Decoding non-coding RNAs in fatty liver disease

Atanasovska, Biljana

APPENDICES

Summary

Non-alcoholic fatty liver disease (NAFLD) encompasses a range of liver disorders, from simple deposition of fat in the liver (hepatic steatosis) to more severe phenotypes characterized by the presence of inflammation, ballooning and fibrosis (non-alcoholic steatohepatitis or NASH). Obesity is the major risk factor for NAFLD and, driven by the global obesity epidemic, NAFLD has become the leading cause of chronic liver disease worldwide. Notably, NAFLD progression to NASH is reversible up to a certain point, currently mainly via lifestyle interventions. However, 10-20% of NASH patients will progress further towards cirrhosis and hepatocellular carcinoma (HCC) which, in view of their increasing prevalence, will become frequent indications for liver transplantation. It is therefore important to understand the mechanisms involved in NAFLD etiology, in order to prevent its development as well as its progression towards more severe conditions.

The completion of the Human Genome Project in 2003 and the advance of high-throughput sequencing technologies have led to a revolution in biomedical research. It has been estimated that protein-coding genes represent less than 2% of the human genome, while more than 98% of human genome is now considered as the non-coding genome. Interestingly, a large part of non-coding genome is found to be transcribed into non-coding RNAs (ncRNAs) that can participate in a number of critical biological processes, such as chromatin remodeling, gene transcription and protein transport and trafficking, thus implicating ncRNAs in a wide range of complex human diseases. However, the involvement of ncRNAs in the liver and in NAFLD development and progression is not well understood. In this thesis we aim to understand the role of ncRNAs in NAFLD by combining transcriptome profiling in a patient cohort, functional genomics in *in vitro* models to mimic disease progression, and follow-up functional studies using various molecular techniques. This research highlights the importance of ncRNAs in NASH.

Firstly, the importance of the non-coding genome in complex diseases and traits has been revealed by genome-wide association studies (GWAS). Obesity is not only the risk factor for NAFLD, it is also associated with an increased susceptibility to type 2 diabetes (T2D) and cardiovascular diseases (CVD). In **chapter 2**, we provide an overview of the 755 single nucleotide polymorphisms (SNPs), encompassing 366 independent loci, that have been associated to various cardiometabolic phenotypes, including obesity, T2D, CVD and NAFLD. By prioritizing candidate genes and performing pathway enrichment analysis, we show that there is a strong connection of lipid traits with obesity, diabetes-related traits and CVD, since lipid-trait-associated loci are the most commonly shared regions between cardiometabolic phenotypes. Furthermore, more than 90% of cardiometabolic SNPs are located in non-coding regions and are expected to have regulatory roles. Therefore, we

need to link genetic SNP variation to effects on gene expression levels to understand the mechanisms behind the associations identified in GWAS studies.

In recent years, regulatory roles of ncRNAs in various diseases have been emerging. In particular, a number of studies have revealed that long non-coding RNAs (lncRNAs) can be involved in liver diseases. However, the involvement of lncRNAs in NAFLD and NASH was largely unexplored. In **chapters 3, 4** and **5**, we conducted various transcriptome analyses in liver biopsies from an obese cohort and *in vitro* cell models that mimic progression of NASH in order to detect and characterize lncRNAs associated with NAFLD and NASH phenotypes. We further conducted *in vivo* and *in vitro* functional analyses to understand the role of selected candidates.

Hepatocyte apoptosis is a major feature of NASH that can lead to fibrosis and cirrhosis, which permanently damage and scar the liver, thereby disrupting essential hepatic functions. Preventing NASH from progressing to fibrosis and cirrhosis is therefore crucial and understanding the regulation of hepatocyte apoptosis will contribute to the identification of molecular targets that prevent NASH progression. In **chapter 3,** we report the discovery of lnc18q22.2 (Liver cell Viability Associated lncRNA - *LIVAR*), a liver-specific lncRNA involved in cell viability that shows elevated expression in the liver of NASH patients. Silencing the expression of *LIVAR* resulted in either a lethal phenotype or decreased cell viability in four hepatocyte cell lines. Pathway analysis of *LIVAR* downstream genes indicated that *LIVAR* might be involved in mRNA translation, cell death, apoptosis and oxidative reduction. These results show that *LIVAR* plays a crucial role in hepatocyte viability and is likely to play a regulatory role by inhibiting hepatocyte apoptosis and necrosis. The discovery of *LIVAR* may provide new insights into the regulation of hepatocyte viability in NASH.

Liver inflammation is a key feature when benign steatosis has progressed to steatohepatitis. However, the underlying mechanisms are still poorly understood, which severely limits treatment options. In **chapter 4**, we performed RNA sequencing on the livers of 60 obese individuals with different degrees of NAFLD and report the expression levels of 19,894 protein-coding and 11,843 lncRNA genes. The correlation analysis between gene expression levels and NAFLD phenotypes revealed 854 lncRNAs that showed association to NAFLD phenotypes, mainly to NASH grade and lobular inflammation. Co-expression analysis of lncRNAs and mRNAs of protein-coding genes suggested downstream effects of lncRNAs. Finally, we identified an antisense lncRNA at the locus of the *HNF4A* gene (*HNF4A-AS1)* that was strongly suppressed in human livers depending on the degree of NASH. In line with our observation in human samples, the mouse *HNF4A-AS1* homolog lncRNA also showed down-regulation in the livers of mice with diet-induced NAFLD/NASH. *In vitro* experiments in HepG2 cells showed that *HNF4A-AS1* was strongly down-

regulated upon TNFα exposure and knock-down studies revealed that *HNF4A-AS1* may regulate the transcription factor HNF4A and its downstream pathways.

Our human obese cohort with varying degrees of NASH can only identify proteins and non-coding RNAs associated to various NASH phenotypes. It remains a challenge to characterize their role in the progression of NASH. Therefore, in **chapter 5,** we used the human hepatocyte cell line HepG2 as a model for NAFL and NASH, which were exposed to free fatty acids to induce cellular steatosis. This was followed by stimulation with tumor necrosis factor alpha (TNFα) to mimic an inflammatory condition. Hepatocytes are the most abundant liver cell type and are strongly affected during NASH development. NASH phenotypes like steatosis and ballooning occur in the hepatocytes. In addition, many stress signals (e.g., lipotoxicity, oxidative stress, endoplasmic reticulum stress and inflammation) can affect hepatocyte function. We conducted whole genome RNA-sequencing upon stimulation at four time points and identified 4,367 genes showing significant response to stimulation, with 109 being lncRNAs. For 18 lncRNAs, the hepatic expression was also significantly associated to NASH phenotypes in our 60 obese individuals. Moreover, our data identified a lncRNA in the TNFα/NF-κB signaling pathway, which we named *lncTNF* (RP11-91K9.1). *lncTNF* showed 20-fold upregulation upon TNFα stimulation and was positively correlated with lobular inflammation in human livers. TNFα is a cytokine that can activate the NF-κB signaling pathway, one of the main signaling pathways linked to liver inflammation. lncTNF silencing in hepatocytes resulted in lower NF-κB activity and subsequent downregulation of *A20* and *IKBA*, suggesting involvement in NF-κB activation pathways. Additional studies are needed to further validate these findings.

In addition to lncRNAs, increasing evidence shows that enhancers can be also transcribed to generate non-coding enhancer RNAs (eRNAs). Enhancers are among the most important gene regulators in the cell. It has been shown that activation of enhancers and their transcribed eRNAs is highly tissue- and context-specific. However, the role of eRNAs in the liver and in NAFLD is not known. In **chapter 6,** we report 1,490 intergenic enhancers that are abundantly expressed in liver biopsies from 60 individuals. Among these, 289 eRNAs showed association with NAFLD and co-expression with nearby genes. These genes were enriched in disease-related pathways, including inflammatory pathways and response to lipopolysaccharide, suggesting potential involvement of enhancers in regulation of underlying genes and pathways. Moreover, eRNAs were affected by genetic variants associated with cardiometabolic and liver traits, including 119 expression quantitative trait effects at FDR<0.05. The expression of enhancers may thus have an important biological impact on regulation of cellular processes, making them a potential target for disease prevention and treatment.

In conclusion, our studies have highlighted regulatory role of non-coding RNAs in NAFLD progression and we have characterized potential functions of several lncRNAs in liver viability, liver inflammation and hepatic function. Further functional studies are needed to better understand the mechanism of action of the ncRNAs described in this thesis. Altogether, the findings reported in this thesis, together with future studies on ncRNAs, in combination with the current knowledge on coding genes will increase our understanding of NASH pathogenesis and development. This will ultimately lead the way towards better therapeutic treatment for NAFLD and NASH patients.

Samenvatting

Niet-alcoholische leververvetting ("non-alcoholic fatty liver disease" of NAFLD) omvat een verzameling van leveraandoeningen, variërend van simpele leververvetting (niet-alcoholische leververvetting ofwel NAFL) tot de meer ernstige vorm die gekarakteriseerd wordt door leverontsteking (niet-alcoholische leverontsteking ofwel NASH), die uiteindelijk kan leiden tot leverfibrose, cirrose en leverkanker. Overgewicht en obesitas zijn de grootste risico factoren voor het onstaan van NAFLD en door de huidige obesitas epidemie is NAFLD de meest voorkomende chronische leverziekte wereldwijd geworden. Leververvetting bij NAFLD patiënten is tot een zeker punt omkeerbaar, hetgeen in de meeste gevallen worden bereikt middels levensstijl interventies. Echter, bij 10-20% van de NASH-patiënten ontwikkelt de ziekte zich verder tot cirrose en hepatocellulaire carcinoma (HCC), in de naaste toekomst waarschijnlijk de meest voorkomende indicaties voor levertransplantatie. Vandaar dat het van belang is om de mechanismen te begrijpen die betrokken zijn bij het ontstaan van NAFLDen de progressie ervan naar ernstigere leveraandoeningen.

De voltooiing van het Human Genome Project in 2003 en de ontwikkeling van *high-throughput sequencing* technologieën hebben geleid tot een revolutie in biomedische onderzoek. Er wordt geschat dat eiwit-coderende genen minder dan 2% van het menselijke genoom vertegenwoordigen, terwijl meer dan 98% van het menselijk genoom wordt gezien als niet-coderend. Interessant is dat een groot gedeelte van dit niet-coderende genoom vertaalt kan worden in niet-coderende RNA's (ncRNA's). Deze ncRNA's blijken betrokken te zijn bij verschillende belangrijke biologische processen, zoals chromatine remodelering, regulatie van gen expressie en eiwit transport. Hierdoor is het dus goed mogelijk dat ncRNA's betrokken zijn in verschillende ziekte processen. Echter, de betrokkenheid van ncRNA's in de progressie van NAFLD is nog onduidelijk. In dit proefschrift proberen we de rol van ncRNA's in NAFLD te begrijpen door het combineren van transcriptoom-analyse van een patiënten cohort, functionele genetica en *in vitro* ziektemodellen. Dit onderzoek heeft het belang van ncRNAs in de chronische leverziekte NAFLD aangetoond.

Allereerst hebben *genome-wide associatiion studies* (GBAS) aanwijzingen opgeleverd voor het belang van het niet-coderende genoom bij complexe aandoeningen en eigenschappen. Obesitas is een risicofactor voor NAFLD maar is ook geassocieerd met diabetes type 2 (T2D) en hart- en vaatziekten. In **hoofdstuk 2** geven we een overzicht van de 755 *single-nucleotide polymorphisms* (SNPs), gelegen op 366 verschillende loci, die zijn geassocieerd met cardiometabole fenotypes waaronder obesitas, T2D, hart- en vaatziekten en NAFLD. Door middel van het prioriteren van kandidaat genen en het uitvoeren van *pathway enrichment* analyses tonen we aan dat bepaalde SNPs vaker voorkomen

in *lipidtrait*-geassocieerde loci. Hieruit concluderen we dat er een sterke connectie bestaat tussen *lipid traits* en obesitas, diabetes en hart- en vaatziekten. Bovendien zijn meer dan 90% van de cardiometabole SNPs gelegen in niet-coderende regionen van het genoom die naar verwachting een regulerende functie hebben. Daarom moeten we, om de mechanismen achter de gevonden GWAS associaties te verklaren, genetische SNP variatie vertalen naar effecten op gen expressie.

In de afgelopen jaren is aangetoond dat ncRNAs bijdragen aan verschillende aandoeningen. Uit verschillende studies is gebleken dat met name lange niet-coderende RNAs (lncRNAs) betrokken te zijn in het ontstaan van leverziekten. Echter, de rol van lncRNAs in NAFLD en NASH is nog onduidelijk. In **hoofdstuk 3, 4 en 5** hebben we lncRNAs ontdekt en gekarakteriseerd die betrokken zijn bij NAFLD en NASH. In deze studies hebben we gebruikt gemaakt van gekweekte cellen die de progressie van NAFLD naar NASH nabootsen en van verschillende transcriptie analyses in lever biopten van patiënten met zeer ernstig overgewicht. Om de rol van deze lncRNAs verder te begrijpen hebben we *in vivo* en *in vitro* functionele analyses uitgevoerd.

Apoptose van hepatocyten is een belangrijk kenmerk van NASH en kan bijdragen aan het ontstaan van leverfibrose en uiteindelijk levercirrose. Fibrose en cirrose kunnen de lever permanent beschadigen waardoor essentiele leverfuncties worden verstoord en het is daarom van cruciaal belang om de ontwikkeling tot fibrose en cirrose te voorkomen. Daarnaast is het ook belangrijk om te begrijpen hoe apoptose in hepatocyten is gereguleerd, omdat dit mogelijk nieuwe therapeutische targets kan opleveren die de ontwikkeling van NASH kunnen voorkomen. In **hoofdstuk 3** rapporteren we de ontdekking van lnc18q22.2 (Lever cel Levensvatbaarheid Geassocieerde lncRNA - *LIVAR*), een lever-specifieke lncRNA betrokken bij de levensvatbaarheid van cellen. Expressie van lncRNA is verhoogd in de lever van patiënten met NASH. Uitschakelen van de expressie van LIVAR leidt tot een afname in de levensvatbaarheid van vier verschillende levercellijnen. Gen expressie analyses suggereert dat LIVAR een rol speelt in mRNA translatie, celdood, apoptose en oxidatieve reductie. Samenvattend laten deze resultaten zien dat LIVAR waarschijnlijk een cruciale rol speelt in het remmen van apoptose en necrose in hepatocyten. De ontdekking van LIVAR kan mogelijk bijdragen aan nieuwe inzichten in de regulatie van de levensvatbaarheid van levercellen tijdens de ontwikkeling van NASH.

Leverontsteking is een belangrijk kenmerk van de overgang van steatose naar steatohepatitis. De onderliggende mechanismen hiervan zijn echter nog steeds onduidelijk, hetgeen de diagnose en behandelingsmogelijkheden ernstig beperkt. In **hoofdstuk 4** beschrijven hoe we RNA-sequencing hebben toegepast op de levers van 60 personen met zeer ernstige overgewicht en met verschillende gradaties van NAFLD. In deze studie rapporteren we de expressieniveaus van 19.894 eiwit coderende en 11.843

lncRNA genen. De correlatieanalyse tussen de genexpressieniveaus en NAFLD fenotypes onthulde 854 lncRNA's die associatie tonen met verschillende NAFLD fenotypes, voornamelijk met NASH en lobulaire ontsteking. Co-expressie analyse van lncRNA's en mRNA's van eiwit-coderende genen suggereerden "stroomafwaartse" effecten van de lncRNA's. Ten slotte identificeerden we een antisense lncRNA gelokaliseerd in het *HNF4A* gen (*HNF4A-AS1*). De expressie van dit lncRNA is zeer verlaagd in NASH en de mate van verlaging is gecorreleerd aan de ernst van NASH. In overeenstemming met deze waarneming in humane levermonsters vertoonde het muizen *HNF4A-AS1* homoloog lncRNA ook verminderde expressie in de levers van muizen met via het dieet-geïnduceerde NAFLD/NASH. *In vitro* experimenten in HepG2 cellen toonden aan dat *HNF4A-AS1* sterk neerwaarts werd gereguleerd na blootstelling aan TNFα en uit knock-down-studies bleek dat *HNF4A-AS1* mogelijk de transcriptiefactor HNF4A en daardoor de door deze factor gecontroleerde genen kan reguleren.

Met behulp vanns obesitas-cohort met daarin personen met verschillende stadia van NASH, kunnen we alleen eiwitten en (non-coding RNAs) identificeren die geassocieerd zijn met diverse NASH-fenotypen. Het blijft een uitdaging om hun rol in de progressie van NASH vast te stellen en nader te karakteriseren. Daarom hebben we in **hoofdstuk 5** de menselijke levercellijn HepG2 als een model voor NAFLD en NASH gebruikt. Deze cellen werden blootgesteld aan vrije vetzuren om leververvetting te induceren. Vervolgens werd de tumor necrose factor-alfa (TNFα) gebruikt om ontsteking te simuleren. Hepatocyten zijn het meest voorkomende levercel type en belangrijk in de ontwikkeling van NASH. Fenotypen van NASH zoals steatose en '*ballooning*'**,** vinden in de hepatocyten plaats. Bovendien kunnen veel stress-signalen (bijvoorbeeld lipotoxiciteit, oxidatieve stress, stress van het endoplasmatisch reticulum en ontsteking) het functioneren van hepatocyten beïnvloeden. We hebben *whole genome RNA-sequencing* gedaan op vier verschillende tijdspunten tijdens inductie van steatose met en zonder TNFα en hebben daarin 4,367 genen geïdentificeerd die significant reageerden op debehandeling, hiervan waren 109 lncRNAs. Voor 18 lncRNAs, was de lever expressie ook significant geassocieerd met NASH fenotypes in onze 60 zwaarlijvige proefpersonen. Een van deze lncRNAs bleek een rol tespelen in de TNFα/NF-κB signaalroute, en daarom hebben we deze lncRNA *lncTNF* (RP11-91K9.1) genoemd. *lncTNF* liet een twintigvoudig hogere transcriptie zien na TNFα stimulatie en was positief gecorreleerd aan lobulaire ontsteking in menselijke levers. TNFα is een cytokine die de NF-κB signaalroute, een van de belangrijkste signaalroutes tijdens ontstekingsreacties, kan activeren. Onderdrukking van *lncTNF* transcriptie in hepatocyten resulteert in een lagere NF-κB activiteit, wat vervolgens resulteerde in een lagere transcriptie van *A20* en *IKBA*. Dit suggereert dat *lncTNF* mogelijk betrokken is in de activatie route van NF-kB. Aanvullende studies zijn nodig om deze bevindingen verder te valideren.

Groeiend bewijs laat zien dat, behalve lncRNA's, ook enhancer regios vertaalt kunnen worden in RNA moleculen, ook wel niet-coderend enhancer RNA (eRNA) genoemd. Enhancers behoren tot belangrijke regio's in het genoom die de transcriptie van genen reguleren. Het is aangetoond dat de activatie van enhancers en hun afgeschreven RNA's (eRNA's) sterk weefsel- en context-specifiek is. Het is echter onbekend wat de rol van eRNA's in de lever en in het ontstaan van NAFLD is. In **hoofdstuk 6** rapporteren we over 1.490 enhancers die zich tussen genen bevinden die tot expressie kwamen in leverbiopten van 60 personen met overgewicht. Hieronder waren 289 eRNA's die geassocieerd waren met NAFLD en die co-expressie met naburige genen lieten zien. Deze genen waren verrijkt in ziekte-gerelateerde metabole processen, waaronder processen in ontstekingsreacties en werking van lipopolysacchariden. Verder werden de expressie van deze eRNA's beïnvloed door genetische varianten die geassocieerd zijn met cardiometabole- en leverfuncties, waaronder 119 kwantitatieve expressie-eigenschap effecten met een FDR<0.05. De expressie van enhancers zou zo een belangrijk effect op de regulatie van cellulaire processen kunnen hebben, wat hen een potentieel belangrijk doel voor ziektepreventie en behandeling maakt.

De bevindingen in dit proefschrift, dragen sterk bij aan onze kennis van de ontwikkeling van NAFLD en NASH in de menselijke lever. Verdere functionele studies zijn nodig om de mechanismen te achterhalen hoe deze ncRNA's aangrijpen op verschillende biologische processen. Deze kennis zal hopelijk leiden tot nieuwe therapeutische aangrijpingspunten om patiënten met NAFLD en NASH te behandelen.

Acknowledgements

During the four years of my PhD studies at the UMCG (Departments of Pediatrics – Molecular Genetics Section and Department of Genetics), I had the opportunity to meet many inspirational people. To all of them I would like to express my deepest gratitude for being a part of this journey and providing me with great support during this period.

First of all, I would like to thank my supervisor Prof. Marten Hofker. Dear Marten, I remember our first discussion during my interview. Your friendly and enthusiastic approach made me feel comfortable and relaxed during our talk, both then and also during my whole PhD period. I was really excited when I met you in person and immediately realized that you are a great mentor. I learned a lot from you and was willing to learn even more. Unfortunately, we lost you too early. You established the foundation of this project, together we worked on its growth, and later we implemented our ideas and followed your example. I hope you are proud of the work. Thank you for giving me the opportunity to do my PhD in your lab. I am grateful to have known you and to have had you as a mentor. Thank you for all the scientific discussions, suggestion, criticism and advice. Your impact is eternal and of great importance for me and my future career!

My daily supervisor, now my promoter, Prof. Jingyuan Fu. Dear Jing, thank you for accepting me as your PhD student and for your excellent guidance throughout my PhD projects. From the first day I met you, I knew we would be a great team! And, indeed, we could openly discuss anything. You are one of the most positive and persistent people I know. Thanks to you I learned a lot more about data analysis and bioinformatics but also about writing papers, summarizing data and making great stories. In parallel, you gave me the space and freedom to work independently, which is of great importance in science. Thanks for believing in me. You are a hardworking and dedicated scientist, and I am grateful to have you as my supervisor and my promoter.

I was fortunate to work closely with one of the great scientists in the Netherlands and worldwide, Prof. Cisca Wijmenga. Dear Cisca, I am honored to have you as my promoter. I am also grateful that I could learn so much from you. Your suggestions were always clear and to the point; I admire how dedicated and focused you are. Even with the many obligations you had, you could always find time for your students. Thank you for all the constructive discussions and for having such a great impact on my scientific development. You and Marten were great hosts, thank you for all the events you organized in your home!

Dear Bart, you were always available and willing to help with every question I had. Because of that I had the feeling that you are also my supervisor. Thank you for being actively involved in all the projects that I worked on, for all the discussions and suggestions, and

for guiding me through lab experiments when needed. I very much appreciate your honesty, advice and critical thinking. You are a great scientist and an inspiration for all your students. Also, thank you for hosting amazing dinners at your place. We always had a lot of fun!

I would like to thank the members of the reading committee, Prof. A.K. Groen, Prof. A. van den Berg and Prof. J.A. Lisman, for their availability and willingness to read and review my thesis. Together with Prof. J.W. Jonker, you were part of my yearly AIO committee, and I am thankful for all the discussions we had throughout these four years.

This work was a result of a fruitful collaboration with Prof. Sander Rensen from Maastricht University. Dear Sander, not only did you provide the human liver biopsies, you were actively involved in every step of this project. Thank you very much for sharing your expertise and your knowledge of NAFLD and NASH. Your comments and suggestions were truly valuable for the whole project. I am grateful to have had you as a collaborator.

Being a part of the Departments of Pediatrics and Genetics gave me the opportunity to learn and combine knowledge on molecular biology, genetics and bioinformatics in my projects. Therefore, I would like to thank all the members from both departments for their help, support and advice.

From the Department of Pediatrics and Molecular Genetics section: Dear Debby, Kuif, Marit, Barbara and Kathrin, thank you for the nice discussions we had during meetings and when needed. You are all such inspiring scientists and great supervisors. Dear Prof. Folkert Kuipers, your suggestions improved the papers substantially as well as my knowledge of liver metabolism and diseases. Dear Prof. Klaas Nico Faber and Prof. Han Moshage, thank you both for the nice collaboration we had on chapter 3. To all the technicians, thank you all for your support in designing and performing experiments. Dear Niels, Daphne, Nicolette, Marieke and Mirijam, you are a great support to all the PhD students: your knowledge, in general, but also your specific expertise, makes our lives much easier. Thank you for always being available and helpful. I have learned a lot from you. Dear Vincent, thank you very much for keeping me up to date with the literature and for sharing your experience with lncRNAs. Many thanks to the secretariat office, to Paula, Hilde, Marijke and Evelin, for organizing paperwork, account access and anything else needed. I appreciate your help and kindness.

I am truly grateful I have met many talented Master and PhD student during this period. Dear Alina and Paulina, thank you for all the advice at the very beginning of my PhD. Tobias, Johanna and Melinde, I enjoyed sharing the same side of our open office table with you. Dear Melinde, I also enjoyed being your roommate at all conferences we attended

together! Thanks for all the support (including during ice-skating ☺) and for being a very pleasant colleague and a good friend. Dear Federico, Antoine and Eelke, thank you for the helpful discussion and for always being cheerful and positive. Dear Venetia, Anouk, Natalia and Aline, you girls have a great personality and that is inspiring. Thank you for the nice conversations during lunch and coffee breaks. Venetia, thanks for being a wonderful neighbor and for accepting the invitation to be my paranymph! Dear Andrias, thank you for continuing my work! I hope you will discover many more interesting findings. Dear Glenn, you were a wonderful student. Thanks for your contribution to the lncTNF story. I hope you learned a lot as well! And thanks for helping me with the Dutch summary! Furthermore, many thanks to all members of System Biology and Metabolic Signaling groups: Anne- Claire, Sarah, Xiang, Agnieszka, Marcel, Melany, Chris, Marty, Mirijam, Alex, Tamara, Stefi, Patricia and all the others for sharing your knowledge and for all the after-work drinks we had together.

Being a part of the Department of Genetics was a great pleasure for me. To all the PIs, technicians and students there, thank you for your help, support and advice. I had the opportunity to work closely with some of you. Dear Sebo, I learned a lot about lncRNAs from you. Thank you for your guidance and detailed discussions. Dear Vinod, Iris, Sasha and Yang, I am grateful I could work with inspiring people like you. Iris, thanks to you I learned much more about transcriptional regulation. To all the members of the Genomic Facility and GCC, thank you very much for generating and maintaining our RNA sequencing datasets. Dear Kate and Jackie, thank you for all your help with English editing, structuring the manuscripts and the whole thesis. Dear Zuzanna, Kieu, Vicky, Raul, Maria, Roeland and all the other students, I appreciate your help whenever needed. Thanks for organizing wonderful events: dinners, barbeques, drinks and more. I enjoyed every moment spent with you! Dear Marijke, I was so glad to work with you on many projects. Thanks for teaching me a lot about bioinformatics and for being a wonderful person and friend. I miss our deep discussions and hope there will be many more!

To all the lovely friends I met in Groningen, thank you all for making my days and weekends full of laughter and adventures! Dear Vere, thanks for your hospitality and for introducing me to Groningen when I arrived! Dear Mina and Max, I admire your pleasant personalities. Thanks for all the events and parties you organized. Dear Kala, you are so sweet and I always enjoyed your company. Dear Viki and Nikola, thank you for all the game nights we had together! I enjoyed every moment with you and your positive energy! Dear Marija, thanks for all the advice you gave me. I appreciate your openness and independence. It's time to finally celebrate together in Amsterdam! Dear Ilche, your dedication and enthusiasm were an inspiration for all of us! Thank you for all the fun moments we had together! Dear Jovan (Joki), thanks for the positive energy (and salsa moves) you brought into our Macedonian community! Dear Ena, you are an example of how hard work and

persistence can lead to the final results! Thank you for all the nice talks and advice! Dear Jane, thank you for sharing all those jokes. You make the rainy days brighter! To my friends from far, far away (Rotterdam ☺). Dear Olivera, Kate, Dina and Carolina, thank you for all the chats, short visits and always being there for me!

I have to thank my supervisor and colleagues from the Netherlands Cancer Institute. Dear Tineke, Ineke, Aleksandra, Heta, Gert-Jan, Wim and Jos, thank you all for your support during this last year! And thank you for your help in translating the summary!

Finally, to all my friends and family in Macedonia, thank you all for your love and support! Драги мои тета, Тања, Ане, Леонора и Душица ви благодарам за сите убави моменти што ги делевте со мене и за сите посети (а ќе има уште многу). Драга баба, ти благодарам за топлите прегратки и долгите разговори секогаш кога ќе дојдев дома. Драг мој вујче, иако беше на пат околу светот не заборави да се јавиш и да ме насмееш! Ти благодарам за се! Моја Еми, пријателка како сестра, ти благодарам за целата поддршка и за сите видео разговори со Мила и Марко, ви се радувам бескрајно! Сандра моја најдрага, distance makes no difference when it comes to friends! Ти благодарам за сите долги разговори и што беше секогаш со мене! Ева ми е насмевка и радост!

My lovely family, my sisters and my parents, my everything. Ви благодарам за довербата, поддршката и љубовта што ми ја давате секојдневно! Алек, ти порасна во прекрасна личност и си бескрајна инспирација. Ти благодарам за сите посети, за сите прекрасни Мишелин (***) вечери, уживав во секоја секунда помината со тебе. Еци, ти зрачиш радост и среќа! Ти благодарам што ми носеше позитивни емоции секој ден! А најмногу од се, ви благодарам тебе и Даниел за најубавото нешто, за нашата Емилиа! Со неа секој ден е љубов и радост! Драги мама и тато, без вас ништо од ова немаше да биде возможно. Ви благодарам што не донесовте на правиот пат, што ни ги покажавте правите вредности и што не научивте да бидеме вредни и упорни! Ве сакам бескрајно!

Biljana

April 2019

List of Publications

[1] **B. Atanasovska**, G. Bozhinovski, L. Chakalova, S. Kocheva, O. Karanfilski, and D. Plaseska-Karanfiska, "Molecular Diagnostics of β-Thalassemia.," *Balkan J. Med. Genet.*, vol. 15, no. Suppl, pp. 61–5, Dec. 2012.

[2] **B. Atanasovska**, G. Bozhinovski, D. Plaseska-Karanfilska, and L. Chakalova, "Efficient detection of Mediterranean β-thalassemia mutations by multiplex single-nucleotide primer extension.," *PLoS One*, vol. 7, no. 10, p. e48167, 2012.

[3] Y. Wang, S. van der Tuin, N. Tjeerdema, A. D. van Dam, S. S. Rensen, T. Hendrikx, J. F. P. Berbée, **B. Atanasovska**, J. Fu, M. Hoekstra, S. Bekkering, N. P. Riksen, W. A. Buurman, J. W. Greve, M. H. Hofker, R. Shiri-Sverdlov, O. C. Meijer, J. W. a Smit, L. M. Havekes, K. W. van Dijk, and P. C. N. Rensen, "Plasma cholesteryl ester transfer protein is predominantly derived from Kupffer cells.," *Hepatology*, vol. 62, no. 6, pp. 1710–22, Dec. 2015.

[4] **B. Atanasovska**, V. Kumar, J. Fu, C. Wijmenga, and M. H. Hofker, "GWAS as a Driver of Gene Discovery in Cardiometabolic Diseases," *Trends in Endocrinology and Metabolism*, vol. 26, no. 12. Elsevier Ltd, pp. 722–732, 2015.

[5] **B. Atanasovska**, S. S. Rensen, M. R. van der Sijde, G. Marsman, V. Kumar, I. Jonkers, S. Withoff, R. Shiri-Sverdlov, J. W. M. Greve, K. N. Faber, H. Moshage, C. Wijmenga, B. van de Sluis, M. H. Hofker, and J. Fu, "A liver-specific long non-coding RNA with a role in cell viability is elevated in human non-alcoholic steatohepatitis.," *Hepatology*, vol. 34, no. 1, pp. 67–79, Jan. 2017.

[6] K. Trajanoska, J. A. Morris, L. Oei, H.-F. Zheng, D. M. Evans, D. P. Kiel, C. Ohlsson, J. B. Richards, F. Rivadeneira, GEFOS/GENOMOS consortium, the 23andMe research team, Forgett V, Leong A, Ahmad OS, Laurin C, Mokry LE, Ross S, Elks CE, Bowden J, Warrington NM, Kleinman A, Willems SM, Wright D, Day FR, Murray A, Ruth KS, Tsilidis KK, Ackert-Bicknell CL, Bassett JHD, van der Eerden BCJ, Gautvik K, Reppe S, Williams GR, Medina-Gómez C, Estrada K, Amin N, Bis JC, Breda S, Chasman, Demissie S, Enneman AW, Hsu YH, Ingvarsson T, Kähönen M, Kammerer C, Lacroix AZ, Li G, Liu CT, Liu Y, Lorentzon M, Mägi R, Mihailov E, Mlani L, Moayyeri A, Nielson CM, Nerea A, Sham PC, Siggeirsdotir K, Sigurdsson G, Thorsteinsdottir U, Trompet S, Thorleifsson G, Vandenput L, van der Velde N, Viikari J, Xiao SM, Hua Zhao J, Akesson KE, Andersen M, **Atanasovska B**, Balcells S, Eriksson J, Formosa MM, Garcia-Ibarbia C, Gonzalez-Macias J, Garcia-Giralt N, Hallmans G, Karlsson M, Khusainova R, Kim, Lee SH, Leung PC, Mallmin H, Kwok TCY, Masi L, Melin BS, Mencej-Bedrac S, Nethander M, Olmos JM, Kollia P, Prezelj J, Van Schoor, Svensson O, Szulc P, Valero C, Woo J, Brandi M, Cheng S, Chapurlat R, Christiansen C, Cooper C, Dedoussis G, Eisman JA, Frost M, Giroux, Grinberg D, Goltzman D, Hocking LJ, Van Hul W, Koh JM, Rejnmark L, Jensen JB, Langdahl B, Lewis JR, Lorenc RS, Khusnutdinova E, Marc J, McGuigan FE, Mellström D, Michaelsson K, Nogues X, Nordström P, Obermayer-Pietsch B, Pettersson-Kymmer U, Prince RL, Reeve J, Reid DM, Riancho, Rousseau F, Tang NLS, Xuereb-Anastasi A, Leslie WD, Evans DS, Cummings SR, Cauley J, van Duijn CM, Brown M, Duncan EL, de Groot LC, Esko T, Gudnason V, Harris TB, Jackson RD, Jukema JW, Ikram MA, Karasik D, Kaptoge S, Khaw KT, Kung AW, Lehtimäki T, Lyytikäinen LP, Lips P, Luben R, Metspalu A, van Meurs

JBJ, Minster RL, Orwoll E, Oei E, Psaty BM, Raitakari OT, Ralston SH, Ridker PM, Robbins JA, Smith AV, Spector TD, Styrkarsdottir U, Zmuda J, Tranah GJ, Stefansson K, Uitterlinden AG, Zillikens MC, Ntzani EE, Evangelou E, Ioannidis JPA, Perry JRB, Tung JY, Hinds DA, Scott R, Agee M, Alipanahi B, Auton A, Bell RK, Bryc K, Elson SL, Fontanillas P, Furlotte NA, Huber KE, Litterman NK, McIntyre MH, Mountain JL, Noblin ES, Northover CAM, Pitts SJ, Sathirapongsasuti JF, Sazonova OV, Shelton JF, Tian S, Tian C, Vacic V, Wilson. CH., "Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study.," *BMJ*, vol. 362, p. k3225, 2018.

[7] D. V. Zhernakova, T. H. Le, A. Kurilshikov, **B. Atanasovska**, M. J. Bonder, S. Sanna, A. Claringbould, U. Võsa, P. Deelen, L. Franke, R. A. de Boer, F. Kuipers, M. G. Netea, M. H. Hofker, C. Wijmenga, A. Zhernakova, and J. Fu, "Individual variations in cardiovascular-disease-related protein levels are driven by genetics and gut microbiome," *Nat. Genet.*, vol. 50, no. 11, pp. 1524–1532, 2018.

Curriculum vitae

Biljana Atanasovska was born on April 18th, 1985 in Skopje, Macedonia. In 2003 she started her bachelor studies at the Institute of Biology, Faculty of Natural Sciences and Mathematics, University 'Ss. Cyril and Methodius" Skopje, Macedonia, graduating in 2007. After her graduation, she worked as a research scientist at the Research Centre for Genetic Engineering and Biotechnology, Macedonian Academy of Sciences and Arts in Skopje, Macedonia. In 2011 she started her Master studies in Human Genetics at the Institute of Biology, Faculty of Natural Sciences and Mathematics in Skopje, received Erasmus Mundus scholarship (ERAWEB program) and continued her Master studies at the Erasmus Medical Center in Rotterdam, the Netherlands. In 2013, she obtained MSc degree in Health Sciences (Genetic Epidemiology). She started her PhD studies in 2013 at the Department of Pediatrics (Molecular Genetics Section) and the Department of Genetics, University Medical Centre Groningen, the Netherlands, under supervision of Prof. Marten Hofker, Prof. Jingyuan Fu and Prof. Cisca Wijmenga, working on detection and characterization of non-coding RNAs in fatty liver diseases, described in this thesis. During her PhD she was presenting her work at numerous national and international conferences. From June 2018, she started her Postdoctoral research at the Netherlands Cancer Institute, Amsterdam, the Netherlands, where she is working on understanding molecular mechanism of gene transcription under supervision of Tineke Lenstra.