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A suit of armor around ADAMTS13

Exploring paths towards resistance against autoantibodies of immune TTP patients

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Chapter 8

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Summary

Von Willebrand Factor (VWF) is an important plasma protein responsible for initiating some of the main events leading to blood clotting. Briefly, upon injury, VWF interacts with collagen and recruits platelets to the injury site to form a platelet plug and immediately dampen any blood loss. VWF is formed as a very large multimeric protein of variable size (ranging from a molecular weight of 250 kDa – the basic monomer size – up to >10 000 kDa – the largest of multimers; for perspective, albumin, the most abundant protein of the blood, is ~65 kDa in molecular weight; antibodies of IgG type are also highly abundant and have ~150 kDa). The greater the size of VWF multimers, the greater is their capacity for platelet recruitment, and the greater the danger of unwanted blood clotting. The VWF size must be kept in check to prevent blood clotting from happening in the absence of injury. This is achieved by the plasma protein ADAMTS13, which acts on VWF to reduce its multimeric size. If ADAMTS13 activity is lost for any reason, VWF multimers remain abnormally large in size (Unusually-Large-VWF or UL-VWF). This leads to unwanted formation of thrombi inside the blood vessels of the entire organism. Platelets are consumed in this process (thrombocytopenia), and the disseminated thrombi provoke ischemia (which leads to organ failure of variable intensity), petechiae (caused by incapacity to efficiently stop bleeding), a hemolytic anemia (where the red blood cells are shredded in pieces called schistocytes at the passage of blood vessels partially occluded by hyaline thrombi), and a high fatality rate (80-90%) if left untreated. This condition is called Thrombotic Thrombocytopenic Purpura (TTP).

TTP has almost 100 years of history, and throughout time the knowledge surrounding this disease entity saw great transformations. The technical terms associated to it can be somewhat confusing for any new-comers, regardless of their scientific expertise. In **Chapter 1**, the story behind this disease is told with an historical perspective. Today, TTP is recognized as a dual entity: it can be "congenital TTP" – where patients have hereditary ADAMTS13 mutations that either reduce the protein's activity *per se*, or reduce its synthesis or secretion – or "acquired TTP" – where ADAMTS13 activity loss is acquired at some point in life, being in many cases associated with autoantibodies that target ADAMTS13 ("immune TTP", autoimmune in nature). In any case it may either be associated with identifiable co-morbidities and/or triggering factors ("non-idiopathic TTP") or not ("idiopathic TTP"). TTP is a sea of information that benefits from a "nautical chart" to help steer the ship and keep it afloat. Literature has attempted to provide some orienting algorithms, but they all fail to show the intricate relationship between the diagnosis process and the treatment process. **Chapter 1** is an attempt to provide such a chart. The current state-of-the-art in clinical practice is explained, and integrated with the remaining goals of this thesis. Current treatments for TTP may be effective, but they haven't changed much the long-term outcomes of mortality and

morbidity. Many haven't yet had a true impact on the most relevant clinical outcomes for patients themselves. This thesis sets out to put the main unmet medical and patient needs out in the open, and to contribute for solving two of those unmet needs: 1) the need to increase the awareness about this rare disease entity and how to treat these patients (possibly also contributing to patients understanding of their own disease); 2) the existing need for an easy to administer and safer treatment that may improve both short- and long-term mortality rates, reduce the rates of complications associated to the main treatments, as well as their refractory rates (that is, lack of response), as well as having a real long-term impact in the sequelae associated with development of this disease.

In **Chapter 2**, we focus on immune TTP and its pathophysiology. The current knowledge in the field is reviewed more thoroughly and serves as a complement to Chapter 1. We show the main characteristics of the immune response of these patients, the antibody types most commonly present, their functional features and value as biomarkers for prognosis, the known genetic features of most immune TTP patients (with a focus on HLA alleles and the current evidence for these alleles as risk factors for immune TTP), and what are the main theories behind immune TTP disease development.

In **Chapter 3**, we investigate the spacer domain of ADAMTS13 as the most prevalent ADAMTS13 epitope target. The classic spacer domain exosite-3 (R568/F592/R660/Y661/Y665) has been considered the main epitope region of the spacer domain. Do all anti-spacer autoantibodies target this sole epitope region? Or do patients' autoantibodies target other regions within the spacer domain as well? We provide evidence that the spacer domain harbors 3 main hotspot regions for autoantibodies: the regions spanned by amino-acid residues 588-592, 602-610 and 657-666.

In **Chapter 4** we work with the classic ADAMTS13 spacer domain exosite-3 residues and insert progressive mutations of 4 types – ranging from highly-conservative, semi-conservative, non-conservative and classic alanine scan mutations – to see how these impact on autoantibody binding to ADAMTS13 and, simultaneously, how to these impact on ADAMTS13 activity. We show that the most conservative mutations tend to conserve both binding and activity, while the less conservative mutations abrogate both binding and activity. This has implications for the field, as one of the main goals has been to find ADAMTS13 mutants that resist binding of autoantibodies, but retain high levels of activity.

In **Chapter 5** we show that by inserting an artificial N-glycan site into one of the spacer domain epitope hotspots found in **Chapter 3**, we can create an autoantibody resistant ADAMTS13 that retains VWF processing activity. This successful variant may have a large impact in the future treatment of this disease, considering that efforts are being made to employ the wild-type recombinant ADAMTS13 as a treatment for acquired TTP patients, which will likely face many challenges posed by the already

present autoantibodies in immune TTP patients in terms of drug pharmacokinetics and efficacy.

In **Chapter 6** we present a more structural basic study of ADAMTS13 and the possible ways in which its conformational status may be altered. It is known that introducing mutations in exosite-3 can trigger an opening of ADAMTS13's conformation, resulting in partial loss of activity specificity. This is because the interactions between the spacer exosite-3 and the CUB domains of ADAMTS13 are lost. It is known that anti-C-terminal mouse antibodies can open ADAMTS13. In immune TTP patients with an acute TTP disease bout (or prior to a relapse), ADAMTS13 is in open conformation status. Furthermore, treatment of these patients with rituximab reverses the conformation back to the naturally closed status, which provides compelling evidence that patient antibodies can open ADAMTS13. We explore the possibility that mutations in CUB domains of ADAMTS13 can also trigger an open-conformation. In this bioinformatics-driven study, we show evidence that for certain mutations of CUB1 and CUB2 this is the case.

Finally, in **Chapter 7** we provide an in-depth discussion of the content of this thesis. Specifically, we stress the importance of having well-organized patient registries and biobanks to advance knowledge in this field; we discuss the main questions that remain unanswered regarding the field of autoimmunity, with a focus on the immune TTP case: we point what could be the next steps in basic research for this disease and we focus on what are the main implications of the findings in **Chapters 3-6** and the potential impact they may have in the field during the upcoming years.

Samenvatting

Von Willebrand factor (VWF) is een bloedstollings eiwit dat een belangrijke rol speelt bij de vorming van bloedstolsels die zeer snel gevormd worden na beschadiging van de vaatwand. Direct na een beschadiging van de vaatwand zorgt VWF er samen met collageen voor dat bloedplaatjes gerekruteerd worden. De bloedplaatjes binden aan de beschadigde vaatwand en vormen een bloedplaatjesprop die ervoor zorgt dat het bloedverlies beperkt blijft. VWF wordt gevormd als een zeer lang polymeer eiwit met een grootte die varieert van 500 kDa tot meer dan 10.000kDa. De lange VWF-polymeren kunnen zeer effectief bloedplaatjes rekruteren. Om spontane binding van bloedplaatjes aan VWF te voorkomen is het belangrijk dat de grootte van de VWF-polymeren gereguleerd wordt. In plasma is een protease aanwezig dat VWF-polymeren in kortere ketens kan knippen. Dit protease is ADAMTS13; ADAMTS13 knipt een peptide-binding in het A2 domein van VWF. Bij patiënten met trombotische trombocytopenische purpura (TTP) werkt ADAMTS13 niet goed of is het simpelweg niet aanwezig. In plasma van patiënten met TTP zijn VWF-polymeren met een zeer groot molecuulgewicht aanwezig. Dit kan leiden tot ongewenste vorming van bloedplaatjes-rijke trombi in de kleine bloedvaten die spontaan aan de grote VWF-polymeren binden. Bloedplaatjes worden tijdens dit proces geconsumeerd en hierdoor neemt de hoeveelheid bloedplaatjes in de circulatie sterk af (trombocytopenie, een tekort aan bloedplaatjes). Door het optreden van stolsels in de kleine vaten kan er onvoldoende zuurstof naar de weefsels en organen worden getransporteerd; dit kan leiden tot orgaan-schade. Bij patiënten waarbij ADAMTS13 afwezig is kunnen kleine paarse vlekjes (purpura) op de huid voorkomen; dit worden petechiën genoemd. Dit zijn kleine bloedingen die optreden omdat er te weinig bloedplaatjes zijn om bloedingen in de kleine vaten te corrigeren. In het bloed van patiënten met TTP komen gefragmenteerde rode bloedcellen voor die schistocyten of fragmentocyten worden genoemd. Door de aanwezigheid van stolsels in de kleine vaten komen de rode bloedcellen vast te zitten en fragmenteert een deel van de rode bloedcellen. Dit resulteert in een hemolytische anemie. TTP is een levensbedreigende ziekte waarbij een snelle diagnose en behandeling van groot belang is.

In **Hoofdstuk 1** wordt een overzicht gegeven van de ontwikkelingen op het gebied van de diagnose en de behandeling van TTP vanuit een historisch perspectief. TTP kan worden veroorzaakt door mutaties in het ADAMTS13 gen; in dit geval spreken we van de aangeboren vorm van TTP. Deze aandoening is zeer zeldzaam. Mutaties in het gen coderend voor ADAMTS13 interfereren met de secretie van ADAMTS13; mutaties kunnen ook tot gevolg hebben dat het eiwit dat gemaakt wordt niet meer actief is. De “verworven” vorm van TTP wordt veroorzaakt door de ontwikkeling van auto-antistoffen die gericht zijn tegen ADAMTS13. De antistoffen die gevormd worden remmen de activiteit van ADAMTS13 of zorgen ervoor dat ADAMTS13 snel weer uit het bloed verdwijnt. Op dit moment is er zeer veel informatie beschikbaar in de

wetenschappelijke literatuur met betrekking tot de diagnose en de behandeling van TTP. De beschikbare informatie wordt in dit hoofdstuk besproken. Daarnaast wordt in dit hoofdstuk aangegeven wat op dit moment de belangrijkste nieuwe ontwikkelingen en onderzoeksvragen zijn met betrekking tot het verder verbeteren van de huidige behandeling van patiënten met TTP.

In **Hoofdstuk 2** beschrijven we de pathofysiologie van de immuun-gemedieerde, verworven vorm van TTP (afgekort als iTTP). De huidige kennis met betrekking tot de immuunrespons van deze patiënten wordt samengevat. Risicofactoren die de kans op het ontstaan van iTTP verhogen worden besproken en op grond van de huidige kennis wordt een model gepresenteerd waarmee het ontstaan van iTTP mogelijk verklaard kan worden.

In **Hoofdstuk 3** onderzoeken we welke delen van het spacer domein van ADAMTS13 betrokken zijn bij de binding van pathogene antistoffen die ontstaan in patiënten met iTTP. De in deze studie gerapporteerde gegevens tonen aan dat verschillende regio's in het spacer domein betrokken zijn bij de binding van antistoffen.

In **Hoofdstuk 4** wordt op systematische wijze gekeken naar de vijf aminozuur-residuen die betrokken zijn bij de binding van antistoffen gericht tegen het spacer domein. De effecten van conservatieve, semi-conservatieve, niet conservatieve en klassieke alanine substituties op de binding van auto-antistoffen en de activiteit van ADAMTS13 is bestudeerd. Uit de resultaten komt naar voren dat ADAMTS13 varianten die minder goed aan pathogene antistoffen binden ook minder actief zijn.

In **Hoofdstuk 5** beschrijven we een aantal spacer-domein varianten waaraan we een extra consensus-site hebben toegevoegd waaraan een "N-gebonden suiker-molecuul" kan worden bevestigd. Door toevoegen van een extra suiker-molecuul kan dit deel van ADAMTS13 mogelijk afgeschermd worden zodat antistoffen niet meer kunnen binden. Eén van de geconstrueerde varianten werd inderdaad minder goed herkend door antistoffen maar was nog steeds in staat om VWF te knippen. De ontwikkelde ADAMTS13 variant kan mogelijk in de toekomst ingezet worden bij de behandeling van patiënten met TTP.

In **Hoofdstuk 6** wordt de conformatie van ADAMTS13 bestudeerd. ADAMTS13 kan in een "open" of een "gesloten" conformatie voorkomen. De "open" conformatie van ADAMTS13 wordt aangetroffen in plasma van TTP patienten gedurende een acute aanval van TTP. De "gesloten" conformatie van ADAMTS13 komt voor bij gezonde individuen en bij TTP patiënten in remissie. Op dit moment is niet bekend hoe de conformatie van ADAMTS13 gereguleerd wordt. Op grond van moleculaire modelling studies zijn er aantal aminozuur residuen in de CUB1 en CUB2 domeinen van ADAMTS13 geïdentificeerd die betrokken zijn bij intra-moleculaire interacties die ervoor zorgen dat ADAMTS13 onder normale condities in een "gesloten" conformatie aanwezig is.

In **Hoofdstuk 7** wordt de inhoud van dit proefschrift besproken en worden

suggesties gedaan voor vervolg-onderzoek. Tevens wordt aangegeven op welke wijze de in dit proefschrift gedocumenteerde studies kunnen bijdragen aan een betere diagnose en behandeling van patiënten met TTP.

PhD Portfolio

PhD Period: March 2016 – October 2020
First Hosting Institution: Icosagen AS, Estonia (Non-Academic)
Second Hosting Institution: Sanquin – AMC, Amsterdam, The Netherlands (Academic/Non-Academic)
Promotors: prof. dr. Jan Voorberg and prof. dr. Karen Vanhoorelbeke
Co-Promotors: dr. Andres Männik and dr. Gerry A. F. Nicolaes

Congresses/Symposia	Year	ECTS	Presentation
Réunion Annuelle CNR-MAT – Paris, France	2016	0,25	
Journee Annuelle "Maladies Rares Immune-hématologiques" – Paris, France	2017	0.25	
9th International BIC Conference – Rome, Italy	2017	1.00	Oral
Bootcamp in Entrepreneurial Innovation in Orphan Diseases – Leuven, Belgium	2017	0.75	Organization
5th European Congress of Immunology – Amsterdam, The Netherlands	2018	1.00	
Sanquin Science Day – Amsterdam, The Netherlands	2018	0.25	Poster
TTP Contact Dag – Meander Medisch Centrum, Amersfoort, The Netherlands	2019	0.25	Poster
10th International BIC Conference – Genoa, Italy	2019	1.00	Oral
Innovation Bootcamp in Rare Diseases – Leuven, Belgium	2019	0.50	Organization
Walk for TTP – Kortrijk, Belgium	2020	0.25	Poster

Courses/Internships	Year	ECTS	-
Clinical Training at the CNR-MAT – AP-HP, Paris, France (6-weeks)	2016	8.57	
Laboratory for Thrombosis and Hemostasis – KU Leuven, Kortrijk, Belgium (1 week)	2017	1.42	
Advanced Immunology (AMC PhD Program)	2019	1.93	
ITN Training in IP Protection – Leuven, Belgium	2019	0.12	
Entrepreneurship in Health and Life Sciences (AMC PhD Program)	2019	0.75	

Seminars, Workshops and Master Classes	Year	ECTS	Presentation
H2020 Consortium ITN PROFILE Workshops			
PROFILE Workshop 1 – Paris, France	2016	0.70	Oral
PROFILE Workshop 2 – Barcelona, Spain	2016	0.60	Oral
PROFILE Workshop 3 – Tallinn/Tartu, Estonia	2017	0.60	Oral
PROFILE Workshop 4 – Amsterdam, The Netherlands	2018	0.70	Oral
PROFILE Workshop 5 – Leuven, Belgium	2018	0.50	Oral
PROFILE Workshop 6 – Copenhagen, Denmark	2019	0.50	Oral
Department Meetings	2018-2020	1.00	6x Oral
Sanquin Research Seminars	2018-2020	1.00	1x Oral

Teaching/Mentoring	Year	ECTS	-
Master Student: Nynke de Leeuw	2018	0.30	
Master Student: Ruby Schipper	2019	0.40	

Miscellaneous	Year	ECTS	Presentation
Science and Cocktails: The First Image of a Black Hole (speakers: prof. dr. Heino Falcke, prof. dr. Sera Markoff, and prof. dr. Feryal Özel) – Paradiso, Amsterdam, The Netherlands	2019	0.05	

Publications List

Published

- Hrdinová J*, D'Angelo S*, **Graça NAG***, Ercig B, Vanhoorelbeke K, Veyradier A, Voorberg J, Coppo P, *Dissecting the pathophysiology of immune thrombotic thrombocytopenic purpura: interplay between genes and environmental triggers*, Haematologica, 2018, vol. 103(7),1099-1109, doi:10.3324/haematol.2016.151407
- **Graça NAG**, Ercig B, Pereira LCV, Kangro K, Kaijen P, Nicolaes GAF, Veyradier A, Coppo P, Vanhoorelbeke K, Männik A, Voorberg J, *Modifying ADAMTS13 to modulate binding of pathogenic autoantibodies of patients with acquired thrombotic thrombocytopenic purpura*, Haematologica, 2019, vol. 104, doi:10.3324/haematol.2019.226068 *(published as Early view)*
- Pereira LCV, Roose E, **Graça NAG**, Sinkovits G, Kangro K, Joly B, Tellier E, Kaplanski G, Falter T, Von Auer C, Rossmann H, Feys HB, Reti M, Prohászka Z, Lämmle B, Voorberg J, Coppo P, Veyradier A, De Meyer SF, Männik A, Vanhoorelbeke K, *Immunogenic hotspots in the spacer domain of ADAMTS13 in immune-mediated thrombotic thrombocytopenic purpura*, J Thromb Haemost, 2021; 19: 478-488; doi:10.1111/jth.15170
- Ercig B*, **Graça NAG***, Kangro K*, Wichapong K, Hrdinová J, Kaijen P, van Alphen FPJ, van den Biggelaar M, Vanhoorelbeke K, Veyradier A, Coppo P, Reutelingsperger C, Nicolaes GAF, Männik A, Voorberg J, *N-glycan mediated shielding of ADAMTS13 prevents binding of pathogenic autoantibodies in immune-mediated TTP,* Blood 2021; (First Edition, 5th February 2021); doi: 10.1182/blood.2020007972

In Preparation

- **Graça NAG**, Voorberg J, Vanhoorelbeke K, Joly B, Stepanian A, Veyradier A, Coppo P, *The "Nautical Chart" of Thrombotic Thrombocytopenic Purpura*, 2020
- Ercig B, Arfman T, **Graça NAG**, Wichapong K, Roose E, Kaijen P, Reutelingsperger C, Vanhoorelbeke K, Nicolaes GAF, Voorberg J, *Modelling-assisted identification of CUB1/2 domain residues involved in maintaining ADAMTS13 in a closed conformation*, 2020

Other Publications

- Vanhoorelbeke K, Pereira LCV, Kangro K, **Graça NAG**, *PROFILE: Early-Stage Researchers Advancing Insights in TTP Through a Unique PhD Track*, HemaSphere, 2018, vol. 2(1), e22, doi:10.1097/HS9.0000000000000022

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PROFILE: Early-Stage Researchers Advancing Insights in TTP Through a Unique PhD Track

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Introduction by Karen Vanhoorelbeke, PROFILE Coordinator

The PROFILE project (www.itn-profile.eu) is funded as Innovative Training Network within the Horizon 2020 Marie Skłodowska-Curie Actions program. Its primary focus is on fine tuning the diagnosis of patients suffering from acquired thrombotic thrombocytopenic purpura (TTP) through immunoprofiling.

The project kicked off in October 2015, and groups top experts from across Europe: academic researchers (Prof Karen Vanhoorelbeke, KU Leuven, Belgium; Prof Jan Voorberg, Sanquin, The Netherlands), clinical academic centers (Prof Paul Coppo and Prof Agnès Veyradier, Assistance Publique-Hôpitaux de Paris, France), industrial partners (Dr Andres Männik, Icosagen, Estonia; Dr Kaia Palm, Protobios, Estonia; Dr Liselotte Brix, Immudex, Denmark; and Dr Marta Palicio, Biokit, Spain), and nonprofit organizations (Dr Rob Fijnheer, TTP Contact group, The Netherlands; Robin Doeswijk, European Hematology Association) (Fig. 1). Coordination is handled by Prof Vanhoorelbeke, based at KU Leuven's Kortrijk campus (Fig. 2).

In this consortium, 6 early-stage researchers (ESR) were recruited to embark on a unique PhD track, consisting of 5 learning paths. Besides receiving thematic training and performing research tasks in both academic labs and industry, all students have received front-line clinical training by Prof Veyradier and Prof Coppo at Assistance Publique-Hôpitaux de Paris. In addition, a translational training program has been developed by Dr Nick Geukens, the PROFILE training manager, in which ESR are learning to deal with challenges linked to the development of new diagnostics or therapeutics.

On top of this, the students are trained in management, business, ethical, legal, career development, and personal skills. To that end, numerous network wide events with top invited lectures are organized within PROFILE. In October 2017, we organized the first edition of the Entrepreneurial Innovation Bootcamp on Rare Diseases in Leuven. More than 20 experts from research, clinical, industry, and policy shared their insights on breakthroughs and hurdles in innovation across the broad spectrum of rare diseases.

"With what expectations did you enter the project?"

Q&A with early-stage researchers Carolina Velàsquez Pereira, Kadri Kangro and Nuno A.G. Graça

The project is interesting due to the industry–academia fusion. We expected for a solid "4 × 4" formation in a translational research project that could yield more than knowledge. Of course, we expected the project to involve a lot of traveling, due to work being done in different laboratories. We will be able to come into contact with both patients and companies, a unique experience! It is a great opportunity to be part of an interdisciplinary team, to share experiences, and to improve language and expression skills as well, while building a network of connections, important and necessary in science.

"How have you experienced it so far?"

We have had the chance to work in several European countries. Our scientific and presentation skills have improved, and networks have expanded. We received a clinical training by experts in patient management at the Public Hospitals of Paris. It was a privilege to see firsthand the day-to-day activities of doctors diagnosing and treating TTP patients, to come into direct contact with some of the patients and witness their fears, hopes, and expectations. This is vital in shaping the perspective of the laboratory research being conducted.

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Figure 1. **The PROFILE early-stage researchers, from left: Kadri Kangro, Nuno A.G. Graca, Silvia D'Angelo, Boğaç Ercig, Johana Hrdinova, and Carolina Velasquez Pereira.**

Figure 2. **Karen Vanhoorelbeke en Wim Maes.**

On another level, for some of us it is the first time living abroad. We experienced cultural differences, and met people from all over the world. Naturally, we encountered some language barriers! We can say the project is an interesting intellectual and practical challenge that constantly forces us to break down our own barriers and broaden our horizons, and not just on the research level. It feels like doing a PhD is an adventure that also takes you on a trip to your very core and it demands malleability to adapt and evolve.

"What are you hoping to have achieved upon completion of your training program?"

We are hoping to become independent scientists with a good overall view on different topics. We hope to have connections and a very competitive formation to achieve a post-doctoral fellowship in a high-level international laboratory, or immediately enter the job market meeting industry's needs. Until the end we expect to further develop a range of hard and soft skills you cannot learn by reading textbooks, etc. And if we are extremely successful in our research—hopefully we will be!—maybe we will be able to say we made a crucial positive impact to this field and in people's lives.

About the Author

Nuno Alexandre Gomes Graça was born in Lisbon, Portugal, on the 13th December 1988. The childhood and early adulthood up to the age of 24 was integrally spent in Lisbon and outskirts, living in the district of Setúbal, in the area of Seixal, with his parents, where his headquarters are still located. He attended the Faculty of Pharmacy of the University of Lisbon from September 2006 to March 2012, graduating from the Integrated Master Degree in Pharmaceutical Sciences. He is a member of the Portuguese Pharmaceutical Society (*Ordem dos Farmacêuticos Portugueses*) since the 11th July 2012 with Member Number L-12260 and Pharmacist Professional License 18959.

He wished to pursue a PhD in Life Sciences for which he began collaborating in research projects in the Pharmacological Sciences Department of his *alma mater* Faculty of Pharmacy for extra experience and to build up his resume. He worked on animal models of type II diabetes and the efficacy of traditional herbal extracts in its treatment. In late 2013 there was the opportunity to move to Porto's Instituto de Biologia Molecular e Celular (IBMC, now a part of the "i3s" institute) to participate in research projects on *Trypanosoma brucei* and animal models of African Sleeping Sickness for drug-discovery/repurposing, where he collaborated in these research projects for a year-and-a-half. In late 2015, Nuno secured a PhD position at the University of Amsterdam under the guidance of Prof. Jan Voorberg and Prof. Karen Vanhoorelbeke in a European Industrial Doctorate funded by the European Union's Horizon2020 Framework Programme, through the Innovative Training Network "PROFILE". These programs demand that the student spends at least half of his/her time outside academia (i.e. in an industry setting). On the 29th February 2016, he moved to Tartu (Estonia) to begin this non-academic part in a company called Icosagen, under guidance from his Co-Promotor Dr. Andres Männik. Nuno initiated his PhD at Icosagen, and then, in 2018 moved to Amsterdam to fulfil the academic part of the training programme at Sanquin. Apart from the publication list inserted in the previous pages, Nuno is also author of other 3 publications that refer to his previous time spent in Lisbon's and Porto's laboratories, which are placed here and showcase his expertise also with laboratory animals models (mouse models of type II diabetes and African Sleeping Sickness; see below).

In his spare time, he enjoys playing boardgames with his friends, as well as several solo hobbys like watching movies, playing video games, and reading books. Nuno is an avid reader, and a fan of the works by famous Astrophysicist Carl Sagan.

Nuno wishes to establish himself in industry, possibly performing R&D in preclinical or already linked to the clinical setting, or on the regulatory sector. After his PhD he will be investing in himself further following formation by the Cheeky Scientist Association and will be out looking for a job where he can capitalize his skills.

Other publications not related to this PhD

- **Graça NAG***, Gaspar L*, Costa DM*, Loureiro I, Thoo-Lin PK, Ramos I, Roura M, Pruvost A, Pemberton IK, Loukil H, MacDougall J, Tavares J, Cordeiro-da-Silva A. Activity of Bisnaphthalimidopropyl Derivatives against Trypanosoma brucei. *Antimicrob Agents Chemother.* 2016; 60(4):2532-2536. doi:10.1128/AAC.02490-15.
  *authors contributed equally to this work
- Encarnação S, Mello-Sampayo C, **Graça NAG**, Catarino L, Silva IBM, Lima BS, Silva OMD. Total phenolic content, antioxidant activity and pre-clinical safety evaluation of an Anacardium occidentale stem bark Portuguese hypoglycemic traditional herbal preparation. *Industrial Crops and Products,* 2016; 82:171-178. doi:10.1016/j.indcrop.2015.11.001
- **Graça NAG**, Soares MA. Terapêutica da Insuficiência Cardíaca Revisitada - Passado, Presente e Futuro, *Revista Portuguesa De Farmacoterapia*, 4(4), 23-35. doi:10.25756/rpf.v4i4.182
  (review article on the past, present and future of heart failure therapy, written in Portuguese with an abstract in English).

Acknowledgements / Dankwoord

It took almost 5 years to have this PhD finished, a cycle that started, unbeknownst to most people, not in 2016, but a few years before. Since close to the end of my Master Degree I wanted to go as far as it was humanely possible regarding an academic degree, and this battle started almost 9 years ago. Along the way I met many people – in science and outside of it – many with whom I've learned much, some of which I am eternally indebted to.

My *alma mater* was the Faculty of Pharmacy of the University of Lisbon, which hosts the iMed.ulisboa. From here it's mandatory to mention two people: Prof. Olga Silva, and Prof. Beatriz Lima, both of whom shared many hours of their wisdom with me, and were crucial people (along with their associates) in my first steps within the world of scientific research. To them I must leave the first word of appreciation for their time, patience, investment and belief in my qualities and capacities.

The second word of appreciation must be addressed here to another two great mentors I had while I did research at the old IBMC in Porto (today called "i3s"): Nuno Santarém, and Joana Tavares. I learned a lot of techniques under their guidance, and I am very grateful for all of their support, including the sympathy and support I had from fellows and colleagues both from the same laboratory – in particular my late-hour lab-buddies Joana Faria and Inês Loureiro – or from others within the institute. The time I spent there was relatively short, but crucial for my scientific development, and therefore a word of gratitude is in order.

It was after these two experiences (which lasted a total of almost 4 years) that I had the unique opportunity to participate in research abroad, away from my homecountry, with a completely new setting and a fresh re-start to my carreer and life perspectives. This began already at the age of 27. Today I am 32. I had never been abroad before, except once - but it was so short and focused on work that I couldn't really appreciate the differences. Looking back I was still an ignorant of my own home country despite 26 years spent in it. I had to leave my homecountry to get to know it better.

Despite having left the country behind, it is still - as I usually say - my main "headquarters" because that's where my family resides and where I have the biggest support, a place to return to - although I doubt it regarding future work (because it is not an industry powerhouse, and it has lacked the political vision of Historic times).

Yet, there are a number of people from there I wish to address here:

My mother Maria Ilda and my father Vitor Manuel, to whom I owe almost everything I came to be, who suffer with the physical distance of their only son, and who shared many of the good moments and also frustrations that come with science. I don't know what would be of me without them.

The same goes for my longtime upstairs neighbours, the Nunes family, who know me since I was only 6 years old and who are, in a way, also my family. Twenty-six years

living in the same place, and I cannot overstate the good memories shared together while I grew up, as well as all the help given to us in our moments of affliction. Cristina is, as I say, my "second mother". Lucas, Silas and Jonas my "little brothers". Paulo, unfortunately, is no longer in this world to witness the climax of this battle that he still witnessed in the beginning. He was my "second father". My mother's father, Amândio, also saw the very beginning of this cycle, but is also not with us since late 2012 , and my father's mother, my grandmother Maria Ribeiro Quialheiro, still saw me leave but passed away recently on February 2nd 2021 (non-COVID causes), so it is another element of my already small family who unfortunately won't be able to witness this journey's climax. This achievement is also in honor of their memories. At least mother's cousin José Francisco, has endured all these years. He helped my parents raise me. For him too, I must leave a thank you word.

Life is as it is, and it teaches hard lessons. It gives some and it takes some.

On the "friends" department, Life has done just that. Some lifetime friends I've had the privilege to keep since the day we met. Some of them sadly got lost along the odd roads of life. I've had the good fortune of still having my best and inseparable friend Dário Martins, who has been like another brother to me all these years and who has been an amazing supporter throughout this whole cycle. Another great friend I've had the privilege to keep for a long time, despite the distance and lower frequency of interaction, has been Sara Couto, who always had a friendly word and also a keen scientific curiosity on this PhD project. All these people helped me develop my Layman skills in one way or another.

In Tartu, where I lived for roughly 2 years, I made more friends than in my entire life. I owe several thank you notes to many of the people I met there outside Icosagen. The first has to go to Martin Krssak and to Hasan Sait Arslan, from whom I ended up having much support from in a few darker moments. The same goes to my mates in the Üliõpilasküla of Raatuse 20 (University Residence) old room 547: Giuseppe Attinasi, Sergey Kim, Gabriel Rezzonico, and later Michael Gurmessa. They were the most fantastic house mates I could have ever asked for. Here I also have to mention several groups of people that brought a lot of color to my life while residing in Estonia: 1) Sofia Oliveira, Lisandra Meneses, Nemailla Bonturi, Guilherme Pinheiro, Jhonny Capichoni, Katyane Anastacia, Jerome Geoffrey, Eka Rusadze and Alaleh Rahimi; 2) The "Mafia gang" of which Martin is also a member: Asif Sattar, Margarita Shmavonyan, Lilit Stepanyan, "Abed Ma", Umesh Abhat, Daniel Blixt, Christian Pfeifer and Nadja Habertz – may you all be drinking Margaritas and Martinis for many years!; 3) Adriano Augusto, Mia Prokopiv, Yauhen Ula Yakimenka, Jayaruwan Gunathilake, Bertalan Vida, Antoniuz, David Kral, Ana Kuznetsova, Gaygysyz Asyr, Mehmet Alpay Demiral, Anna Vittaus, Meline Avagyan, Elias Riad, Marina Braga (all based in Tartu), and Jorge Arena, based in Tallinn; 4) and also Estonian locals whom I befriended while over there: Alla Dudina, Raini Pert, Anton Klink, Pilleriin Arge, Ingrid Prigoda, Hanna-Eliisa

Madilainen, Andres Jaanson, Meeri Lembinen, Irene Teinemaa, Agnieszka Kunz, the regular folks at the Agape Kohvik, Janus Pinka and Maarja Mustimets, and others… All of you, one way or another, were vital in ensuring the 2 years I spent in Estonia were the happiest I've had in my entire life!

Leaving Estonia, even if for a previously set professional obligation, was far more difficult than what I initially imagined. I faced many challenges that most PhD students don't face, which can make even the most fervent enforcer of the European Union (EU) become a political Euro-Skeptic - and that's a great criticism towards the bodies of the EU behind these programs that I wish to leave registered here. It's not all wine and roses to move between countries without boarders. When it comes to an employee living and working in EU member state A, then being sent to live and work in member state B while still being paid a salary from an employer physically present in member state A, halfway a PhD project, and the employee is from nationality C (where nationality C pertains to an EU member state) it's an administrative and bureaucratic chaos. There is no good mechanism to deal easily with the extremely confusing mix of taxing systems throughout the Euro-Zone, which seriously hinders these programs in my opinion – and no one knows about it, until someone ends up in this entanglement. I went through 2 months without a salary after moving because of this, and had to waste plenty of energy and time trying to solve these bureaucratic problems while simultaneously having to secure housing, adapting to a new culture, a new environment with much higher cost of life, a new mentality of the people, way of work, a new laboratory... While being demanded to keep the same output of productivity. I can't keep track of the infinite number of calls and e-mails made back and forth to try to solve this issue. Not even the Euraxess contact points served of much, nor was there any experience within the general channels of communication between the different Tax Authorities of cases like mine. There is social security, IRS, health insurances, pension funds, structural barriers to regular payments between MasterCard/Maestro payment systems, regardless if the currency at stake is exactly the same… Everything that concurs to a basic life structure. This is something that the EU should strive to correct, otherwise the Schengen area remains as nothing more than a tourism hub. This was very detrimental, my mental health suffered tremendously with this – I was several times under high pressure and at a brink of simply giving up. For these reasons I may have failed with many things here and there, and may have seemed like a different person than expected to many people around me, even obnoxious at times.

Upon my move to Amsterdam I was very lucky of having had for a full year a friendly landlord in Martin Olšanský, and nice roommates in Ian Malony and Andrea Sanna. Cool guys who definitely deserve good fortune, and who are making a living elsewhere within the city now. Also during this time I was fortunate enough of being able from time to time to keep up with a hobby of playing boardgames and having had a few good friends here to boot and who helped me keep my mental sanity: Meïdy Chazeau, Woud

Hulshof, Hugo, and Jakub Hutner, who recently moved back to his homecountry.

The final words are to the people directly and indirectly involved in the PROFILE consortium, mostly in Icosagen and in Sanquin. Many of them had a vital influence in keeping me in the project, even if they didn't notice themselves.

First and foremost, my word of gratitude must go to each of my direct supervisors – Andres Männik, from Icosagen, and Jan Voorberg, from Sanquin – for having selected me for this position and giving me the opportunity to pursue this Academic degree I had sought for a long time. Jan in particular has been like a father to me around here in Amsterdam, as he helped me a lot solving my problems with the Belastingdienst and SVB, and even moving to a new place when it was needed again in early 2019.

The second must go to my fellow Early Stage Researchers who shared these whole projects with me – including former ERS3, Silvia D'Angelo, who unfortunately had to leave sooner that we wished. All 6 of us had a formidable time in the Assistance Publique-Hopitaux de Paris (AP-HP) under the guidance of Prof. Paul Coppo, Prof. Agnès Veyradier, Béragère Joly, Alain Stépanian, and both Sandrine's during the early summer of 2016, and kept many good memories of our time there. My thanks to all of them, including to their patients who volunteered to provide samples to their Reference Center, and without whom all these studies would not have been possible. Our host Patrick, at Rue de Charenton no 246 was also very friendly and provided us with a good environment for our first time meeting in person. As one of the only 2 guys among the 6 ESRs, and having shared almost all papers with him – and bedrooms too… and even once the bed itself (a hotel's mistake) – it's mandatory to explicitly mention Monsieur Boujacques Ergic… uhm… Bogacci Erciguini… uhm… Bogashev Ercigov… uhm… Bocas… bah, whatever, you know who I'm referring to! – for… leaving the lights on during the night when he arrived at late hours to our shared bedroom in Paris to wake me in the middle of the night! :P :P :P Es true! Hahahaha! Jokes aside, he has been a good comrade throughout these years, and has also been a listener to my frustrations and anxieties, which is something to keep in good memory, besides the shared drinks and conversations – scientific and others). Of course, the other ESRs Kadri Kangro, Carolina Velasquez, and Johana Hrdiravo… uhm, pardon me :P … Johana Hrdinová… have been also great "sisters in arms" with whom to share these projects and consortium, having played very important parts in the whole project and sharing the "weight" along the way. Without them, none of this could have come to any fruition.

The third, goes to the people in KU Leuven where the consortium was being led from. In particular Wim Maes and Nick Geukens for their efforts for cohesion, Prof. Karen Vanhoorelbeke and her team from the KU Leuven laboratories, Elien Roose, An-Sophie Schelpe, Sara Horta, Kim Martinod, Sirima Kraisin, and the technicians, all of whom contributed with raw and processed materials vital to these projects, and with whom I shared also some nice moments along the way.

Still among the consortium but outside of any of the places I've been in

secondments, it's mandatory to mention Gerry Nicolaes for his scientific insights, and Chris Reutelingsperger, also very insightful – and both very good humored! A huge thanks to them, and to both Marta Palício (hopefully still a fan of António Zambujo) for all her teachings and contributions, and to Liselotte Brix, who besides having made similar contributions gave Silvia, Carolina and me a friendly shoulder, listened to our anxieties and fears, and provided a calming pep-talk in a moment of bigger despair and disorientation regarding our near future (back in May 2019) – something that is a definite plus to carry into any of these projects.

Next I wish to thank many of the Icosagen people, starting with Anne Noop for all the trips she did with me to the Estonian bureaus where I needed to register to settle in Estonia, making everything a lot quicker and easier to deal with while circumventing the language barrier – a very important job! From Icosagen, of where I keep very good memories and had the honor to call "home" for a while, I want to thank in particular the CEO Mart Ustav Sr., a great human being, and extend my thanks to the rest of the people in general for making for a wonderful stay and making me feel like I belonged there (my apologies if I forgot anyone, there were so many!): Raini Pert, Radi Tegova, Andres Tover, Sille Näkk, Meelis Kadaja, Nelli Rebane, Liina Taal, Erkki Jüronen, Airi Alajan, Alar Süüden, Kaupo Teesalu, Anneli Plink, Triinu Kuuba, Tiiu Männik, Dagni Krinka, Ene Talpsep, Silver Olesk, Mart Ustav Jr., Mart Toots, Eva-Maria Tombäk, Kadri Õunap, the cleaning ladies I never knew the name of (both the operator of the autoclave and the ones who usually still caught me in the late afternoons, evenings and weekends finishing my work), and a very, very special thanks to Urve Toots, a pool of knowledge with whom I learned a lot!

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and tips and for being good listeners; to Floris van Alphen, Carmen van der Zwaan, and Maartje van den Biggelaar for their important contributions and efforts dispensed with these projects; to Aicha Laghmouchi for all the efforts in trying to arrange materials and extra collaborations with the blood donors and her colleagues from Leiden, and now lately with Çan Kesmir from Utrecht; to Tom Arfman for the comradery and availability to help us with some of these projects, with much merit to his account; to Anne-Marieke van Stalborch and Paul Verkuijlen for their hints and tips on some things we do at the molecular biology and VMT lab; and to the nice people in the U building with whom we had collaborations with, like Anja ten Brinke, Jana Koers and Rocco Sciarillo (Theo Rispen's group), Maximillian Brinkhuis and Thijs van Osch (Gestur Vidarsson's group), and remaining folks from those departments we had fun with on the borrels, of which I wish to emphasize Rick Kapur, also for having listened to my problems with some friendly words (hopefully one day soon these borrels will return).

To any person I came across with in this path and whose name I may have either forgotten to write or who I had to leave out of this already quite extensive list.

And last but not least: to Alexandra Elbakyan, whom has helped many PhD students more than anyone will ever imagine - me included.

Each one of you had some importance in this journey, and deserve my gratitude.

Finally, a critic to the academic system is in order. We are now in the digital age. In any field of Science, technological progress has been a driver for advancements, which have also brought further demands and higher standards. There used to be problems of how research was done that we don't face anymore (like the way you do your research on a topic and how quickly you can access information). The general rules of Science have not changed... but it's general standards have. In this sense, despite technological easiness to achieve some things, the task of proving something to the level of satisfaction that is required nowadays demands a higher "weight of evidence". Therefore, each publication nowadays carries more of that than, say, most publications from 20 years ago. An example can be given with the use of RNA interference (similar to shRNA), discovered in 1998. Once something gets discovered and established, the standards increase, regardless of the work load they entail. It's still in accordance with the general rules of Science, which is also the core of achieving a PhD (Doctor of Philosophy), a degree that means you are capacitated to do the process of scientific critical thinking, and you specialize yourself in a particular field/theme. Yet, in some Academies, the system is still eskewed towards how many publications are achieved. Get 3, you can defend your thesis. Get 2, it's 1 too short. This does not play well for everybody. Some projects are more laborious than others. Some allow you to publish in high impact journals with ease, others force you to burn all your data in a single publication to make it there. Should we bet more on lower-tier journals, then? Let's not even discuss negative results (suffice it to say there are specialized journals for those). There is Science and there are Politics and Policies. In my personal view, an Academic System that focuses more on

output numbers of publications rather than on their quality (not always measured by such a reductionist thing like an impact factor) is doomed to fail more often on the mid-to-long term. Serious practical consequences for the lives of those involved begin to be evident, especially in a saturated special job market. It's not very productive, nor motivating. You can't complain for people not wishing to get into Science more often when social needs such as stability and work load are deemed as less relevant than the overall product by which almost all of research is being measured: publications. Science must bear in mind a concept just as relevant, if not more: Human Capital. This is failing, in my view, at many levels.

I endured many of the hardships, such as the uncertainty, the stress, the impossibility of proper rest, the impostor syndrome... What made me endure to finish this PhD was but a single trait of my mind: I don't consider myself an Academic.

For all these reasons, I must also thank myself. For having persevered in the face of challenges that a lesser person could have simply opted to give up from now we stand at the doorstep of the Unknown Future. Let's see where Life takes me.

Nuno Alexandre Gomes Graça
Amsterdam, 22nd March 2021

Original	**English translation**
"Se souberes adormecer	"If you know how to fall asleep
Com o dia no olhar	With the day in your sight
O teu sonho viverás,	Your dream you will live,
E ao chegar o amanhecer	And when dawn arrives
Não terás que aceitar	You won't have to accept
Entrar no jogo a perder	Getting into the game from behind
Para procurar um lugar, um	To search for a place, one
Um lugar ao sol, sempre teu	One sunny place, always yours
Sei que é um sonho e não pertence a mais ninguém	I know it's a dream and doesn't belong to anyone else
O teu lugar ao sol	Your sunny place
Se souberes acreditar	If you know how to believe
Que sonhar é só viver	That dreaming means to live
E viver imaginar	And living is imagining
Vais conquistar um lugar	You will conquer a place
Um lugar ao sol, sempre teu	One sunny place, always yours
Sei que é um sonho e não pertence a mais ninguém	I know it's a dream and doesn't belong to anyone else
O teu lugar ao sol	Your sunny place
Não pares de lutar	Don't ever stop fighting
Agarra o dia ao nascer	Seize the day as it dawns
Há uma batalha a travar	There's a battle to be fought
Que só tu podes vencer	One that only you can win
Um lugar ao sol	A sunny place
O teu lugar ao sol	Your sunny place
Um lugar ao sol	A sunny place
Um lugar ao sol	A sunny place
Um lugar ao sol	One sunny place
O teu lugar ao sol"	Your sunny place"
Canção: 1 lugar ao sol	Song: A sunny place
Artista/Banda (País): Delfins (Portugal)	Artist/Band (Country): Delfins (Portugal)
Álbum: O caminho da Felicidade – O melhor dos Delfins	Album: The Path to Happiness – The Best of Delfins
Ano: 1995 (canção originalmente gravada como single em 1988)	Year: 1995 (Song originally recorded as a single in 1988)

And a final word in honor of the late Doctor Jasper Evan Sadler III (known in the field of TTP and von Willebrand Disease as J. Evan Sadler), deceased on the 13th December 2018 – coincidentally this PhD candidate's 30th birthday. A scientific mind from this field that I never had the chance to directly meet, but taught me from a distance with his written works and foresights.