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GLP-1 SECRETION AND SIGNALING IN PATIENTS WITH ANEURYSMAL ENLARGEMENT OF THE THORACIC AORTA – A POTENTIAL CONTRIBUTOR TO REDUCED PREVALENCE OF ANEURYSM IN DIABETES

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GLP-1 secretion and signaling in patients with aneurysmal enlargement of the thoracic aorta – a potential contributor to reduced prevalence of aneurysm in diabetes
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my mum

‘We never counted floods
We went through everything and passed’
Odysseus Elytis, ‘The crazy boat’

‘Κατακλυσμούς ποτέ δε λογαριάσαμε
Μπήκαμε μέσ’ στα όλα και περάσαμε’
Οδυσσέας Ελύτης, ‘Το τρελοβάπορο’

POPULAR SCIENCE SUMMARY OF THE THESIS

An aneurysm is a dilation of an artery (blood vessels that carry blood) when it is greater than 50% of its normal size. The aneurysm located in the aorta in the chest is called thoracic aortic aneurysm (TAA) and is fatal when it ruptures, making it a big clinical problem. The normal aortic valve in the heart has three cusps, i.e. tricuspid aortic valve (TAV), however in 1-2% of the population two of the cusps are merged, leaving only two cusps, a condition termed bicuspid aortic valve (BAV). BAV is the most frequent cardiac anomaly, and involves a higher risk of TAA formation, although the nature of this relationship is unclear. Importantly, type 2 diabetes (T2D) is associated with lower frequency of TAA among BAV and TAV patients alike, an association that may involve effects of current anti-diabetic therapy.

Glucagon-like peptide-1 (GLP-1) is a peptide that can lower blood sugar (glucose) by stimulating insulin secretion, when needed. Since 2006 the European Union has approved drugs that mimic GLP-1 (GLP-1 analogs) and drugs that prevent the degradation of GLP-1 (DPP-4 inhibitors) for T2D management. Of note, GLP-1 has been shown to also regulate mechanisms involved in TAA formation/progression and to protect from aortic aneurysm in animal models. Consequently, the use of GLP-1 analogues in anti-diabetic therapy and reports of increased fasting plasma levels of GLP-1 in association with T2D provokes the hypothesis that GLP-1 signaling may underlie the reduced frequency of TAA in T2D.

The focus of this thesis is to understand the potential role of GLP-1 signaling in the reduced frequency of TAA among T2D patients. We aim to identify potential correlations between elevated levels of GLP-1 in the blood and mechanisms involved in TAA formation, including inflammation, and the expression of a protein functioning as a master regulator of inflammation (called Syndecan-1 [Sdc-1]). In addition, we studied the potential direct effects of GLP-1 signaling on cells of the aortic wall. Patients with TAA represent a new patient group that may benefit from the above-mentioned GLP-1-based drugs, which would mean a first pharmacological intervention to reduce the frequency of TAA or prevent small TAAs from progressing into life-threatening conditions.

There is normally a balance between inflammatory markers and inhibitors of inflammatory markers in your body. If an imbalance between the two arises, this may lead to inflammatory disease. TAA has been associated with relatively more inflammatory markers in the blood. In **Paper I**, we showed that T2D patients have increased levels of inhibitors of inflammatory markers in relation to inflammatory marker, where TAA patients, in agreement with previous reports, have reduced levels of these inhibitors. Furthermore, the ratio of inhibitors of inflammatory markers and inflammatory markers was increased in T2D in association with increased GLP-1 levels in the blood.

In **Paper II**, we demonstrated that T2D is associated with increased Sdc-1 expression in the aortic wall, which has previously been indicated to prevent aneurysm formation in animal models, and may be a contributing factor to the reduced frequency of TAA among T2D patients. Nevertheless, no significant association was detected between aortic Sdc-1 expression

and levels of GLP-1 in blood. We also showed that TAA patients had increased aortic Sdc-1 expression in association with the increased invasion of immune cells in the aortic wall, supporting previous reports of induced Sdc-1 expression in TAA that balances out the inflammatory processes.

In **Paper III**, we evaluated whether Sdc-1 may also play a role in BAV patients. However, we did not observe an increase in Sdc-1 expression in the aorta of BAV patients with T2D.

Finally, in **Paper IV**, we performed studies on cells isolated from the aortic wall and cultured in the lab. These aortic smooth muscle cells normally regulate the contractility of the aorta. In patients with TAA, these cells lose their contractile function. Importantly, Sdc-1 expression maintains the contractile function of these cells, and can be induced by effectors also activated in response to GLP-1. Here, we show that diabetic conditions do not change Sdc-1 expression, although in a simulated aneurysmatic environment GLP-1 analogs prevent the reduced Sdc-1 expression observed in smooth muscle cells.

In conclusion, this thesis showed that T2D is associated with a relative increase in inhibitors of inflammatory markers, in relation to inflammatory marker, and increased levels of aortic Sdc-1 expression, which may play a part in the lower frequency of TAA in T2D patients with TAV.

ΕΠΙΣΤΗΜΟΝΙΚΗ ΠΕΡΙΛΗΨΗ ΤΗΣ ΔΙΠΛΩΜΑΤΙΚΗΣ

Όταν η αορτή (αγγείο που διοχετεύει αίμα) διασταλεί κατά 50% του αρχικού του μεγέθους μπορεί να προκαλέσει ανεύρυσμα. Συγκεκριμένα το ανεύρυσμα που βρίσκεται στην αορτή του στήθους ονομάζεται ανεύρυσμα θωρακικής αορτής (TAA). Το TAA είναι θανατηφόρο όταν σπάσει, με την έλλειψη φαρμακολογικής θεραπείας και προληπτικού ελέγχου να το καθιστά ένα μεγάλο κλινικό πρόβλημα. Η φυσιολογική βαλβίδα έχει τρεις πτυχές (γλωχίνες) και ονομάζεται τρίπτυχη αορτική βαλβίδα (TAV), ωστόσο το 1-2% του πληθυσμού έχει δύο από τις τρεις γλωχίνες συγκολλημένες; αυτό ονομάζεται δίπτυχη αορτική βαλβίδα (BAV) και είναι η πιο κοινή καρδιακή ανωμαλία. Οι ασθενείς με BAV έχουν υψηλότερο κίνδυνο ανευρύσματος από ό, τι τα άτομα με TAV, αλλά η φύση αυτής της σχέσης είναι ασαφής. Έρευνες έχουν δείξει ότι ο διαβήτης τύπου 2 (T2D) σχετίζεται με μειωμένη συχνότητα TAA τόσο μεταξύ ασθενών με BAV όσο και με TAV, μια συσχέτιση που μπορεί να συνδέεται με την τρέχουσα αντιδιαβητική θεραπεία.

Το γλυκαγονοειδές πεπτίδιο-1 (GLP-1) είναι ένα πεπτίδιο που μπορεί να μειώσει το σάκχαρο του αίματος. Η ταχεία αποικοδόμηση του GLP-1 οδήγησε στην ανάπτυξη φαρμάκων που μιμούνται την δράση του GLP-1 (ανάλογα GLP-1) και φαρμάκων που αποτρέπουν την αποικοδόμηση του GLP-1 (αναστολείς DPP-4), τα οποία έχουν εγκριθεί από την Ευρωπαϊκή Ένωση από το 2006 και χρησιμοποιούνται με επιτυχία στη διαχείρηση του T2D. Το GLP-1 ρυθμίζει πολλούς μηχανισμούς που εμπλέκονται στο σχηματισμό και την εξέλιξη του TAA, προστατεύοντας παράλληλα από ανεύρυσμα αορτής σε ζώα. Κατά συνέπεια, η χρήση αντιδιαβητικής θεραπείας που έχει ως βάση το GLP-1 και αναφορές στα αυξημένα επίπεδα GLP-1 στο αίμα, σε ασθενείς με T2D, οδηγούν στην υπόθεση ότι το GLP-1 μπορεί να αποτελεί τη βάση της μειωμένης επικράτηση του TAA στον T2D.

Σ’ αυτή τη διατριβή επικεντρωθήκαμε στο να κατανοήσουμε τον πιθανό ρόλο της σηματοδότησης του GLP-1 στη μειωμένη επικράτηση του TAA στον T2D. Στόχος μας είναι να εντοπίσουμε πιθανές αλληλεπιδράσεις μεταξύ των αυξημένων επιπέδων GLP-1 στο αίμα και των διεργασιών που εμπλέκονται στο σχηματισμό TAA, συμπεριλαμβανομένου της φλεγμονής, και της έκφρασης μιας πρωτεΐνης που λειτουργεί ως κύριος ρυθμιστής της φλεγμονής (της συνδεκάνης-1 (Sdc-1)). Επιπλέον, διερευνήσαμε πιθανές άμεσες επιδράσεις της σηματοδότησης του GLP-1 στα κύτταρα του αορτικού τοιχώματος. Οι ασθενείς με TAA αντιπροσωπεύουν μια νέα ομάδα ασθενών που μπορεί να ωφεληθεί από τη θεραπεία που έχει βάση το GLP-1, η οποία θα σήμαινε μια πρώτη φαρμακολογική παρέμβαση έτσι ώστε να μειωθεί η συχνότητα του TAA ή στην πρόληψη της εξέλιξης μικρών TAA σε καταστάσεις απειλητικές για τη ζωή.

Υπάρχει συνήθως μια ισορροπία μεταξύ των δεικτών φλεγμονής και των αναστολέων τους στο σώμα μας. Μια πιθανή ανισορροπία μεταξύ των δύο, μπορεί να οδηγήσει σε φλεγμονώδη νόσο. Στο Άρθρο I, δείξαμε ότι το αίμα ασθενών με T2D έχει αυξημένα επίπεδα αναστολέων δεικτών φλεγμονής σε σχέση με τους δείκτες φλεγμονής σε αντίθεση με τα μειωμένα επίπεδα αυτών των αναστολέων που ανιχνεύονται στο αίμα ασθενών με TAA. Περαιτέρω, η αναλογία

αναστολέων δεικτών φλεγμονής και δεικτών φλεγμονής ήταν αυξημένη στους ασθενείς με T2D σε συνδυασμό με τα αυξημένα επίπεδα GLP-1 στο αίμα.

Στο Άρθρο II, δείξαμε ότι ο T2D σχετίζεται με αυξημένη έκφραση της Sdc-1 στο αορτικό τοίχωμα. Έχει προηγουμένως αποδειχθεί ότι η αυξημένη αορτική έκφραση της Sdc-1 αποτρέπει το σχηματισμό ανευρύσματος σε μοντέλα ζώων και η παρατηρούμενη αυξημένη αορτική έκφραση Sdc-1 σε συνδυασμό με τον T2D μπορεί να συμβάλει στη μειωμένη συχνότητα του TAA στον T2D. Ωστόσο, δεν καταφέραμε να εντοπίσουμε μια συσχέτιση μεταξύ της έκφρασης της Sdc-1 στην αορτής και του GLP-1 στο αίμα. Δείξαμε επίσης ότι οι ασθενείς με TAA είχαν αυξημένη αορτική έκφραση της Sdc-1 σε συνδυασμό με αυξημένη εισβολή ανοσοκυττάρων στο αορτικό τοίχωμα, υποστηρίζοντας προηγούμενες αναφορές που δείχνουν ότι αυτή η αυξημένη έκφραση της Sdc-1 αντισταθμίζει τις φλεγμονώδεις διεργασίες στο TAA.

Στο Άρθρο III, αξιολογήσαμε εάν η Sdc-1 μπορεί να παίζει ρόλο σε ασθενείς με BAV. Ωστόσο, δεν παρατηρήσαμε αύξηση στην έκφραση της Sdc-1 στην αορτή των ασθενών με BAV και T2D.

Τέλος, στο Άρθρο IV, πραγματοποιήσαμε μελέτες σε κύτταρα που απομονώθηκαν από το τοίχωμα της αορτής και καλλιεργήθηκαν στο εργαστήριο. Αυτά τα ‘κύτταρα λείου μυός αορτής’ σε φυσιολογικές συνθήκες ρυθμίζουν τη συσταλτικότητα της αορτής, όπου σε ασθενείς με TAA αυτά τα κύτταρα χάνουν τη συσταλτική τους λειτουργία. Δείχνουμε ότι ενώ οι διαβητικές συνθήκες δεν αλλάζουν την έκφραση της Sdc-1, τα ανάλογα της GLP-1 επαναφέρουν τη μειωμένη έκφραση της Sdc-1 που παρατηρείται στις συνθήκες ανευρύσματος.

Συμπερασματικά, αυτή η μελέτη έδειξε ότι ο T2D σχετίζεται με τους αναστολείς των δεικτών φλεγμονής και με την αυξημένη αορτική έκφραση της Sdc-1, που μπορεί να συμβάλει στη μειωμένη συχνότητα του TAA στον T2D.

ABSTRACT

An aortic aneurysm is a localized dilation of the artery, greater than 50% of the normal size. It is the result of a weakening of the aortic wall, and untreated or unrecognized aortic aneurysms may be fatal due to massive internal bleeding, as a result of dissection or rupture of the aneurysm. The aorta is divided into two major sections; the thoracic and the abdominal aorta. Approximately 25% of all aneurysms are Thoracic aortic aneurysms (TAA) and the remaining are Abdominal aortic aneurysms (AAA). Most commonly, TAAs are located in the ascending aorta (AscAA). There are different conditions that involve increased risk of TAA formation, for example, bicuspid aortic valve (BAV), a congenital condition where the aortic valve has two instead of three (tricuspid aortic valve (TAV)) cusps. The mechanisms underlying the increased prevalence of TAA among BAV patients are largely unknown. However, differential mechanisms have been indicated in aneurysm formation in BAV and TAV patients. Interestingly though, the prevalence of AAA and TAA among BAV and TAV patients alike is reduced among those with type 2 diabetes (T2D). The reduced prevalence of TAA in T2D has been speculated to involve pharmacological treatment.

The peptide hormone, Glucagon-like peptide-1 (GLP-1) is an intestinal hormone and neuronal peptide with an important role in glucose metabolism, primarily through enhanced glucose-induced insulin secretion, wherefore GLP-1 also forms the basis of current anti-diabetic therapy. Although studies have indicated defective postprandial secretion of GLP-1, T2D is also associated with increased fasting plasma levels of GLP-1. Of note, the GLP-1 receptor is expressed in various different tissues including the vasculature, and GLP-1-based therapy prevents aneurysm formation *in vivo*. Furthermore, clinical studies indicate that GLP-1 governs many of the mechanisms implicated in TAA formation/progression, such as inflammation, oxidative stress, and proteolytic activity. Smooth muscle cells (SMCs) play a central role in TAA pathology, where the transition from a differentiated (with contractile function) to a de-differentiated (synthetic) phenotype is important in the formation/progression of TAA. Some of the indicated beneficial effects of GLP-1-based therapy on TAA pathology may be speculated to involve the proteoglycan, syndecan-1 (Sdc-1), whose expression is regulated by intracellular targets of the GLP-1 receptor (GLP-1R). Sdc-1 modulates pro-inflammatory processes and has a protective role in aortic aneurysm. Nevertheless, it is not known whether T2D or enhanced fasting plasma GLP-1 in T2D is associated with altered inflammatory profile, proteolytic activity, or Sdc-1 expression, nor are the effects of GLP-1R activation on SMCs in the presence/absence of diabetic conditions well characterized.

Our hypothesis was that elevated fasting plasma GLP-1 levels, in T2D patients with valve disease, may contribute to the reduced risk of AscAA in T2D patients. The aim of this project was to further understanding of a potential role for GLP-1 in the reduced prevalence of AscAA in T2D, by assessing potential correlations between elevated fasting plasma GLP-1 and processes involved in aortic aneurysm formation, including inflammation, proteolytic activity, and Sdc-1 expression. In addition, we aimed to determine potential direct effects of GLP-1 signaling on aortic SMCs.

In **Paper I**, we show that patients with T2D are characterized by a shift towards an anti-inflammatory profile, which is associated with elevated fasting plasma GLP-1. Furthermore, a potential role for this anti-inflammatory shift in the reduced prevalence of AscAA in T2D patients is indicated by the inflammatory Th1 bias of immune responses in patients with AscAA.

In **Paper II**, the results show elevated expression of Sdc-1 in the aorta of T2D patients, facilitating a possible role for this increase in T2D- associated reduced prevalence of AscAA. We also demonstrate increased Sdc-1 expression in AscAA patients in association with increased infiltration of macrophages, substantiating data from *in vivo* studies indicating a role for Sdc-1 upregulation on immune cells as a counterbalancing response to TAA formation.

In **Paper III**, results do not indicate an association between T2D and increased Sdc-1 expression in aortic adventitial tissue among BAV patients. Nor was increased macrophage infiltration or Sdc-1 expression detected in association with AscAA among BAV patients.

Finally, in **Paper IV**, we show that GLP-1R is highly expressed in differentiated aortic SMCs, as compared to de-differentiated SMCs, and its activation attenuates Angiotensin II induced downregulation of Sdc-1 expression. In addition, a diabetic milieu increased the gene expression of the differentiation markers (calponin and myosin).

In conclusion, this study indicates that T2D is associated with a Th2 bias, and increased aortic expression of Sdc-1, which may play a role in the reduced prevalence of AscAA in T2D.

LIST OF SCIENTIFIC PAPERS

This thesis includes the work of the following papers and from now on they will be referred to by their Latin numbers as shown below:

I. **Stelia Ntika**, Harshitha Jois, Karin Lång, Christian Olsson, Anders Franco-Cereceda, Hanna M. Björck and Camilla Krizhanovskii.
Elevated glucagon-like peptide-1 and a Th2 shift may support reduced prevalence of thoracic aortic aneurysm in patients with diabetes.
J. Cardiovasc. Dev. Dis. 2021, 8, 143. https://doi.org/10.3390/jcdd8110143.

II. **Stelia Ntika**, Linda M Tracy, Anders Franco-Cereceda, Hanna M Björck, Camilla Krizhanovskii.
Syndecan-1 Expression Is Increased in the Aortic Wall of Patients with Type 2 Diabetes but Is Unrelated to Elevated Fasting Plasma Glucagon-Like Peptide-1.
Biomedicines 2021, 9, 697. https://doi.org/10.3390/biomedicines9060697.

III. **Stelia Ntika**, Linda M. Tracy, Anders Franco-Cereceda, Hanna M. Björck and Camilla Krizhanovskii.
Type 2 Diabetes is not associated with elevated levels of Syndecan-1 in adventitia among patients with bicuspid aortic valve. *Manuscript.*

IV. **Stelia Ntika**, Linda M. Tracy, Anders Franco-Cereceda, Hanna M. Björck and Camilla Krizhanovskii.
Glucagon-like peptide-1 receptor activation counteracts Angiotensin II induced reduction of Syndecan-1 expression in aortic smooth muscle cells. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AAA	Abdominal Aortic Aneurysm
AKT	Protein Kinase B
ANOVA	Analysis of Variance
AngII	Angiotensin II
AoSMCs	Aortic Smooth Muscle Cells
ASAP	Advanced Study Of Aortic Pathology
AscAA	Ascending Aortic Aneurysm
BAV	Bicuspid Aortic Valve
cAMP	Cyclic Adenosine Monophosphate
CI	Confidence Interval
CRP	C Reactive Protein
CREB	cAMP-Responsive Element Binding
CVD	Cardiovascular Disease
DAVAACA	Disease Of The Aortic Valve, Ascending Aorta And Coronary Arteries
DPP-4	Dipeptidyl Peptidase-4
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular-Signal-Regulated Kinases
GLP-1	Glucagon-Like Peptide-1
GLP-1R	Glucagon-Like Peptide-1 Receptor
GSIS	glucose-stimulated insulin secretion
HbA1c	Hemoglobin A1c
hsCRP	High Sensitivity-C Reactive Protein
IFN-γ	Interferon γ
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6

IGF-1	Insulin-Like Growth Factor-1
MMP	Matrix Metalloproteinase
MMP-2	Matrix Metalloproteinase-2
MTT	Methyl Thiazolyltetrazolium
PKA	Protein Kinase A
PVDF	Polyvinylidene Fluoride
ROS	Reactive oxygen species
RT	Room Temperature
Sdc-1	Syndecan-1
SMC	Smooth muscle cells
SM MHC11	Smooth Muscle Myosin Heavy Chain 11
T2D	Type 2 Diabetes
TAA	Thoracic Aortic Aneurysms
TAV	Tricuspid Aortic Valve
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor B
Th	T cells
Th1	Pro-inflammatory cells
Th2	Anti-inflammatory cells
TNF-α	Tumor Necrosis Factor A

1 INTRODUCTION

An aneurysm is a disease, which was first described in the late 1st century AD and then in the 16th century was recognized as a common pathology [1]. Most patients with thoracic aortic aneurysms (TAA) are asymptomatic and untreated aneurysms can be fatal due to dissection or rupture [2]. In addition, there is a lack of screening programs or pharmacological treatments. The aortic aneurysm death rate is around 200,000 people/year worldwide [3].

For the above-mentioned reasons, it is very important to be able to identify biomarkers of and pharmaceutical targets for TAA. This doctoral thesis focuses on the observation that TAA prevalence is reduced among patients with type 2 diabetes (T2D). Data from the two cohorts used for this project support the reduced prevalence of TAA in T2D reported in numerous clinical studies [4-13]. The idea to use the two cohorts Disease of the Aortic Valve, Ascending Aorta and Coronary Arteries (DAVAACA) and Advanced Study of Aortic Pathology (ASAP) to study potential mechanisms of T2D- associated reduced prevalence of TAA was suggested in 2015 by Prof. Anders Franco-Cereceda and Prof. Per Eriksson at Karolinska Institutet. Both Anders Franco-Cereceda and Per Eriksson were involved in data collection from these surgical patients and created a 'library' of samples that could be used for research purposes. The idea that altered glucagon like peptide-1 (GLP-1) signaling, may underlie the reduced prevalence of TAA in T2D was conceptualized by Ass. Prof. Camilla Krizhanovskii. The studies conducted after that demonstrated that fasting plasma GLP-1 levels were increased in surgical bicuspid and tricuspid aortic valve (BAV and TAV, respectively) patients with T2D from the two cohorts [14]. Around the same time, the first *in vivo* study demonstrating decreased aortic aneurysm formation in response to incretin therapy was published [15]. Our promising results [14] provided us with the curiosity to look further and study whether elevated fasting plasma GLP-1 in T2D and/or GLP-1-based therapy could help prevent aneurysm formation.

As I studied chemistry and then neuroscience, this was something very different from my background, but at the same time quite interesting to further understanding of if and how T2D pathology or GLP-1 receptor (GLP-1R) signaling could counteract the pathological processes of this disease, which can today only be 'treated' by surgery.

2 LITERATURE REVIEW

2.1 THORACIC AORTIC ANEURYSM

2.1.1 History

An aneurysm is derived from ancient Greek and means expansion. Aorta is the main vessel of the left ventricle of the heart and it was first described by Aristotle. Although, the first written proof of the use of 'aortic aneurysms' was 4000 years ago in the Ebers Papyrus [16]. Antyllus, a Greek physician was the first who tried to treat aneurysms; the methods he used were described in the notes of Oribasius [17, 18]. In detail, he mentions that 'the violent tension of the arterial pneuma often displaces the ligatures' [18]. The treatment of the disease has changed and evolved a lot since then. Osler [19] mentions that Vesalius was later the first to recognize both abdominal and thoracic aneurysms.

2.1.2 Pathophysiology

An artery is aneurysmatic when the diameter of the blood vessel is 1.5 times bigger than normal, taking into consideration also the age of the individual. Morphologically the aneurysms are fusiform or saccular [20], and both of them could cause weakening of the arterial wall and are fatal if dissection or rupture occurs.

There are two main types of aortic aneurysms: abdominal aortic aneurysm (AAA), which accounts for approximately 75% of all aneurysms, and thoracic aortic aneurysm (TAA), which accounts for the remaining 25%. Although any vascular entity could develop aneurysm the aorta is the most common one [20]. The abdominal aorta is located below the diaphragm and the thoracic aorta above the diaphragm. The thoracic aorta, in which we are interested in this thesis, has three parts; the ascending aorta; which is the part closest to the heart, the aortic arch, and the descending aorta (Figure 1). TAAs most commonly occur in the ascending aorta (i.e. ascending aortic aneurysms, AscAA) and are typically fusiform [21]. The aneurysm of the ascending aorta expands on average 1 mm/year [22, 23]. The aortic wall is composed of the adventitia, media, and intima (Figure 2). The outer layer of the aortic wall, the adventitia, contains fibroblasts, the *vasa vasorum*, and is rich in collagen fibers [24]. The media is the middle layer, which encloses the smooth muscle cells (SMCs) and has two layers of elastic fibers that separate the layers between them [25]. Finally, the intima constitutes the endothelium and is the layer that has contact with the blood [26].

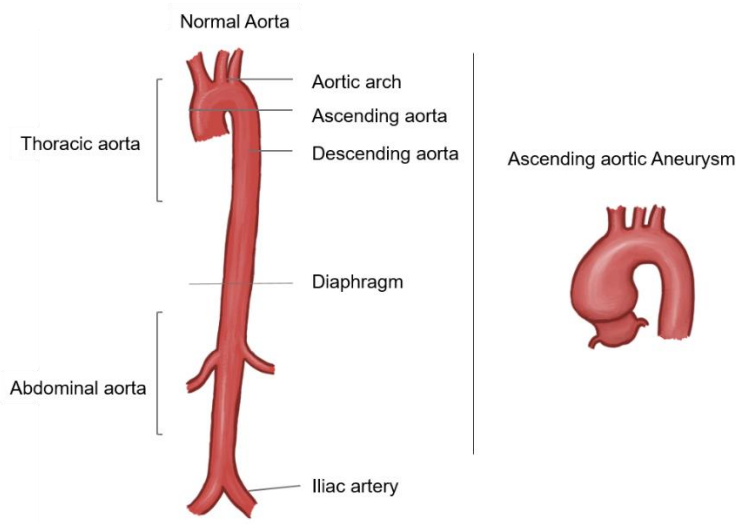

Figure 1. Schematic representation of the thoracic and abdominal aorta (Left) and representation of the ascending aortic aneurysm (Right).

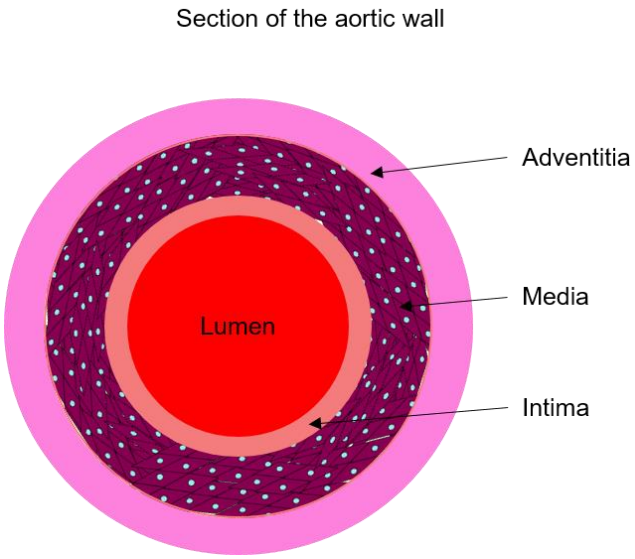

Figure 2. The structure of the aortic wall. The outer layer is the adventitia, then the media layer follows, and finally, the intima is the layer closer to the lumen.

Interestingly, TAA generally appears in younger patients than AAA and is associated with medial degenerative changes [27] in contrast to AAA, which is linked to atherosclerosis. However, both conditions have hypertension and smoking as risk factors [28-30]. Twenty percent of TAAs are associated with heritable genetic changes [31-33]. Ninety-five percent of TAAs are asymptomatic and no screening programs are available, wherefore they may remain undiagnosed or misdiagnosed, unless the patients have undergone data tomography for other reasons [23, 34, 35]. AAA and TAA together are the 20th most common causes of death according to the Centers for Disease Control for the year 2019 [36], although this may be artificially low due to the reason stated above [34]. If the TAA is identified early, and elective surgical intervention is performed, TAA does not affect survival [37].

2.1.3 Mechanism of Aneurysm

The mechanisms of aortic aneurysm development are a series of events that result in changes of the aortic wall. Although AAA mechanisms have been extensively studied, the mechanisms of TAA have been less studied. However, it is known that TAA is the result of changes in both cellular and extracellular environments [31]. Numerous genes have been suggested to be associated with TAA [25, 38, 39]. The majority of these genes are encoding proteins for the SMC differentiated phenotype and metabolism, extracellular matrix, or transforming growth factor β (TGF-β) pathway proteins [38].

During TAA, there is a loss of collagen and elastin and accumulation of a proteoglycan-rich mucoid material in the extracellular matrix. This loss and mucoid material accumulation compromises the mechanical integrity of the aortic wall and leads to SMC loss, and to the dismantling of the elastic layer [40-42] (more details about SMCs can be found in the next section). Collectively these events contribute to increased material stiffness, which increases the risk of cardiovascular events [43] and correlates with aortic dilation in TAA [44].

Matrix metalloproteinases (MMPs), inflammation, and reactive oxygen species (ROS) also play a pivotal role in TAA. Inflammatory cells have been found in TAA in the adventitia and media layers of the aortic wall [45]. The upregulation of different pro-inflammatory cytokines mediates increased release of MMPs in the aneurysmal wall [46-48] and contributes to increased matrix turnover (i.e. the balance between degradation and production of proteins) during aneurysm [49]. Further, MMPs are also produced in response to oxidative stress and matrix degradation products [25].

Angiotensin II (AngII) is a hormone, which has numerous effects on the cardiovascular system, such as ROS production, proliferation, extracellular matrix (ECM) production, MMP activation, inflammatory cell intrusion, and macrophage activation [31, 50-53]. Interestingly, a study from Sweeting et al. showed that patients who were administered angiotensin-converting enzyme (ACE) inhibitors had an increased growth rate of AAA compared to patients without ACE inhibitors [54].

2.1.3.1 Smooth Muscle cells in TAA

As mentioned above, the media layer of the aorta is composed mainly of SMCs. The SMCs are a type of muscle cell that can be found in different organs, such as the aortic wall and the urinary bladder. They are responsible for maintaining homeostasis and different physiological processes, such as synthesizing the ECM, proteolysis, and clearance during proteolytic injuries [55, 56]. Specifically, in the vascular system, they are longitudinally oriented creating multiple layers. By contracting and relaxing, the SMCs alter the diameter of the media layer, which helps in regulating blood circulation and pulse pressure [57, 58].

The diverse functions of the SMCs are dependent on their phenotypic stage. SMCs can hold both differentiated and various de-differentiated phenotypes, changing from one to the other (Figure 3) [57]. The transition from a differentiated to a de-differentiated phenotype may play

an important role in the formation/progression of TAA. However, the role of SMCs during TAA development/progression is not well defined. In differentiated SMCs, proliferation is decreased and contractility is the main function [59]. For the SMCs to differentiate various contractile proteins, ion channels, and signal transduction molecules are needed [59]. Generally, during differentiation the SMCs express: i) the α-smooth muscle actin (α-SMA), which oligomerizes, and forms thin filaments [60], ii) the smooth muscle cells myosin heavy chain 11 (SM MHC11), which composes the thick filaments [61] and iii) calponin (among other contractile proteins), which regulates the actin filaments by binding to actin with high affinity [62-64]. Thin and thick filaments together create a unit that helps in the development of tension and shortening of SMCs [61]. The polymerization, and organization of the actin cytoskeleton maintain vascular plasticity [65].

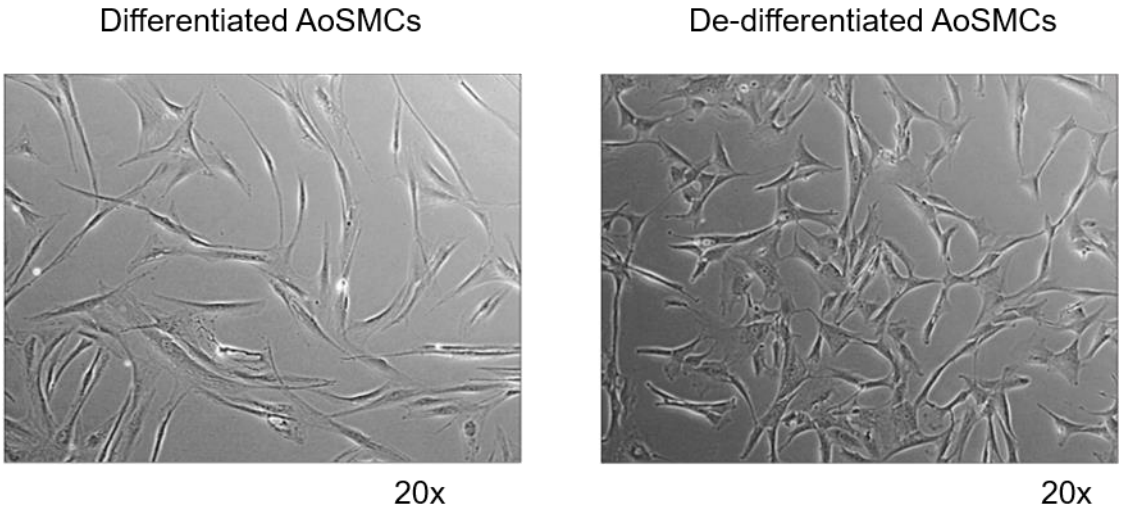

Figure 3. Example of differentiated (left) and de-differentiated (right) human aortic SMCs (AoSMCs). The cells were plated for 4 days in the appropriate medium before taking these pictures. The pictures were taken with 20x magnification, in a light microscope. Abbreviation: AoSMCs: aortic smooth muscle cells.

Alteration in the genes encoding the above-mentioned proteins may underlie heritable TAA as they play an important role in the structure of the ascending aorta [66, 67], where TAA involves a loss of elastic fibers and SMCs in the aortic wall, as well as accumulation of proteoglycans. As mentioned above, SMCs of the aneurysmal aorta typically display a de-differentiated phenotype with increased expression of inflammatory mediators and MMPs (involved in dismantling the proteins in the aortic media matrix [40, 68]), as well as insulin-like growth factor-1 (IGF-1) [40]. Factors that induce this de-differentiated phenotype include; tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) [69], where TNF-α stimulates the proliferation of vascular SMC in a mitogen-activated protein kinase-dependent manner [70] and decreases the differentiation marker α-SMA gene and protein expression *in vitro* [71].

In addition, AngII, can stimulate superoxide formation through NADH/NADPH oxidase activity in cultured SMCs [28, 72], induce AscAA development *in vivo* [73], and has effects on the growth and contractility of SMCs [50, 74]. Furthermore, during TAA the expression of

the differentiation markers (such as the contractile proteins i.e. α-SMA, calponin, SM MHC11) decreases [75, 76].

2.2 BICUSPID AND TRICUSPID AORTIC VALVE

2.2.1 Pathophysiology

A normal aortic valve has three valvular cusps and is called a tricuspid aortic valve (TAV). Bicuspid aortic valve (BAV) with two instead of three cusps is a common congenital cardiac impairment occurring in 1-2% of the population (Figure 4). There are three different types of BAV depending on the orientation of the sinuses and the cusps from the aortic root [77, 78]. The pathogenesis of the cardiac anomaly is not yet known however it has been demonstrated that it is partly heritable [79], and it associates with congenital and genetic disorders, like the Turner syndrome [80]. BAV is a risk factor for aneurysm (27) as well as aortic valve diseases, such as aortic stenosis or insufficiency [81-83], and approximately 50 % of BAV patients will have to undergo aortic valve surgery [84]. It has been suggested that BAV patients have reduced biosynthesis and weakened structure of collagen in the aortic wall contributing to increased prevalence of aneurysm in these patients [85]. For the above-mentioned reason, BAV patients may need surgery at a younger age, and when the aneurysmal diameter is smaller than in TAV patients [86-88].

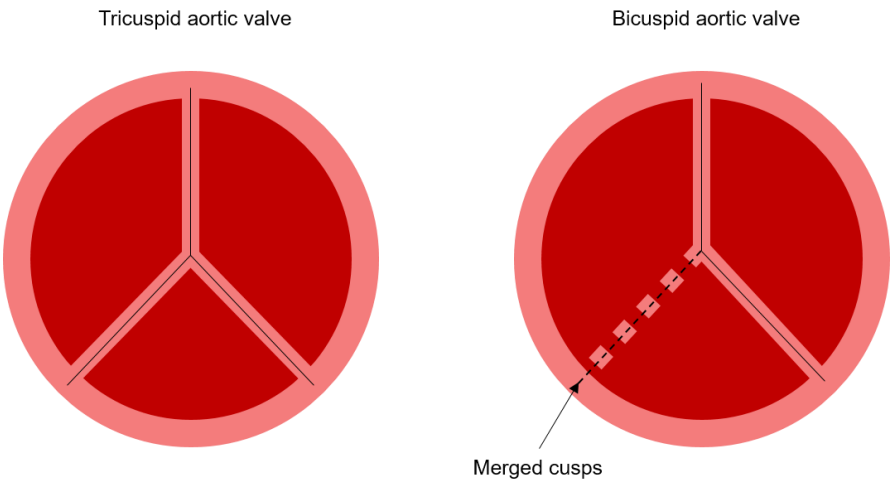

Figure 4. Schematic representation of tricuspid aortic valve (TAV, on the left) and an example of a bicuspid aortic valve (BAV, on the right).

Interestingly, it has been reported that inflammation is associated with TAA development in TAV patients, but not in BAV [89], possibly due to different genetic, cellular, and molecular pathways that are involved in these patient groups [86]. Moreover, matrix metalloproteinase-2 (MMP-2) expression and oxidative stress differ between BAV- and TAV- associated TAA, with increased MMP-2 expression [90-93] and oxidative stress [94] in BAV individuals with TAA. Ignatieva et al. showed that specific smooth muscle gene expression of SMCs derived

from BAV patients differs from the ones derived from TAV patients [95], and BAV- derived SMCs have been suggested to be less mature than TAV ones [96].

2.3 T2D AND ITS PROTECTIVE EFFECT ON TAA

2.3.1 T2D characteristics

T2D is a systemic disease described as disordered metabolism causing hyperglycemia (high blood sugar). Diabetes is one of the most common causes of death; 1.6 million people died in 2016 from diabetes and, as stated by the World Health Organization, approximately 400 million people worldwide have diabetes and its prevalence is rapidly increasing in middle- and low-income countries [97, 98]. Some of the risk factors for T2D are; obesity, hypertension, dyslipidemia, age, heredity, and lack of exercise [99]. According to American Diabetes Association, there are several ways to diagnose diabetes, such as the; hemoglobin A1 (HbA1c) test, which measures the mean levels of the fasting blood glucose from the last 2 to 3 months. An HbA1c level of ≥ 48 mmol/mol or higher on two separate tests means an individual has diabetes, or likewise, fasting blood glucose ≥ 7 mM on two separate occasions means an individual has diabetes. An oral glucose tolerance test may also be used, following an overnight fast; blood glucose ≥ 11.1 mM 2 h after glucose intake suggests diabetes. Finally, random blood glucose measurements, ≥ 11.1 mM suggest diabetes, especially if in combination with other signs and symptoms [100].

Under physiological conditions, insulin is released from the pancreatic β-cells when blood glucose levels increase (glucose-stimulated insulin secretion, GSIS). The main role of insulin is to decrease blood glucose levels by transporting glucose into specific target cells, like skeletal muscle, liver, and fat cells [101]. T2D is a heterogeneous group of conditions, characterized by a combination of peripheral insulin resistance (IR) and impaired β-cell function, contributing to hyperglycemia [99]. The pancreatic β-cells can normally compensate for IR, but when the β-cell function is impaired and increased secretion of insulin to compensate for IR is no longer possible, T2D characteristic hyperglycemia develops [102]. The hypersecretion of insulin that occurs to compensate for IR contributes to β-cell stress, β-cell failure, and reduced β-cell mass [103].

In a healthy control person, 70% of the insulin response to food intake is contributed to by the release of an intestinal hormone; GLP-1 [99]. GLP-1 is released after food consumption and stimulates GSIS, while also exerting many other anti-diabetic effects on target tissues (see below in the chapter about GLP-1) [104].

T2D is characterized not only by IR and defective insulin secretion but also by abnormalities in the secretion of other hormones, including GLP-1 [105]. Obesity is a major risk factor for diabetes and is associated with altered secretion of most of these hormones, while also characterized by altered lipid profiles, and low-grade systemic inflammation — all factors that

contribute to IR and T2D [106]. For instance, chronic IR is associated with inflammation and specifically with the inflammatory cytokine TNF-α [107], and other inflammatory markers, like C-reactive protein (CRP), and interleukin-6 (IL-6) [108].

Diabetic hyperglycemia may lead to complications of the kidneys, feet, eyes, nerves, and cardiovascular diseases (CVDs). Explicitly, adults that suffer from T2D are two to four times more likely to die from CVD than patients without diabetes, according to the American Heart Association [109]. Importantly, although T2D is a risk factor for CVD, patients with T2D have reduced prevalence of both AAA [4-9] and TAA [10-13]. Furthermore, a Swedish observational study by Avdic et al. [110] included patients with T2D and showed a lower risk of hospitalization due to aortic aneurysm among these patients, as compared to control patients.

2.3.2 The mechanisms of the reduced prevalence of TAA in T2D are unknown

Factors contributing to the reduced prevalence of TAA in T2D are currently unknown but have been speculated to involve T2D pathology, as well as the effects of anti-diabetic therapy. Understanding the mechanisms at work may aid in identifying targets for pharmacological interventions to limit the progression of small aneurysms.

It has been suggested that the reduced prevalence of aortic aneurysms in T2D, could involve pathological mechanisms that control the aortic structure [111], as well as the effects of anti-diabetic therapy. Patel et al. [112] mention that surgeons have noticed that the aorta in diabetic patients seems to be thickened, dense and fibrous [113]. Specifically, the mechanisms that have been speculated to underlie the protective effect of T2D in TAA include effects on ECM remodeling, glycation, vascular SMC homeostasis, and neo-angiogenesis [114, 115]. Further, it has been postulated that these effects are mediated by increased TGF-β signaling [113], altered nitric oxide levels mediating elastin degradation through MMPs [111], or increased collagen cross-linking. However, research has not proved a relation between serum glucose, advanced glycation end-products (AGE), and aneurysm formation [116].

2.3.2.1 Anti-diabetic therapy and aneurysm prevention

There are many ways to manage T2D, such as through a healthy diet, exercise, but also, anti-diabetic therapy, like insulin injections and oral anti-diabetic drugs. Of interest for this research project, incretin therapy (based on the actions of GLP-1) is a successful way to treat T2D. Specifically, the anti-diabetic effects of the endogenous hormone and neurotransmitter GLP-1 stimulating GSIS and improving insulin sensitivity underlie the success of this therapeutic strategy. However, due to the rapid degradation of endogenous GLP-1 (1-2 min [117]), incretin therapy includes drugs administered as pills or injections that either mimic the effects of GLP-1 or prevent its degradation. Stable GLP-1 analogs mimicking the actions of GLP-1 include exenatide (brand name Byetta®), liraglutide (Victoza®), and lixisenatide (Lyxumia®) [118-123]. Exenatide or synthetic exendin-4 is a 39-amino acid peptide, which has a longer half-life (60-90 min) than GLP-1 [124] and is the first GLP-1 analog approved for clinical trial. Dipeptidyl peptidase-4 (DPP-4) inhibitors, preventing the degradation of GLP-1 and increasing

levels of its active form, include; sitagliptin (Januvia®), linagliptin (Trajenta®), vildagliptin (Galvus®), alogliptin (Nesina®), and saxagliptin (Onglyza®). GLP-1-based drugs are used as monotherapy or in combination with other anti-diabetic drugs (usually metformin). Both, stable GLP-1 analogs and DPP-4 inhibitors have been indicated to prevent aneurysm formation in many recent *in vivo* studies [15, 125-131].

Interestingly, the first-line drug in T2D therapy, metformin (dimethylbiguanide), is indicated to exert some of its glucose-lowering effects through GLP-1. How metformin works is not very clear yet, but it is known to increase AMP-activated protein kinase (AMPK) activity [132]/phosphorylation [133]. The main effect of this drug is to lower blood glucose through reduced gluconeogenesis (production of glucose in the liver) [134]. However, metformin treatment is also associated with increased plasma levels of GLP-1, and it has been demonstrated to induce GLP-1R expression [135-138]. Similarly to incretin therapy, metformin has been indicated to prevent aneurysm formation [139] in clinical and *in vivo* studies [140-142], although our study did not show any association between metformin treatment and reduced prevalence of AscAA in T2D patients [143].

2.3.3 GLP-1 peptide hormone

GLP-1 is, secreted from the enteroendocrine L-cells, in response to nutrient intake. GLP-1 secretion is stimulated by fatty acids, glycerol, saccharides, aminoacids as well as bile acids. GLP-1 is an incretin hormone, where the incretin effect is characterized as the capability of hormones like GLP-1, secreted from the intestine, to stimulate a glucose-dependent insulin secretion from β-cells [144]. However, GLP-1 has, as mentioned above, pleiotropic effects on the body. In addition to its role in GSIS, it decreases glucagon secretion from α-cells, protects from β-cell apoptosis, increases β-cell proliferation [145], inhibits gastric emptying [146] and food intake [147, 148] (Figure 5). GLP-1 has also anti-inflammatory and anti-oxidant effects [149, 150], and exerts direct effects on the cardiovascular system [150]. It exerts its effects on target tissues via the GLP-1R, which is a G-protein coupled receptor.

The peptide has two biologically active forms, the GLP-1 (7-36)-NH_2 and the GLP-1 (7-37) (amidation is not always important for its activity). The peptide was first discovered at the beginning of the 1980s following the cloning of the proglucagon gene [151]. The proglucagon gene encodes preproglucagon, which contains transcripts for glucagon, GLP-1, and GLP-2 peptide hormones. The active form of the GLP-1 peptide is rapidly degraded (half-life approximately 2 min [117]) at the N-terminal, by DPP-4 resulting in GLP-1 (9-36), which does not activate the classical GLP-1R, and is rapidly cleared from the circulation [152], through renal excretion. Degradation of the peptide starts before it enters the systemic circulation, as DPP-4 is also located in the endothelium close to L cells [153]. GLP-1 diffuses into capillaries and more than 50% is degraded before reaching the portal vein, and additional degradation in the liver leaves only approx. 10% of intact GLP-1 to enter the systemic circulation [154]. Although it was initially believed that GLP-1 (9-36) was an inactive metabolite [152, 155] and GLP-1 (9-36) administered in healthy individuals did not affect the insulin secretion and

glucose metabolism [156], today there is evidence for cardiovascular effects of GLP-1 (9-36)-NH_2 [157-160].

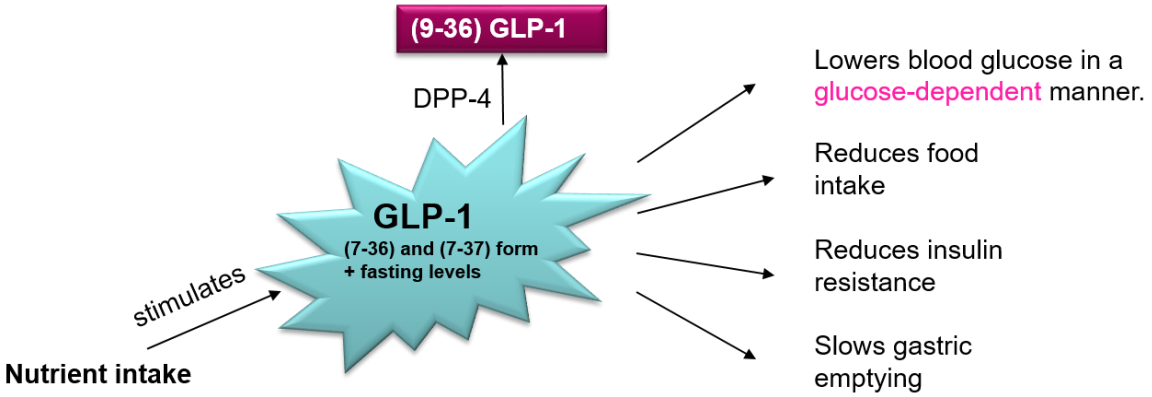

Figure 5. A simple representation of some of the pleiotropic effects of GLP-1 in the body. More details can be found in the text. Abbreviations: GLP-1: Glucagon-like peptide-1, DPP-4: Dipeptidyl peptidase-4.

The GLP-1R has been found in the gastrointestinal tract, kidneys, pancreas, heart, SMCs, endothelial cells, and in the central and peripheral nervous system [161, 162]. Activation of the GLP-1R stimulates the adenylate cyclase pathway, which induces the cyclic adenosine monophosphate (cAMP) formation [163], and is followed by the activation of protein kinase A (PKA) [164]. The PKA levels are important for normal GSIS, which is driven by Ca^{2+} influx [165].

GLP-1 mediated cAMP formation also promotes β-cell survival and increased β-cell regeneration, by activating the pro-survival cAMP-responsive element binding (CREB) [166]. The activation of the cAMP pathway by GLP-1 could also contribute to other signaling pathways, such as the protein kinase C, extracellular-signal-regulated kinases (ERK), phosphoinositide 3-kinase/(Protein kinase B) AKT, and change the levels of peroxisome proliferator-activated receptor gamma [167]. In addition, GLP-1 (9-36)-NH_2 can also activate the CREB, ERK, and AKT pathways [168].

Finally, endogenous GLP-1 secretion is dysregulated in T2D, where defective secretion in postprandial plasma samples [169] or no change or increase on fasting plasma levels [14, 170, 171] has been shown.

2.3.4 GLP-1 and Aneurysm

It was recently shown that GLP-1 prevents aneurysm formation *in vivo* [15, 126, 127, 129, 130, 172, 173]. Although these studies focused on AAA, the models used and the mechanisms indicated are relevant also for TAA. Many clinical trials have focused on the effects of GLP-1 analogs or DPP-4 inhibitors on the cardiovascular outcome, such as the LEADER, ELIXA, EXSCEL, and SUSTAIN-6 studies [174-177]. The LEADER trial was the first trial to show

the cardiovascular benefit of treatment with a GLP-1 analog in patients with a high risk of CVD [175].

Further, *in vitro* and clinical studies [14, 15, 175, 178, 179] indicate effects of GLP-1 on processes that are thought to govern TAA formation. Specifically, GLP-1 has effects on blood pressure [180-182], exerting vasodilation by activating the endothelial nitric oxide synthase (eNOS) and activating the production of the nitric oxide. Richter et al. [183] were the first to show the vasorelaxant effect of GLP-1 in rats, probably through the eNOS pathway. Since then, others have followed and shown that all GLP-1 compounds and exendin-4 cause vasorelaxation, through a cAMP pathway [184].

Moreover, GLP-1 has anti-inflammatory and antioxidant effects that alter the composition of aortic media and may be of importance for TAA prevention [15, 129]. GLP-1 also increases elastin content in the aortic wall of rats [172] and increases gene expression of elastin [185]. In turn, elastin regulates SMC proliferation [186] and is the dominant ECM protein in the aortic wall. In addition, DPP-4 inhibitors decrease MMP-2 and MMP-9 production [15]. The important role of MMPs in aortic dilation further indicates that targeting GLP-1 may be a way of modulating the processes that govern TAA formation/progression.

2.3.4.1 Inflammation and GLP-1

The homeostasis of the aortic wall and changes in the structural components play a very important role in the progression of TAA. Inflammatory markers (eg. cytokines) may affect these processes and promote TAA formation. Specifically, one model suggests that inflammation participates in TAA by the movement of the immune cells from the adventitia to the tunica media [187]. During this migration different cytokines (IL-6, TNF-α, interferon γ (IFNγ)) are produced [188]. Cytokines (cyto + kines ie. cell movement) are small secreted proteins with a specific effect on cell-cell interactions. They are mainly produced by T cells (Th) and macrophages. Cytokines are divided into pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines. Th1 cytokines are involved in the upregulation of inflammation, while Th2 cytokines are immunoregulatory molecules that control the response to Th1 cytokines [189].

DPP-4 inhibitors (i.e. sitagliptin and linagliptin) reduce plasma expression of inflammatory markers elevated during aneurysm formation like TNF-α [190], CRP [191], IL-6, IL-1β, IFNγ [190]. In addition, the DPP-4 inhibitor sitagliptin downregulated the expression of the cytokines IL-6 and TNF-α in the heart of diabetic rats [128]. These anti-inflammatory actions are also replicated by exendin-4 and other GLP-1 analogs and occur through suppression of activation of the nuclear factor-κB in macrophages [192].

2.3.4.2 Smooth muscle cells and GLP-1

It has been shown that SMCs express the GLP-1R [192-194]. *In vivo* and *in vitro* studies indicate that GLP-1R agonists have biological actions in SMCs. For example, they modulate

oxidative stress in the vasculature and exendin-4 reduces intimal thickening and attenuates neointimal hyperplasia after vascular injury via direct effects [131, 195-198]. Furthermore, exendin-4 and liraglutide reduce the proliferation of rat and mouse vascular SMCs respectively, through activation of the cAMP/PKA pathway [196, 199]. Importantly, this reported effect on proliferation indicates that de-differentiation is prevented (see above for details of SMC function) in response to GLP-1R activation. Further, Torres et al. [200] demonstrate that GLP-1 inhibits the de-differentiation of SMCs through a PKA mitochondrial fusion, decreasing platelet-derived growth factor PDGF-BB-induced cell migration and proliferation. Collectively, these data indicate a direct effect of GLP-1 on the vasculature. In addition, exendin-4 and other GLP-1-based therapies have anti-inflammatory effects, decreasing pro-inflammatory markers, like TNF-α [201-208], which may serve to indirectly modulate these processes (see above).

In summary, these data support the possibility of using GLP-1 analogs for therapeutic reasons in CVDs [209] and suggest that GLP-1 analogs/activation of the GLP-1R or downstream targets may also be able to regulate one of the most prominent features of TAA formation/progression, which is SMC de-differentiation and loss.

2.3.4.3 Syndecan-1 and GLP-1

The proteoglycan syndecan-1 (Sdc-1) is expressed by several cell types, such as epithelial, fibroblasts, endothelial, SMCs, and macrophages [210]. Sdc-1 is a cell surface protein/co-receptor that plays a very important role in cell–ECM and cell-cell adhesion and migration, as it provides sites for the ECM ligands to bind so to increase the strength of cell adhesion [211]. Furthermore, it modulates tissue repair by changing the activity of different proteases, growth factors, elastase, and chemokines [212].

Under certain conditions, such as in response to oxidative stress and inflammation, continuous shedding of Sdc-1 from the cell surface is significantly increased by protease activity [213], resulting in increased concentrations of ectodomains in plasma. Specifically, TNF-α, and IL-1β decrease Sdc-1 gene expression and increase shedding [214, 215]. Sdc-1 on the cell surface and the ectodomains in plasma alike can bind to growth factors and other peptides. When Sdc-1 is shed from the cell surface in response to inflammation, the ectodomains in plasma sequester these factors and help to alleviate the inflammation. The ectodomains of shed Sdc-1 bind — through their heparan sulfate chains — different chemokines, cytokines, and proteases, and neutralize any potential pro-inflammatory effects by balancing the progression of inflammation [216, 217]. Specifically, it has been suggested that the absence of Sdc-1 affects the leukocyte-adhesion process by increasing the adhesion [218]. In line with this, it has been observed that agents that increase shedding are released during tissue repair and wound healing [219, 220]. Many studies show the protective effect of increased expression of Sdc-1 in inflammation, for example through its control of different cytokines, like TNF-α and IL-6 [217, 221-224].

However, the role of Sdc-1 is complex and may be cell type specific. The expression of Sdc-1 on SMCs can modulate pro-inflammatory processes [225], and Sdc-1 knock-out in SMCs

reduces the protein expression of the differentiation markers α-SMA and calponin [226], indicating that it promotes a differentiated phenotype of SMCs. Of note, Sdc-1 also has a protective role in aortic aneurysm, as its deficiency in the aortic wall increases aneurysm formation in mice models [227].

As part of the rationale for including Sdc-1 expression as one of the outcomes analyzed for this project, Sdc-1 expression is induced in a cAMP/PKA dependent manner [228], and GLP-1 is a cAMP/PKA activator [229, 230]. It may, thus, be hypothesized that GLP-1 increases Sdc-1 expression in the aortic wall. Such a direct effect of GLP-1 on Sdc-1 expression in the aortic wall may contribute to the anti-inflammatory effects of GLP-1, through maintaining SMC differentiation in response to GLP-1. In addition, anti-inflammatory effects of GLP-1 may reduce the cleavage of Sdc-1 ectodomains from the cell surface, through regulation of MMP activity (see below) and thereby contribute to increased Sdc-1 expression.

2.3.4.4 MMP-2 activity and GLP-1

MMP-2 (or gelatinase A or type IV collagenase) is an enzyme involved in the degradation of the ECM. It degrades elastin and types I, II, and III collagens in the vasculature [231] together with other zinc MMPs. The MMP-2 is secreted by macrophages, fibroblasts, and SMCs [232-234], and can help coordinate many aspects of the inflammatory response and tissue repair, through direct and indirect effects [235]. Moreover, the MMP-2 gene produces a pro-MMP-2 protein [236], which in turn, is activated by proteinases or *in vitro* by chemical molecules, such as the mercurial compound 4-aminophenylmercuric acetate [237, 238].

Plasma MMP-2 levels are dysregulated in T2D, where Lewandowski and colleagues [239] show that it is decreased. However, the mechanisms are not known, and other studies have found increased plasma levels in T2D [215, 240]. MMP-2 levels and activity are increased in AAA tissue [234, 241], where targeted MMP-2 gene deletion in the aorta of murine knock-out models inhibit aneurysm formation [242]. Increased gene expression of MMP-2 is also observed in TAA [243], where MMP-2 levels are also relatively increased in BAV- associated TAA, as compared to TAV- associated TAA [244].

In vitro studies have shown that GLP-1 decreases MMP-2 activity [15] and *in vivo* models demonstrated that MMP-2 activation can be triggered by pro-inflammatory cytokines [245-249], where GLP-1 analogs and DPP-4 inhibitors decrease MMP-2 expression in the aorta [15, 129]. It is thus possible that GLP-1 through direct and indirect effects reduces MMP-2 activity.

Considering the above and the increased GLP-1R activation in T2D, as a result of pathology and/or anti-diabetic therapy, GLP-1 becomes a potential candidate in the search of factors that may govern reduced prevalence of aortic aneurysms in T2D. If such a role is confirmed for GLP-1 signaling, modulating GLP-1 signaling may also provide a means of future pharmacological intervention for small aneurysms. However, clinical studies identifying possible differences in processes of importance for TAA formation between patients

with/without T2D, and how these changes may associate with elevated fasting plasma GLP-1/GLP-1R activation have not previously been performed.

3 RESEARCH AIMS

The overarching aim of the thesis was to investigate the role of the hormone GLP-1in the reduced prevalence of AscAA in patients with T2D.

The specific aims were:

- In **Paper I** we aimed to determine whether fasting plasma GLP-1 correlates with T2D-associated changes in systemic inflammation or proteolytic activity among patients with TAV. We also compare and contrast the changes associated with T2D to the inflammatory profile and proteolytic activity of TAV patients with established AscAAs.

- In **Paper II** the objective was to elucidate whether Sdc-1 expression in aortic tissue is altered in TAV patients with T2D and whether such a potential change correlates to fasting plasma GLP-1, inflammation, or proteolytic activity in the same patients. Finally, we aimed to elucidate the potential clinical relevance of Sdc-1 expression as a counterbalancing response to AscAA formation indicated *in vivo*, by assessing Sdc-1 expression and the infiltration of macrophages in the adventitia of AscAA TAV patients.

- In **Paper III** we studied whether Sdc-1 expression in adventitia is altered in T2D BAV patients, and any possible correlations with T2D- associated changes in proteolytic activity or fasting plasma GLP-1 among patients with BAV. Finally, we aimed to clarify any possible relevance for Sdc-1 expression in BAV patients, as a counteracting response to AscAA formation indicated *in vivo*, by assessing macrophage infiltration and Sdc-1 aortic expression in BAV patients with established AscAAs.

- Finally, in **Paper IV** we aimed to demonstrate the direct effects of GLP-1R activation on viability, phenotype, and Sdc-1 expression of human aortic SMCs (AoSMCs) in the presence/absence of diabetic conditions and in the presence/absence of aneurysmatic conditions [in differentiated cells simulated by AngII (promoting TAA formation) and in de-differentiated cells].

4 MATERIALS AND METHODS

This section briefly describes the material and methods used for the studies included in this thesis. Further details can be found in the Material and Methods section of **Papers I-IV**.

Table 1. Overview of the papers and the methodology in **Papers I-IV**.

Paper	**I**	**II**	**III**	**IV**
Participants	TAV individuals with AscAA, or T2D and control group	TAV individuals with AscAA, or T2D and control group	BAV individuals with AscAA, or T2D and control group	*In vitro* study with Human AoSMCs
Design	Systemic Inflammation and Proteolytic activity	Proteolytic activity and Sdc-1 expression in the aortic wall and shedding	Proteolytic activity and Sdc-1 expression in the aortic wall and shedding	Potential effects of GLP-1R analogs on the expression of differentiation markers and Sdc-1 expression.
Main Methods	ELISA, caspase 3	ELISA, Western blot	ELISA, Western blot	Western blot, qPCR, caspase 3, MTT
Data analysis	Pearson correlation linear regression, student's t-test, ANOVA, ANCOVA	Pearson correlation linear regression, Mann-Whitney, student's t-test, ANCOVA	Pearson correlation linear regression, Mann-Whitney, student's t-test, ANCOVA	ANOVA, student's t-test

Abbreviations: ANOVA: Analysis of variance, ANCOVA: Analysis of covariance, AscAA: Ascending aortic aneurysm, BAV: Bicuspid aortic valve, ELISA: enzyme-linked immunosorbent assay, MTT: Methyl thiazolyltetrazolium, T2D: Type 2 diabetes, TAV: tricuspid aortic valve.

4.1 PARTICIPANTS

Papers I-III of the present thesis included patients from the ASAP [89] and the DAVAACA cohorts. ASAP has enrolled 600 patients and DAVAACA is an ongoing study, which up until this thesis was going on had recruited more than 2000 patients. Both studies include surgical patients with aortic valve disease and/or ascending aortic aneurysm. The patients included in these cohorts underwent surgery at the Cardiothoracic Surgery Unit at Karolinska University Hospital in Solna. Plasma, serum, and tissue samples were collected from all the patients. Both studies were approved by the ethical committee of the Stockholm Region and they were

conducted in accordance with the Helsinki declaration. All patients included in the cohorts had signed written informed consent. Patients with TAV were included in **Papers I and II** and BAV were included in **Paper III**.

The ascending aorta was classified as normal/non-dilated when the diameter was less than 40 mm and aneurysmatic/dilated when it was larger than 45 mm [250]. The participants were excluded when the dimension of the ascending aorta was between 40 and 45 mm. Other exclusion criteria were Marfan (**Papers I-III**) or Turner (**Paper III**) syndrome, unicuspid valve, atherosclerosis, and type 1 diabetes. The patients were divided into three groups: patients without AscAA and T2D (control group), patients with T2D (T2D - group), and patients with AscAA (AscAA - group). The studies did not include T2D patients with AscAA, partly because there were not enough patients with both AscAA and T2D (due to the reduced prevalence of AscAA in T2D), and partly because of the difficulty in interpreting any data from such a group, due to the impossibility of discriminating cause from consequence. For further details and discussion around this, refer to the individual papers. In addition, the BAV patients that were included in **Paper III** were not separated based on BAV type.

4.2 CELL CULTURE MODELS

4.2.1 Murine GLP-1 secreting cells

The GLP-1-secreting GLUTag cells were used in **Paper I** to study GLP-1 secretion *in vitro* and the caspase-3 activity, after treating with palmitate and the cytokines IL-6 and TNF-α. These are murine enteroendocrine L cells. The GLUTag cells were donated by Dr. Neil Portwood at Karolinska Institutet and they were originally from Dr. Daniel J. Drucker, Lunenfeld - Tanenbaum Research Institute, Mount Sinai Hospital, Canada. They were cultured in DMEM medium supplemented with 10 % fetal bovine serum, 10 mU/ml penicillin, and 10 mg/ml streptomycin (Thermo Fisher Scientific, Massachusetts) and 5.5 mM glucose (Sigma Aldrich, Missouri) in a humidified incubator at 37 °C and 5 % CO_2. The cells were plated at a density of 250.000 cell/ml (for GLP-1 secretion) or 180.000cells/ml (for caspase-3) in a 6-well plate and incubated with palmitate (to induce hyperlipidemia), and IL-6 and/or TNF-α for 48 h.

4.2.2 Human aortic smooth muscle cells

Human aortic smooth muscle cells (AoSMCs) were used for **Paper IV** and were bought from Lonza (Art.No.: CC2571, Switzerland). The cells are primary cells that can be cultured for up to 15 doublings. *In vitro* the AoSMCs are not differentiated; consequently, they need to be differentiated before treating them. AoSMCs are an excellent model for studies in diabetes and aneurysm. The cells were cultured in SMGM-2 medium, supplemented with 5% fetal bovine serum, 0.1% insulin, 0.2% hFGF-B, 0.1% GA, 0.1% hEGF (Lonza, Switzerland), 10 mU/ml penicillin and 10 mg/mL streptomycin (Thermo Fisher Scientific, Massachusetts) under 5% CO_2. No extra supplements were added in the differentiation medium. For the differentiation,

the cells were plated for 9 days at a density of 15.000 cell/cm^2 and the differentiation medium was added a day after plating them and changed every 3 days. The de-differentiated cells were plated at a density of 6000 cells/cm^2 for the same amount of days, and the experiments were run in parallel. The cells were used from passage 2-13 and they were treated on the 9th day of differentiation for 24 h.

4.3 PROTOCOLS

4.3.1 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a technique used for detecting and quantifying different soluble substances. ELISA was used for detecting total GLP-1 secretion in plasma (Merck, Massachusetts, **Papers I-III**), active GLP-1 levels in plasma (Merck, Massachusetts, **Papers I and III**), Sdc-1 expression in plasma (shedding) (Diaclone, France, **Papers II-II**), endogenous active MMP-2 activity in plasma (Quickzyme Biosciences, The Netherlands, **Papers I and III**) and in tissue (**Papers II and III**). A multiplex ELISA kit was also used from MesoScale Discoveries (Maryland), for measuring different cytokines (**Papers I-II**), where up to ten analytes can be detected. Here seven cytokines were analyzed: TNF-α, IL-6, IL-1β, IFN-γ, IL-12p70, IL-4, and IL-5.

The ELISAs were carried out in a 96-well plate, where the antibody or the antigen can be bound to the surface. The ELISAs that were used for this project were sandwich ELISAs, which are the most common ones. The procedure is stepwise and it starts by coating the plate with the specific capture antibody, if not already coated by the manufacturer. After washing the plate from the excess antibody, the sample is added. The sample could be plasma, serum, cell supernatant, cell lysate, etc. and it should be prepared as described by the instructions of the manufacturer. The capture antibody will bind to any antigen found in the sample and after washing any excess sample, the detection antibody is added. In turn, the detection antibody will bind to the antigen bound to the plate. To measure the plate a substrate is added, for example, TMB, and then a stop solution is added that turns the substrate into a colored product. The absorbance was measured in all the ELISAs using the appropriate wavelength, except in the active GLP-1 ELISA where the relative light units at 425nm were measured in a luminometer. The DPP-4 inhibitor had been added in these samples where active GLP-1 was measured when they were collected.

However, the multiplex ELISA has a bit more complex system using a 10-spot U-PLEX plate and linkers that were assembled onto specific spots on the plate. The biotinylated capture antibody was first coupled to the specific linker and then the plate was coated with the coupled antibody. The analytes from the sample are then bound to the capture antibody and in turn detection antibodies conjugated with electrochemiluminescent labels bind to the analyte and the sample was measured in an MSD instrument.

A standard curve was prepared for each plate that was run.

4.3.2 Protein assay

For the western blot and the caspase 3, the protein assay was used to measure how much we need to load or to normalize the results, respectively. We used the DC total protein assay (Bio-Rad Laboratories, California). For the preparation of tissue, the adventitia layer was used and it was homogenized on TissueLyser II and lysed in Tris HCl 50mM with 0.1%Trition X 100 buffer (Sigma Aldrich, Missouri), pH=7 - 8. The samples from the AoSMCs were lysed for 30min in RIPA buffer with 1% protease and 1% phosphatase inhibitor cocktail (all from Sigma Aldrich, Missouri). After centrifuging to take the cell lysate we followed the protocol as described by the manufacturer. Together with our samples, a BSA protein standard curve was added to the plate and the absorbance was read at 750nm.

4.3.3 Western blot

Western blot was applied to quantify the amounts of Sdc-1, GLP-1R (Abcam, UK, **Papers II-IV**), and the macrophage marker CD68 (Abcam, UK, **Papers II and III**). Western blot starts by separating the macromolecules using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins are separated by their molecular weight, as the smaller ones 'travel' faster. Eight or 10% acrylamide gel was prepared for the experiments depending on the molecular weight of the protein we wanted to detect. After the proteins were separated they were transferred to a polyvinylidene fluoride (PVDF, Bio-Rad Laboratories, California) membrane, which is a hydrophobic thermoplastic fluoropolymer with 0.2μm pore size and has less background than a nitrocellulose membrane. The membranes were then blocked with 5% w/v milk/tris-buffered saline -0.1% tween 20 (TBS-T), to prevent any unspecific binding. The appropriate primary antibody was added overnight at 4°C. Then the appropriate secondary antibody (Santa Cruz Biotechnology, Texas) was added and was incubated for 1h at room temperature (RT). After washing with TBS-T the enhanced chemiluminescence (GE Healthcare, Illinois) reagents were used to detect the proteins. The images were developed using the ChemiDoc XRS+ version 4.6.5 (Bio-Rad Laboratories, California) and they were analyzed with the Image Lab software (Bio-Rad Laboratories, California) by measuring the density of each band. For total protein normalization, the PVDF membranes were either incubated with β-actin (Santa Cruz Biotechnology, Texas) or they were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories, California).

4.3.4 Viability and apoptosis assays

4.3.4.1 Methyl thiazolyltetrazolium (MTT) cell viability assay

A colorimetric non-radioactive MTT assay, from Promega (Wisconsin), was used to measure the viable cells after treatment in the *in vitro* studies, **Paper IV**. The cells were cultured in a 96-well plate and an optimized dye solution, containing growth factors or a test substance, was added to the wells. The cells were then incubated up to 4 h at 37°C in a humidified, 5% CO_2 environment, and the living cells converted the MTT tetrazolium salt into an insoluble formazan product, which was easily detected. Then the stop solution was added to solubilize

the formazan and the absorbance was measured at 570nm with a reference wavelength at 630nm.

4.3.4.2 Caspase 3 assay

The apoptosis of the cells in **Papers I and IV** was measured utilizing a caspase 3 assay from Thermo Fisher (Massachusetts). Caspase 3 belongs to a family of proteases that are associated with cell apoptosis and is specifically important for the initiation of apoptosis. A substrate called 7-amino-4-methylcoumarin-derived substrate Z-DEVD-AMC is the basis of this assay. This substrate is fluorescent upon proteolytic cleavage and it was measured in a fluorescence microplate reader at excitation/emission, 342/441nm. The results were normalized by the DC total protein assay (Bio-Rad Laboratories, California).

4.3.5 Gene expression

Quantitative, real-time polymerase chain reaction (qPCR) was used in **Paper IV** to detect the gene expression of the differentiation markers (Calponin and SM MHC11), GLP-1R, Sdc-1, and the anti-oxidant catalase in differentiated, de-differentiated AoSMCs and under different treatments. AoSMCs were lysed and the Aurum total RNA mini kit from Bio-Rad Laboratories (California) was used to extract the mRNA as described by the manufacturer. After the mRNA was extracted the complementary DNA (cDNA) was prepared by reverse-transcription PCR using the iScript Reverse Transcription Supermix - RT-qPCR (Bio-Rad Laboratories, California). The cDNA was then used to run the qPCR together with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, California). Each forward and reverse primer for the different genes was designed in the OligoPerfect software (Thermo Fisher, Massachusetts) with the help of BLAST and Primer3Plus tools. SYBR Green was used as a dye for the qPCR, which is binding to double stranded DNA and increases the fluorescent yield of the dye. The intensity of the fluorescence was measured after each cycle with the Bio-Rad CFX Maestro 2.0 software (Bio-Rad Laboratories, California). Forty cycles were run for each gene and GAPDH was used as a housekeeping gene (See Table 2 for the thermal cycle protocol). For the analysis, the ΔC_t ($C_{t\,\text{target gene}} - C_{t\,\text{GAPDH}}$, where C_t is the threshold cycle at which fluorescent exceeds the threshold [251]) was used for normalization and it was then converted to $2^{-\Delta Ct}$ to calculate the mRNA for each target gene. To calculate the relative changes in gene expression for each treatment group compared to the control group the ratio of the average $2^{-\Delta Ct}$ is calculated ($2^{-\Delta\Delta Ct}$).

Table 2. Thermal cycle protocol

	Polymerase Activation and DNA denaturation	Amplification	
		Denaturation	Annealing/Extension and Plate Read
Time	30 sec	5 sec	30 sec
Temperature	95°C	95°C	60°C

4.4 DATA ANALYSIS

4.4.1 Statistical analysis

In **Papers I-III** all data from patients were collected to a local registry and anonymized before receiving them for analysis. All the analysis that was performed were quantitative and the GraphPad prism 6 or the R studio software version 4.0.3 (Massachusetts) were used (**Papers I-IV**). The data throughout this thesis were considered significant when $p < 0.05$ and they were represented in ± SEM (standard error of mean). Pearson correlation coefficients with 95 % confidence interval and linear regression were assessed for any possible correlations. Unpaired two-tailed student's t-test or Mann-Whitney was used for two-sample comparisons, where appropriate, and one-way ANOVA (Analysis of variance) was performed for comparison between multiple groups. ANCOVA (Analysis of covariance) was analyzed with the R studio when we wanted to control for one or more covariates. GraphPad Prism 6 (GraphPad Software, California) was used to identify outliers in **Papers I-IV**. All the graphs throughout the thesis have been plotted with GraphPad Prism 6. For the analysis of the western blot data, we used the Image Lab software version 5.2.1 (Bio-Rad Laboratories, California) to measure the volume of the bands, and β-actin was used to normalize them.

4.4.2 Sample size

All *in vitro* studies were run in duplicates and they were repeated at least 3 times (**Papers I and IV**). For the clinical analysis, a power analysis was performed at the beginning of the study taking also into consideration previously published data from our group that is related to this thesis [14]. As many different variants were measured this could increase the number of the patients that needed to be used to have significance depending on the expression of a specific variable. Power analysis was performed for the approximate expected concentration (from previous studies [14, 171, 252-255]) of fasting plasma GLP-1 levels (with 80% power, 5% level of significance). After searching in the bibliography for the tissue experiments, we found other studies that have used around 10 patients per group in similar experiments to ours [241,

256-258] with significant results. In this thesis, we used approximately 10-12 patients for the tissue analysis.

4.5 ETHICAL CONSIDERATIONS

For the clinical studies, **Papers I-III**, the ethical permissions were acquired by the Stockholm Regional Ethical Committee: (Dnr:2006/78431/1; approved:2006-09-15) and (Dnr:2012/1633-31/4; approved:2012-10-24). The ethical permission was in place before this project started, as these samples are actively collected to create two different Biobanks; the ASAP and DAVAACA cohorts, this is why there were two different ethical permissions.

In accordance with the above ethical permit, biopsies were taken from all the patients that were included in the study, where minimal harm was ensured by taking the smallest possible amount. Blood samples were also collected, in turn plasma and serum samples were prepared following the ethical permit. Another aspect considered when approving the ethical application for the ASAP and DAVAACA cohorts was that the cardiac exams for the patients included in the cohorts were a bit longer than normal.

The clinical studies included in this thesis were observational retrospective cohort studies including patients from the ASAP/DAVAACA cohorts. The consent form from the patients had been collected before the patients were admitted for surgery. The first ethical consideration with regards to the present studies would be if we had to go back to the patients and ask them for informed consent with respect to these studies. However, this study was covered by the first consent form, so we did not have to ask them again.

Another consideration for us was if the patients voluntarily participated in the studies of this thesis. However, the consent form signed specified plasma and tissue sample analysis and covered studies such as those included in this thesis. Further, patients were asked after being accepted for surgery if they would like to participate in the study, this did not influence if they would have surgery or the needed treatment. In addition, the patients can ask for their samples to be destroyed at any given point without explanation.

Studies such as the ones included in this thesis may benefit the patients included, but will more likely benefit future patients with TAA through a better understanding of the pathology and possibly also facilitating a future pharmacological intervention for small TAAs.

Paper IV is an *in vitro* study utilizing commercially available cells from Lonza, and no ethical permission is needed. No primary cell cultures were used.

5 RESULTS

This section is providing the main results of each paper. Further details and graphs can be found in the Result section in **Papers I-IV**.

5.1 PAPER I

In **Paper I**, we used patients with TAV. We demonstrated that the T2D patient group was characterized by HbA1c above 48 mmol/mol and fasting plasma glucose above 7 mM. Next, our analysis of high sensitivity CRP (hsCRP), Th1, Th2 cytokines, and their relative plasma levels in the different patient groups, demonstrated that T2D was associated with an increased IL-6/TNF-α ratio, indicating a Th2 bias. However, no other changes were detected in association with T2D. We also demonstrated data in support of a contrasting association between AscAA and a Th1 bias of inflammatory responses, as patients with AscAA had significantly decreased levels of IL-4 and IL-5 as compared to control patients. Our group previously demonstrated increased total and active fasting plasma GLP-1 in association with T2D [14], and we further confirmed these data in this study. Although no correlation between elevated fasting plasma GLP-1 and an elevated IL-6/TNF-α ratio was found in T2D patients, we demonstrated that fasting plasma GLP-1 was positively associated with the IL-4/IFN-γ ratio, which is frequently utilized to represent a Th2 shift [259]. However, we did not find a significant change in plasma MMP-2 activity in association with T2D, nor an association between fasting plasma GLP-1 and MMP-2 activity in any of the patient groups.

5.2 PAPER II

TAV patients were also included on **Paper II**. The three patient groups can be found in Table 1. Our findings showed that T2D is associated with increased adventitial Sdc-1 expression in the ascending aorta. Further, T2D was correlated to low plasma Sdc-1, as compared to the control group, indicating reduced shedding of Sdc-1 from cell surfaces. However, no correlation was detected between plasma and adventitial Sdc-1. Further, no change was observed in terms of MMP-2 activity in adventitia in association with T2D. Nor was an association between plasma or tissue Sdc-1 expression and increased fasting plasma GLP-1 levels found in association with T2D. However, GLP-1R in the adventitial tissue was positively correlated to Sdc-1 in plasma in the same patient group.

Further, we demonstrated data in support of recent *in vivo* studies indicating increased Sdc-1 expression on infiltrating macrophages as a counterregulatory response to AscAA development. Specifically, we observed that Sdc-1 expression in the adventitial tissue was, as compared to control subjects, increased in patients with established AscAAs, in association with increased expression of the CD68 macrophage marker. Further, plasma Sdc-1 was

unaltered in the AscAA group, as compared to the control group. Of note, macrophage infiltration of the ascending aorta was not detected among T2D patients.

5.3 PAPER III

In **Paper III**, we studied the BAV patients of the cohort studies, as T2D is associated with reduced prevalence of TAA also among these patients. The three patients groups can be found in Table 1. Similarly, to T2D TAV patients from **Paper I**, T2D BAV patients were characterized by elevated HbA1c and fasting plasma glucose levels ($\geq$ 48 mmol/mol and $\geq$ 7 mM, respectively). However, Sdc-1 expression in adventitia was significantly lower in BAV T2D patients compared to control patients. No significant change in GLP-1R expression, MMP-2 activity, fasting plasma GLP-1, or plasma Sdc-1 was detected in association with T2D.

Moreover, aortic Sdc-1 expression was not significantly changed in association with AscAA, and the CD68 macrophage marker was not detected in the aorta from BAV patients with AscAA. However, aortic MMP-2 activity showed a trend towards an inverse correlation to Sdc-1 expression in the adventitia of the AscAA BAV patients.

5.4 PAPER IV

For **Paper IV** we analyzed a potential role for GLP-1 in the reduced prevalence of TAA in T2D, through effects on AoSMCs. For these *in vitro* studies, we successfully differentiated cultured AoSMCs, as indicated by increased mRNA expression of calponin, SM MHC11, as well as decreased levels of IL-1β. We showed that differentiated AoSMCs express lower levels of Sdc-1 mRNA and significantly increased GLP-1R mRNA expression while also displaying a trend toward higher protein levels of GLP-1R in lysate when compared to de-differentiated cells.

Diabetic conditions (treatment with palmitate and glucose) increased the expression of SM MHC11, and IL-1β. Furthermore, the addition of exendin-4 in the presence of diabetic conditions increased the expression of the endogenous antioxidant catalase. However, we did not detect a significant effect of exendin-4, in the presence/absence of diabetic conditions, on viability, phenotype, or Sdc-1 expression of AoSMC. The latter despite increased mRNA expression of Sdc-1 in response to the PKA activator forskolin.

When simulating a milieu promoting TAA formation using the addition of AngII, we observed that Sdc-1 mRNA levels decreased, as compared to control. However, importantly, pre-treatment with exendin-4 counteracted this effect. We utilized de-differentiated AoSMCs used as a model of the AoSMCs found in patients with TAA. Although forskolin increased Sdc-1 mRNA expression in this phenotype as well, exendin-4 did not have an effect.

6 DISCUSSION

6.1 THESIS FINDINGS

In **Paper I**, we confirm a previously reported increase in both total and active fasting plasma GLP-1 levels among patients with T2D [14]. Other reports have identified decreased GLP-1 secretion in T2D, although they show defective secretion in postprandial plasma samples [169], which we have not analyzed here. Whereas postprandial secretion may be of most interest for the effects of GLP-1 on glucose metabolism, fasting levels of GLP-1 are implicated in energy expenditure, fat oxidation, and cardioprotective effects [171]. In terms of fasting levels, a similar increase in association with T2D has been reported also by other groups [14, 170, 171, 260]. However, careful consideration should be paid to the form of GLP-1 analyzed (active or total), as well as patient characteristics and medication when comparing studies. We noticed an increase in both total and active fasting plasma GLP-1 levels in T2D patients, and a positive correlation between the two. However, due to a limited set of samples with DPP-4 inhibitor added, and to exclude potential effects of differences in plasma DPP-4 activity between patients, we decided to continue analyzing only total fasting GLP-1 levels.

The increased ratio of IL-6/TNF-α in patients with T2D as compared to the control group remained also after controlling for HbA1c, and is an important finding of **Paper I**. IL-6 blocks Th1 responses and promotes activation of Th2 responses [261, 262]. This relative increase in an early response cytokine with anti-inflammatory properties in relation to a Th1 cytokine thus indicates a Th2 bias of immune responses. This Th2 bias may bear relevance to the reduced prevalence of TAA in T2D, as TAA is associated with a Th1 bias of immune responses. Specifically, the significantly reduced levels of the Th2 cytokines, IL-4 and IL-5, demonstrated in patients with AscAA in **Paper I**, further confirm previous reports of a Th1 bias in patients with TAA [263, 264].

It has previously been reported that IL-6 increases secretion of GLP-1 from the intestine, while TNF-α may block this effect of IL-6 [265, 266], rendering the IL-6/TNF-α ratio a potentially important regulator of GLP-1 secretion. However, although our *in vitro* experiment confirmed a role for the IL-6/TNF-α ratio in GLP-1 secretion, no significant association was detected between fasting plasma GLP-1 and the IL-6/TNF-α ratio. We have previously shown that fasting plasma GLP-1 inversely correlates with HbA1c in these T2D patients [14], wherefore we also controlled for HbA1c when we assessed a potential correlation between the cytokine ratio and GLP-1. That we could not detect a correlation indicates that the elevated fasting plasma GLP-1 does not come as a result of this T2D- associated shift in inflammatory profile. However, the positive correlation detected between fasting plasma GLP-1 levels and the IL-4/IFN-γ ratio indicates that, elevated GLP-1 levels in T2D are associated with a Th2 bias, as an increase in the IL-4/IFN-γ ratio is commonly used to demonstrate such a Th2 bias [259].

As reported in **Papers I** and **II** respectively, we were not able to detect a significant change in plasma or adventitial active MMP-2 levels between any of the patient groups, despite previous studies of decreased total plasma MMP-2 in patients with T2D [239]. However, it should be mentioned that we measured active MMP-2, which may not reflect changes in total plasma MMP-2 levels.

In **Paper II**, we showed that plasma Sdc-1 was decreased in patients with T2D and aortic Sdc-1 was increased in the same patient group. These findings are supported by reports showing that shed plasma Sdc-1 is increased due to inflammation and oxidative stress. Specifically, in response to inflammatory Th1 cytokines or other factors such as oxidative stress, the cell membrane proteoglycan Sdc-1 is cleaved off the cell membrane by proteases such as MMP-2 and MMP-9 [267], which results in increased plasma levels of the Sdc-1 ectodomains. Consequently, considering the detected anti-inflammatory Th2 bias in association with T2D, the reduced plasma levels of Sdc-1 were expected. Likewise, it may be hypothesized that reduced shedding of Sdc-1 may contribute to the detected increased levels of Sdc-1 in aortic tissue. Nevertheless, no association was detected between Sdc-1 in plasma and aortic Sdc-1 expression in T2D patients. Nor was an association between reduced MMP-2 activity and increased Sdc-1 expression in adventitia observed. These results may argue against reduced Sdc-1 shedding from the cell surface as a mechanism of increased adventitial Sdc-1.

However, Sdc-1 is secreted by various cells/organs and mostly by kidneys and liver [268, 269], and aortic tissue may be a small contribution to the total plasma Sdc-1, wherefore we may not detect an association between Sdc-1 in plasma and aortic Sdc-1. Further, it may be that altered activity of another gelatinase, such as MMP-9, or other proteases results in less Sdc-1 being shed from the cell surface.

Consequently, our results do not rule out that the reduced shedding of Sdc-1 from the adventitial cell surfaces contributes to the detected increase of Sdc-1 in adventitia associated with T2D.

Increased aortic tissue expression of Sdc-1 protects from aortic aneurysm *in vivo* [227], and the findings reported in **Paper II** may thus be of relevance for the reduced prevalence of TAA in T2D. Although we do not detect a significant association between adventitial Sdc-1 expression, and fasting plasma levels of GLP-1, we must consider the limited number of tissue samples available, and the subsequent difficulty in statistically identifying a correlation. The strong positive correlation detected between adventitial GLP-1R expression, and Sdc-1 in plasma may seem contradictory to reduced shedding of Sdc-1 in response to GLP-1 signaling. GLP-1R expression is not synonymous with increased GLP-1 signaling and the correlation observed may be the result of GLP-1R upregulation in response to GLP-1 deficiency (hypothetically involving increased Sdc-1 shedding). However, the correlation may also be due to confounding variables and not indicative of a direct relationship between GLP-1R expression and plasma Sdc-1. Considering that dyslipidemia is associated with increased plasma levels of Sdc-1 [270], increased GLP-1R expression may be secondary to lipid-lowering drugs, such as metformin, known to promote GLP-1R expression [271, 272]. This is speculative and future larger studies

need to be conducted to elucidate the correlation detected herein. The increased Sdc-1 expression that was observed in the adventitial layer of the aorta from AscAA patients was expected, considering similar data from *in vivo* studies [227].This increase in aortic Sdc-1 expression stems from increased infiltration of macrophages where Sdc-1 is upregulated as part of a counterbalancing response to the aneurysm and ongoing inflammatory changes [227]. We demonstrate increased aortic expression of the macrophage marker CD68 in association with the increased expression of Sdc-1, which supports these processes also in patients with AscAA. Of note, we did not detect increased aortic expression of CD68 in association with the increased aortic Sdc-1 expression in T2D. While increased expression of Sdc-1 on infiltrating macrophages is a response to AscAA, the increased Sdc-1 expression preceding AscAA formation in patients with T2D may serve to protect from aneurysm formation.

The rationale for **Paper III** was that BAV- and TAV- associated AscAA pathologies differ. For instance, inflammation does not play an important role in BAV- associated AscAA, but MMP-2 activity is indicated to be a more significant feature [244]. Specifically, previous studies have shown that proteolytic activity is increased in association with AscAA among BAV patients [90, 91]. However, we did not observe any alterations of MMP-2 activity in these patients.

In contrast to what was observed for TAV patients, in **Paper III** we report that T2D is associated with lower levels of aortic Sdc-1 expression among BAV patients. This difference may not necessarily stem from T2D pathology and/or anti-diabetic therapy, such as GLP-1-based therapy — exerting differential effects on Sdc-1 expression in BAV and TAV patients — but may be correlated to other differences between the groups. For instance, the BAV T2D group was significantly older than the BAV control group, and although the difference detected remained also after controlling for age, the effect may be secondary to age-related inflammatory changes that this study did not investigate. However, our data indicate that altered aortic expression of Sdc-1 may not contribute to the reduced prevalence of AscAA among T2D BAV patients.

The fact that AscAA was not associated with increased aortic infiltration of macrophages, nor increased aortic Sdc-1 expression among BAV patients, is in agreement with the fact that inflammation and infiltration of immune cells is not a prominent feature of BAV- associated AscAA pathology.

Of note, comparing **Papers II** and **III** illustrates and emphasizes differences between BAV- and TAV- associated AscAA pathology, while also indicating that the reduced prevalence of AscAA in T2D BAV *vs.* TAV patients may be contributed to by different factors. In summary, these data argue the importance of separating BAV and TAV patients, when studying AscAA pathology and/or its reduced prevalence in T2D.

Of importance to consider when interpreting the results of **Paper I-III,** is that very small amounts of active fasting plasma GLP-1 reach the GLP-1Rs at the site of the aneurysm. In addition, only 10% of the active GLP-1 metabolite manages to reach the systemic circulation,

as it has a half-life of 1-2 minutes [154, 273]. Consequently, that an association with fasting plasma GLP-1 was not found, does by no means exclude direct effects of incretin therapy.

In **Paper IV**, we go back to focusing on Sdc-1 as a potential mediator of the suggested contribution of GLP-1 signaling to T2D- associated reduced prevalence of TAA. In **Paper II** we assessed alterations of Sdc-1 expression in adventitial samples in association with T2D, as well as in association with AscAA, and potential correlations to fasting plasma GLP-1, while in **Paper IV** we study isolated AoSMCs from the media layer of the aorta. We focus on the direct effects of GLP-1R activation on AoSMCs, and its implications on AoSMC phenotype and Sdc-1 expression under diabetic/non-diabetic conditions, as well as in an environment promoting aortic aneurysm formation. As AoSMC de-differentiation and loss characterizes TAA and Sdc-1 is implicated in AoSMC resilience to de-differentiation, we were surprised to see increased Sdc-1 expression in de-differentiated cells compared to differentiated ones. However, the increased Sdc-1 expression may be speculated to be a response to the de-differentiated state.

In **Paper IV**, the relatively low GLP-1R expression reported in de-differentiated cells may argue against the relevance of GLP-1-based therapy to treat TAA by targeting AoSMCs. However, the abundant expression of the GLP-1R in differentiated AoSMCs (physiological state of AoSMCs) facilitates GLP-1 mediated effects, where GLP-1R activation also upregulates the expression of the GLP-1R in these cells, compared to the de-differentiated cells. That diabetic conditions (simulated by addition of glucose and palmitate) increased the inflammatory profile of the cells, while also increasing the expression of one of the differentiation markers analyzed, agrees well with reports of increased inflammation, but reduced proliferation and migration of SMCs in response to palmitate [274]. While co-treatment with a GLP-1R agonist did not alter this effect, it did increase the expression of one of the main antioxidants (catalase) in AoSMCs, which has been suggested to prevent aortic aneurysm formation [275]. The fact that we were not able to detect an effect of GLP-1R activation on Sdc-1 expression, despite increased Sdc-1 expression after treating with forskolin, which is an adenylate cyclase activator [276, 277], may be due to differences in the timing, extent, or duration of cAMP and PKA activation in response to GLP-1R activation *vs.* forskolin [276, 278-280]. When treating differentiated AoSMCs with AngII or TNF-α to simulate an environment promoting aortic aneurysm formation, we did not observe the expected effect of either of them on the differentiation markers analyzed [50, 281]. This discrepancy of results is probably due to differences in the duration of AngII treatment, and/or the time of analysis. However, the decreased Sdc-1 expression detected after treating with AngII agrees with previous studies [227] and may contribute to de-differentiation of the cells with prolonged exposure to AngII [226, 227]. The fact that the effect of AngII was attenuated by pre-treatment with a GLP-1R agonist, implies that GLP-1R activation may, through effects on Sdc-1 expression, prevent de-differentiation in response to prolonged AngII exposure, which may contribute to reduced prevalence of TAA in T2D. Finally, we also used de-differentiated AoSMCs as a model of the AoSMCs found in patients with TAA. However, the fact that we

did not detect an effect of GLP-1R activation on the AoSMC phenotype or Sdc-1 expression of these cells may result from the relatively low expression of the GLP-1R.

6.2 METHODOLOGICAL CONSIDERATIONS

6.2.1 Cell cultures

The AoSMCs that were used for **Paper IV** were commercially available primary cells. The disadvantage of using primary cells involves the fact that they, in contrast to cell lines, have a finite lifespan and cannot be cultured in the lab indefinitely. However, primary cell lines are more representative of endogenous cells. Further, these primary AoSMCs were guaranteed by the manufacturer for 15 passages, under recommended culture conditions, making the planned experiments feasible. Herein we simulated T2D/AscAA conditions to study them. However, having primary cells from patients with AscAA or T2D would have been an advantage when characterizing the AoSMCs found in T2D or AscAA and potential differences between them.

Of note, *in vitro* experiments offer controlled conditions, allowing for assessment of potential direct effects of an agent on the cell type of interest. However, this is also a very artificial milieu, as we are missing any interactions with other factors, cells, and organs. Specifically, the aorta does not only have AoSMCs, and interactions with, for example, endothelial cells may be very important for their response to different environments or factors. It would be very interesting to study a system where we would 'simulate the aorta' with both AoSMC and endothelial cells. However, to the best of my knowledge, although there are studies that try to create such systems, they have not been optimized yet. Perhaps with future advances in organoid culture development this would be possible. In addition, it would be more difficult to sustain such a culture. When simulating diabetic conditions, we added palmitate and glucose although more fatty acids are present and could interact with each other. However, palmitate is often used to study the effects of hyperlipidemia, as it is the fatty acid for which serum concentrations increase the most in association with obesity. In similarity, aneurysmatic conditions are characterized not only by increased AngII or TNF-α but increased levels of other cytokines, proteolytic activity, etc. have also been observed.

In addition, for a small part of **Paper I** we used the murine GLP-1 secreting GLUTag cells. GLUTag cells are well characterized in scientific literature and are a good model of the intestinal GLP-1 secreting cells. However, they are an immortalized cell line and the artificial nature of the *in vitro* setting applies also here.

6.2.2 A Comparison of Methodologies

Going through the literature and trying to compare and contrast our findings with what has been found before we noticed that the levels of the analytes differ, depending on which ELISA kit was used. We also found some papers comparing ELISAs from different manufacturers, where differences were also found in the standards provided by different manufacturers [282-

284]. For that reason, we used the same ELISAs throughout all of the studies included in this thesis.

In addition, we tried to use ELISA to analyze protein expression whenever possible, as it is a quantitative method, whereas western blotting is only a semi-quantitative method. However, for some analysis, this was not possible due to unavailability.

6.3 LIMITATIONS OF THE STUDY

Data collected from an observational study is more generalizable to the general population, as compared to data generated from other types of clinical studies. I. e. for an observational study, the effect of a risk factor, diagnostic test, treatment, or other intervention is observed without changing who is or is not exposed to it. However, observational studies are for the same reason less controlled, and a major limitation of such studies is the presence of various confounders (such as in this case anti-diabetic therapy, age, gender, aortic valve pathology, duration of T2D, etc.), and errors while collecting the data (when not the same person was performing the surgery or was collecting the plasma or tissue). Further, selection bias may make results less generalizable to the population of interest [285]. In addition, observational studies such as the ones included in this thesis cannot be used to show causality [285].

Oxidative stress is an important factor in TAA pathology, governing also Sdc-1 expression, and the anti-oxidative effects of GLP-1 are well documented. However, oxidative stress was not studied in any of the **Papers I–III,** as some of the plasma samples that were obtained for this study were too old for such analysis.

All the patients that were included in the studies of this thesis were cardiac surgery patients, so the study lacked a healthy control group. In addition, all patients were included irrespective of T2D or AscAA duration, and medication (statins or anti-diabetic therapy). It was a deliberate choice to not remove anti-diabetic therapy that may enhance/alter fasting plasma GLP-1, as we did not seek the cause but rather the effect of elevated fasting plasma GLP-1. However, statins or other medications may contribute to the changes in the systemic inflammation in patients with T2D, hiding a potential association with fasting plasma GLP-1. However, as the studies were underpowered to do so, we were not able to control for any medications. Another limitation is that we did not separate the patients with aortic stenosis and aortic insufficiency. In addition, both men and women were included in our study, despite reports indicating that TAA does not develop with the same frequency in men and women, where women are usually older than men at the onset of symptoms [286-288] and have a poorer outcome of the disease [286]. Finally, it would be very interesting if we had access to samples from the media aortic layer of the patients, something that was not possible for this study.

7 CONCLUSIONS

- From the studies of **Paper I**, we can conclude that among surgical patients with aortic valve pathology, T2D is associated with a Th2 shift of the inflammatory profile, which may contribute to the reduced prevalence of AscAA in T2D patients. Further, fasting plasma GLP-1 is associated with this Th2 bias.

- From the studies of **Paper II**, we conclude that T2D is also associated with elevated levels of Sdc-1 in adventitia — an association unrelated to increased fasting plasma GLP-1 — which may contribute to the lower prevalence of AscAA in T2D patients. Further, elevated Sdc-1 expression in the AscAA patients occurs in association with macrophage infiltration and may be part of a counterregulatory response to AscAA formation.

- From the studies of **Paper III**, we come to the conclusion that, in contrast to what was found for TAV patients, Sdc-1 expression is not indicated to be involved in the reduced prevalence of BAV- associated AscAA in T2D.

- From the studies of **Paper IV**, we draw the conclusion that GLP-1 signaling may not exert significant effects on de-differentiated cells of the aneurysmatic aorta, but may contribute to decreased prevalence of TAA in T2D by maintaining AoSMCs in a differentiated state, through attenuation of the effects of AngII on Sdc-1 expression.

8 POINTS OF PERSPECTIVE

Although AAA is well studied the data for TAA are limited and mortality is underestimated because of many undiagnosed/misdiagnosed cases, where only autopsy could show the cause of death [289]. Of note, Olsson et al. found that from 1987 to 2002, the prevalence of TAA and dissection increased by approximately 50% in men and 30% in women [290]. Consequently, although the mortality rate has improved in the last years with hospital mortality to be less than 10% [289, 291] there is a great need to find useful biomarkers that can help in the detection of TAA even in asymptomatic patients. Although many biomarkers have been studied, the best method for disease diagnosis available today is molecular imaging, like PET and SPECT [292, 293]. Studies have suggested MMPs, hsCPR, and cytokines as biomarkers, but none of these have been proven good enough [294-297]. Moreover, there is a great need to find a successful pharmacological prevention or treatment, which could prevent aneurysm formation/limit progression of small aneurysms into life-threatening conditions.

This thesis adds relevance to *in vivo* studies, by providing clinical data, which may support a protective role for GLP-1 signaling in AscAA. At the same time, it adds relevance for larger registry-based/multicenter studies, designed to determine whether GLP-1-based anti-diabetic therapy associates with reduced prevalence of TAA among T2D patients. As we were not able to control for the type of anti-diabetic therapy, it leaves to debate whether the alterations of the inflammatory profile detected herein could be relevant only for a group of T2D patients receiving a specific type of anti-diabetic therapy (such as GLP-1 analogs/DPP-4 Inhibitors/Metformin).

Due to its beneficial effects in terms of CVD the European association of cardiology recommend GLP-1 analogs to everyone with high CVD risk, independent of HbA1c status [298]. Current Swedish guidelines indicate GLP-1 analogs as a drug of choice if metformin therapy does not achieve recommended HbA1c levels or in the case of Metformin intolerance [299].

Future studies should be designed to understand if/how fasting plasma GLP-1 levels/GLP-1-based therapy and systemic inflammation are of importance for local inflammatory processes and SMC differentiation in the aortic wall of patients with T2D. In addition, clinical studies should determine whether Sdc-1 expression is increased in AoSMCs of diabetic patients, and the possible association with GLP-1R signaling.

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