Scratching the surface: Regulation of cell surface receptors in cholesterol metabolism
Nelson, Jessica

Citation for published version (APA):
Nelson, J. K. (2016). Scratching the surface: Regulation of cell surface receptors in cholesterol metabolism
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SUMMARY

Cholesterol homeostasis is tightly regulated through transcriptional and post-translational mechanisms. This thesis elaborates on the post-translational pathways that govern cell surface LDLR, which is key determinant of plasma LDL-cholesterol levels. Through genetic, molecular and biochemical characterization, we identify novel regulators of LDLR pathway. The initial discovery and characterization of the LDLR pathway by Brown and Goldstein laid the foundation of our current understanding of cholesterol metabolism, as described in Chapter 1. Briefly, this chapter elaborates on the SREBP- and LXR-driven transcriptional pathways that govern intracellular sterol concentrations. Beyond these transcriptional pathways, the post-translational regulators PCSK9 and IDOL have emerged as key determinates of LDLR protein abundance and circulating LDL-cholesterol levels.

Chapter 2 describes quantitative and qualitative methods to assess LDLR protein abundance and function. The described methodology can be applied to different cell-based models to measure LDL binding, internalization and/or trafficking of ligand-bound LDLR. Moreover, the use of the described methodology also allows one to distinguish LDLR protein abundance and localization at the plasma membrane. These methods were used in the following chapters to elucidate the molecular mechanisms that govern the LDLR pathway, the main research focus of this thesis.

Chapter 3 expands our understanding of the endosomal trafficking route of IDOL-mediated ubiquitylation of the LDLR. Genetic-, pharmacological- and functional-based approaches revealed that this route is distinct from the “classical”, clathrin-mediated endocytic pathway required for lipoprotein uptake by the LDLR and for PCSK9-mediated degradation. This cellular route does not require the alternative dynamin-, caveolin-, or macroautophagy-mediated internalization pathways. Instead, sorting of the ubiquitylated-receptor to the lysosome requires the collaborative actions of the endocytic adaptor Epsin, the ESCRT complex and the deubiquitylase USP8.

Chapter 4 identifies NDRG1 as a novel regulator of endosomal trafficking of the LDLR, following IDOL-mediated ubiquitylation. This chapter reports that NDRG1 depletion causes reduced cellular uptake of LDL-cholesterol through increased IDOL-mediated ubiquitylation of the LDLR at the plasma membrane. However, in the absence of NDRG1, ubiquitylation of the receptor by IDOL does not lead to lysosomal degradation. Rather, it leads to enhanced accumulation of the ubiquitylated-receptor in modified endosomes/MVBs. Mechanistically, depletion of NDRG1 in cells induces formation of ceramide-rich intraluminal vesicles, a reduction of ESCRT components and dysregulation of endosomal Rab GTPases. Collectively, these effects impair MVBs morphology and LDLR trafficking. As a result, these findings suggest that impaired endocytic trafficking is the underlying cause of the demyelinating neuropathy Charcot-Marie-Tooth disease 4D (CMT4D), a rare disorder in humans linked to loss-of-function mutations in the NDRG1 gene. In murine oligodendrocytes, we show that Ndg1 deficiency impairs LDL uptake and neurite outgrowths. High levels of cholesterol are required for myelin formation; therefore, dysregulation of the LDLR pathway in myelinating cells, due to may be the underlying pathology of CMT4D.
Chapter 5 describes the first loss-of-function IDOL mutation in human individuals with low circulating levels of LDL-cholesterol. This variant was identified by sequencing the IDOL coding sequence in individuals with extreme LDL phenotypes in a large Dutch cohort. Evaluation of this rare missense variant revealed a premature stop codon in IDOL mRNA. If not subject to nonsense-mediated RNA decay, this IDOL variant would encode a truncated protein, IDOLR266X, which lacks the RING domain required for its ubiquitylation activity. Functional analysis of this truncated IDOL variant showed that it is unable to promote ubiquitylation and degradation of the LDLR, or attenuate LDL uptake into cells. Accordingly, this variant was identified in two related individuals with low circulating LDL. These findings provide the first evidence that IDOL may contribute to variation in circulating levels of LDL-cholesterol in humans, and supports the notion that inhibiting IDOL activity may provide a novel therapeutic approach for treating dyslipidemia.

Chapter 6 identifies the deubiquitylase USP2 as an endogenous inhibitor of IDOL-mediated ubiquitylation of the LDLR. Through genetic, molecular and biochemical-based approaches, USP2 was identified as a novel binding partner of IDOL. This interaction promotes IDOL deubiquitylation and subsequent protein stabilization. Paradoxically, this preserves LDLR and LDL uptake despite elevated IDOL protein levels with USP2 overexpression. Reciprocally, silencing of USP2 in cells leads to increased IDOL-mediated degradation of the LDLR and attenuation of LDL uptake. Mechanistically, USP2 interacts with the LDLR in an IDOL-dependent manner to deubiquitylate and stabilize both LDLR and IDOL.

Chapter 7 reports that short-term pharmacological pan-inhibition of cellular DUB activity leads to a rapid and robust induction of IDOL, mirrored by enhanced IDOL-mediated degradation of the LDLR and attenuation of LDL uptake. This inducible expression of IDOL by DUB inhibition occurs in a time- and dose-dependent manner in numerous cell lines and in primary human cells. In contrast to the established sterol-dependent transcriptional regulation of IDOL, this process is LXR-independent and was mapped to a 70-bp region in the proximal promoter of IDOL. This is the first report to demonstrate a sterol-independent mechanism to regulate IDOL expression, and will direct future studies towards elucidating the underlying transcriptional mechanism.

Chapter 8 identified (P)RR as a novel regulator of LDL metabolism by controlling SORT1 and LDLR protein abundance. Using proteomic- and molecular-based approaches, SORT1 was identified as a high-confidence (P)RR-interacting partner. Functional characterization of this interaction revealed that silencing of (P)RR in hepatocytes decreased SORT1 and LDLR protein abundance without changes in their corresponding transcript level. Subsequently, (P)RR depletion severely attenuated LDL uptake, which appeared to be selective, as other clathrin-mediated endocytosis events were unaffected. Mechanistically, the (P)RR-SORT1 complex does not perturb LDLR on the cell surface, but seems to be essential for proper trafficking of the nascent LDLR protein towards the plasma membrane.
Chapter 9 describes an intricate interplay between the E3 ubiquitin ligases IDOL and MARCH6, which together, uncouple cholesterol synthesis from lipoprotein uptake in hepatocytes. In these cells, silencing MARCH6 increases the expression of SREBP-2 target genes (i.e. HMGCR, LDLR) and flux across the mevalonate pathway. Unexpectedly, this was also associated with an increase in LXR signaling and induced expression of the LXR-responsive E3 ligase IDOL. As a result, despite enhanced expression of the LDLR, overall LDLR protein and LDL uptake were reduced in a MARCH6-dependent manner.

Chapter 10 characterizes the transcriptional response of human macrophages to a comprehensive set of endogenous and synthetic LXR ligands. Through this screen, EEPD1 was identified as a novel LXR target gene in human and mouse macrophage. As a myristoylated protein, anchored to the cytosolic side of the plasma membrane, EEPD1 influences ABCA1 protein stability. We report that depletion of EEPD1 in LXR-treated macrophages leads to decreased ABCA1 protein and subsequently reduced ApoA1-dependent cholesterol efflux. As such, EEPD1 seems to act as an LXR-regulated gene that facilitates cholesterol efflux from macrophages, likely by regulating stability/trafficking of ABCA1.

Chapter 11 summarizes the findings presented in this thesis on novel mechanisms to regulate the plasma membrane abundance of key cell surface receptors and transporters in cholesterol metabolism. Further, in this chapter future directions related to these findings are discussed.
SAMENVATTING

Cholesterolhomeostase is onderhevig aan zeer precieze regulatie door zowel transcriptionele als post-transcriptionele mechanismen. Dit proefschrift wijdt uit over post-transcriptionele netwerken die de hoeveelheid LDLR aan het celoppervlak reguleren; een belangrijke determinant van cholesterolniveaus in het bloed. Door middel van genetische, moleculaire en functionele karakterisering hebben wij nieuwe regulators van het LDLR-netwerk weten te identificeren. De ontdekking en initiële beschrijving van het LDLR-netwerk door Brown en Goldstein liggen ten grondslag aan het huidige wetenschappelijke onderzoek naar cholesterolhomeostase, zoals staat beschreven in Hoofdstuk 1. In het kort beschrijft dit hoofdstuk de SREBP- en LXR-gedreven transcriptionele netwerken die intracellulaire sterolniveaus bepalen. Naast deze transcriptionele regulatie zijn de post-translationele regelaars PCSK9 en IDOL aan het licht gekomen als essentiële regulatoren van LDLR hoeveelheden en de niveaus van circulair LDL-cholesterol.


Hoofdstuk 4 identificeert NDRG1 als een nieuwe regelaar van het endosomale transport van de LDLR na IDOL-gemedieerde ubiquitinilering. Dit hoofdstuk beschrijft hoe depletie van NDRG1 zorgt voor verminderte cellulaire opname van LDL-cholesterol. Het onderliggende mechanisme hiervan is verhoogde IDOL-gemedieerde ubiquitinilering van de LDLR op het celmembraan. Echter, in afwezigheid van NDRG1 leidt de ubiquitinilering van de receptor door IDOL niet tot afbraak in het lysosoom, maar tot verhoogde ophoping van de geübiquitinileerde receptor in gemodificeerde endosomen/MVBs. De depletie van NDRG1 zorgt in cellen voor de vorming van ceramiderijke intraluminale blaasjes, de reductie van ESCRT-componenten en ontregeling van endosomale Rab GTPasen. Samengenomen verhinderen deze effecten MVB-morfologie en het transport van de LDLR. Deze bevindingen suggereren dat verhinderd endocytotisch vervoer de onderliggende oorzaak is van demyelinerende neuropathie, Charcot-Marie-Tooth Disease 4D (CMT4D), een zeldzame
mensenlijke aandoening gelinkt aan mutaties in het NDRG1 gen. In een experiment met muis-oligodendrocyten tonen wij aan dat NDRG1 deficiëntie LDL-opname verhinderd, alsmede de uitgroei van neurieten. Hoge cholesterolniveaus zijn nodig voor myelineformatie. Hierdoor is het aannemelijk dat de onderliggende pathologie van CMT4D gemedieerd is door ontregeling van het LDLR-netwerk in myelinerende cellen.

Hoofdstuk 5 beschrijft de eerste IDOL-mutatie, die leidt tot functie verlies van het eiwit, in mensen met een laag circulair LDL-cholesterolniveau. Deze variant was geïdentificeerd door de sequentie te bepalen van het coderende deel van het IDOL-gen, in individuen uit een Nederlandse cohortstudie met een extreem laag LDL-gehalte. Analyse van deze zeldzame missense-variant onthulde een prematuur stopcodon in het IDOL mRNA. Zolang dit mRNA niet onderhevig is aan nonsens-gemedieerde degradatie zou deze variant een korter eiwit opleveren: IDOLR^{266X}, welke het RING-domein mist wat noodzakelijk is voor de ubiquitinileringsactiviteit. Functionele analyse van deze ingekorte IDOL-variant liet zien dat deze niet in staat is om de LDLR te ubiquitinileren en te degraderen, noch om LDL opname in cellen te verbeteren. Daarnaast is deze variant gevonden in twee verwante individuen met een laag LDL-plasma gehalte. Deze bevindingen omvatten het eerste bewijs dat IDOL bij kan dragen aan variatie in circulatoire LDL-niveaus in mensen, en ondersteunen het idee dat het remmen van IDOL-activiteit een nieuwe therapeutische strategie kan verschaffen voor de behandeling van dyslipidemie.

Hoofdstuk 6 identificeert de de-ubiquitinilase USP2 als een endogene remmer van IDOL-gemedieerde ubiquitinilatie van de LDLR. Door middel van genetische, moleculaire en biochemische technieken is USP2 geïdentificeerd als een nieuwe bindingspartner van IDOL. Deze interactie stimuleert IDOL-deubiquitinilering met als gevolg stabilisatie van het eiwit. Schijnbaar tegenstrijdig behoudt dit LDLR-niveaus en LDL-opname, ondanks de verhoogde aanwezigheid van IDOL-eiwit door USP2 overexpressie. Aan de andere kant leidt het inactiveren van USP2 tot een verhoogde IDOL-gemedieerde degradatie van de LDLR en verminderde LDL-opname. USP2 gaat een IDOL-afhankelijke interactie aan met de LDLR om de LDLR en IDOL beiden te deubiquitinileren en te stabiliseren.

Hoofdstuk 7 beschrijft de observatie dat farmacologische pan-inhibitie van cellulaire DUB-activiteit op korte termijn leidt tot een snelle en forse inductie van IDOL, met in parallel verhoogde IDOL-gemedieerde degradatie van de LDLR en verminderde LDL opname. In verscheidende celllijnen en primaire humane celllijnen vindt deze geïnduceerde expressie van IDOL door middel van de remming van DUBs plaats in een tijd- en doseringsafhankelijke wijze. Dit proces is LXR-onafhankelijk en is gerelateerd aan een 70-bp groot gebied in de proximale promotor van het IDOL gen, wat in contrast staat tot de bekende sterol-onafhankelijke transcriptionele regulatie van IDOL. Dit is de eerste keer dat een sterol-onafhankelijk mechanisme gedemonstreerd wordt waarmee IDOL-expression is gereguleerd. Toekomstige studies kunnen hierop voortbouwen om het onderliggende transcriptionele mechanisme te ontrafelen.

In Hoofdstuk 8 is (P)RR geïdentificeerd als een nieuwe regelaar van LDL-metabolisme via de regulatie van SORT1 en LDLR eiwitheeveelheid. Door het gebruik van proteomics en moleculaire technieken is SORT1 geïmpliceerd als een significante interactiepartner van (P)RR. Functionele analyse van deze interactie heeft onthuld dat het onderdrukken van (P)RR in hepatocijten SORT1 en LDLR eiwitheeveelheden vermindert, zonder een
corresponderende reductie in hun transcriptniveaus. Bovendien vermindert (P)RR-depletie LDL opname selectief, aangezien andere clathrin-gemedieerde endocytose onaangetast bleef. Het (P)RR-SORT1 complex lijkt essentieel te zijn voor geschikt vervoer van nieuw aangemaakt LDLR-eiwit naar het plasmamembraan en heeft geen mechanistisch effect bij het cel membraan zelf.

**Hoofdstuk 9** beschrijft het ingewikkelde samenspel tussen de E3 ubiquitin ligasen IDOL en MARCH6. Deze ligasen ontkoppelen samen cholesterolsynthese en lipoproteïne-opname in hepatocieten. In deze cellen zorgt een vermindering van March6 expressie tot verhoogde expressie van SREBP-2 gereguleerde genen (bijvoorbeeld HMGCR, LDLR) en flux door het mevalonaat productie proces. Onverwachts bleek dit ook geassocieerd met een verhoogde LXR-signaltransductie en geïnduceerde expressie van de door LXR gereguleerde E3 ligase IDOL. Als resultaat was algehele LDLR-eiwit en LDL-opname verminderd ondanks verhoogde expressie van de LDLR. Dit effect was March6 specifiek.

**Hoofdstuk 10** karakteriseert de transcriptionele respons van humane macrofagen op een set endogene en synthetische LXR-liganden. Door middel van deze screen is EEPD1 geïdentificeerd als een nieuw LXR-target gen in de macrofagen van mensen en muizen. EEPD1 functioneert als een gemyristoileerd eiwit wat ingebed is aan de cytosolische kant van het plasmamembraan, en beïnvloedt ABCA1 eiwitstabiliteit. In dit hoofdstuk wordt beschreven dat depletie van EEPD1 in LXR-geïnduceerde macrofagen leidt tot een vermindere hoeveelheid ABCA1 eiwit en verminderde ApoA1-afhankelijke export van cholesterol. EEPD1 lijkt aldus te functioneren als een LXR-gereguleerd gen dat de export van cholesterol uit macrofagen medieert, waarschijnlijk door de stabilititeit en/of het vervoer van ABCA1 te beïnvloeden.

**Hoofdstuk 11** vat de bevindingen van dit proefschrift samen over nieuwe mechanismen om celmembraanhoeveelheid van essentiële receptoren en transporteurs in cholesterolmetabolisme te reguleren. Verder zal dit hoofdstuk toekomstige onderzoekslijnen bediscussiëren gerelateerd aan deze bevindingen.
CURRICULUM VITAE

Jessica Kristine Nelson was born on September 4, 1983 in Minnesota, USA. She attended Champlin Park High School, in her hometown of Champlin, Minnesota, where she graduated with Honors in the top 5% of her graduating class. In 2002, she moved to southern Minnesota to pursue an undergraduate degree in Human Physiology and Chemistry at Minnesota State University-Mankato (Mankato, Minnesota, USA). During her time there, she represented the College of Science and Technology as a student senator, volunteered as a biology and chemistry tutor, and worked as a certified nursing assistant in the local hospital. Additionally, she participated in a six-month study abroad program at Macquarie University (Sydney, Australia), and she completed a scientific internship at the Department of Experimental Cardiology at Erasmus Medical Center (Rotterdam, the Netherlands) under the supervision of Prof. Dr. Dirk J. Duncker. In the spring of 2007, Jessica obtained her Bachelor of Science with honors (Magna Cum Laude), after which she taught Science and English at Taegang Sahmyook Elementary School in Seoul, South Korea. In 2009, she was awarded a Huygens Scholarship from the Dutch Ministry of Education, Culture and Science to pursue her Master of Science degree at the University of Amsterdam (Amsterdam, the Netherlands). After choosing the Biochemistry specialization track, she undertook two scientific internships at the Academic Medical Center of the University of Amsterdam: one at the Laboratory of Experimental Intensive Care and Anesthesiology (LEICA) under the guidance of Dr. Coert J. Zuubier, and the other at the Department of Medical Biochemistry under the supervision of Dr. Marco van Eijk and Prof. Dr. J.M.F.G. Aerts. Afterwards, she completed her literature thesis under the supervision of Prof. Dr. Noam Zelcer, during which she first became interested in ubiquitylation and its role in cellular lipid metabolism. She graduated with honors (Cum Laude) in the summer of 2011 and started her PhD training in the lab of Prof. Dr. Noam Zelcer at the Department of Medical Biochemistry of the Academic Medical Center. After completion of her PhD, she will commence her postdoctoral research on the role of ubiquitylation in (cancer) stem cell homeostasis, in the lab of Dr. Axel Behrens at the Adult Stem Cell Laboratory of the Francis Crick Institute (London, United Kingdom).
PORTFOLIO

Jessica K. Nelson

Duration: September 2011 - September 2016
PhD Supervisor: Prof. Dr. Carlie de Vries
PhD Co-Supervisor: Dr. Noam Zelcer

Courses

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PUBLICATIONS


'I don’t know where I’m going from here, but I promise it won’t be boring,‘

-David Bowie
ACKNOWLEDGEMENTS

And here I am, after a long and adventurous journey I want to thank all the people that have helped me achieve this milestone. Without the support of my family, friends and colleagues, completion of this thesis would never have been possible.

First and foremost, my deepest gratitude to my promoter Prof. dr. Zelcer. Noam. I am honored to be your first PhD graduate under your new Prof. title! These past years have been a great and stimulating scientific experience. Thank you for devoting so much of your time and enthusiasm to my research, and for always demanding my best. You are an inspiring mind, and working with you has shaped me as a scientist and pushed me to become a more independent researcher. I appreciate that you always made time for me, not only professionally, but also personally. I look forward to working together in the future, and wish you all the best not only with your growing lab and research, but also with your children and your lovely wife Irith.

Secondly, I would also like to express my deepest gratitude to my second promoter Prof. dr. de Vries. Carlie. Thank you for all the invaluable comments, suggestions and encouragements throughout my PhD. I am extremely grateful for your patience, time, and energy spent in helping me plan and finalize my thesis. Also, thank you for making me feel welcome in your group. I had a great time joining your lab for the Nuclear receptor conferences in Belgium and in the Netherlands, as well as the moments celebrating accepted manuscripts (which usually ended with no dinner and many bottles of bubbles!).

I would also like to extend my gratitude to my PhD committee members: Prof. dr. S. Urbe, Prof. dr. A. Tall, Prof. dr. J.M.F.G. Aerts, Prof. dr. M.P.J. de Winther, Dr. E.A.J. Reits, and Dr. G.K. Hovingh. Thank you for taking the time to critically read and revise my thesis. Dear Sylvie, thank you for your knowledgeable suggestions and advice on my research. Your collaboration was essential for completing our USP2 manuscript, and since I am continuing in the ubiquitin field I look forward to collaborating with you again in the future. Dear Alan, it was a pleasure to meet you in Copenhagen during the Keystone meeting, as well as during the IAS meeting in Amsterdam. Thank you for your support and interest in my research. Dear Hans, you have known me since I was an eager Master student working in your department, and I am very grateful that you have been a part of my PhD training from start to finish. I loved our metabolism literature discussions on Friday mornings, and learned so much during those lively meetings. Menno, thank you for your help and enthusiasm for my work. I appreciate that you were always available and eager to discuss all my macrophage-related question. Kees, thank you for sharing your thoughts and fruitful collaboration in our human genetics research. Dear Erik, I appreciate all your helpful tips and advice for moving my projects forward. It was a pleasure to join the ubiquitin work and literature discussions. I learned a lot from working with you and your group.

My gratitude goes out to our collaborators. A special thanks to Sabine, Katrine, Alicia, Karen, and Anne for all your help with the confocal microscopes, optimizing the “art” of sub-cellular fractionation and discussing all ubiquitin-related matters. I am also very thankful to Geesje and
Alinda for providing us with essential reagents for our research and for your scientific input. Thank you Jeroen, I really enjoyed our conversations ranging from CRISPR and neddylation, to life in the USA. And thanks for the borrel invites, Pathology knows how to party! Dear Igor and Prof. Peter Hordijk it was pleasure working with you at the Sanquin, thank you for your help in the HUVEC experiments and your scientific input on our research. Thank you Prof. Huib Ovaa, for your expertise and collaboration in our DUB research.

During my years at the AMC I have made incredible friends and colleagues who have made my time spent here extraordinary and unforgettable. Without your help and support the completion of this thesis would have been immeasurably more difficult. We shared so many great memories, and I appreciate all the time spent together working late hours in the lab as well as celebrating borrels, labdays, Sinterklaas, Epstein bar Thursdays, Christmas lunches, birthdays, housewarmings, Haloween, Carnivals and BBQs. I first would like to thank the members of the Zelcer lab, who have been an important part of my life for the last five years. Dear Vincenzo (the original motha fuckaaaaaa), words cannot express how much I miss working with you in the lab (especially your inappropriate sense of humor). During these past years I have learned so much from you, scientifically and in life. You are a brilliant scientist and true friend, and I look forward to share more laughs and good times with you in the future. Thank you Lilith and Ana for making my start in the Zelcer lab possible in the first place and enjoyable at the same time. Martina and Saskia, the lab would crumble without your constant support and maintenance. I know I am not the easiest person to work with, as I barely followed safety rules and almost never wore my lab coat, but thank you for putting up with me and for your generosity and help. Martina, I enjoyed both working with you in the lab and your great taste in music. Saskia you were my “guardian angel” in the lab. I appreciate your willingness to always help me find a reagent, or optimize a FACS protocol. And thank you for inviting me to Foo Fighters. To this date, it is still the best concert I have ever been to! Anke, it has been such a pleasure to work and learn from you. You are such a skillful scientist and a kind and caring person. I am honored to have been a part of you and Frederik’s wedding day. It was a very special moment for the whole lab. Emma, I really enjoyed your company, both in and out of the lab. Together we have faced so many scientific challenges and achievements. You are a very dedicated researcher and I wish you all the best in these next months with finishing your PhD! Thank you Sigrid (Zig-Zag) for always patiently answering all my naive questions related to genetics, and for your sassy jokes. It really lab work more fun for me. Xifeng (Erik), thank you for joining our lab (if only for a short time). It was a pleasure working with you on the P-R-R study, and I wish you great success back in China. Nienke, Josephine (Pep), and Remco, it has been a great pleasure to help you start in the lab as new PhD students, I wish you great success with your projects. A special thanks to Nienke for all your positivity during the writing of this thesis. I know being my roommate was a challenge at times, especially since both Marc and I were finishing our books at the same time! Thank you Luca and Franziska for joining the lab, sharing your expertise, and for making the lab more social and enjoyable. Luca I enjoyed our lively IDOL discussions, and wish you great success in solving its structure!

Many thanks also to the other people of the department of Medical Biochemistry (MBIOC). Thank you Dave Speijer for your unwavering support, especially towards the end of my PhD. With your
guidance you helped me to focus and see the “light at the end of my PhD tunnel”. I will miss your sense of humor and somewhat funny jokes 😊. Vivian, I am grateful for your support and interest during my PhD. I appreciate that you were not only concerned about my scientific progress, but also in my well-being during the completion of my thesis. A special thanks to Pieter, Mariska, Cindy and Roelof for managing the labs and animal experiments, and for your willingness to always help out and share a laugh. Boris and Annett, thank you for all your expertise in the lab and for your advice for optimizing all our protein-related experiments. A special thanks to Ben. It has been a wonderful experience working with you and your group. Thank you for all your support in my research, and for your good advice that moved my projects forward. Thank you Janny and Marlene for you all your patience and help, be it from borrowing lab supplies, optimizing a PCR, to finding rare digestion enzymes! Guy you made my time in the lab very enjoyable with you entertaining stories and songs. Stephan, it’s been a pleasure to meet you since joining the department. I wish you the best in starting up your own research group. Thank you Esther for all your support during in my PhD studies, as well as helping me plan for my postdoctoral research. Annette Opdam and Romana, thank you for your help in the organization of the lab, and thank you Annemarie for your help with organizing all the administration surrounding my graduation. A special thanks to Duco Zonneveld, you have been a dear friend throughout the years, thank you for all your support. A special thanks to the Aerts lab: Marco, Rolf, Marri, Wouter, Mina, Arthur, Frans, Judith, Milka Anneke, Wilma and Tineke for all your contributions and support over the years. Best of luck in Leiden and elsewhere!

To my fellow PhDs of MBIOC and the AMC, in your own (special and unique) way you made my time spent in the lab and in Amsterdam extraordinary and unforgettable. Our Belgium beer-infused nights got us through the best and worst of times, and at the very least proved to ourselves that we were never too old to dance on the tables, drink tequila, hit on young medical students, or challenge them to a Jillz-off on the bar. These wild nights brought us together, not only as colleagues but as friends, and I am grateful for all the laughs, memories and hangovers. Claudia, Saskia O., Tanit, Daniela, Khang, Shaynah, Tsveta, Romain, Teresa, and Sharina you have made my time in MBIOC so memorable, thank you for all the good times. Lejla, my hand-twin, know that if I was allowed to have a fourth paranimf you would be it! You have been such a great and supportive friend, in and out of the lab. I wish you all the best with finishing your PhD. Stijntje, thank you for all the great memories and awesome parties in your beautiful canal house. Goran and Babu, thanks for the good times, the many laughs and the great impersonations you did of each other. Anouk thank you for all your help in the B-Lab, and for the hours spent with the cholesterol efflux experiments, I wish you great success in the USA. Iker, we have shared many joys and frustrations during our PhD, and I wish you all the best with your future plans in Spain. Rossella (Roxy) working with you has been so enjoyable, your positive attitude and energy made any ordinary day exciting. I will always remember our trip to Turkey together for the Ubiquitin conference, with us running around Istanbul and the sights of Ephesus. Best of luck Stijn with your projects, you are a much needed addition (a dude) to our side of the department. Teja, Areti, and Yudho thank you for all the good fun in and out of the lab. Maria thank you for being a great friend and for your willingness to meet for a beer after work. I cherish the time spent with you and your family in Portugal, and to this day, I still miss your mom’s baked octopus (Muito Bom!). André, I am grateful for all the shared memories during our Masters and PhD, I wish you and
Eelke all the best in Germany. A special thanks to Mr. Volendam, Marc, who I have had the pleasure of being roommates with for the last leg of our PhDs. I never got bored of hearing about Bnip3! 😊 You always provided valuable opinions and suggestions, and I know you will achieve great things in the USA. Thank you Marieke, Jan, Susan, Suzanne, Quinte, Oliver, Jeroen, Annette, Myrthe, Pascal, Annelie, and Saskia for all the support in the lab as well as the fun times spent at borrels and the Epstein bar. Thank you Marten for collaborating in our research and for patiently teaching me how to use the InDesign software. Thijs, where do I start. I remember when you first started as an eager Master student and now you are all grown up. We have had a lot fun times at parties, festivals, or just going out drinking. You are a great friend and I wish you all the best with your PhD. A special thanks to Helene, Carlos, Chris, and Charo for all the special nights together in Amsterdam, either in a pub or at home cooking delicious Spanish food. Helene, you are a dear friend and I am glad to have been one of your paranimf, I wish you all the best with your new job in German! I also want to thank Marcel, Robert, Dmitri, Vincent, Quinn, Elena, Mahnoush, Timon, Ana, Nazanin, Evita, Fabio, Laila, and Emanuele thanks for being good friends in and outside of work.

To my friends in the Netherlands and rest of the world: thank you for your patience and support over these last five years. Yes, I have finally finished my thesis! A special thanks to Julius, you have been a great friend and support while finishing this thesis. To Jelle, Xuna, Ashish, Ann, Jelmer, Lowie, Jeory, Koen, Melissa, Joost, Chris, Steve, Jennifer, and Ronald: thank you for all the great memories over the years. I have enjoyed all the Nepali Momo nights, festivals, and beers shared in and around Amsterdam. To my Minnesota crew: thank you Cole and Nicole for coming to visit Amsterdam, I had an amazing time showing you my home here and completing our epic bike trip in the rain and snow to and from Volendam! Thank you Jenna Obrycki for your constant support, love and friendship over the many years that we have known each other (going on 17 years now!).

From the bottom of my heart, I would like to thank my Weerdestein (aka Beerdestein) housemates, who have made me feel truly at home in Amsterdam. We have shared so many incredible memories in Amsterdam (and in Utrecht, Maastricht, Berlin, Naples, Brussels, and Milan). We have thrown some unforgettable parties, including our housewarming Halloween cross-dress party, where all the “ladies” that night looked absolutely famous! Dear Emma, I am happy you decided to leave your old house Utrecht and join ours. It has been an incredible year with you Gianni, and I have enjoyed all the laughs, bottles of wine and making awesome Spanish tortilla! Mahnoush, it was such a pleasure to have you living with us (even for a short period of time), I really enjoyed your Iranian food! Paulo, you are a dear friend, thank you for the great time in the lab and in Weerdestein. I miss your crazy sounds effects and songs, and obrigado for introducing us to Picadinho. Dear Matthijs, you are the older brother I never had. You have the incredible ability to turn the ordinary and mundane into the extraordinary. You played a key role in making Weerdestein a home, and I thank you for all the support you have shown me throughout the years. One of my favorite memories with you is when we (but especially you) got banned from Epstein bar for a year because you were too tall (and also not a medical student), while Robert was dancing on the pool table, Vincenzo was trying to pick up medical students, and I was chanting “loser!!!” over and over at the angry bar staff that were trying to kick you and Robert out.
That was the night we started the “Belgique group”, and all the good times which followed after. **Alessandra** (Ale), you made our home into a proper Italian family (the love, the drama, and the food!). We have had such an incredible journey and I could not have made it this far without your love and support. Ale, you bring life into every conversation, every party and even every meal we cook! Someday I look forward to retiring the pipette and opening a restaurant with you! I love how chairs were optional in our house, and that we always felt more comfortable talking with each other sitting on the kitchen floor. I am so happy that you have met **Jorge** and brought him into our lives. Jorge thank you for being our resident Dr. and for sharing in all the good times. It is great to have you in our home! One of the happiest family memories I have with all of us is when your cousins Ivano, Valentina and her boyfriend were staying with us in Weerdestein. That night, we decided to stay in and make Neapolitan pizzas. Emanuele was playing guitar, you were singing an Italian song, Ivano was making the pizzas, and Duco and I were sitting on the kitchen floor eating and drinking Duvels. It was super gezellig! I am so going to miss these Weerdestein moments. Dear **Duco**, schatje, every day I realize more and more how important you are to me. I know I am not the easiest person to deal with sometimes, but thank you for standing by me, calling me out on my bullshit and making me laugh more than anyone else. You have a brilliant mind and a beautiful personality, and I am so grateful to have you in my life. I have so many great memories of us, from you always needing to do a squat in every foto (the pooping pose) to your Justin Bieber impersonation whenever any of his songs are played. One moment that stands out the most, is the time you wore your grey suit to Vincenzo’s PhD promotion. That night we had such a wild party, and at some point you got incredibly drunk (like everyone else, especially Vinci!). It was then that we found you walking around the bar with your fancy tie inside your beer! When someone told you, you quickly pulled it out (that’s what she said), only for it to immediately splash back in again! By the end of the night, your tie had soaked up an entire beer. Needless to say, Vinci’s promotion party left a mark on all of us, especially on your suit! I would also like to extend my gratitude to the Koenis clan, for you all your love and support. Thank you **Roxanne** for all the fun times in the Netherlands and in Belgium, you are such a beautiful person and I appreciate your friendship.

Finally, from the bottom of my heart I want to thank my family. Your constant support and acceptance of my life abroad has helped carry me through to the end. Special thanks to my parents: Robin, Tim, Matt and Kay, to my siblings: Jenny (and Trevor), Katie, Zack, and Alysha and to my grandparents. I am grateful for the visits you’ve made to experience my life and home in Amsterdam. Thank you all for you love!