Long non-coding RNAs (lncRNAs) and cardiovascular disease (CVD)

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Project thesis

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Abstract

Long non-coding RNAs (lncRNAs) have broken into the limelight in the wake of advances in sequencing technologies in recent years. Progression in uncovering functions and properties of these transcripts, have provided initial insights into their roles in both physiological and pathological states. While they have been linked to the development of cancers, association in other disease groups such as cardiovascular diseases (CVDs) has also been found. This review aims to shed light on mechanisms and functions of lncRNA, their role in CVD, and explore their potential use in diagnostic and therapeutic strategies.

Preface

Genetic diagnostics has mainly focused within the exome, the genetic material that codes for mRNA and ultimately, proteins. Well working proteins are absolute necessities for the normal functioning of cells and organ systems, and a fault in their blueprints could yield consequences possibly disastrous for the organism. Much work has thus been done to unveil mutations that cause disease by faulty or no protein product at all, as traditionally proteins are canonically considered the effector whilst mRNA simply function as intermediate templates.

Despite the well-deserved attention the exome has received, it only makes up roughly 1% of the total human genome[1; 2]. Historically, technological limitations dictated feasibility and set boundaries for subjects of research. Mutations are by sheer probability bound to occur more frequently in vast non-exome parts. However, even with de novo mutations being relatively rare[3], importance of the genomic "dark matter" in biology and disease is not entirely hinged on this circumstance, as they seem to be intertwined in many important pathways of both regular physiology and disease, whether in mutated form or not[3-5].

Over the recent years, an explosion of technologies has enabled possibility for discovery and functional investigation of a large variety of long non-coding transcripts. A common notion that, to some extent, still exists today is that the non-coding portion of the genome is of limited clinical importance. The increasing amount of studies are being done on these non-coding transcripts, warrants elucidation of their functions and an exploration of their uses. The majority of efforts so far has been focused on neoplastic diseases[6-11]. Here, I wish to explore their importance in a non-neoplastic disease group, namely cardiovascular diseases, and lastly, look into how lncRNAs might be able to serve as potential biomarkers or even targeted in therapeutic strategies.

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1 Intro

Overall global mortality rates of cardiovascular diseases (CVDs) have significantly decreased over the past 25 years. However, in the most recent 5 years, this decline has reached a plateau[12]. Recently, long non-coding RNAs (lncRNAs) have been shown to serve roles in the development and pathologies of the cardiovascular system. Research in this area might therefore aid to disrupt the stagnation currently experienced, by providing novel targets and strategies for prevention and treatment.

Genetics play a central role in the pathophysiology of numerous diseases, some of which are caused by mutation in a single gene, giving rise to over 6000 known phenotypes[13; 14]. Most of the well-studied lncRNAs so far are found to be crucial in regulating cellular processes such as the cell cycle, growth, and apoptosis, tying them directly into the hallmarks of cancer[6; 9; 11]. The burden of disease caused by cancers is globally among the highest, along with cardiovascular diseases (CVDs), as ranked by the Global Burden of Disease (GBD) study[15]. Efforts have thus been put towards looking for novel strategies in diagnostics and treatment for these non-communicable diseases, including research in the non-coding genome. This initially led to the mapping of lncRNAs' significance in neoplastic diseases, exposing possibilities for future diagnostic and therapeutic strategies[10; 16; 17]. Likewise, lncRNAs involved in CVD might hold similar value in disease prevention and treatment.

Research interest in lncRNA has apparently increased drastically, as inferred by the number of publications on lncRNAs. The number of publications mentioning lncRNA has been doubling once every two years since 2010 (Figure 1), dwarfing the amount in the previous decade.

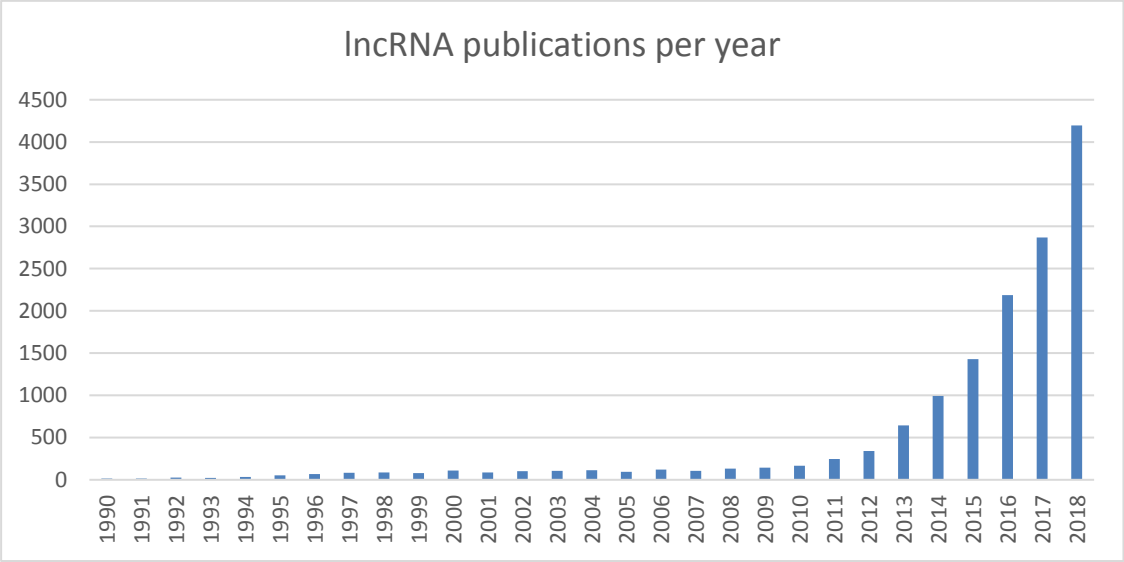

Figure 1: Number of publications retrieved in PubMed searching with the term "lncRNA", non-cumulative totals per each year.

1.1.1 The non-coding RNAs

The central dogma of molecular biology, originally stated by Francis Crick, but also restated in altered renditions, states that genetic information follows the straightforward flow of DNA → RNA → protein. On the other hand, scientists have since the late 50's noted the existence of RNAs that do not coding for proteins, but are functional in themselves, transcripts comprising the category later to be termed non-coding RNAs (ncRNAs).

Analyses of the human transcriptome revealed that most of the genome is transcribed in one setting or another, with transcripts mapping mostly to non-coding genomic regions, initially in the FANTOM consortium[18] and later in ENCODE[2]. Around 93% of the human genome seems to be transcribed at some point. From outermost start to their stop codon, protein-coding genes (PCGs) span 33% of the genome, with PCG exons making up about 1%[2]. Non-coding RNAs make up the remaining transcribed proportion, which is over half (>50%). Organisms of relatively less complexity are also found to transcribe most of their genome, like mice and *Saccharomyces cerevisiae*, transcribing about 87% and 85%, respectively[19; 20]. This extensive transcription is dubbed as "pervasive transcription", and the phenomenon seems widespread among eukaryotes[21; 22]. Complex organisms also tend to both have a total and proportionally larger amount of non-coding transcripts than their counterparts. Whether this represents

transcriptional noise or, rather in some way contributes to the complex regulation of genes perhaps required in such an organism is largely still debated in the scientific community[23].

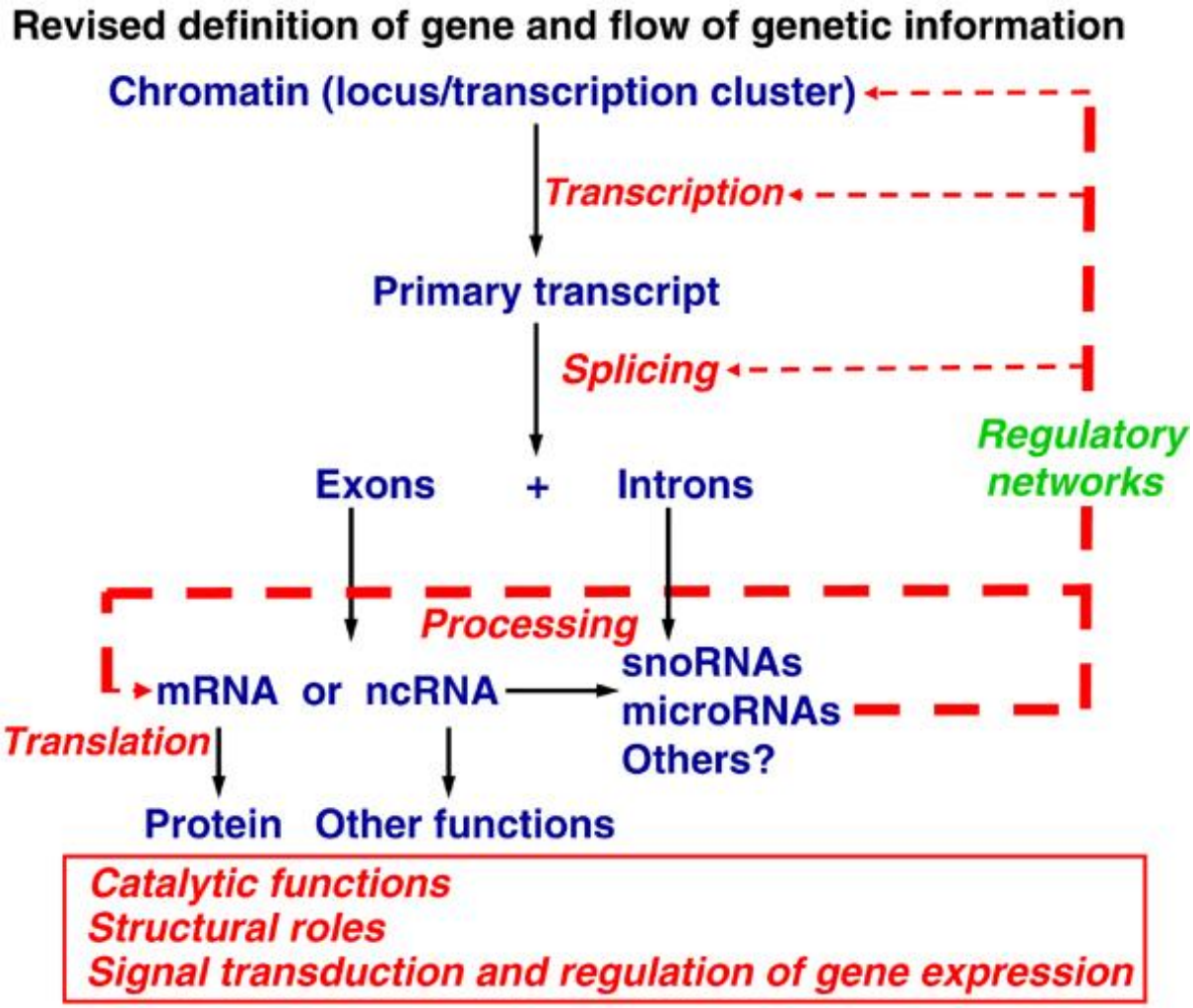

Figure 2: **ncRNAs may be processed into functional RNAs with structural or signaling functions**, participating in the gene regulatory networks or as parts of signaling pathways, e.g. metabolic or inflammatory.

From Kenzelmann and Mattick, original text: "A revised view of the flow of genetic information in the higher eukaryotes. Primary transcripts may be (alternatively) spliced and further processed to produce a range of protein isoforms and/or ncRNAs of various types, which are involved in complex networks of structural, functional and regulatory interactions." [24; 25]

Some ncRNA already have well-established functions such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), whilst some are more recently discovered and our understanding

of their functions less complete. This includes small nuclear RNAs (snRNA), as well as other short ncRNA species like microRNAs (miRNA), small interfering RNAs (siRNA), and Piwi-interacting RNA (piRNA) which are key mediators in the RNA interference (RNAi) pathway[26]. ncRNAs have been shown to participate in the control chromosome architecture, mRNA turnover and the developmental timing of protein expression, along with regulation of transcription and alternative splicing[24], and although many of them have been subject of extensive study, a full review of their workings is beyond the scope of this project thesis. Their discovery has however prompted a revised view of the central dogma and flow of genetic information (Figure 2). Several ncRNA species are themselves end products of transcription, and may exhibit properties similarly to proteins, e.g. with structural or signaling function, as well as being involved in gene regulatory networks[24; 25], bending the original dogma even further. This changes traditional conceptions of what constitutes a functional gene, and may allow for novel points of attack in research, diagnostics or therapy.

1.1.2 History of lncRNAs

Long non-coding RNAs (lncRNAs) were already described in the pre-genomic era but largely remained exceptions until the 2000s[23]. H19, an oncogene transcribed from the maternal allele exclusively as a result of imprinting, was the first one to be described back in the early 1990s[27]. X-inactive specific transcript (Xist) that mediates X-inactivation to achieve dosage compensation in humans[24], has also been long recognized as a functional RNA[28; 29].

The Human Genome Project and next generation sequencing (NGS) technologies were important milestones and laid early foundations for the study of non-coding RNAs. Facilitated by these advances, transcript groups like microRNA became the subject of extensive study in the past decade[30-32]. More recently, much research focus has been directed towards lncRNAs as well.

Initial de novo identifications of lncRNAs utilized complementary DNA (cDNA) cloning followed by Sanger sequencing. Other methods include identifying chromatin signatures like trimethylation marks (K4-K36) and utilizing tiled microarrays across non-coding regions[33]. However, RNA-Seq technology is possibly the most influential technique for lncRNA discovery so far, and variations combining it with chromatin immunoprecipitation (ChIP) or chromatin interaction analysis (ChIA) have been developed to identify interacting chromatin

regions[33]. Further, applying methods able to probe protein interactions such as cross-linking and immunoprecipitation (CLIP), Capture Hybridization Analysis of RNA Targets (CHART) and Chromatin Isolation by RNA Purification (ChIRP) have also been important, especially in inferring possible lncRNA mechanisms[34]. Initially, the FANTOM project at RIKEN launched in the early 2000s catalogued approximately 16000 novel transcripts in mouse, about 70% of which were ncRNAs[18]. For the human genome, the ongoing GENCODE project (v29) has annotated 16066 lncRNA gene loci, with the number of lncRNA transcripts totaling to 29566 as of 2018 in their most recent assembly[35], meaning approximately 27% of annotated human genes encode lncRNAs[7; 35].

1.1.3 lncRNA definition

LncRNAs are defined as non-coding RNA transcripts that exceed 200 nucleotides in length as per convention. This definition traces back to Okazaki et al. that first identified the transcripts in 2002 in the FANTOM project sequencing mouse cDNA library[18]. The cutoff is somewhat arbitrary, but it conveniently excludes small RNAs based on purification protocols of RNA, and is still the definition used as per convention to this day[22; 23; 36]. lncRNA may originate from intergenic regions or from known annotated genes. In the latter case, their source may be simultaneously exonic and intronic in either sense or antisense direction (Chapter 1.1.4). Unlike messenger RNA (mRNAs) they have little or no protein-coding capacity, but may on exceptional occasions give rise to small peptides[11]. They share some similarities with mRNA in that they are predominantly transcribed by RNA polymerase II, and may be subject to post-transcriptional modifications such as 5'-capping, polyadenylation, and intron splicing[9; 37; 38].

1.1.4 Classification of lncRNAs

LncRNA are initially classified according to their distribution in genomic location and context, and may be referred to as intergenic, intronic, sense, antisense, bidirectional, overlapping, or possible combinations of these (Figure 3).

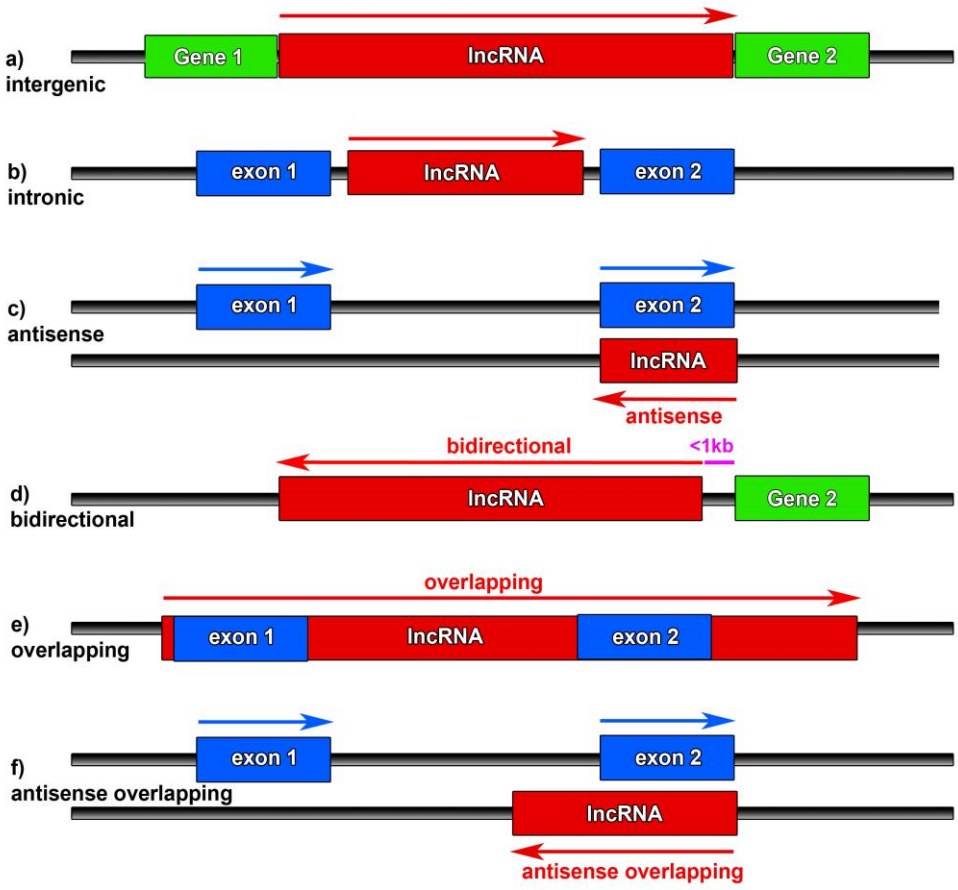

Figure 3: lncRNA classification based on genomic location and context. Green box: protein-coding gene (PCG). Red box: lncRNA. Blue box: exons of PCGs. Arrows indicate transcriptional direction. **Intergenic**: a) Also known as "lincRNA". Located between two genes, from either DNA strands at least 1kb away from the nearest PCG. **Intronic**: b) Transcribed solely from introns of protein-coding genes. **Sense**: "Transcribed from the sense strand of protein-coding genes and contain exons from protein-coding genes, overlapping with part of protein-coding genes or covering the entire sequence of a protein-coding gene through an intron"[39] such as in a), b) e). **Antisense**: "Transcribed from the antisense strand of protein-coding genes, overlapping with exonic or intronic regions or covering the entire protein-coding sequence through an intron."[39] such as in c) or f) **Bidirectional**: A bidirectional lncRNA is oriented head to head with a protein-coding gene <1kb away. May also be referred to as divergent lncRNA. **Overlapping**: e) Part of the transcript is an exon.

(Based on figure in "On the classification of long non-coding RNAs" by Ma et al.[39] and figures by Lanzafame et al.[5; 40]. Definitions in quotation marks are directly recited.)

In this way of classification, some lncRNA may fall into more than a single group. E.g. transcribed from the antisense strand and overlapping an exonic region of a protein-coding sequence such as in 3f) [39]. Genomic context remain as a main way to classify lncRNA. New methods of classification arise as research is still developing. They may also be classified in several other ways, such as the effect exerted on DNA sequences, mechanism of function, and target mechanism[39]. As reviewed by Cao et al. lncRNAs are methodologically, a very challenging group of transcripts to study[41]. They identify hurdles at almost every level of their annotation, from the initial genomic annotation and basic functional annotation, to a more in depth mechanistic investigation, and lastly their biological relevance. Better annotations are expected to become more common as mechanisms are more wholly mapped and annotation methods are further streamlined.

1.1.5 Localization and expression

Subcellularly, most lncRNA transcripts persists in the nucleus, clustered in sub-nuclear foci[42-44]. Some can be found in the cytosol, while a few transcripts are found almost exclusively in the cytosol, as shown by Cabili et al. by single-molecule RNA fluorescence in situ hybridization (RNA-FISH)[42]. Overall, they observed a strong bias towards nuclear localization of lncRNA, with 95% of transcripts having a higher nuclear fraction than mRNA[42]. Localization is of importance as the lncRNA transcript is the final product from the transcription site and thus their initial function is tied to their subcellular localization, unlike coding RNAs. Determining the transcripts spatial distribution is consequently important in the initial exploration of its function.

LncRNAs expression can be highly tissue- and cell specific, and may be dynamically expressed according to biological context, correlating to specific stages in development, differentiation and disease[7; 45; 46]. Some lncRNAs are transcribed in response to developmental cues, cellular signals and other stimuli, and affect downstream signaling in a tissue-specific and stage-dependent manner[47; 48]. Their specificity allows for their usage as markers in these different contexts by assessment of expression profiles in the study of basic biology or pathological states[49].

1.1.6 Functions, mechanisms and secondary structure

Functions of lncRNA appear as highly heterogeneous, including their underlying mechanisms. They are found to be intertwined in important pathways of both regular physiology and in disease. LncRNA influence genetic expression on various scales from epigenetic chromosome modification to transcriptional regulation, as well as participating in post-transcriptional processes. A whole host of biological processes involve lncRNAs including imprinting, organogenesis, cell growth and differentiation[50], and specific ones have been identified in normal and pathological development, including cardiovascular (Table 1). There are many ways to categorize their diverse mechanisms. Some of the more well-known lncRNA functions, are summarized below and illustrated in Figure 4. Different lncRNA may:

1. Recruit chromatin modifiers mediating the deposition of activatory or repressive histone marks (Figure 4A). E.g. in *Xist*, recruiting polycomb repressive complex 2 (PRC2), initiating gene silencing through methyltransferase activity to providing dose compensation[51], maintaining silencing on the inactive X-chromosome.
2. Recruit specific transcription factors (Figure 4B). E.g in *linc-HOXA1*, repressing *Hoxa1* by recruiting the protein PURB[52].
3. Regulate alternative splicing by modulating splicing factors (Figure 4C)[53].
4. Bind cis-regulatory elements, establishing contacts between enhancers and cognate promoters to activate transcription through chromatin looping (Figure 4D)[54; 55].
5. Initiate formation of subnuclear bodies, by acting as platforms in recruiting proteins to assemble paraspeckles. E.g as in NEAT1_2 (Figure 4E)[56; 57].
6. Act as decoys, influencing the folding of complex 3-dimensional structures affecting mRNA cytoplasmic shuttling and localization[54].
7. Sequester miRNA (Figure 4H), impeding their silencing effects on translation, dubbed as "sponging"[58], essentially working as competing endogenous competing RNAs (ceRNAs)[9; 59; 60].

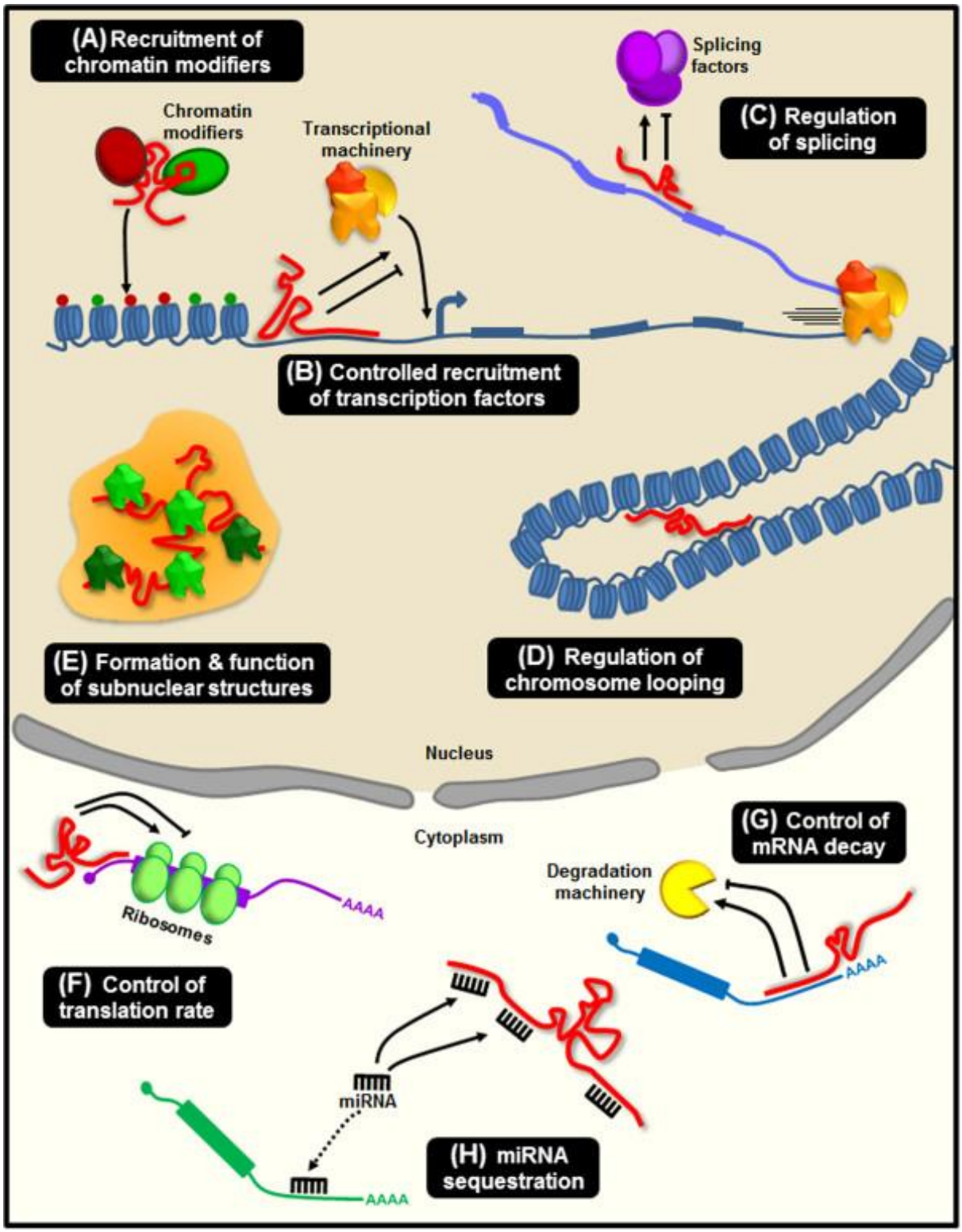

Figure 4: Mechanisms for long noncoding RNA (lncRNA) function. LncRNA are shown in red.

(Figure from works of Neguembor et al.[61], unchanged)

Another element of importance for the transcripts' function is their secondary structure, as they do not code for proteins[62; 63]. Structures are formed by base-pairing within and between sequences similarly to other functional ncRNA. Many actions of lncRNAs are highly reliant on the formation of secondary or even tertiary structures, like when acting as assembly centers for other structures. Validating domains and motifs in their structure gives clues to their binding compatibility with possible DNA, RNA or protein targets. Functions of lncRNA are multifarious and may have unrelated and opposing effects, some act to initiate silencing, while others direct an increase of gene expression[64]. Their occasionally debated biological functionality[23] will be touched on in chapter 5.1 "On the functionality of lncRNAs".

1.2 Cardiovascular disease

Cardiovascular disease (CVD) is a class of diseases that include diseases of the heart, vascular diseases of the brain and diseases of blood vessels. "Global Atlas on cardiovascular disease prevention and control" published by WHO groups CVDs into two major groups: those due to atherosclerosis, such as ischemic heart disease (IHD) or coronary artery disease (CAD), cerebrovascular disease (e.g. stroke), and diseases of the aorta and arteries, including hypertension and peripheral vascular disease. The other group comprises congenital heart disease rheumatic heart disease, cardiomyopathies and cardiac arrhythmias.[65] This project thesis will mainly focus on the former group, as they represent a large fraction of the global disease burden, necessitating and warranting developmental efforts in prevention and treatment strategies.

1.2.1 Pathophysiology of atherosclerotic disease

Atherosclerosis is a complex pathological process in the walls of blood vessels that develops over time, typically years. Lipid material and cholesterol are deposited inside the lumen of arteries, and may turn into fatty streaks. Subsequent plaque formations cause the inner surface of the blood vessels to become irregular and the lumen to narrow, constricting blood flow. Blood vessels can also become sclerotic as a result. Eventually, the plaque can rupture, triggering the formation of a blood clot. If the blood clot develops in a coronary artery, it can cause a myocardial infarction (MI); if it develops in the brain, it can cause a cerebrovascular accident (CVA). [65]

Endothelial injury is thought to be central in the initiation of atherosclerotic formations. Risk factors such as hyperlipidemia, abnormal glucose metabolism, hypertension, smoking, as well as immune reactions, toxins, and other hemodynamic factors contribute to this dysfunction [66]. Monocyte adhesion is facilitated by the injured endothelium, followed by their extravasation to the intima. Here they differentiate to macrophages and eventually, with the uptake of oxidized LDL via scavenger receptors combined with excessive cholesterol esterification, turn into foam cells[67]. Growth factors from macrophages aid proliferation and migration of fibroblasts and vascular smooth muscle cells (VSMCs), and further accelerates the development of the atherosclerotic lesion. Moreover, cytokines are also released from macrophages and nearby immune cells, causing inflammation and immune responses and to form a vicious cycle of endothelial injury. [66]

1.2.2 LncRNAs in cardiovascular development and differentiation

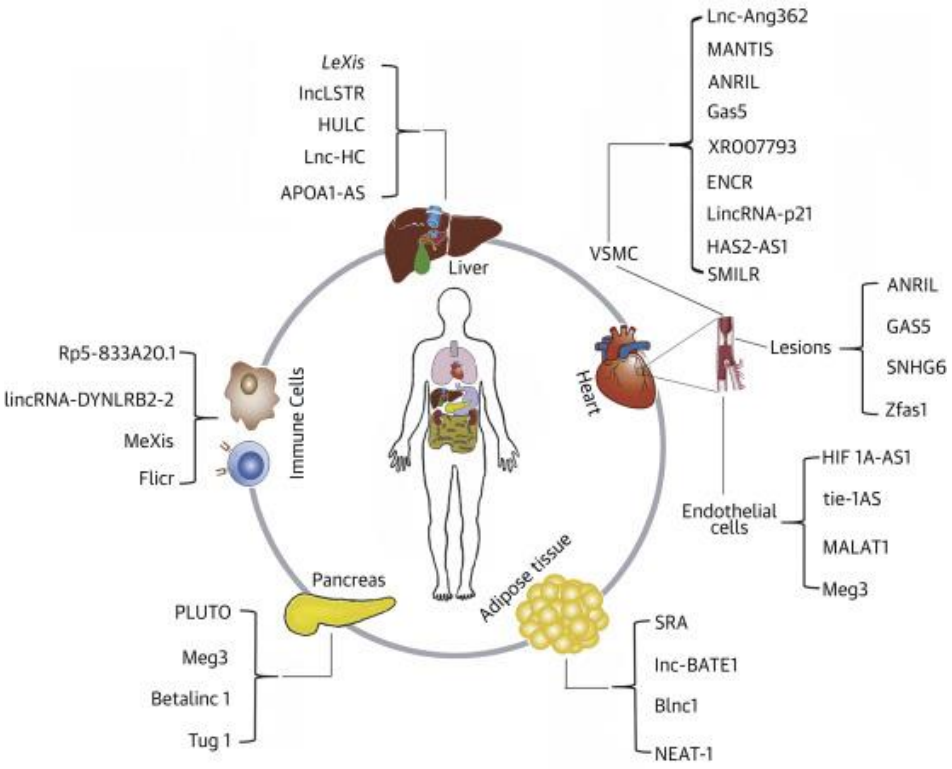

Figure 5: A collection of mammalian long noncoding ribonucleic acids (lncRNAs) expressed from various tissues or cell-types involved in atherosclerosis pathways, from Zhang, 2018[54]. Original text:
Summary of mammalian long noncoding ribonucleic acids (lncRNAs) expressed from various tissues or cell-types involved in atherosclerosis pathways. ANRIL = antisense noncoding RNA in the INK4 locus; APOA1-AS = APOA1 antisense RNA; Betalinc1 = β-cell long intergenic noncoding RNA 1; Blnc1 = brown fat lncRNA 1; ENCR = smooth muscle and endothelial cell enriched migration/differentiation-associated long noncoding

RNA; Flicr = FOXP3 regulating long intergenic noncoding RNA; Gas5 = growth arrest specific 5; HAS2-AS1 = HAS2 antisense RNA 1; HIF1A-AS1 = HIF1A antisense RNA 1; HULC = hepatocellular carcinoma up-regulated long noncoding RNA; LeXis = liver-expressed LXR-induced sequence; Lnc-BATE1 = brown adipose tissue enriched long non-coding RNA 1; lncLSTR = liver-specific triglyceride regulator; Meg3 = maternally expressed 3; MeXis = macrophage-expressed LXR-induced sequence; NEAT-1 = nuclear paraspeckle assembly transcript 1; SMILR = smooth muscle enriched long noncoding RNA; SNHG6 = small nucleolar RNA host gene 6; SRA = steroid receptor RNA activator; Tie-1AS = tie-1 antisense RNA; Tug1 = taurine up-regulated 1; XR007793 = long noncoding RNA XR007793; Zfas1 = ZNFX1 antisense RNA 1.

As highlighted above, cells with importance in atherosclerosis pathology include endothelial cells (ECs), vascular smooth muscle cells (VSMCs), various immune cells including macrophages (MΦ), and cells of metabolic importance such as adipocytes, hepatocytes and pancreatic islet cells, and are displayed in Figure 5. Cardiomyocytes may be taken into consideration for lesions in myocardial infarction (MI) and later stages of heart disease like chronic heart failure (CHF). This underlines the related tissues, cellular pathways and lncRNAs of interest in CVD development, which look to be numerous. Yet, although many lncRNAs have been found to be associated with, or identified in various tissues relevant for CVD, their actual significance whether in disease development itself or as novel markers remain far from expounded for the vast majority of these, and their precise roles remains poorly understood[54]. While novel or less studied transcripts may be of significance, Table 1 contains putative lncRNA involved in CVDs that most likely will be subject to further investigations in the near future.

Table 1: List of lncRNA involved in tissue development and organogenesis

From chapter 6, "Long Noncoding RNAs in Mammalian Development and Diseases" in Long Non Coding RNA Biology[49], highlighting table section of tissue development and organogenesis:

Cardiac development			
lncRNA	**Expressed in**	**Function**	**Refs**
aHIF	Human	Associated with cardiac pathology (Hypoxia-inducible factor 1A antisense RNA)	[68]
Kcnq1ot	Human	Involved in cardiogenesis	[69]

		Regulates chromatin reorganization at imprinted loci	
ANRIL	Human	Involved in atherosclerosis, carcinomas, and inflammatory response Interacts with CBX7 of PRC1 complex (antisense noncoding RNA in the INK4 locus)	[70; 71]
SENCR	Human	Regulation of endothelial differentiation from pluripotent cells Controls the angiogenic capacity of human umbilical vascular endothelial cells (HUVEC) (Cytoplasmic lncRNA)	[72; 73]
LIPCAR	Human	Biomarker for myocardial infarction (Mitochondrial lncRNA)	[74]
CARL	Human	Inhibits anoxia-mediated mitochondrial fission and apoptosis Acts as mir-539 sponge (Cardiac apoptosis-related lncRNA)	[75]
Mhrt (Myheart)	Human Adult heart	Protects against cardiomyopathy A chromatin-remodeler and antagonizes Brg1 (myosin heavy-chain-associated RNA transcript)	[76]
MIAT	Human	Regulates diabetes mellitus-induced microvascular dysfunction Regulates expression of vascular endothelial growth factor and miR-150-5p (myocardial infarction-associated transcript)	[77-80]
Braveheart	Mouse Cardiac cells	Regulates cardiovascular lineage commitment Epigenetic regulator that interact with Suz12	[81]
Fendrr	Mouse	Involved in differentiation of multiple mesenchyme-derived tissues Associates with PRC2	[82; 83]

2 Methods

2.1 Search strategy

Both systematic and non-systematic search strategies were employed in this project thesis. Refining the search topic into a single answerable question might not be appropriate for this project as it aims to elucidate current knowledge on lncRNA, and explore current and future possibilities with lncRNA. Furthermore, the topic is a relatively new frontier of research. A less constrained search strategy can be desirable in this case, increasing overall search sensitivity. Explorative "predictor finding studies" are of interest in this project, and it brings upon challenges in the search and filtering phase, as current generic search filters are found to be not very useful in researching these kinds of studies.[84; 85].

Table 2: PICO table

Patient/Problem	Intervention	Comparison	Outcome
All patient groups	lncRNA as biomarker	Traditional biomarkers, diagnostically and prognostically	Feasibility compared to traditional biomarkers

In the formulation of key questions, "PICO" is a commonly used method in conducting literature searches for evidence, guiding the scope of research for systematic reviews[86-88]. PICO was used in this project thesis (Table 2), as some questions in the project thesis are of clinical nature. The framework was overall fairly useful in framing and clarifying the problem of interest, as well as limiting the search.

2.1.1 Databases and criteria

Databases used in the search are McMaster PLUS[89], Cochrane Library [90] and PubMed[91].

Predefined search words: "lncRNA" and "cardiovascular disease".

Criteria for inclusion: No formal quality assessment tool was used. Publications were eligible if:

1) it is a review article or meta-analysis;

2) investigating long non-coding RNAs in regards to cardiovascular diseases;

3) include trials in human patients in the base material

Major exclusions were:

1) publications in language other than English;

2) duplicated publication

3 Results

3.1 Search results and included articles

McMaster Plus search yielded no summaries, synopses of synthesis, syntheses or synopses of studies based on their 6S Pyramid model. Only non-appraised references are listed.

Cochrane Library search yielded no reviews and 2 controlled trials (Table 3).

Table 3: Cochrane Library (04.01.2019) search results

Authors	Title	Action	Ref
Gao et al. (2016)	"Long Noncoding RNAs and Their Regulatory Network: potential Therapeutic Targets for Adult Moyamoya Disease"	Included	[92]
Zhao et al. (2018)	"Expression profiles of long noncoding RNAs and mRNAs in peripheral blood mononuclear cells of patients with acute myocardial infarction"	Excluded, duplicate in PubMed search	[93]

To specify the PubMed search it was built using confirmed MeSH terms from the National Institutes of Health (NIH) MeSH Browser in the advanced search.

Table 4: PubMed (04.01.2019) search results:

Search details	Restriction	Total number of articles	Included
rna, long noncoding AND cardiovascular diseases	none	619	
rna, long noncoding"[MeSH Major Topic] AND "cardiovascular	Search filter: Review article or systematic review	62	9

diseases"[MeSH Major Topic]			
rna, long noncoding"[MeSH Major Topic] AND "cardiovascular diseases"[MeSH Major Topic]	Meta-analysis	3	3

Initial search with the predetermined search words yielded 619 articles. Restriction the search using the PubMed's search filtering using the input: *" rna, long noncoding"[MeSH Major Topic] AND "cardiovascular diseases"[MeSH Major Topic] AND Review[ptyp]"*, reduced the number of articles down to 62, out of which 3 were meta-analyses (Table 4). These 3 articles satisfied the inclusion criteria in 2.1.2 and were all included for investigation (Table 6).

Articles from the remaining 59 articles were then non-systematically selected according to their coverage on lncRNA and CVD, using the criteria for inclusion in chapter 2.1.2 "Databases and criteria" as a general framework, with an emphasis on atherosclerotic heart diseases. Main articles serving a foundation for the project thesis are listed in Table 5.

Table 5: Review articles

Authors	Article title	Main topic	Ref
Liu et al. (2017)	Emerging roles and mechanisms of long noncoding RNAs in atherosclerosis	Atherosclerosis (AS)	[94]
Zhang, Z et al. (2018)	Long Noncoding RNAs in Atherosclerosis: JACC Review Topic of the Week.	Atherosclerosis (AS)	[54]
Wang, Y et al. (2018)	Long non-coding RNAs in coronary atherosclerosis.	Atherosclerosis (AS)	[95]
Yu et al. (2018)	Long Noncoding RNAs: New Players in Ischaemia-Reperfusion Injury	Ischemia-reperfusion injury	[48]
Zhang, HN et al. (2018)	Endothelial dysfunction in diabetes and hypertension: Role of microRNAs and long non-coding RNAs	Endothelial dysfunction in diabetes and hypertension	[96]

Xu, Z et al. (2018)	Angiogenic lncRNAs: A potential therapeutic target for ischaemic heart disease	Ischeamic heart disease (IHD)	[97]

Table 6: Meta-Analyses

Meta-analysis	Polymorphism of interest	Calculated association	Patient material	Patient group numbers	Population
Huang (2014) [98]	*ANRIL* (*CDKN2B-AS1*) SNP rs4977574	CAD: risk of CAD (P<.0001, odds ratio [OR]=1.27, 95% CI 1.22–1.31)	Case-control	36,452 cases and 39,781 controls	Asian and Caucasian
Xu, B (2018) [99]	*ANRIL* polymorphism rs4977574	CAD: allelic model (OR=1.18, 95% CI 1.04–1.34, P=.010), the dominant model (OR=1.28, 95% CI 1.13–1.44, P<.0001), the recessive model (OR=1.27, 95% CI 1.01–1.60, P=.04),the homozygous model (OR=1.46, 95% CI 1.15–1.86, P=.002), and the heterozygous model (OR=1.17, 95% CI 1.07–1.28, P=.0004)	Case-control	5683 CAD patients and 6322 controls.	Asian
Wang, P (2016) [100]	*ANRIL* polymorphism rs2383207	CAD: associated with a significantly increased risk of CAD (OR=1.47; 95% CI 1.33–1.62), including Caucasians (OR=1.51; 95% CI 1.28–1.77) and Asians (OR= 1.42; 95% CI 1.26–1.61).	Case-control	6796 cases and 9956 controls	Asian and Caucasian

The meta-analyses (Table 6) all identified significant association between investigated SNPs (rs4977574 and rs2383207) and coronary artery disease (CAD) in Asian and Caucasian populations. Certain alleles displayed higher susceptibility of both CAD and myocardial

infarction (MI). These meta-analyses utilize a relatively large base material, with combined patient and control populations totaling between 12005 and 76233, but are heavily based on genome-wide association studies (GWASs), and supplementary functional studies are needed to provide further evidence to their value in predicting CAD, as some of the authors also suggests[99].

Gao et al.[92] (Table 3) studied long noncoding RNA expression profiles in moyamoya disease (MMD) based on samples from peripheral blood. They identified multiple signaling pathways that are closely associated with immune response, vasculogenesis and smooth muscle contraction to interact with lncRNAs regulatory mechanisms. Microarrays were initially used to get expression profiles, and confirmed using real-time polymerase chain reaction (RT-PCR). Molecular function was predicted using bioinformatics database analysis. The mitogen-activated protein kinase (MAPK) signaling pathway, well studied for the treatment of many other cardiovascular diseases, was especially identified as a core regulatory pathway in MMD targeted by lncRNAs.

4 lncRNAs associated with cardiovascular disease

Table 7: lncRNA associated with cardiovascular diseases, from chapter 6: "Long Noncoding RNAs in Mammalian Development and Diseases" in Long Non Coding RNA Biology[49]:

Cardiac diseases and disorders	**lncRNA**	**Refs**
Heart failure	*Mhrt*	76
Cardiac hypertrophy	*CHRF*, *Novlnc6*	101
Myocardial infarction	*MIAT*, *LIPCAR*	74; 77
Spectrum of cardiac disorders	*FENDRR*, *Braveheart*, *CARL*, *KCNQ1OT*, *MALAT1*	76
Blood and circulatory system disorders and syndromes		
Atheromatosis and atherosclerosis	*ANRIL*	102

Specific lncRNAs have been found to be associated with certain CVD states (Table 7). Many of the transcripts only have associations through genome-wide association studies (GWAS), while some have been further investigated, e.g. either using bioinformatics analysis or immunoprecipitation techniques to identify their interactions. At length, transcripts may be subjects to in vitro or in vivo knockdown studies, or investigated as potential novel markers.

In mouse, ***Bvht*** *or "Braveheart"* regulates certain core cardiovascular gene networks and is necessary for functions of MesP, a transcription factor needed for epithelization and development of cardiac mesoderm. Bvht mediates epigenetic regulation required for cardiac commitment in differentiation, through interaction with SUZ12 protein of the PRC2 complex[81]. Bvht promotes the differentiation of murine MSC to cardiogenic phenotype in vitro[103]. A human lncRNA orthologue has not been found for *Bvht*.

MHRT (Myosin Heavy Chain-Associated RNA Transcript) seem to be part of a cardioprotective pathway (Mhrt-Nrf2 pathway), possibly via upregulation of the transcription factor NFE2L2, leading to inactivation of proapoptotic proteins. The regulation is through prompting combination of Nrf2 promotor and H3 histone, thus enhancing Nrf2 gene transcription and Nrf2 protein expression. Initial studies of *MHRT* by Han et al., 2014[76] identified several transcripts differing in size (from 709 to 1147 nt), and some of them were especially downregulated following transaortic constriction (TAC) models, concomitant with the induction of cardiomyopathy. They propose the mechanism lncRNAs cardioprotective

mechanisms to be through antagonization of the chromatin-remodeling factor Brg1, usually upregulated in stress conditions. ChIP was used to confirm transcription factors occupying *MHRT* promotor in stressed hearts. In a heart failure (HF) model, its levels correlated with the cardioprotective hormone obestatin, and the silencing of *MHRT* reversed the protective effects of administered obestatin[104]. A different study measured circulating MHRT at significantly higher levels in patients experiencing HF[105], and also identified another transcript, ***NRON***, which was found to have an even higher increase difference at 3.17 ± 0.30 *versus* 1.0 ± 0.07 in non-HF subjects, ($P < 0.0001$). They propose *NRON* as a novel biomarker in heart failure, and using ROC analysis to evaluate its predictive power, find it comparable to NT-proBNP, with an AUC of ROC curve of 0.865 vs 0.844 of NT-proBNP.

KCNQ1OT1 (KCNQ1-Overlapping Transcript 1), is an unspliced intronic antisense lncRNA from the opposite strand of *KCNQ1*. The CDKN1C/KCNQ1OT1 domain contains genes that are expressed from parent-of-origin manner, including *KCNQ1* that codes for the primary subunit of a cardiac cell potassium channel[106]. *KCNQ1OT1* interacts with chromatin and regulates transcription of multiple gene targets in the domain, including *KCNQ1*, likely through recruitment of H3K9- and H3K27-specific histone methyltransferases G9a and PRC2 complex members[107]. In chronic diabetes mellitus, *KCNQ1OT1* is found to be part of the caspase-1/TGF-β1 pathway important in pyroptosis (programmed cell death associated with inflammation), involved in the pathogenesis of MI[108; 109] and progression of diabetic cardiomyopathy[110; 111]. In this situation, the mechanism of KCNQ1OT1 seem to be through acting as a competing endogenous RNA to regulate the expression of caspase-1 by sponging the microRNA miR-214-3p[112]. The silencing of KCNQ1OT1 represses the TGF-β1 pathway and in cardiac fibroblasts, and may present as a novel therapeutic target, as previous studies have shown that inhibition of pyroptosis can improve cardiac function in diabetic mice[111; 113-116]. *KCNQ1OT1* expression was significantly upregulated in an atrial fibrillation (AF) model, and its knockdown suppressed the progression of AF by regulating the proposed miR-384/CACNA1C axis[117], with CACNA1C being a previously shown biomarker for AF.

4.1.1 *MALAT1*

In humans, *MALAT1* is highly expressed in most cell types[118], and sometimes even more than many housekeeping genes in certain cells[119]. *MALAT1* starts as a precursor transcript that is stabilized with a 3' triple helical structure, by ribonuclease P cleavage of a small tRNA-like

ncRNA from its 3' end. The mature transcript lacks a poly(A) tail, and is retained in the nucleus, localized to nuclear speckles, domains involved in pre-mRNA processing[106]. Here it regulates levels and activity of SR splicing factors, modulating alternative splicing of a subset of pre-mRNAs[33]. *MALAT1* also facilitates spatial movement of growth control genes by binding Polycomb 2 proteins (Pc2), relocating it from repressed to active environments[120]. *MALAT1* seems required for proper G1/S checkpoint transition and mitotic progression. Transcript depletion results in arrest of cell cycle, and leads to activation of p53 and its target genes, indicative of p53 being a major downstream mediator of MALAT1 activity[121].

In regards to CVD development, upregulation of MALAT1 may recruit PRC2 to the promoters of anti-inflammatory genes and epigenetically repress these targets, which might subsequently allow for increased transcription of inflammatory genes. Another pathway is also proposed, where MALAT1 directly interacts with inflammatory genes to invoke an inflammatory response.[122] *MALAT1* interacts with signal transduction pathways of apoptosis involving the microRNA miR-145 in myocardial I/R injury. Upregulated in response to oxidative stress, miR-145 is important in protection of cardiomyocytes from apoptosis[123; 124]. The role of MALAT1 in this pathway thought to be sponging of miR-145 (mechanism as shown in Figure 4H), reducing the microRNAs effective expression and cardioprotection. Additionally, overexpression of MALAT1 attenuates cardioprotective effect of fentanyl, shown in I/R injury models[125]. Fentanyl is a potent opioid agonist drug with cardioprotective effects[126-128], and also shown to reduce infarct sizes in mice[129; 130]. A cardioprotective mechanism of fentanyl is likely by inducing downregulation of MALAT1 and upregulation of miR-145[125].

4.1.2 *MIAT*

MIAT (Myocardial Infarction Associated Transcript) is a spliced transcript from a susceptibility locus for myocardial infarction (MI), and seems to regulate several microRNA involved in atherosclerosis (AS) development, mainly through sponging, with high levels of MIAT contributing to disease progression. It may possibly also upregulate the TGF-β signaling pathway, found at significantly higher levels in diabetic retinopathy patients compared to healthy controls[113]. Regulation of this pathway is noteworthy as substantial evidence indicates TGF-β as a key modulator of vascular repair and that dysfunctions in this pathway promote a pro-inflammatory, pro-fibrotic and pro-atherosclerotic environment[131; 132].

Initially, investigations at RIKEN found that six SNPs in its locus, showed markedly significant association with MI (chi2=25.27, P=0.0000005) in a study of 3435 affected individuals versus 3774 controls[77].

The sponging effect of MIAT on miR-149-5p, inhibits efferocytosis, the removal of apoptotic cells by macrophages in advanced atherosclerosis, through CD47 upregulation in macrophages. MIAT levels were markedly elevated in the serum of patients with symptoms of vulnerable atherosclerotic plaque and in atherosclerotic mouse models. In the mice, increased levels of MIAT in macrophages of necrotic cores were also identified. Knockdown model of MIAT through MIAT-shRNA delivered with an adenoviral vector also reduced the progression of atherosclerosis and necrotic core size, while increasing plaque stability in vivo.[133]

MIAT expression is found to be upregulated in serum of atherosclerotic patients, whilst microRNA miR-181b was downregulated. In cell culture model of ox-LDL stimulated atherosclerosis (AS) cells, MIAT has a pro-proliferative and anti-apoptotic effect.[134] Relation between *MIAT* and miR-181b was also investigated, suggesting its sponging effect upregulated STAT3 expression, a transcription factor and target of miR-181b. STAT3 is previously known as a mediator in chronic inflammatory diseases[135-137], upregulated in inflammatory human atherosclerotic lesions, and contributing to fatty streak formation[138]. *MIAT* accelerates the proliferation of cells involved in atherosclerotic formations, while its knockdown induces apoptosis in human aortic vascular smooth muscle cells (HA-VSMCs) and may reduce progression. Altogether, these studies expose possible lncRNA pathways in atherosclerotic disease, with mechanisms such as miRNA sponging, presenting *MIAT* as a potential novel therapeutic target in atherosclerosis.

4.1.3 *ANRIL / CDKN2B-AS1*

CDKN2B-AS1 (Cyclin-dependent kinase inhibitor 2B antisense RNA 1) is an antisense lincRNA commonly referred to as ***ANRIL*** (antisense non-coding RNA in the INK4 locus) in lncRNA studies[70; 71; 99; 100; 102; 139-143]. The INK4 locus on chromosome 9p21 is a genetic susceptibility locus for CVD[71; 102; 139], even when adjusted for traditional CVD risk factors[144]. Several SNPs in this locus are associated with CVD, and they are found to mainly influence expression of *ANRIL*[145]. The transcript is found in pathways of metabolism[143] and inflammation[142], and is also found to be associated with hypertension[146].

The 3 meta-analyses (Table 6) studying SNPs (rs4977574 and rs2383207) in *ANRIL* show significant association with certain variants and coronary artery disease (CAD) in both Asian and Caucasian populations. Wang et al. also found that the rs2383207 polymorphism also influenced myocardial infarction (MI) risk (OR=1.75; 95% CI, 1.24-2.47)[100].

The main mechanism of action for *ANRIL* is through regulation of *CDKN2A* and *CDKN2B* expression, by binding to components of polycomb repression complexes 1 and 2 (PRC1 and PRC2) and mediating transcriptional repression by H3K27 trimethylation (general mechanism as shown in Figure 4A)[95; 147], epigenetically silencing these tumor suppressor genes.

ANRIL can affect the proliferation and cell growth of VSMC and fibroblasts by modulating *CDKN2B* expression[145; 148]. Animal models for coronary artery disease (CAD) show that overexpressed ANRIL through its methylation of *CDKN2B* and reduces VSMC proliferation, possibly by inhibition of the p53 pathway[140; 149].[148] Expression of the genes involved in glucose and fatty acid metabolism such as *ADIPOR1*, *VAMP3*, and *C11ORF10* have also found to be regulated by ANRIL in a time-dependent manner[143].

In hypertensive patients, SNP alleles in the ANRIL locus were found at significantly higher frequencies[146]. Risk for atherothrombotic and hemorrhagic stroke and recurrences were found in a large prospective study to be higher in patients with certain SNP alleles, especially the rs10757278GG, correlating with *ANRIL* expression, [141]. Within inflammatory modulation, *ANRIL* has also been shown to be part of the NF-κB signaling pathway[142]. Through activation of this pathway it has also been shown to promote angiogenesis by upregulating VEGF in a model of diabetes complicated by cerebral infarction[150].

Investigation of lncRNA from patients with MI treated by primary percutaneous coronary intervention (PCI) revealed 5 lncRNAs with especially associated with cardiac pathology, as found by Vausort et al.[68]. ANRIL and KCNQ1OT1 were found to be most significantly lowered, and their levels were found to be univariable predictors of left ventricular dysfunction. They could also correctly reclassify a significant proportion of patients misclassified by a current multivariable clinical model. Lower levels of ANRIL (and KCNQ1OT1) were also found in STEMI compared with NSTEMI ($p<0.001$). In all, expression levels of the lncRNAs selected for investigation were either found up- or downregulated in peripheral blood cells of patients with acute myocardial infarction.

ANRIL seems to play an integral part in CVD, being involved in many pathways central in CVD development, including metabolic, inflammatory and differentiation of key cells. The transcript is one of the few lncRNA that has been explored in clinical studies. Being measurable in blood samples, enables its utilization in diagnostic and prognostic approaches that are relatively non-invasive.

4.1.4 *LIPCAR*

LIPCAR (long intergenic noncoding RNA predicting cardiac remodeling) is a circulating lncRNA of mitochondrial origin associated with myocardial infarction (MI)[151]. The mechanisms of LIPCAR is still uncertain, but it is thought to regulate mitochondrial pathways such as oxidative phosphorylation[74]. While the precise function is unknown, leakage of this lncRNA into the circulation following MI similarly to cardiac marker proteins enables its usage as a biomarker.

A study by Kumarswamy in 2014 shows LIPCAR levels can predict death in patients with HF after anterior myocardial infarction. Circulating numbers are low shortly after MI, but elevated in advanced disease. LIPCAR levels independently predicts STEMI, and improves on current prediction models. Lower levels of LIPCAR found after MI are prognostic for patients experiencing future remodeling. For patients with chronic HF, levels are elevated, higher even than those experiencing left ventricular remodeling, consistent with their more advanced disease[74]. A more recent study of similar design (by Li et al) find similar expression patterns, encouraging its possible application as a novel biomarker. To account for the observed increased MI risk, they propose possible linkage between higher LIPCAR expression and the promotion of coronary lesion formation[152]. Subclinical cardiac changes and dysfunction may also be predicted in patient groups with elevated risk for future major adverse cardiovascular events, as demonstrated in patients with well-controlled type-2 diabetes using LIPCAR as a biomarker[153].

5 Discussion

5.1 On the functionality of lncRNAs

Considering the functionality of ncRNAs historically has largely been debated[154], this chapter will give some insight into points commonly brought up in the reasoning for their function (or non-function), perhaps valuable in incitation for further investigation of lncRNAs significance in biology and disease.

For instance, regarding their functionally, one may allude to their low expression or short-livedness to infer non-functionality, and conclude that they simply are transcriptional noise that the cells are trying to get rid of. As for the low expression being reflective of non-function: For mRNA, high levels may be necessary in sufficient levels to produce required quantities of protein, e.g. for core structural or metabolic functions, regularly found in most types of cells[155]. Conversely, only small amounts of ncRNA may be required for regulatory signals to trigger cascades of significant downstream effects, similarly to the mode of transcriptional factors or hormones[156]. lncRNA may also interact directly with specific gene loci, and in this circumstance the minimal required amount may be at a ratio as low as 1:1 per allele (1-2 copies per cell). Investigations so far find lncRNAs to commonly interact with transcription factors and other global regulators, supporting this rationale[157-160].

As for the effects of diffusion on top of the already low expression, regulatory lncRNA remain and act locally, a trait common for most lncRNA[42; 52; 70], minimizing significant limitations attributable to diffusion. In regulation of differentiation and development, seemingly low expression may also be observed due to a dimension of such settings, namely strictness in spatial and temporal placement. In murine brain, a large fraction of long ncRNAs (849 out of 1328 examined by Mercer et al.) are expressed in particular cells in hippocampus, cortex or cerebellum[45]. However, taking the whole brain into account, they end up accounting only for a small portion of all the transcripts. This may give impression of overall low levels in specific tissues or the organism as a whole.

Non-coding RNAs may be rapidly degraded[161-163], which may be used to suggest the cell is actively trying eliminate them[164]. However, while mRNAs might need to persist for a sufficient period to be exported and translated multiple times, nuclearly localized and acting lncRNAs can exert their functions immediately. Indeed, many of lncRNA functions are of

recruitment variety and do not need to persists after engagement between targets, like the epigenetic silencing effects of suppressors like *ANRIL*, resulting in heterochromatin formation, persisting even after the lncRNA is subsequently inhibited or removed[165]. Such triggering mechanisms to induce responses need only be transient and do not require large amounts to achieve. Moreover, this may represent an intrinsic advantage of RNA regulation given that RNA signals can both be produced and eliminated swiftly, providing efficient and dynamic changes to the system. E.g. in non-coding transcripts induced by DNA damage, where they recruit and locally activate RNA-binding proteins[166].

Many challenges arise in the quest of clarifying lncRNAs functions, and as some questions are answered, new road blocks arise, some of which will be presented in the coming chapters.

5.2 Challenges in studying lncRNAs

There are inherent limitations and technical noise associated with methods currently in use, while methodologies for annotation lack an established standard[54]. Integration of information from several platforms and biological conditions seem necessary to effectively categorize lncRNA transcripts[41], and such integration may be crucial to validate real and biologically-meaningful properties of lncRNAs, and grouping of them based on shared features.

Reduction of noise and bias in the information gathering is necessary for meaningful integration. With currently abundant technologies, there are limitations in mapping and quantifying long, non-polyadenylated and low abundance transcripts at single-cell level. Fourth-generation single-molecule sequencing technologies such as nanopore sequencing-based methods may answer this need, enabling direct and ultra-long reads, without the need of prior chemical labeling or PCR amplification[167-169]. While this may address quantification issues of lncRNA, proper investigation of molecular functions remains as an obstacle, which may be important in identifying targets for therapeutic strategies.

CRISPR (clustered regularly interspaced short palindromic repeats) were first discovered as part of the defense against invading phages and plasmid DNAs in prokaryotes[170]. Several CRISPR/Cas (CRISPR-associated protein) systems exists, with the type II system consisting of two components in its simplest form: the Cas9 nuclease enzyme and a single guide RNA (sgRNA) directing Cas9 to its target DNA site. Cas9 can induce double-stranded breaks (DSBs), and lead to gene inactivation of genes through non-homologous end joining (NHEJ),

or introduction of donor DNA through homologous recombination. The sequence-specific manner which Cas9 can bind and cleave DNA, provides a powerful genome editing tool and has been employed in genomic studies and model organisms over the last six years[171; 172]. There are also other Cas forms that can interfere with either transcription initiation or elongation to reduce transcription, or even induce an increased expression of target genes, used in CRISPRi (CRISPR interference)[173] or CRISPRa (CRISPR activation)[174] strategies. Tiling arrays using CRISPR-Cas technologies have been demonstrated as unbiased approaches in mapping functional regulatory regions in the genome[175], and methods using CRISPR have been developed and shown to be able to map functional domains of lncRNA, in an unbiased fashion and with potential of being high-throughput[176; 177], adding to the toolbox for investigations of lncRNA functions.

Related to intrinsic characteristics of ncRNAs, a challenge appears in the lack of sequence conservation between species. Whether in context of disease causing mutations or orthologous conservation, preservation of base pairing properties appear more important than that of nucleotide sequence, since reciprocal mutations can maintain the same secondary structure[118]. Thus, overall usefulness and transferability of animal studies is somewhat diminished. While RNA structure also play important roles in some facets of mRNA metabolism, the relatively clear linear logic of the genetic code allows precise delineation of functional domains of protein-coding transcripts into untranslated regions (UTRs) and open reading frames (ORFs) via in silico analysis of primary sequence[41]. Such analysis has proved to have tremendous predictive power, and may be considered a cornerstone in molecular biology. On the other hand, the process of prediction and validation of biologically meaningful secondary structures in lncRNAs is very challenging. While functional prediction of proteins and miRNA by looking at their homology domains or target sites is possible, lncRNA functions are not tied to their sequence in the same manner. In addition, the longer the transcript, the more possibilities exist and predictions can become less reliable. Interpretation of sequence conservation, or largely lack thereof for most lncRNAs, is thus made exceedingly ambiguous since the conservation mainly lies at the level of RNA structure rather than that of primary sequence[178].

Reverse-genomics might thus be especially important in further analysis of lncRNA. Knock-in or knockout studies examining phenotypic effects can give insight in specific lncRNA functions. A challenge however lies in the lack of currently known functional domains in

lncRNAs, and thus investigations should aim to include entire lncRNA loci[41]. This however, introduces technical and interpretation challenges because many lncRNAs are very long, and may contain repeats, as well as overlap with other genomic elements and protein-coding genes. Knockdown can be achieved by RNA interference or antisense oligonucleotides (ASOs), and these methods have advantages in not interfering with transcription or promoter activity. However, they have limitations in incomplete depletion of transcripts, partly due to the nuclear localization of lncRNA, and potential off target effects[179]. CRISPR/Cas9 mediated gene knockout strategies have also been successfully been used to deplete genomic transcription of specific lncRNA and addresses some of these issues by providing highly specific ablation of desired genes[180].

5.3 In diagnostic strategies

The journey from discovery to meaningful clinical use for a biomarker can be lengthy and strenuous. Take the Bence Jones protein, a marker now in routine diagnostic test for multiple myeloma, though taking 140 years from discovery to FDA approval[181]. A biomarker needs to fulfill several conditions in order to be useful in the clinic. Does the biomarker: Provide reproducible results; have the ability to distinguish between patient groups; have outcomes change based on their usage; or improve cost-efficiency in reducing either risk or cost? In addition, psychological or ethical implications may need to be considered. To truly be valuable, a biomarker needs to provide clinically relevant information outside of what is currently available, or improve on patient risk or financial cost.[182] Proof of concept needs first to be established, which has been done in human patients for *ANRIL* and *LIPCAR*, but lack any extensive clinical trials to compare their predictive power against traditional biomarkers, or investigating outcomes based on their usage[68; 74; 152]. As found in this project, so far, the only meta-analyses so far comprises studies of CVD association with certain genetic variants (Table 6).

LncRNAs do offer some advantages to traditional markers. E.g. having been found to be more stable compared to protein markers in circulation[183; 184]. Yang et al. credits stability of one such circulating lncRNA to its localization in extracellular vesicles (EVs), likely originating from monocytes[185]. An additional advantage, proposed by Xuan et al.[105] is the high sensitivity of methods used, like real-time PCR.

For diseases outside of the CVD sphere, the lncRNA PCA3 (Prostate Cancer Antigen 3) already exists as a clinically used marker in prostate cancer[8; 11; 186; 187]. It is used in combination with the widely used PSA (Prostate-Specific Antigen), correlating with tumor aggressiveness and grading. Improving diagnostic accuracy, PCA3 score outperforms PSA alone in predicting biopsy diagnosis of prostate cancer, reducing indication for biopsy to avoid overdiagnosis and overtreatment[188]. As for lncRNA associated with CVD, studies are currently sparse, and whether lncRNAs have significant diagnostic value in CVDs to justify their implementation, needs to be validated with further investigations.

5.4 In therapeutic strategies

LncRNA transcription is normally tightly regulated, but may be dysregulated in disease[48; 189]. Therapeutic strategies that involve lncRNA revolve around modulation of their levels. Knockout or knockdown of overly abundant expression has been widely implemented in murine models so far, but loss-of-function cases may also be treated by introducing gene product[48]. Transfection of lncRNA in murine models can decrease myocardial infarction sizes in response to ischemia-reperfusion (IR) injury by interaction with specific miRNAs and inhibit mitochondrial fission and apoptosis in vivo in mice[75; 190].

On the other hand, knockdown can be achieved with RNA interference, using miRNA transfection to mediate silencing of lncRNA[191]. Antisense oligonucleotides (ASOs) may also be utilized for inhibition of lncRNAs. Treatment by ASO-based silencing has been successfully used in mouse models of Angelman syndrome, partially ameliorating associated cognitive deficits[192]. Additionally, there are methods employing “GapmeRs”, ASOs containing a stretch of DNA complementary to its target and flanked by blocks of 14-16 locked nucleic acids, forming a stable DNA:RNA heteroduplex to elicit ribonuclease H (RNase H)-mediated target degradation[193]. In transverse aortic constriction (TAC)-operated mice, GapmeR-mediated silencing of the lncRNA *Chast* was able to attenuate pathological cardiac remodeling[194].

Delivery may be achieved with several available viral and non-viral approaches, such as polyplexes, lipoplexes, peptide- or protein based systems[195]. A whole range of viral vectors may be implemented in delivery systems including retroviruses, adenoviruses, adeno-associated virus (AAV) and herpes simplex, but they are not always able to carry the large

load that lncRNA usually represent[196]. Numerous diseases are caused by mutations in genes with a gene coding sequence (CDS) exceeding 3.5kb[197], whilst many lncRNA have a mean length of 10kb[23], with some lncRNA genes and transcripts exceeding 100 kb[198]. Most viral vectors are limited to less than 10kb, while some have capacities over >30kb[196], with variability in immune response and toxicity. AAV is attractive as a vector due relatively low immunogenicity, but has a limited packaging capacity of ~5kb[197]. Efforts in addressing such issues include engineering of dual AAV vectors[199; 200]. No single universal vector seems suitable for the treatment of all indications[196]. This might also be the case for strategies involving lncRNA, in part due to their heterogeneous mechanisms and functions. While level of cell specificity and potential side effects are concerns with these methods of delivery, safety is improving, and even modest predictions suggest viral vectors to be attractive delivery vehicles in upcoming gene therapy strategies[196].

The CRISPR/Cas9 system can handle large genetic cargos and may be part of the solution to these challenges, and has been already been demonstrated to be able to introduce or delete lncRNA that modifies disease[201]. Practical use of the powerful tool may however be limited by abhorrent risks such as of deregulation of neighboring genes, which are possibly unavoidable. Estimated safe loci amounts to less than half of known lncRNA, as shown in a genome-wide analysis.[171] This also impacts reverse-genomics studies investigating lncRNA functions, as phenotypical change might be due to changes in neighboring or overlapping genes, rather than gain or loss of lncRNA function.

6 Conclusions

To a great extent, the diverse functions of lncRNAs are only clarified to varying degree. While the process of investigation itself presenting many challenges such as the lack of methodological standards for data analysis and cost efficiency, these obstacles are anticipated to be transitory for the most part. Intrinsic hurdles rooted in the nature of ncRNAs themselves add another layer of difficulty, like their mode of function and lack conservation between species. While the substantial catalogue of lncRNAs remain far from fully expounded, those with putative biological importance or possible key players in disease have had many aspects of their workings exposed[36]. However, alongside numerous transcripts still in their initial assessment, their exhaustive analysis is still necessitated.

In the clinical setting, strategies based on lncRNAs are only starting to move from proof of concept to practical implementation in the clinic. Diagnostic tools improving on existing biomarkers already exist in fields outside of heart disease, like s-PCA3 in prostate cancer[186-188]. However, for CVD, no such biomarker has been taken into clinical practice as of yet[95], with the amount of clinical studies being presently sparse. While concept proving studies for human application as biomarkers already show some promising results, many still seek to clarify lncRNA functions via in vitro or animal models. Therapeutic strategies have approaches shown to work in animal models, but have challenges regarding transferability. Besides, unanswered questions exist regarding possible toxicity or unwanted side effects associated with changes to lncRNA expression levels, the lncRNA gene loci, or with the delivery methods themselves.

Our evolving understanding of the lncRNAs are simultaneously dependent on and enabled by development in future prediction tools and systems of investigation and classification. Moreover, improvements in gene therapy delivery systems will be necessary if lncRNAs are to be taken advantage of in eventual upcoming therapeutic strategies. Both apparent and validated biological and clinical significance attributed to lncRNAs are being increasingly recognized, accordant with the effort and frequency of studies currently observed. lncRNAs will likely become progressively more relevant in the clinic[7; 202-205], including in the area of cardiovascular diseases (CVDs)[96]. However, although they may appear as attractive targets, many hurdles still exist in the course to their implementation in diagnostic and treatment strategies.

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