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 IncRNAs. For 18 IncRNAs, 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 IncRNAs, 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 IncRNAs 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.
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 risicofactoren 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 carcinooma (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 NAFLD en 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 association studies (GBAS) aanwijzingen gegeven 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 (IncRNAs) betrokken te zijn in het ontstaan van leverziekten. Echter, de rol van IncRNAs in NAFLD en NASH is nog onduidelijk. In hoofdstuk 3, 4 en 5 hebben we IncRNAs 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 leverbiotpen van patiënten met zeer ernstig overgewicht. Om de rol van deze IncRNAs 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 essentiële 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 Inc18q22.2 (Lever cel Levensvatbaarheid Geassocieerde IncRNA - LIVAR), een lever-specifieke IncRNA betrokken bij de levensvatbaarheid van cellen. Expressie van IncRNA 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 levercelllijnen. 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
IncRNA genen. De correlatieanalyse tussen de genexpressieniveaus en NAFLD fenotypes onthulde 854 IncRNA’s die associatie tonen met verschillende NAFLD fenotypes, voornamelijk met NASH en lobulaire ontsteking. Co-expressie analyse van IncRNA’s en mRNA’s van eiwit-coderende genen suggereerden “stroomafwaartse” effecten van de IncRNA’s. Ten slotte identificeerden we een antisense IncRNA gelokaliseerd in het HNF4A gen (HNF4A-AS1). De expressie van dit IncRNA 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 IncRNA 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 tijdpunten tijdens inductie van steatose met en zonder TNFα en hebben daarin 4,367 genen geïdentificeerd die significant reageerden op debehandeling, hiervan waren 109 IncRNAs. Voor 18 IncRNAs, was de lever expressie ook significant geassocieerd met NASH fenotypes in onze 60 zwaarlijvige proefpersonen. Een van deze IncRNAs bleek een rol tespelen in de TNFα/NF-κB signaalroute, en daarom hebben we deze IncRNA 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 resulteerde 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-κB. Aanvullende studies zijn nodig om deze bevindingen verder te valideren.
Groeiend bewijs laat zien dat, behalve IncRNA’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 verrijk 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.
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List of Publications


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.