, and for all of your encouragement and support. Thank you for always understanding various life situations. I feel so fortunate to have had such a motivating, genuine and caring SUPERvisor. Thank you for a great learning experience. Your guidance and counsel was what I needed to reach the end of my PhD.

My Co-SUPERvisor **Tereza Planck** was always so patient and smiling. It has been a true pleasure having you as my SUPERvisor. I have learned so much from you. You have always been available and helpful. I am so glad that I had the opportunity

to work closely with you and under your supervision. Thank you for your guidance, positivity in every situation and great learning experience.

My Co-SUPERvisor **Peter Åsman,** thank you for always providing me with any of the tools I needed to complete my tasks and always being helpful and positive whenever I needed any help.

My opponents at my halftime; **Olga Göransson** and **Marketa Sjögren**, thank you so much for your constructive feedback and encouragement

I want to thank all my local collaborators and coauthors: **Håkan Brorson**, **Kleoniki Tsoumani**, **Ruchi Jain**, **Pernilla Asp**, **Anna Sundlöv**, **Jan Tennvall**, **Ola Lindgren**, **Kristoffer Ström**, **Bengt Hallengren**, **Pernilla S. Johnson and Tania Singh**. Thank you all for your contributions to the manuscripts and smooth collaborations.

"Be grateful for whoever comes,
because each has been sent as a guide from beyond"

Mewlana Jalāl ad-Dīn Mohammad Rūmī

Thank you, **Maria Sterner** and **Malin Neptin** for always being so helpful and thoughtful whenever I needed help or just was too stressed. I have missed you both a lot.

Malin Svensson thank you for always helping out, with technical or practical issues. You have never been too busy to help, have always been available and have kept a smile. **Widet Gallo** thank you for being kind and helpful with any questions regarding RNA extractions, which at times were very frustrating. **Marketa Sjögren** thank you for your kindness, scientific guidance and chats. **Ulrika Blom-Nilsson**, thank you for always "taking care of everything" almost before me asking for it. **Ola Hansson** thank you for being thoughtful and kind. **Esa Laurila**, thank you for always helping out with any technical issues in the lab. **Ylva Wessman** thank you so much for being so flexible and collaborative with the blood samples and for always being available whenever needed. **Jeanette Arvidsson** thank you so much for always being so helpful and caring. You never said you did not have time whenever I came to you, and you were always available on e-mail. When I was analyzing on CytoFlex you would just come by to check on me and ask if I needed help; you are just the kindest person ever. I am so glad that you were there helping me out. Thank you, **Ana Persson**, for always being available, helpful and kind. **Rosmari Geidenstam,** thank you for always taking care of all administrative work with a smile. **Bengt Hallengren**, when I had my first oral presentation at the ETA,

you were so supportive and kind, I can never forget that. Thank you so much for being this humble and caring human being you are.

There are for sure many other coworkers and former PhD students that have helped me out in some way. It is difficult for me to mention them all here, but you all know who you are, and I will always appreciate and be thankful for your help and discussions.

"Friend, our closeness is this:
anywhere you put your foot,
feel me in the firmness under you."

Mewlana Jalāl ad-Dīn Mohammad Rūmi

Thank you **Iram** for always being so caring and thoughtful. For helping out in situations where I had to bring Aniyah to work (apparently you are on her BEST friend list). Thank you for bringing me tea and cookies during my writing so I did not forget to eat, and our makeup shopping. I am so fortunate to have met such a lovely and sincere friend like you here at CRC.

Thank you **Ruchi** for the fruitful scientific discussions and sharing your gorgeous vegetarian recipes, for being a good and caring friend. The pep talks during my writing has been very appreciated, Thank you!

Dear **Tania** and **Vaibhav,** I am not sure if I will be able to tell you in words how much I appreciate you both in my life. Thank you, for your true support, sincerity, love and help in every way. I have known you since we moved to Sweden; thanks for being our family here. **Tania** thanks for all the time during my thesis, and for the pep talks and support. I am truly blessed to have met you. You are a true friend.

Thank you, **Pradeep** for being a good friend, always so helpful and kind; **Gad,** you are always so thoughtful, thank you, for all your help with technical issues, musical inspiration and for making Aniyah interested in piano. I am so grateful, that I have you as my friend, thank you for everything**. Njaniday** for your pep talks and always being happy to help with anything, our lovely chats during lunch and for being a good friend.

“Always search for your innermost nature in those you are with,
as rose oil imbibes from roses.”

Mewlana Jalāl ad-Dīn Mohammad Rūmī

My oldest friends, **Noreen** and **Bushra,** for always being my energy boost when everything seemed so stressful and unreachable. Tak for jer mine basser. Hvor er det fantastisk at have venner som jer, som altid støtter en og giver en det friske pust, man lige behøver for, at komme op på hesten igen

Rihanna min kæreste ven. Tak for alt. Du var der og er der altid. Uden dig ville jeg ikke have kunnet skrive min Ph.d. afhandling. Tak for at være den du er. Det er en velsignelse at have dig i mit liv.

”Be with those who help you being”

Mewlana Jalāl ad-Dīn Mohammad Rūmī

Thank you, my dear sister **Tuba** for always supporting me and believing in me, for being there whenever I needed you. My dear brother **Mansoor,** thank you, for believing in me and always being supportive. My dear sister-in-law **Maham,** thank you, for your support and kindness. I love you all very much. You are the ultimate source of motivation for me. I cannot thank the Almighty enough for giving me such caring and loving family, that have been my support in every way. My lovely nieces, **Yemina** and **Mayra** and nephews **Binyamin** and **Maaz** who make every day so beautiful. May you all always be blessed with every good thing in life. I er alle mit livs farver og rosen duft.

Thank you, dear mother in-law for your endless support and love. For always being there, whenever needed.

"I love you neither with my heart nor with my mind.
Just in case the heart might stop, the mind can forget.
I Love you with my soul.
Soul never stops or forgets"

Mewlana Jalāl ad-Dīn Mohammad Rūmī

My dearest kids; for being patient and for accepting a halftime mother during the writing of the thesis. My firstborn, kindest and most caring **Ayaan,** thank you so much for always being so thoughtful and understanding. My beautiful little lady **Aniyah** for being so understanding and patient. I love you both with every part of my soul; you both are a true delight for my heart and eyes. May God always protect you and shower His tremendous blessings upon you both. You are a true gift from Him that I can never thank Him enough for.

My dear husband **Irfan**, you have been the biggest support. Your love, care, and support complete me in every way. You have always thought about my needs before your own, you are the kindest and most humble person I know. I am truly blessed to have you in my life. The Almighty has given me the ultimate blessing when He brought you into my life. I love you very much, may you always stay healthy and blessed.

My **grandmother,** for always showering her love and prayers upon me and wishing me every good thing in life. For always being supportive, caring and believing in me. I pray to the Almighty that He gives you a long and healthy life.

Thank you **ammi** and **abu**. My dearest parents thank you for your endless support and guidance. For always believing in me and being there for me when I needed you. You always taught me to be kind and humble, no matter what, you taught me always to increase my knowledge. You taught me that wisdom would come to me through the unlikeliest of sources and lot of times it might be through failures. You taught me that when I will hit rock bottom I should always remember that the rock bottom can also be a solid foundation from which I can build and grow when I am struggling. You taught me to learn from every experience in my life whether success or failure. I cannot thank you enough for the guidance you have given me, and the sacrifices you have made in order that we could have a better life. I pray to the Almighty that He will always bless you with a long and healthy life and that He may have mercy on you. I love you both a lot.

There remain many mysteries about the existence of **GOD**, mysteries beyond science. For me, I have felt the protection and blessing from the Almighty in every step of my life. I thank **GOD** for His tremendous blessings He has always Showered upon me and my loved ones.

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