7-23-2021 2:00 PM

Genetic Approaches for the Study of Complex Human Diseases

Julieta Lazarte, *The University of Western Ontario*

Supervisor: Hegele, Robert A., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biochemistry

Abstract

The field of human genetics has evolved from its initial narrow focus on single-gene Mendelian disorders, which largely affect children, to our current understanding that for most diseases there is continuum of rare to common variants which can exert a range of phenotypic effects. Despite advances in sequencing capabilities and our overall understanding of diseases, there remains a large proportion of heritability unexplained. Through the use of next-generation sequencing technologies and DNA microarray, I have explored a spectrum of genetic variations from rare, single and structural variants to common variants in individuals with i) "lone" atrial fibrillation; ii) familial hypercholesterolemia; and iii) familial partial lipodystrophy. From my research efforts, we implicated rare loss-of-function variants in cardiomyopathy genes to "lone" atrial fibrillation, providing evidence that atrial cardiomyopathy is a genetic sub-phenotype of atrial fibrillation. Additionally, we determined that "lone" atrial fibrillation has a significant accumulation of common variants that together elevate susceptibility to the disease. Also, considering the application of genetics in Medicine, I directly evaluated the increasing responsibility that clinicians have to adjudicate causality of various genetic factors. For instance, having successfully identified a novel apparently pathogenic genetic variant in a family with hypercholesterolemia, I sought to determine its pathogenicity by performing cascade screening and co-segregation analysis in the extended family. My analysis demonstrated that the novel variant was independent of the disease phenotype, preventing a potential misdiagnosis and emphasized the importance of gathering additional confirmatory data in the clinical setting. Further, by studying a well-genotyped and phenotype familial partial lipodystrophy cohort, I uncovered that the prevalence of severe hypertriglyceridemia and its most severe complication, namely acute pancreatitis was more common in affected individuals who had concurrently developed diabetes. In spite of these contributions, significant work remains to explain the full genetic contributions to complex diseases. The benefits of understanding the complete genetic architecture of a disease are potentially immense, allowing advances in pre-symptomatic detection to the development of novel targeted therapies. For the patients this could translate into such benefits as earlier detection, screening for the family, personalized therapies, and a confirmed diagnosis.

Keywords

Genetics, complex diseases, atrial fibrillation, rare and common variants, next-generation sequencing (NGS), familial hypercholesterolemia, lipodystrophy.

Summary for Lay Audience

Despite great advances in our understanding of the role of genetics in complex diseases, most patients with a complex disease have no identified genetic cause. This lack of understanding poses significant limitations on the application of genetic testing in the clinical setting. About 99.9% of the genetic code is practically unchanged among humans, however the remainder 0.01% that is variable is a key contributing factor for differences we observe between people, especially related to disease. The field of human genetics has evolved to our current understanding that for most diseases there is a broad spectrum of rare to common mutations that can determine susceptibility to or expression of a disease. To better understand the contribution of different types of genetic variants – or "mutations" - I used several genetic technologies to identify both rare genetic variants in individuals with i) "lone" atrial fibrillation; ii) familial hypercholesterolemia; and iii) familial partial lipodystrophy. The work conducted over the course of my graduate studies determined that rare mutations in cardiomyopathy genes contribute to atrial fibrillation without affecting the ventricles. Additionally, for a significant portion of "lone" atrial fibrillation patients the accumulation of many inherited common variants from across the genome increases their risk for atrial fibrillation. As a future clinician-scientist, I am also interested in the application of genetics in Medicine and how it can improve patient care. During my research, I used pedigree extension to assess the potential causality of a novel apparently pathogenic mutation in a family with hypercholesterolemia, and then demonstrated how feasible and helpful it is for a clinician to perform such additional work to help determine causality by incorporating such data. Lastly, I analyzed a genetically homogeneous group of familial partial lipodystrophy patients and identified a risk factor for a severe complication, namely acute pancreatitis. From my collective research efforts, we now have a better understanding of the different genetic variants or mutations that can cause or increase risk for "lone" atrial fibrillation. Further, careful use of genetics in the clinic has the potential for benefits in medical care from the perspective of both the provider and patient.

Co-Authorship Statement

For all manuscripts listed in my Dissertation, I am the first-listed author and I contributed towards the study's design, performed most, if not all, data analyses, and wrote each manuscript. In each listed review, I wrote more than 85-90% of each manuscript. Dr. Robert A. Hegele (primary supervisor) provided funding, supervision, contributed to study design, manuscript preparation, and critical revision for all manuscripts.

Technical assistance for each research manuscript was provided by core members of the Hegele Lab, including: Adam D. McIntyre, for extracting and isolating DNA from the patient samples under study; Dr. Henian Cao, for preparing the DNA samples for sequencing; Dr. Jian Wang, for assisting in the sequencing and validation of single nucleotide variants and the copy-number variants (CNVs) described in Chapter 2 and Chapter 4; Matthew R. Ban and John F. Robinson, for support in lab and project management; Ericka Simon, for providing the clinical information from the Lipid Genetics Clinic; and Brooke Kennedy, for consenting family members and obtaining their blood samples described in Chapter 4 and maintaining all ethical protocols.

Additional support was provided by other members of the Hegele Lab and collaborators at Western University, including: Dr. Robert Jason, for the atrial fibrillation samples, supervision, contribution to study design, manuscript preparation, and critical revision for the manuscripts in Chapter 2 and 3; Dr. Jacqueline Dron, for guidance and assistance in the analysis of rare and common variants in Chapter 2 and 3; and Dr. Amanda J. Berberich, for contributing the clinical perspective in Chapter 4;.

External collaborators were also instrumental for the completion of many research studies, including: Dr. Zachary Laksman and Janet Liew (University of British Columbia), for contributing atrial fibrillation samples, sharing clinical data and contacting family members in Chapter 2 and 3; and, Dr. Brett Trost and Dr. Stephen W. Scherer (University of Toronto) for accessing the frequency of our CNV in their WES databases described in Chapter 2.

"If I have seen further, it is by standing on the shoulders of Giants."

— Sir Isaac Newton

Dedication

In loving memory of my grandmother Emilia Brotzu.

For my mom and dad,

my brother,

and Vic.

Acknowledgments

Along this journey, I have had the privilege to work and be mentored by an outstanding group of people that have inspired me and helped me achieve all my goals.

All the projects and collaborations that I worked on, the excellent people that I met and the internship that I was able to pursue, would not have been possible if it wasn't for my supervisor Dr. Rob Hegele. I am thankful for his mentorship in the clinic and in the lab, his unwavering support of all the projects/collaborations/internship I wanted to pursue, and ultimately the excellent training environment that he provided. It was truly an honour to have been mentor by one of the best clinician-scientists that I have met.

I would also like to acknowledge, Dr. Jason Roberts, for letting me spearhead the “lone” atrial fibrillation project. Thank you for your guidance at all stages of the project and your constant support even outside this project. I learned a lot from the “lone” atrial fibrillation projects and I am very thankful for that.

Thank you to Dr. Geoff Pickering and Dr. Tony Chan, for providing me with excellent mentorship and support during all our committee meetings. I genuinely value the feedback and support you have provided me. I also want to sincerely thank Dr. Pickering for trusting me to lead a study on a family with aortopathy and learn from the vascular field.

To the members of the Hegele lab: Dr. Jian Wang, Brooke Kennedy, Adam McIntyre, Matthew Ban, John Robinson, David Carter, Jenn Biltcliffe and Dr. Henian Cao, thank you for your guidance and support. Special thanks to Dr. Jian Wang for her technical expertise that made possible many of the findings presented here and to Brooke Kennedy for her guidance consenting family members. I am also very grateful of the outstanding trainees that I was lucky to meet throughout this time. Special thanks to Dr. Jacqueline Dron, for her friendship and guidance while we trained at Robarts and at the Broad. Thanks to Jacqueline, I pursued an amazing internship at the Broad Institute. Lastly, thank you to Dr. Amanda Berberich for her kindness, frank discussions about medicine and guidance in my first research project on the familial hypercholesterolemia family.

I am grateful to the Department of Biochemistry, Barb Green and Lindsay Ralph for providing me with administrative support.

I also would like to acknowledge the unwavering support from my friends, by either helping me disconnect or by problem solving with me. I am forever grateful to all of you for being in my life.

To my family in Argentina: my grandmother, who was so proud I was studying atrial fibrillation, my aunts, uncles, and my cousins, as well as my extended family in Canada, Diana, Richard, Lala y Lalo, thank you very much for being part of my life and always supporting me.

To Victor, I am so thankful to share my life with you, for your constant support, your passion about my work whether in Medicine or in my PhD, and your love.

To my family, mom, dad, and Franky, thank you for your love, sacrifices and support. I have achieved all of this and more thanks to all of you. I love you three and the dogs.

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List of Abbreviations

1KG 1000 Genome Project

AF Atrial fibrillation

ACMG American College of Medical Genetics and Genomics

ASCVD Atherosclerotic cardiovascular disease

CAD Coronary artery disease

CADD Combined Annotation Dependent Depletion

CI Confidence interval

CNV Copy-number variant

CVD Cardiovascular disease

ddNTPs Dideoxynucleotides

eQTL Expression quantitative trait loci

FH Familial hypercholesterolemia

FPLD Familial partial lipodystrophy

FPLD2 Familial partial lipodystrophy, Dunnigan variety

GWAS Genome-wide association study

HDL High-density lipoprotein

HTG Hypertriglyceridemia

Indel Insertion or deletion

LMNA Lamin A/C

LOF Loss-of-function

LDL Low-density lipoprotein

LOD Logarithm of the odds

MAF Minor allele frequency

MLPA Multiplex ligation-dependent probe amplification

NGS Next-generation sequencing

OR Odds ratio

PC Principal component

PCA Principal component analysis

RVAS Rare variant association study

SD Standard deviation

SKAT-O Optimal unified sequence kernel association test

SNP Single-nucleotide polymorphism

SNV Single-nucleotide variant

SVS SNP & Variation Suite

UTR Untranslated region

Chapter 1 Introduction

1.1. Overview

Materials from the following texts with appropriate modifications, were incorporated in Chapter 1:

1. **Lazarte, J.**, Hegele R.A. (2019) "Can one overcome “unhealthy genes”?. *npj Genom Med*. 4: 24. (PMID: 31602315)
2. **Lazarte, J**., and Hegele, R.A. (2020). DNA sequencing in familial hypercholesterolaemia: the next generation. *Eur J Prev Cardiol*. Advance online publication. (PMID: 33623969)
3. **Lazarte, J.**, Hegele R.A. (2020) Can genetic testing help in the management of dyslipidaemias? *Curr Opin Lipidol*. 31(4): 187-193. (PMID: 32520779)
4. **Lazarte, J**., and Hegele, R.A. (2021). Editorial comment: hazards of interpreting genetic reports. *Curr Opin Lipidol* 32, 81-82. (PMID: 33606402)

1.2. Genetic variations

The human genome is composed of approximately three billion nucleotides found within 23 chromosome pairs in the nucleus and a small amount in the mitochondria. Although 99.9% of the genomic sequence is identical between humans, the remaining 0.01% is the main driver for the phenotypic diversity that is observed among humans (Reich et al., 2002). Most variations in the human genome are hypothesized to be neutral, while some variations introduce an adaptive or deleterious effect (Frazer et al., 2009). The contribution of genotype to phenotype is a subject of intense research in biology and medicine, and the basis of my thesis (Genomes Project et al., 2010). In the following section, I will begin by introducing the various types of genetic variation.

1.2.1. Single nucleotide variants (SNVs)

The most prevalent genetic variation encountered in the human genome is the single-nucleotide variant (SNV), which involves the change of a single nucleotide position (Frazer et al., 2009). It has been estimated that there are about 11 million SNVs in the human genome pool (Frazer et

al., 2009). SNVs can be described by their sequence ontology. Within protein-coding regions of the genome, a SNV can exert a "synonymous" or "non-synonymous" change. A synonymous variant is defined by a nucleotide change that does not translate into an amino acid change, ultimately producing no change to the protein. This is achieved by the redundancy of the genetic code where the same amino acid can be called out by a variety of codons (sequence of three nucleotides). Synonymous SNVs are mostly considered to be silent or neutral variants as they have no impact on the encoded protein product and thus do not modify the phenotype. However, that maybe an oversimplification, as research has shown that in some cases the RNA product does not undergo the same folding process or is subtly different in shape (Kimchi-Sarfaty et al., 2007; Pagani et al., 2005). Conversely, a non-synonymous variant is defined as a nucleotide change that translates into a completely different amino acid or the introduction of a stop codon, commonly described as "missense" or "nonsense" variants, respectively. Missense SNVs modify the protein product while nonsense SNVs cause an early truncation. SNVs that occur within or near the exon/intron boundaries are referred to as "splice-donor" or "splice-acceptor". They can alter the mRNA splice junction and cause an early truncation in the protein product (Cartegni et al., 2002). Lastly, outside of protein-coding regions, SNVs are classified according to the region where they are found, such as within introns, 5' or 3' untranslated regions (UTRs), promoters, enhancers, silencers, non-coding genes, or pseudogenes.

1.2.2. Insertion-deletion (indel) variants

Insertion–deletion variants (indels) are the addition or subtraction of one or more nucleotides in the genome. Indels vary in length and can be up to 50 nucleotides long (Levy et al., 2007). "Frameshift" variants are indels within protein-coding regions; they can have the potential to significantly disrupt the protein product if the codon reading frame is altered. Frameshifts that are out of frame can lead to the early truncation of a protein or an elongated protein with the loss of a stop codon. Conversely, frameshifts within the reading frame, could pose minimal disruption to the protein, given that just a single amino acid would be altered.

1.2.3. Structural variants

In contrast to SNVs, "structural variants" are genetic variations that involve at least 50 nucleotides (Frazer et al., 2009; Kosugi et al., 2019). They can be subclassified into: copy

number variations (CNVs) (deletions and duplications), inversions, translocations, insertions, and aneuploidy (Frazer et al., 2009; Kosugi et al., 2019). Structural variants have been associated with various diseased phenotypes, such as neurodevelopmental disorders, cancers, metabolic disorders, atrial fibrillation, and others (Frazer et al., 2009; Iacocca & Hegele, 2018; Liu et al., 2015; Stankiewicz & Lupski, 2010; Sudmant et al., 2015).

1.2.3.1. Copy number variants (CNVs)

CNVs are large structural changes in the genome that involve deletion or duplication of DNA segments ranging in size from 1 kb to millions of base pairs (Conrad et al., 2010). CNVs are estimated to involve perhaps 5% of the total genomic sequence in healthy individuals and can increase to 10% or more in certain diseases (Conrad et al., 2010; Iacocca & Hegele, 2018). CNVs are particularly relevant in the etiology of complex diseases given their potential impact on gene expression: these large-scale changes can have greater impact than SNVs by altering gene dosage and gene regulation, and by disrupting the DNA sequence through a large deletion or duplications (Stranger et al., 2007). In fact, the effects of CNVs on disease have little overlap with SNVs, making CNV analysis a complimentary and relevant area to explore in the overall understanding of complex diseases (Girirajan et al., 2011; Stranger et al., 2007). Although CNV detection alone would not explain the genetics of the vast majority of complex disease, the contribution that CNVs have further advances our understanding of pathophysiology (Conrad et al., 2010). Further, CNV analysis remains a relatively unexplored field in most human diseases and represents an area with opportunity for much needed investigation.

1.2.3.2. Inversion, translocation, insertion and aneuploidy

An inversion occurs when the nucleotide sequence is reversed and a translocation when a section of one chromosome reattaches to another chromosome. Both inversion and translocations are balanced forms of structural change, meaning that there is no net change in the genomic size (Kosugi et al., 2019). Insertions, on the other hand, like CNVs, are imbalanced forms, changing the net genomic size (Kosugi et al., 2019). These types of chromosomal alterations can be large enough to be identified using fluorescent *in situ* hybridization (Feuk et al., 2006), however not large enough to be visualized with karyotyping. Chromosomal aneuploidy, on the other hand, occurs when there is a loss (monosomy) or addition (trisomy) of a full chromosome altering the

karyotype of the individual from 46 XX/XY (female/males). Aneuploidies can easily be identified at the karyotype level with cytogenic testing (Feuk et al., 2006).

1.2.4. Variant frequency

Genetic variants (SNVs and structural variants) can be classified by their minor allele frequency (MAF). Each variant's MAF is determined from multiple databases containing thousands of healthy controls; the MAF is calculated for the general global population and for each ancestral group separately (Genomes Project et al., 2015; Lek et al., 2016). For a given variant, their frequency, occurrence and population distribution is dictated by human evolutionary forces (mutations, genetic drift and natural selection). Purifying selection decreases the frequency of deleterious variants, however the size of the population in which the variant occurs, and the disease effect of the variant can enhance or diminish the efficacy of purifying selection (Quintana-Murci, 2016). Additionally, the demographic history such as the expansion out of Africa, founder effect and/or population bottlenecks can potentially lead to an increase in the frequency of deleterious variants. Hence, MAF varies by ancestral group due to different evolutionary forces that acted on that population at various points in history, such as purifying selection, founder effect and/or population bottlenecks (Quintana-Murci, 2016).

Rare variants, by definition have a MAF of < 1% in the population (MacArthur et al., 2014). Rare variants tend to be specific to certain populations and have large deleterious effect (Quintana-Murci, 2016). Once the MAF falls below 0.5%, it becomes very difficult to detect a rare variant due to limited statistical power unless it has a disease effect size large and equivalent to variants that cause monogenic diseases (Manolio et al., 2009). In addition, low-frequency variants are defined by a $1\% \leq \text{MAF} < 5\%$ and common variants with a MAF of >5% (Manolio et al., 2009). Low-frequency variants, just like rare and common variants, contribute to disease susceptibility and in fact, explain part of the missing heritability (Manolio et al., 2009). Common variants are sometimes referred to as genetic polymorphisms, e.g. “single nucleotide polymorphisms” (SNPs) or “copy number polymorphisms”. Common variants, being relatively frequent and common across population, are typically phenotypically neutral, or at most have small deleterious effect. However, when their individual effects are considered in aggregate, they can collectively have a large effect on a quantitative or qualitative trait can contribute to susceptibility to a physiological or pathological phenotype (Khera et al., 2018).

Accordingly, variant frequency by ancestry is an important concept to consider when assessing frequency differences of variants between cases and controls. If the ancestral population is different between the cases and controls, then the difference is simply artifactual and not based on true biological reasons.

1.3. Genetic diseases

1.3.1. Monogenic diseases

Monogenic diseases are caused by a rare genetic variation that typically occurs in a single gene and follows Mendelian inheritance pattern (Nehring & Faux, 1999). The foundation of monogenic diseases is based on Gregor Mendel's three laws of inheritance. Briefly, "the law of independent assortment" states that during gamete formation, each paternal and maternal allele will segregate independently from each other; the second law "the law of segregation" states that each gamete will receive a single allele for each gene and "the law of dominance" states that after fertilization, the dominant allele will determine the phenotype and overshadow the effect of the recessive allele (Castle, 1903).

Monogenic diseases follow distinct Mendelian inheritance patterns, such as autosomal dominant, recessive, co-dominant, or X-linked (sex-linked) dominant or recessive. A dominant inheritance pattern requires a single variant allele for the phenotype to be expressed; if a parent is the carrier of dominant allele, each child has 50% chance of inheriting it (Winsor, 1988). Conversely, a recessive inheritance pattern is only expressed when both pathogenic alleles are present; if a parent is a carrier (homozygous for both diseased alleles) and the second parent is a non-carrier (homozygous for normal allele), the chances of a child inheriting one pathogenic allele are 100% but the child would not express the phenotype as it would maximum get one diseased allele. However, if one parent is a homozygous carrier for the pathogenic allele and the second parent is heterozygous for the pathogenic allele, the chances of a child inheriting one pathogenic allele are 100% and the chances of a child inheriting both pathogenic alleles and expressing the phenotype are 50%. On the other hand, if one parent is heterozygous for the recessive allele and the other parent is a non-carrier, then a child has a 50% chance of inheriting the recessive allele and no chance of expressing the disease phenotype. Lastly, if both parents are heterozygote carriers, there is a 25% chance that a child will inherit both pathogenic alleles and express the disease

phenotype, a 50% chance that the child will be a heterozygous carrier and a 25% chance of being a non-carrier. Overall, the chances of a child expressing the disease phenotype are only 25% (Winsor, 1988). Co-dominance occurs when there is co-expression from two distinct dominant alleles resulting in a phenotype that is the mixture of both. X-linked inheritance applies to genes only in the X chromosome, they have different inheritance pattern in each sex given that males have a single X chromosome. Female carriers follow the dominant or recessive inheritance pattern described above, while male carriers express the single allele, regardless of whether it is a dominant or recessive. Hence, males are often affected, and females are usually unaffected carriers (Winsor, 1988). The same situation is observed in variants found on the Y chromosome, the single allele whether dominant or recessive will be expressed by default in the male carriers. These principles are the major cornerstone of clinical genetics.

Monogenic diseases are an integral part of the current approach to decipher the totality of human diseases and disease heritability (Antonarakis & Beckmann, 2006). Monogenic diseases are a more probable consideration when: there is a positive family history; there is a recognized inheritance pattern in the family; secondary factors are absent; age of the patient is relatively young; syndromic features are present; and the biochemical perturbation is relatively extreme (Berberich & Hegele, 2019c).

Monogenic diseases are largely unaffected by environmental factors and thus become perfect cases to investigate with low environmental noise. However, environmental or non-genetic factors can sometimes accelerate or alleviate the phenotype. For instance, individuals with heterozygous familial hypercholesterolemia (FH) most often due to loss-of-function variants in the *LDLR* gene have markedly elevated levels of low-density lipoprotein (LDL) cholesterol and are strongly predisposed to develop premature heart attacks and strokes (Berberich & Hegele, 2019b). Observational studies show that daily statin use by FH patients reduces their atherosclerotic cardiovascular disease (ASCVD) risk by 44% (Besseling et al., 2016). Indeed, it is in individuals with extreme phenotypes (and their families) were scientist can discover a novel gene, understand its functions in the disease pathophysiology, and expand those findings for therapy development (Chakravarti & Turner, 2016).

1.3.2. Polygenic diseases

Polygenic (or complex) diseases are determined by the cumulative impact of many genetic variants in combination with environmental factors and lifestyle choices (Khera & Kathiresan, 2017). Culprit variants can be rare as well as common and originate anywhere across the genome (Choi et al., 2020; Dron & Hegele, 2019). Indeed, the majority of the common variants are located outside of protein-coding genes in non-coding and intronic regions of the genome believed to influence gene regulation (Boyle et al., 2017). Unlike causative variants in monogenic diseases, most of these variants have a small to modest disease effect when considered individually, which makes them challenging to study. However, when the effect of all variants is combined, they cumulatively have been shown to influence susceptibility to many diseases like coronary artery disease (CAD), atrial fibrillation (AF), etc. (Boyle et al., 2017; Choi et al., 2020; Khera et al., 2018). Further in various diseases the aggregate effect of the genetic variants across the genome can often approximate in magnitude the expected impact of a single large-effect rare pathogenic variant in a monogenic disease (Khera et al., 2018). Although the exact number of variants underlying polygenic susceptibility for each disease has not been fully determined, genome-wide association study (GWAS) have associated several hundreds of loci with numerous diverse phenotypes, in each case explaining a portion of the disease susceptibility (Boyle et al., 2017; Manolio et al., 2009). However, the total number of common small-effect alleles contributing to particular traits or diseases may number in the tens or hundreds of thousands

As mentioned, polygenic diseases are also determined by environmental factors and lifestyle choices in combination with the underlying genetic susceptibility. Thus, it is possible to overcome a strong polygenic predisposition to certain diseases with risk factor modification, such as proper diet and increased level of activity, and where appropriate, use of appropriate medications. For instance, a study of 55,685 individuals showed that among those with the highest polygenic risk (top quintile of PRS), a favorable lifestyle was associated with ~ 50% lower relative risk of CAD (Khera et al., 2016a). Conversely, in AF the reduction in risk was not as prominent, individuals with a high polygenic risk but low clinical risk factors had an overall lifetime risk for AF of 44%, compared to individuals with high polygenic risk and high clinical risk factors that had an overall lifetime risk of 48% (Weng et al., 2018). This emphasizes that for

different diseases, the weighting of genetic versus environmental or non-genetic determinants can vary substantially.

1.4. Heritability

Heritability refers to the portion of variance of a trait or phenotype that can be attributed to genetic factors (Manolio et al., 2009). Heritability varies by trait or disease. For atrial fibrillation (AF) about 22% of the heritability can be explained by mostly common variants and a small percentage by rare variants (Weng et al., 2017a). Unfortunately, for AF, the vast majority of those common variants do not surpass the GWAS significance threshold indicating both their small absolute effect size and the need for huge samples for the effects to be detected. This highlights one aspect the missing heritability problem (Manolio et al., 2009). Despite great advances in technology and genetic analysis tools, there remains a large portion of heritability that remains unexplained even when large numbers of common variants are accounted for. The missing heritability is estimated to lie in rare and common variants, including structural variants like copy number variants, that have been mostly missed due to low statistical power and technological limitations (Manolio et al., 2009). Indeed, there is no clear approach as to what should be investigated first and depending on the disease the proportion of each type of variant will differ. For instance, FH patients are more likely to carry rare variants compared to hypertriglyceridemia patients that are more likely to harbour an accumulation of triglyceride raising common variants (Dron et al., 2020). Hence a holistic approach to determine the genetic burden of trait or disease will improve our current understanding of disease etiology and human physiology.

1.5. Approaches to genetic investigations

In this section, I will introduce various approaches to study the genetics of human diseases from rare variants to common variants in related to unrelated individuals. Two key factors in determining the correct approach are a variant's disease effect size and allele frequency.

1.5.1. Rare variant

1.5.1.1. Family studies - Linkage analysis

Linkage analysis was ubiquitous in the pre-GWAS era to map the location of putative causal genes. Recently, this analytic approach is resurging with the accessibility of exome or whole genome sequencing data and the need to objectively identify causality with statistical criteria (Bailey-Wilson & Wilson, 2011; Ott et al., 2015). Linkage analysis studies are powered to detect rare variants with large effect sizes in families with a higher burden of a phenotype. Many causative genes for monogenic diseases have been identified this way.

Classical linkage analysis is a statistical calculation that assesses whether two or more genetic loci are in close proximity on the chromosome; the linkage algorithm determines the probability of whether the two loci are transmitted together during meiosis or not (Bailey-Wilson & Wilson, 2011). If two genetic loci are inherited together at a significantly higher frequency than what would be expected by chance, then they are said to be in genetic linkage. The recombinant fraction considers the distance between the two loci within the same chromosome: if the distance is wide, the likelihood that there will be recombination due to crossing over is 50%, such that they appear to assort independently as if they were located on two different chromosomes. This is due to the Mendel's law of independent segregation. However, if the evaluated loci are directly adjacent to each other or even in close physical proximity, the chance of recombination due to crossing over is close to zero, meaning that they are more likely to be transmitted together as a pair. Here, Mendel's law of independent segregation does not apply. The LOD score (or logarithm of odds) calculates the likelihood of linkage between two genetic loci assuming various recombination fractions and compares the observed linkage with the likelihood of no linkage at the maximal recombination fraction. The recombination fraction that produces the highest LOD score represents the most likely distance between the two genetic loci. A maximum LOD score >3 (i.e. odds of 1000:1 that co-segregation was occurring randomly) is the traditional level accepted as indicating significant linkage, resulting in rejection of the null hypothesis of independent assortment and would imply strong evidence to support co-segregation of the two loci.

Indeed, linkage analysis is re-emerging as a tool to advance the study of rare variants within families, instead of localizing two genetic loci, its contemporary use is to assess whether a genetic locus identified by a DNA variant is linked to a phenotype with an implicit genetic basis. It can also be applied to multiple families with the same phenotype were a joint LOD can be calculated (Sun et al., 2021). The main limitations with these studies is that they are not power to detect variants of modest effect or variants with incomplete penetrance (Altmuller et al., 2001).

1.5.1.2. Population studies - Rare variant association studies

Next-generation sequencing (NGS) technologies have facilitated the investigation of rare variants. Previous to the development of this technology, studies on rare variants were restricted to families (linkage analysis), however with NGS it became possible to do genome or exome-wide association studies in unrelated individuals exploiting the potential that more cases would harbour causative rare variants in genes implicated with a disease compared to controls (Cordell & Clayton, 2005). The low frequency of rare variants significantly hinders the statistical power to detect them across unrelated individuals, forcing the development of novel statistical techniques and analysis such as "rare variant association studies" (RVASs) to boost statistical power (Lee et al., 2012).

Population stratification is a factor that can affect the interpretation of rare variant analysis (Mathieson & McVean, 2012; Tintle et al., 2011). As discussed in section 1.2.4, MAF is specific to ancestries and hence, what may be rare in a population may not be rare in another. This is a confounding factor that can easily inflate type 1 error. Techniques like principal component analysis (PCA) that adjust for population stratification by explaining the variance observe with principal components (PC) are very important to reduce false positive errors (Bansal et al., 2010; Price et al., 2006).

Below I will describe two approaches to investigating rare variants:

1.5.1.2.1. Single-variant approach

For variants with relatively large effect size and MAF not too rare, a "single-variant" approach remains a useful test to evaluate the association of each variant with the disease trait following an

additive genetic model (Lee et al., 2014). Variants with lower effect size can also be detected at the cost of a larger study group.

1.5.1.2.1.1. Candidate gene approach

The candidate gene approach refers to a hypothesis driven study based on experimental data of a biological or physiological relationship between a gene or set of genes and the disease of interest (Jorgensen et al., 2009). This approach allows for the identification of rare genetic culprits with moderate to low effect size and can serve as validation of previous findings (Jorgensen et al., 2009). However, it is not suitable for novel diseases, where there are no established relationships. Additionally, candidate gene analysis is based on our current imperfect understanding of the disease mechanisms and hence, the genes are subject to change with new knowledge. However, it is a hypothesis-driven analysis that can easily test out a signal noted from GWAS.

1.5.1.2.2. Collapsing approach

For variants with moderate to low effect size, a common approach is to collapse rare variants within genes or pre-defined regions (sliding windows) and compare the collective frequency between the cohorts (Bansal et al., 2010). By collapsing the variants, the collective frequency is compared between the cohorts. Collapsed rare variant associations are tested with either a burden or variance-component test (Lee et al., 2014). Briefly, a burden test assumes that all the rare variants have the same phenotypic effect direction, meaning all variants either increase or decrease a quantitative trait or disease risk. Conversely, a variance-component test assumes that variance have opposing effects for the same phenotype, meaning some variants could lead to an increase or decrease in a quantitative trait or disease risk. Hybrid test were developed that include both burden and variance-component (like optimal unified sequence kernel association test [SKAT-O]) to maximize the statistical power to detect an association (Lee et al., 2014). These collapsing methods can greatly increase the statistical power to detect a true difference and lower the requirement of sample size, which are already quite high to detect these variants (Bansal et al., 2010).

For non-coding rare variants, the same collapsing approaches can be applied however several challenges arise. Unlike coding variants, that a natural testing unit is a gene, with non-coding

variants it is a lot more difficult to decipher how to group the variants since the non-coding genome organization is not as well understood (Bocher & Genin, 2020). One approach is to pool variants by functional units (promoter or enhancer region) or apply agnostic approach such as sliding windows. A second challenge is that the test applied must account for the inclusion of neutral variants; for instance, burden tests assumes that all rare variants in a region are causal and statistical power is lost when the assumption is violated while sequence kernel association tests assume that there are a variety of variants that are neutral variants or have opposing effect (Lee et al., 2012). Lastly, quantifying the functional impact of non-coding rare variants on phenotype expression remain a difficult challenge. There are databases that determine the functionality of non-coding variants but these remain limited and not applicable to novel variants (Bocher & Genin, 2020). However, non-coding variants have an important role in disease susceptibility and these challenges will need to be addressed to better understand the effect of all rare variants.

1.5.2. Common variants

1.5.2.1. Genotype-phenotype association

Before GWAS, the disease susceptibility of common variants was investigated in genotype-phenotype association studies; the hypothesis was that the diseases-associated alleles should occur in higher frequency in the disease cases versus controls. However, not all common variants found in the genome can be investigated because many variants are linked in linkage disequilibrium and thus, occur together at a higher frequency than just by chance alone. This is particular common across population, where certain alleles from different loci occur together in individuals of common ancestry. This would lead to an inflation of type I error, hence the International HapMap Project sought to determine in diverse populations how many variants are independent and not in linkage disequilibrium. The project determined that about 1 million loci in the human genome are independent (International HapMap, 2005). This began the study of common variants across large groups of individuals which was technically facilitated by the advent of DNA microarrays, discussed in the following section (Hardy & Singleton, 2009).

1.5.2.2. Genome-wide association studies (GWAS)

GWAS employ DNA microarrays that genotype common variants, typically SNPs with allele frequencies >5% (Manolio et al., 2009). GWAS have allowed for the cost-effective genotyping

of millions of SNPs in large populations. This unbiased approach well powered for large-scale genetic association studies has led to the identification of millions of loci or SNPs that contribute to disease and advanced our understanding of the genetic underpinning of complex diseases (Hardy & Singleton, 2009). Given that there are 1 million independent loci, GWAS have a standard Bonferroni correction threshold of genome-wide significance *(P*- value = <5×10-8) to adjust for the multiple independent tests (McCarthy et al., 2008). Despite the great improvement in detecting genome-wide significant loci, the replication of the findings in an unbiased population remains paramount to clearly demonstrate that the findings are not due to population stratification (McCarthy et al., 2008). Further, it remains challenging to determine the mechanism of disease of a GWAS significant locus. Indeed, the vast majority (~80%) of associated loci fall outside coding regions (Manolio et al., 2009). Within a coding region, it simply involves determining the effect of the amino acid substitution on the protein's function. However, in non-coding regions, the loci itself is not causative, it just serves to identify a region that is associated with the outcome. This is because the locus is a "sentinel" SNP that is linked to a region that is inherited together (Tucker et al., 2016). The challenge is to identify the functional gene among the various genes that can be found in that region (Roselli et al., 2020). One approach is to use expression quantitative trait loci (eQTL) mapping, which links the genotype of a disease associated SNP to the expression of one or more genes in that region (Roselli et al., 2020). The genes found using eQTL are then likely to be causative genes at that locus. Although this approach has been very helpful at narrowing down the list of causative genes, it suffers from a few limitations including that it is tissue specific, availability of tissue samples is variable (in particular for cardiac analysis), and the sample sizes remain low specially if the tissue is hard to obtain (Roselli et al., 2020).

1.5.2.3. Polygenic risk scores

Despite great advances in identifying common variants associated with a particular phenotype, the small effect size limits their predictive power and alone they are not informative when trying to decipher disease heritability. Hence, to understand the overall cumulative impact of numerous small effect variants each genome is quantified using polygenic risk scores (PRS). PRS are derived using mathematical models that aggregates the burden of common variants on the trait of interest. Individuals in the highest decile of a CAD PRS distribution have approximately

threefold increased risk of ASCVD compared with individuals in the lowest decile (Khera et al., 2018). The PRS quantifies an individual's total susceptibility for a phenotype by adding up the disease effect of its common variants (Choi et al., 2020). Because these scores appear to add prognostic information above and beyond traditional variables, they are extensively researched as valuable clinical risk prediction tools and have the potential to serve as diagnostic tools (Khera et al., 2018; Muse et al., 2018).

Each common variant, if biallelic, will have one allele associated negatively with the qualitative trait or associated with a reduction of the quantitative trait and the second allele with the opposing effect (Dron & Hegele, 2019). The disease effect of each SNP is considered probabilistic given that healthy controls will undoubtedly carry some of the effect alleles. Most of the information of estimated disease effect is obtained directly from GWAS summary statistics and they are particular to a phenotype of interest.

Initially only a few SNPs were included in these calculation, only those that pass the stringent genome-wide significant level (Dron & Hegele, 2019). However, as GWAS studies increase in cohort size, more SNPs previously nonsignificant became significant, as it was clearly seen in AF (Boyle et al., 2017; Christophersen et al., 2017b; Roselli et al., 2018). However, this ever-growing list of significant SNPs came with very small effect size. Across different diseases, polygenic scores grew in size by incorporating millions of loci. Khera et al. (2018) compared the predicting performance of a 6 million SNP score for CAD versus smaller scores and found that the genome-wide score of 6 million SNPs outperformed the smaller scores. Clearly, the trivial incremental addition of those very small effect SNPs significantly improved the predicting performance of the score.

Application of polygenic scores in the general population can aid in risk assessment. For instance, a PRS can be applied prospectively to identify individuals at risk that would benefit from early intervention. Despite growing appreciation for the importance of polygenic determinants, for most diseases they remain a research tool. The main limitation is that no consensus exists on how to quantify polygenic effects and there are no accepted reporting standards (Dron & Hegele, 2019). For instance, for LDL cholesterol, at least 15 different polygenic scores have been reported; however, none has been validated across different study

samples. However, we anticipate that the role of polygenic scores in the assessment of various complex diseases will be clarified within a couple of years with extensive research, and that such scores may be shown to have clinical utility at some future time.

1.6. Variant pathogenicity classification

NGS technology has produced an ever-growing list of novel putative disease-causing variants that require further validation to affirm their pathogenicity (Sunyaev, 2012). To address this, the American College of Medical Genetics (ACMG) has recommended a multifactorial approach as the standard for attributing clinical relevance to a previously unreported DNA variant (Richards et al., 2015). The ACMG guidelines consider several lines of criteria: population MAF data, functional data, predictive *in silico* algorithms, segregation data, *de novo* status, and allelic data (Richards et al., 2015). Below I will briefly describe each criteria:

(1) Low allele frequency in the population

Through evolution, deleterious alleles are maintained at a very low frequency by purifying selection (a form of natural selection) because they reduce the reproductive success of individuals (Quintana-Murci, 2016). Hence, a true deleterious variant should be rare in the population.

(2) Functional validation of the genetic variant

Experimental validation using cell lines (*in vitro*), or model organisms (*in vivo*), or patient-derived tissue (*ex vivo*) provides strong evidence of the variant's effect on the protein and disease pathway. However, this is a time-consuming and resource intensive effort and it may no exist for novel variants.

(3) *In silico* predictive algorithm

Prediction algorithms assess the deleteriousness of a variant, by tracking evolutionary conserved regions, changes in amino acid sequence, or changes on protein function and structure. Given that the algorithms are predictive, the evidence is just supporting**.**

(4) Segregation data

Segregation data is one of the ACMG criteria that requires pedigree extension by cascade screening followed by genotype-phenotype co-segregation analysis (Iacocca et al., 2018a; Richards et al., 2015). Consistently observing the presence and absence of the disease phenotype in family members who are carriers and non-carriers, respectively, of a newly discovered DNA variant in a gene already known to cause the disease, helps to build a statistical case favoring causality. Conversely, for a highly penetrant trait such as FH, even a single instance of a family member who deviates from the expected phenotype-genotype relationship can be sufficient to reject a causal role. Indeed, sampling the extended kindred and performing co-segregation analysis is relatively more feasible and comprehensible for a clinician than laboratory experiments; however, it is a time-consuming effort.

All the evidence for each criteria must be considered in aggregate to determine a final variant classification: (i) pathogenic; (ii) likely pathogenic; (iii) uncertain significance; (iv) likely benign; or (v) benign (Richards et al., 2015). Obtaining all the corroborative evidence required by the ACMG is essential to avoid misdiagnosis and false positive reports of causality; however, this can be complex, time and resource intensive (MacArthur et al., 2014).

1.7. Genetic testing technologies

In this section, I will describe the different genetic technologies employed to interrogate from rare variants to common variants in the human genome.

1.7.1. Sanger sequencing

Chain termination sequencing or Sanger sequencing is a sequencing method that creates DNA fragments with variable lengths due to chain-terminating nucleotides (dideoxynucleotides [ddNTPs]) (Sanger & Coulson, 1975). For a relatively small region such as a single exome or small gene, the pre-specified DNA fragment is amplified with polymerase chain reaction, the fragment is sequenced incorporating at random regular deoxynucleotides and ddNTPs that terminate the sequence. The ddNTPs are labeled with fluorescent dyes that emit a different light wavelength for each nucleotide (A, C, T, and G) (Heather & Chain, 2016). During capillary gel electrophoresis, each DNA fragment moves according to its length from smallest to largest across the gel. The fluorescence emitted by each fragment is read from 5' to 3' by a chromatogram. Sanger sequencing was the first-generation sequencing technology and although

it advanced the field tremendously, it was costly and laborious. Currently, it is the gold standard for confirming a variant found with NGS technology (Berberich & Hegele, 2019c). It can also be easily employed when performing family cascade screening.

1.7.2. Next-generation sequencing (NGS)

NGS is a massively parallel, high-throughput sequencing approach that amplifies and sequences millions of fragments of DNA in parallel (Pfeifer, 2017). NGS has greatly advanced variant discovery across all human diseases by providing a cost-effective method to do large-scale sequencing and rapid access to large amount of data (Pfeifer, 2017). NGS is highly versatile, it can sequence a targeted panel of genes, or all the exomes (protein-coding regions that amount to 1-2% of the human genome), or the whole genome (Rehm et al., 2013).

Given that each DNA fragments is amplified multiple times, those fragments once aligned to the reference genome, become multiple overlapping reads for each region. The overlapping reads form the NGS coverage and help elucidate allelic zygosity. Intuitively, as NGS coverage increases variant calls become more accurate; for whole-genome sequencing a minimum of 30X reads is reasonable for laboratories to use (Rehm et al., 2013). Further, NGS coverage can be exploited to do CNV analysis, CNV detection methods have evolved from multiplex ligation-dependent probe amplification (MLPA) to NGS-based detection algorithms improving the accessibility and feasibility to detect CNVs (Iacocca et al., 2017; Wang et al., 2005). Detection algorithms that are sequence based are much more amenable to detecting a broad range of structural variants, not just CNVs, and provide the opportunity to determine the breakpoint location (Kosugi et al., 2019). Indeed, our laboratory compared the concordance of a CNVs detection algorithm, VarSeq-CNV® caller algorithm (Golden Helix, Inc., Bozeman MT, USA) that utilizes NGS data versus the gold-standard MLPA, and demonstrated that VarSeq-CNV® caller algorithm had 100% concordance with MLPA (Iacocca et al., 2017).

1.7.2.1. LipidSeq

The relatively complete understanding of some monogenic diseases makes them prime candidates for clinical application of NGS technology, particularly targeted DNA sequencing (Hegele, 2019). Monogenic dyslipidemias are particularly amenable to DNA analysis because there are many different possible causative rare variants and DNA sequencing is the preferred

method to maximize their detection rate (Berberich & Hegele, 2019c; Hegele et al., 2015). Dr. Hegele's laboratory designed a targeted sequencing panel, termed "LipidSeq" to detect rare causal variants in 69 genes underlying 25 monogenic dyslipidemias as well as the simultaneous evaluation of polygenic forms by micro-sequencing 185 SNPs; the complete list of genes included in the LipidSeq panel: *LDLR, APOB, PCSK9, STAP1, APOE, LDLRAP1, LIPA, ABCG5, ABCG8, MTTP, SAR1B, ANGPTL3, LPL, APOC2, APOA5, LMF1, GPIHBP1, GPD1, CETP, LIPC, SCARB1, LIPG, ABCA1, APOA1, LCAT, LMNA, PPARG, PLIN1, CIDEC, ZMPSTE24, LIPE, LPIN1, AGPAT2, BSCL2, CAV1, PTRF, LMNB2, AKT2, DYRK1B, POLD1, WRN, HNF4A, GCK, HNF1A, PDX1, HNF1B, NEUROD1, KLF11, CEL, PAX4, INS, BLK, KCNJ11, ABCC8, SORT1, MYLIP, NPC1L1, GALNT2, MLXIPL, TRIB1, APOA4, PPARA, GCKR, CREB3L3, PLTP, ABCG1, CAV2, PNPLA2*, and *APOC3* (Dron et al., 2020; Hegele et al., 2015). Patients with suspected FH can access genetic testing with LipidSeq, that would scan not just the three major FH-causing genes, but also 'minor' FH genes; allowing for the interrogation of monogenic (SNVs and CNVs) and polygenic causes for FH (Dron et al., 2020). Further we can identify the exact breakpoint of a CNV utilizing Sanger sequencing which helps us determine the degree of disruption caused by a CNV (Newman et al., 2015). In theory, NGS technology should improve the diagnostic yield and improve patient care and from our experience with LipidSeq, we have observed an increase in diagnostic yield of rare variants by 10% as a result of simultaneous screening for CNVs (Iacocca et al., 2019); increased diagnostic certainty, specifically upgrading patient diagnosis from 'possible' or 'probable FH' to 'definite FH', which in turn; provided motivation to initiate cascade screening of family members; and helped patients to obtain reimbursement for proprotein convertase subtilisin kexin type 9 inhibitor drugs. Although there is the propensity to obtain as much sequencing data as possible, there are drawbacks to whole-exome or whole genome sequencing compared to targeted panels in the context of diseases like monogenic dyslipidemias. For instance, there is a greater per-sample cost than with a targeted panel; increased risk of incidental or secondary findings unrelated to dyslipidemia, which the physician could be obliged to report; and diminished resolution to detect certain types of genetic variants, specifically CNVs (Johansen et al., 2014).

1.7.3. DNA microarray

DNA microarray technology genotypes common polymorphic variants (single nucleotide polymorphisms or SNPs) that are spaced by an average <1 kilobase across the genome (Bumgarner, 2013). This technology at a relatively economical cost produces data from hundreds of thousands to more than 1 million loci genome-wide (Bumgarner, 2013). DNA microarrays have been extensively used for GWAS studies that primarily target common variants in thousands of individuals and it has proven to be very successful to have mapped many loci that are associated with literally thousands of phenotypes, including complex diseases (Christophersen et al., 2017b; Nielsen et al., 2018; Roselli et al., 2018).

1.8. Atrial fibrillation (AF)

Atrial fibrillation (AF) is a complex heritable disorder (Fatkin et al., 2017; Lubitz et al., 2010a). It is the most common sustained cardiac arrhythmia, affecting 30 million people globally and its prevalence is estimated to double in the near future (Kannel & Benjamin, 2009). AF causes an irregularly irregular heart rhythm originating in the atria that leads to a loss of synchronized contraction which in turn decreases cardiac output and increases predisposition to blood clot formation. AF increases the risks of stroke, heart failure, and death independent of other cardiac conditions (Benjamin et al., 1998; Lin et al., 1996; Stewart et al., 2002). AF mortality risk does not change with increasing age (Benjamin et al., 1994). The majority of AF cases develop in the presence of established clinical risk factors, including advancing age, obesity, hypertension, diabetes mellitus, and cardiac diseases including coronary and valvular heart disease, and ventricular cardiomyopathy (Huxley et al., 2011). There is also an increased risk for males and individuals of European ancestry to develop AF over females and individuals of African, Hispanic or Asian ancestry (Dewland et al., 2013; Magnussen et al., 2017).

Heritability studies have confirmed a genetic contribution to AF. A first-degree family members with AF is a risk factor for AF especially among individuals with early-onset forms of AF, and can confer a 40% increased hazard risk (Alzahrani et al., 2018; Fox et al., 2004; Lubitz et al., 2010a). The heritability of AF has been estimated to be as high as 62% in a study on monozygotic Danish twins (Christophersen et al., 2009). Most recently, the heritability of AF in individuals of European ancestry has been estimated to be ~22% (Weng et al., 2017a). Indeed, a

number of reports have identified rare and common variants that predispose to the condition, though a significant portion of AF heritability remains unexplained.

1.8.1. "Lone" AF

"Lone" AF refers to a subset of AF patients who are diagnosed early in life (< 60 years of age) and have no clinical or echocardiographic evidence of cardiopulmonary disease or hypertension (Wyse et al., 2014a). Consequently, "lone" AF is predicted to have a strong genetic etiology (Choi et al., 2018b). Like AF, "lone" AF is more frequent in males and its prevalence is estimated to be around 3% but has ranged between 1.6% and 30% (Potpara & Lip, 2014; Wyse et al., 2014a). The prognosis of "lone" AF patients from a 12-year follow-up study was relatively favourable, as long as no other comorbidity developed (Potpara et al., 2012). Indeed, most of the rare variants discussed in the preceding section have been identified in "lone" AF families where AF segregates like a Mendelian trait and the variants have large disease effect sizes (Fatkin et al., 2017).

1.8.2. Rare variants

The first genetic study on a "lone" AF family identified a novel missense gain-of-function variant in the *KCNQ1* gene, which encodes a voltage gated potassium channel (Chen et al., 2003a). The variant likely shortened the atrial refractory period which increased the susceptibility for AF by making it easier for impulse re-entry (Chen et al., 2003a). Since then, numerous candidate genes with rare heterozygous variants of large disease-causing effect have been identified, helping elucidate portions of the pathophysiology of AF (Fatkin et al., 2017). In particular, it established a key role for ion channel defects with both gain-of-function and loss-of-function (LOF) variants that either shorten or lengthen atrial action potential duration (Fatkin et al., 2017). Atrial arrhythmogenesis is also associated with variants near cardiac transcription factors however the disease mechanism of these variants remains inconclusive. Lastly, genes associated with myocardial structure have also been implicated in atrial arrhythmogenesis. Two separate reports have identified cases of early-onset AF that were found to be secondary to mutations within *MYL4*, which encodes a sarcomere protein and whose expression is restricted to the atria (Gudbjartsson et al., 2017a; Orr et al., 2016). Consistent with their chamber-specific expression, the ventricles of these patients appeared normal. Since these original reports, other

myocardial structural genes also implicated in ventricular cardiomyopathy have been associated with AF. For instance, cohort studies have identified rare variants from *TTN* to associate with "lone" AF incidence (Ahlberg et al., 2018; Choi et al., 2018b; Gudbjartsson et al., 2017b). *TTN* encodes a sarcomere protein called titin that has a critical role in cardiac contractile function, is expressed in both the atria and ventricles, and is a well-established culprit of various forms of ventricular cardiomyopathy (Herman et al., 2012). Further, patients with "lone" AF were identified with impaired myocardial energetics and LV dysfunction that would not correct post ablation, indicating that in these patients AF presence possibly resulted from an occult cardiomyopathy (Wijesurendra et al., 2016). Similar findings with *TTN* variants have been reported in other ancestral populations, such as African American and Hispanic/Latinx individuals (mostly individuals of Mexican heritage) (Chalazan et al., 2021). Indeed, the notion that a gene implicated in ventricular cardiomyopathy could give rise to an atrial arrhythmia is perhaps not surprising given that many genes are expressed in both the atria and ventricles. Hence these reports lead us to ponder, do genes implicated in ventricular cardiomyopathy also contribute to "lone" AF? Does the increased burden of these variants in "lone" AF patients indicate a shared predisposition between AF and DCM? What implications would ventricular cardiomyopathy genes have on AF? Considering the spectrum of genetic variants, what is the contribution of rare CNVs to "lone" AF?

1.8.3. Common variants

The genetics underlying AF are slowly being unravelled, indeed like many complex diseases, both rare and common variants have been implicated in AF (Choi et al., 2020). Upwards of hundreds of common variants have been associated with AF susceptibility through GWAS (Christophersen et al., 2017b; Khera et al., 2018; Nielsen et al., 2018; Roselli et al., 2018; Roselli et al., 2020). Indeed, the application of a single common variant to predict AF is limited, thus the value of GWAS was to identify novel genes and pathways not previously implicated in AF pathogenesis (Bapat et al., 2018). For instance, Nielsen et al. (2018) identified loci near genes for structural integrity and function of cardiac muscle to associate with AF susceptibility. However, bridging the gap from a GWAS significant locus to a functionally relevant gene is not straight forward, many loci occur in non-coding regions, and that remains a major challenge (Roselli et al., 2020; Tucker et al., 2016).

In this context, polygenic risk scores that incorporate all the GWAS significant loci and stratify AF risk in individuals have had tremendous uptake for their clinical utility (Khera et al., 2018). One of the earliest PRS developed for AF had a modest incremental discrimination of AF over clinical risk factors but it did associate strongly with cardioembolic stroke (Lubitz et al., 2017). Another PRS applied to a community-based cohort identified that individuals in the highest PRS tertile had a significantly higher AF lifetime risk compared to those in the lower PRS tertile (48.2% versus 22.3%, respectively) (Weng et al., 2018). Lastly, Khera et al. (2018) identified that 6% of the European population with a high PRS score had a 3-fold higher risk for AF than individuals with a low PRS, and this risk was equivalent to having a single rare pathogenic AF variant (Khera et al., 2018). Indeed, the role of PRS have begun to be evaluated in AF, but they have yet to be assessed in a "lone" AF cohort, where their potential genetic impact is anticipated to be the most dramatic. "Lone" AF is suspected to have a greater genetic burden than AF alone and, as we have observed with our previous study, a significant portion of "lone" AF heritability was not explained by rare variants alone (Lazarte et al., 2021d). Hence the exploration and comparison of two validated polygenic scores in a "lone" AF cohort should unravel to what extent are polygenic factors contributory in "lone" AF? And further elucidate which polygenic score harbors the greatest predictive power, a concept that has yet to be evaluated in the AF literature?

1.9. Familial hypercholesterolemia (FH)

Familial hypercholesterolemia (FH) is a common disorder with serious public health consequences, characterized by elevated plasma low-density lipoprotein (LDL) cholesterol (≥5 mmol/L for the heterozygous form). FH is an autosomal co-dominant condition; its heterozygous form is the most prevalent, affecting 1 in 300 individuals (Defesche et al., 2017; Hu et al., 2020). A lifetime exposure to high levels of LDL cholesterol augments atherosclerosis build-up and substantially increases the lifetime risk of ASCVD (Defesche et al., 2017). For instance, the risk of premature ASCVD for someone with LDL cholesterol >5 mmol/L and a pathogenic FH mutation is increased by 22-fold compared to a variant negative person with LDL cholesterol <3.4 mmol/L (Khera et al., 2016b). Early statin treatment can reduce lifetime ASCVD risk to near normal levels (Defesche et al., 2017). Furthermore, positive diagnosis of

FH should trigger family cascade screening, since half of first-degree relatives will also be affected (Defesche et al., 2017).

Blood LDL cholesterol levels are determined by environmental, as well as, monogenic and polygenic etiologies (Lazarte & Hegele, 2019). Monogenic FH consist primarily of dominant rare variants in the canonical genes: *LDLR*, *APOB*, and *PCSK9*; or in the minor genes: *LDLRAP1*, *ABCG5*, *ABCG8*, *APOE*, and *LIPA* (Berberich & Hegele, 2019a). Conversely, polygenic FH encompass a multitude of mutations scattered throughout the genome that, combined into a score, determine the susceptibility to elevated LDL cholesterol (Dron & Hegele, 2019). Overall, 47.3% of patients seen in our clinic have a pathogenic SNVs (Wang et al., 2016). Further an additional 6.4% of patients are found to harbour a pathogenic CNV (Wang et al., 2016). Of the remainder, a significant portion of patients carry a high polygenic predisposition to FH (Wang et al., 2016). Despite best efforts, FH remains underdiagnosed and undertreated; in particular, FH patients on statin therapy remain at high risk for developing ASCVD (Nordestgaard et al., 2013; Versmissen et al., 2008).

The growing availability of NGS technology and the relatively easy application of targeted NGS panel for FH diagnosis, has created an ever-growing list of novel variants that clinicians are increasingly expected to interpret for clinical decision-making. Following the Hippocratic oath's central dictum 'primum non nocere' ('first do no harm'), minimizing genetic reporting errors is essential for an accurate molecular diagnosis, family counselling and recommending the appropriate treatment (Brown et al., 2020; Schaefer et al., 2019). Guidelines for variant interpretation have been proposed by the ACMG and are explained in detail in section 1.6 but we wondered, what supporting data from the ACMG guidelines can be easily gathered in the clinic to improve variant assessment? How feasible would be it to be apply it on a novel variant and interpret it?

1.10.Lipodystrophy

Lipodystrophy is a heterogenous group of rare congenital or acquired metabolic disorders characterized by a complete or partial loss of adipose tissue that results in the storage of lipids in the liver, muscle and other organs, and leads to insulin resistance (Brown et al., 2016). Insulin resistance in turn triggers diabetes mellitus type 2, hypertriglyceridemia, polycystic ovarian

syndrome (PCOS), and non-alcoholic fatty liver disease (Brown et al., 2016). The natural progression for patients with lipodystrophy can include the development of cardiomyopathy, heart failure, myocardial infarction, arrhythmia, liver failure, gastrointestinal hemorrhage, hepatocellular carcinoma, kidney failure, acute pancreatitis, and sepsis. Given the heterogeneity of the lipodystrophy group, there is a natural wide range of prognosis and outcomes; lipodystrophies can be broadly classified into "familial or genetic" and "acquired" types (Bhayana & Hegele, 2002). The main distinguishing factor between each form is the unique loss of adipose tissue from specific regions of the body; for instance, congenital generalized lipodystrophy involves loss of adipose tissue in the entire body while acquired partial lipodystrophy spares the lower extremities of the subcutaneous fat loss (Gupta et al., 2017).

One type of lipodystrophy is familial partial lipodystrophy (FPLD) which is an inherited type of body fat loss limited to the extremities, trunk and gluteal region (Brown et al., 2016). The most common subtype of FPLD is Dunnigan-type (or FPLD2) (OMIM 151660) which is caused by autosomal dominant pathogenic rare missense variants in the *LMNA* gene encoding lamin A/C (Hegele et al., 2007). The FPLD2 clinical phenotype develops around puberty, with noticeable loss of subcutaneous fat and a potential accumulation of fat in the face, neck, back and labia majora. In some cases, muscular hypertrophy, acanthosis nigricans, hirsutism, menstrual abnormalities, dyslipidemia, hepatosteatosis and PCOS can also occur. Later in life, many patients develop type 2 diabetes mellitus, which increases the risk for microvascular and macrovascular complications, and coronary heart diseases (Brown et al., 2016). Due to the rarity of the lipodystrophy syndromes, understanding the natural history of each subtype has not been possible and most of what we know originates from small case reports. We wondered, what role, if any, could genetics have in elucidating the natural history of one FPLD subtype?

1.11.Thesis outline

1.11.1. Thesis hypothesis

1) Complex diseases, like "lone" AF, are determined by a mixture of rare and common variants across the genome.

2) Twenty first century genetics has a valuable role in improving clinical medicine and in the advancement of our understanding of diseases, but this must occur alongside a rigorous assessment of novel putative variants.

1.11.2. Thesis aims

Aim 1: I propose to utilize NGS technologies to characterize the burden of rare SNVs and CNVs in a "lone" AF cohort.

Aim 2: I propose to interrogate SNP microarray data to characterize for the overall burden of common variants on a "lone" AF cohort and compare two discriminatory capabilities of two polygenic scores.

Aim 3: I propose to apply cascade screening in the setting of a novel CNV to improve variant assessment in clinical settings.

Aim 4: I aim to investigate the prevalence of severe hypertriglyceridemia and pancreatitis in patients with FPLD2, utilizing genetic and health record data.

1.12.Conclusion

In this PhD Thesis, I describe my research efforts primarily dedicated to elucidating the genetic contribution underlying "lone" AF as well as clinical lessons from the application of genetics on clinical medicine. I have achieved this by investigating both rare and common variants captured with NGS and DNA microarray technologies, on patients with AF, FH and FPLD. Specifically, my findings on the "lone" AF cohort confirmed the contribution of rare variants from classical cardiomyopathy genes and the substantial contribution of common variants (previously unexplored). Collectively, my work has further advanced our understanding of "lone" AF and highlighted the diverse genetic basis of the disease. My investigations on the FH family and lipodystrophy cohort provide valuable clinical lessons about the application of genetics in clinical medicine. In the case of the FH family, the lesson was on the importance of proper variant interpretation. While with the FPLD2 cohort, the lesson was on the increased risk FPLD2 patients with concurrent diabetes have for serious complications and how they should be monitored more frequently.

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Chapter 2. Loss-of-Function and Copy Number Variants in Ventricular Cardiomyopathy Genes in “Lone” Atrial Fibrillation

The work contained in this Chapter has been edited from its original publication in *Europace* for brevity and consistency throughout this PhD Thesis.

ESC European Society of Cardiology

Europace (2021) 00, 1–7
doi:10.1093/europace/euaa421

CLINICAL RESEARCH

Enrichment of loss-of-function and copy number variants in ventricular cardiomyopathy genes in ‘lone’ atrial fibrillation

Julieta Lazarte [1], Zachary W. Laksman[2], Jian Wang[1], John F. Robinson[1], Jacqueline S. Dron[1], Emma Leach[2], Janet Liew[2], Adam D. McIntyre[1], Allan C. Skanes[3], Lorne J. Gula[3], Peter Leong-Sit[3], Henian Cao[1], Brett Trost[4], Stephen W. Scherer[4,5], Robert A. Hegele [1], and Jason D. Roberts[3]*

[1]Department of Medicine, Robarts Research Institute, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada; [2]Department of Medicine, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada; [3]Section of Cardiac Electrophysiology, Division of Cardiology, Department of Medicine, Western University, London, ON, Canada; [4]The Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada; and [5]Department of Molecular Genetics, McLaughlin Centre, University of Toronto, Toronto, ON, Canada

Received 29 September 2020; editorial decision 20 December 2020; accepted after revision 22 December 2020

Aims Atrial fibrillation (AF) is a complex heritable disease whose genetic underpinnings remain largely unexplained, though recent work has suggested that the arrhythmia may develop secondary to an underlying atrial cardiomyopathy. We sought to evaluate for enrichment of loss-of-function (LOF) and copy number variants (CNVs) in genes implicated in ventricular cardiomyopathy in ‘lone’ AF.

Methods and results Whole-exome sequencing was performed in 255 early onset ‘lone’ AF cases, defined as arrhythmia onset prior to 60 years of age in the absence of known clinical risk factors. Subsequent evaluations were restricted to 195 cases of European genetic ancestry, as defined by principal component analysis, and focused on a pre-defined set of 43 genes previously implicated in ventricular cardiomyopathy. Bioinformatic analysis identified 6 LOF variants (3.1%), including 3 within the *TTN* gene, among cases in comparison with 4 of 503 (0.80%) controls [odds ratio: 3.96; 95% confidence interval (CI): 1.11–14.2; $P = 0.033$]. Further, two AF cases possessed a novel heterozygous 8521 base pair *TTN* deletion, confirmed with Sanger sequencing and breakpoint validation, which was absent from 4958 controls ($P = 0.0014$). Subsequent cascade screening in two families revealed evidence of co-segregation of a LOF variant with ‘lone’ AF.

Conclusion ‘Lone’ AF cases are enriched in rare LOF variants from cardiomyopathy genes, findings primarily driven by *TTN*, and a novel *TTN* deletion, providing additional evidence to implicate atrial cardiomyopathy as an AF genetic subphenotype. Our results also highlight that AF may develop in the context of these variants in the absence of a discernable ventricular cardiomyopathy.

Keywords Atrial fibrillation • Copy number variation • Dilated cardiomyopathy • Titin • Loss-of-function • Genetics

Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia. Its prevalence is age-dependent with an overall estimate of 2–3% in the general population that drops to <1% among individuals under 50 years of age, but is upwards of 10% in octogenarians.[1] Beyond symptomatic palpitations, the arrhythmia is associated with increased risks of stroke, heart failure, and death. The majority of AF cases develop in the presence of established clinical risk factors, including hypertension, coronary and valvular heart disease, and ventricular cardiomyopathy. However, cases of so-called ‘lone’ AF that manifest in the absence of known predisposing clinical conditions are well-

* Corresponding author. Tel: +1 519 663 3746 ext 34526; fax: +1 519 663 3782. *E-mail address*: jason.roberts@phri.ca

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2.1. Study rationale

"Lone" atrial fibrillation (AF) is a relatively uncommon phenotype; however, the first ever pathogenic variant (located in the *KCNQ1* gene) was discovered in a "lone" AF family (Chen et al., 2003b). Since then, numerous novel variants in candidate genes, mostly encoding ion channels, have been identified, helping elucidate portions of the pathophysiology of AF (Fatkin et al., 2017). However, much remains to be explored with newer candidate genes that have been mapped with genome-wide association studies (GWAS). For this investigation, we sought to determine if "lone" AF patients carried a higher burden of rare variants in ventricular cardiomyopathy genes.

2.2. Overview

AF is a complex heritable disease whose genetic underpinnings remain largely unexplained, though recent work has suggested that the arrhythmia may develop secondary to an underlying atrial cardiomyopathy. We sought to evaluate for enrichment of loss-of-function (LOF) and copy number variants (CNVs) in genes implicated in ventricular cardiomyopathy in "lone" AF. Whole-exome sequencing was performed in 255 early onset "lone" AF cases, defined as arrhythmia onset prior to 60 years of age in the absence of known clinical risk factors. Subsequent evaluations were restricted to 195 cases of European genetic ancestry, as defined by principal component analysis, and focused on a pre-defined set of 43 genes previously implicated in ventricular cardiomyopathy. Bioinformatic analysis identified 6 LOF variants (3.1%), including 3 within the *TTN* gene, among cases in comparison with 4 of 503 (0.80%) controls (Odds ratio: 3.96; 95% confidence interval [CI]: 1.11-14.2; P=0.033). Further, 2 AF cases possessed a novel heterozygous 8,521 base pair *TTN* deletion, confirmed with Sanger sequencing and breakpoint validation, that was absent from 4,958 controls (P=0.0014). Subsequent cascade screening in 2 families revealed evidence of co-segregation of a LOF variant with "lone" AF. "Lone" AF cases are enriched in rare LOF variants from cardiomyopathy genes, findings primarily driven by *TTN*, and a novel *TTN* deletion, providing additional evidence to implicate atrial cardiomyopathy as an AF genetic sub-phenotype. Our results also highlight that AF may develop in the context of these variants in the absence of a discernable ventricular cardiomyopathy.

2.3. Introduction

AF is the most common sustained cardiac arrhythmia and is associated with increased risks of stroke, heart failure, and death. The majority of AF cases develop in the presence of established clinical risk factors, including hypertension, coronary and valvular heart disease, and ventricular cardiomyopathy. However, cases of so-called "lone" AF that manifest in the absence of known predisposing clinical conditions are well-documented, highlighting our limited insight into its pathophysiology and the need to explore non-conventional risk factors, including genetic contributors. Heritability studies have confirmed a genetic contribution to AF and a mounting number of reports have identified rare and common variants that predispose to the condition, though the majority of AF heritability remains unexplained (Lubitz et al., 2010b). Notably, a potential contribution of copy number variants (CNVs), large genomic structural alterations, has yet to be extensively explored.

Two separate reports have identified cases and families of early-onset AF that were found to be secondary to mutations within *MYL4*, which encodes a protein critical to sarcomeric structure and whose expression is restricted to the atria (Gudbjartsson et al., 2017a; Orr et al., 2016). Consistent with their chamber-specific expression, the ventricles of these patients appeared normal. Since these original reports, loss-of-function (LOF) *TTN* variants have also been implicated in AF (Choi et al., 2018a). *TTN* encodes titin, a sarcomeric protein that has a critical role in cardiac contractile function, is expressed in both the atria and ventricles, and is a well-established culprit of various forms of ventricular cardiomyopathy (Ware et al., 2016). Aligning with this cardiomyopathic theme, recent AF genome-wide association studies have also identified common variants within or near genes associated with ventricular cardiomyopathy (Roselli et al., 2018).

The concept of an underlying atrial cardiomyopathy being responsible for AF vulnerability and potentially leading to a heightened stroke risk has been increasingly discussed in the literature in recent years (Goette et al., 2016; Guichard & Nattel, 2017). Notably, multiple different clinical, environmental, and genetic factors have been purported to give rise to different pathophysiological and histological subtypes of atrial cardiomyopathy (Goette et al., 2016). In this context, the notion that a gene implicated in ventricular cardiomyopathy could give rise to an atrial cardiomyopathy and subsequently predispose to AF is perhaps not surprising given that

many genes are expressed in both the atria and ventricles (McNally & Mestroni, 2017). Motivated by these concepts, we sought to evaluate for a role of LOF variants and CNVs within genes implicated in ventricular cardiomyopathy as causes of "lone" AF.

2.4. Methods

2.4.1. AF study cohort

Patients with early-onset "lone" AF, defined as development of arrhythmia in the absence of known clinical risk factors prior to 60 years of age, were recruited from the referral bases for AF management at the London Health Sciences Centre, London, Ontario, Canada and St. Paul's Hospital, Vancouver, British Columbia, Canada. All AF cases were unrelated and had at least one episode of electrocardiographically documented AF characterized by erratic atrial activity without distinct P waves and irregularly irregular QRS intervals lasting >30 seconds. Exclusion criteria consisted of known risk factors for AF, including hypertension, coronary artery disease, left ventricular ejection fraction <50%, moderate to severe valvular heart disease, hyperthyroidism, obstructive sleep apnea, and presence of a known underlying inherited channelopathy or cardiomyopathy. All patients underwent, at minimum, a clinical history, physical examination, 12-lead ECG, and echocardiogram.

Familial cascade screening was attempted for all AF cases identified to possess an LOF variant or a CNV within a screened cardiomyopathy gene. All family members that agreed to participate in cascade screening underwent, at minimum, a baseline ECG and echocardiogram. Among family members that did not possess AF on baseline ECG, Holter monitoring was performed (minimum duration of 24 hours). Use of clinical and genetic data from family members was restricted to genotype-phenotype segregation analyses and was not included in statistical analyses for variant enrichment. Evidence of genotype-phenotype segregation was defined as 2 or more family members carrying the variant and possessing either an AF or a DCM phenotype.

Participants provided informed written consent under protocols that were approved by the research ethics boards of Western University and the University of British Columbia. This study complies with the Declaration of Helsinki.

2.4.2. Genetics analyses

In preparation for whole-exome sequencing, genomic DNA for AF cases was isolated using the Puregene® DNA Blood Kit (Gentra Systems, Qiagen Inc., Mississauga, Ontario, Canada). Whole-exome enrichment was performed with Roche Nimblegen SeqCap EZ Human Exome capture chemistry and sequencing (NovaSeq 6000 sequencer, Illumina), average coverage depth was 80x. DNA-Seq was implemented with optimized BROAD institute germline best practices standard operating procedures (Van der Auwera et al., 2013). The optimized procedure entailed trimming raw reads derived from FASTQ files followed by alignment to the human reference genome (build hg19), post alignment refinements and variant calling. Trimmed reads were aligned to the reference by the Burrows-Wheeler Aligner, bwa-mem (algorithm) (Li & Durbin, 2009). Refinements of mismatches near indels and base qualities were performed using GATK indels realignment and base recalibration to improve read quality post alignment. Processed reads were marked as fragment duplicates using picard mark duplicates (Van der Auwera et al., 2013). SNP and small indels were annotated using either GATK haplotype callers or samtools mpileup (Li et al., 2009). The Genome in a Bottle (Zook et al., 2016) dataset was applied to select steps and parameters minimizing the false positive rate and maximizing the true positive variants to achieve a sensitivity of 99.7%, precision of 99.1% and F1-score of 99.4%. Finally, additional annotations were incorporated using dbNSFP (Liu et al., 2016) and/or Gemini (Paila et al., 2013) and quality control metrics were collected at various stages and visualized using MultiQC (Ewels et al., 2016).

Following raw data processing into variant call files (.vcf files), AF cases and control .vcf files were uploaded to VarSeq v2.2.0 (Golden Helix Inc, Bozeman, MT, USA).

Microarray analysis of AF cases was performed with Infinium™ Global Screening Array-24 v2.0 (Illumina). GenomeStudio software was utilized to retrieve the microarray data and export it to SNP & Variant Suite (SVS) v8.8.3 (Golden Helix Inc, Bozeman, MT, USA). To improve genotyping accuracy, data points were filtered out if they had a GenCall score cutoff < 0.15, removing unreliable genotypes that had not been assigned. Microarray data was further cleaned by filtering samples with a low rate of autosomal SNP calls over the total number of SNP calls in the dataset to avoid inappropriate results from faulty genotyping calls (Miyagawa et al., 2008). Any sample with a call rate <95% was removed (n=2).

2.4.3. Control cohorts

Various control cohorts were employed depending on the analysis undertaken. For the rare LOF variant analysis, our reference was the European subgroup (503 individuals) of the 1000 Genomes Project (1KG), a multi-ancestry cohort of 2,504 individuals over the age of 18 that self-reported as healthy (sequencing platform: Illumina, average depth of coverage: 7.4) (Genomes Project et al., 2012). SNP genotyping of the 1KG cohort was performed with the Illumina Omni 2.5M microarray. In order to evaluate the frequency of the *TTN* deletion identified in 2 AF cases, whole-genome sequencing data from 4,958 unaffected parents participating in an autism study was assessed (Yuen et al., 2017). Although these study participants had not undergone AF screening, all were less than 50 years of age and hence < 1% would be anticipated to possess AF (Zoni-Berisso et al., 2014). Among these 4,958 unrelated individuals, sequencing was performed on either the Illumina HiSeq X (n=4466), HiSeq 2000 (n=472), or HiSeq 2500 (n=20) platforms.

2.4.4. Principal component analysis on ancestry

SNP & Variant Suite (SVS) v8.8.3 (Golden Helix Inc, Bozeman, MT, USA) was utilized to correct for ancestry applying principal component analysis in order to confine the rare variant analysis to a single population (Zhang et al., 2013). Utilizing X chromosome heterozygosity, samples were removed if positive for sex discordance between clinical data and genotype information (n=4). Linkage disequilibrium (LD) pruning was applied to all autosome chromosomes to prepare the data for identity by descent estimation analysis. One father and son pair was detected and the father was removed from further analysis (n=1). Principal component analysis on patients and 1KG controls was performed on variants found in both datasets post LD pruning using EIGENSTRAT (Price et al., 2006). Five eigenvalue components were formulated, to which the top three were utilized to explain the majority of the stratification (Price et al., 2006). Around the 1K Genome European population cluster, a centroid was mathematically identified and any case sample that fell outside the 1.5 inter-quartile range was excluded from the analysis as it was deemed outside the European population cluster (n= 44).

2.4.5. LOF variant analysis

Bioinformatic analysis of whole-exome sequencing (WES) data was consistent for cases and controls and was initially restricted to a previously used panel of 43 dilated cardiomyopathy (DCM)-associated genes and then, in a subsequent analysis, to a subset of 12 genes recently shown to have robust evidence as highly penetrant and monogenic culprits of DCM (Mazzarotto et al., 2020; Ware et al., 2016). The panel of 43 genes included: *ABCC9, ACTC1, ACTN2, ANKRD1, BAG3, CRYAB, CSRP3, CTF1, DES, DMD, DNAJC19, DSG2, DSP, DTNA, EMD, FKTN, ILK, LAMA2, LAMA4, LAMP2, LDB3, LMNA, MYBPC3, MYH6, MYH7, NEXN, PDLIM3, RBM20, SCN5A, SDHA, SGCB, TAZ, TCAP, TNNC1, TNNI3, TNNT2, TPM1, TTN, VCL, CAV3, FXN, SYNE1,* and *SYNM* (Ware et al., 2016). In the subsequent analysis, we investigated the following 12 genes: *BAG3, DSP, TTN, VCL, LMNA, MYH7, TNNC1, TNNT2, TPM1, ACTC1, NEXN,* and *PLN* (Mazzarotto et al., 2020). Variant calls were filtered for a minor allele frequency of <0.1% or missing according to the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org). A threshold of <0.1% was selected in an effort to capture both high and intermediate penetrant rare variants given that autosomal dominant forms of AF are extremely rare and hence highly penetrant rare variants are likely the exception rather than the rule. Of the remaining variants, filters for *in silico* predictions of deleterious outcome were retrieved from the prediction tool Combined Annotation Dependent Depletion (CADD; http://cadd.gs.washington.edu/score). Variants with a CADD Phred score >20 were selected, belonging to the top 1% of most deleterious variants in the human genome. The final filter applied was for variant types that could be assumed to result in a LOF in the protein with a high degree of confidence; only variants that were frameshift, splice acceptor, splice donor and stop-gain were considered in the analysis.

2.4.6. Sanger sequencing

The validity of each LOF variant identified in AF cases was verified with bidirectional direct DNA (Sanger) sequencing. Amplification of targeted genomic regions was performed using polymerase chain reaction (primer sequences available upon request) followed by DNA sequencing using the ABI PRISM dye terminator method (Applied Biosystems, Foster City, CA, USA).

2.4.7. CNV detection

Sequencing data in the form of FASTQ files for AF cases and the internal reference group were processed utilizing a custom workflow in CLC Genomics Workbench v11.0.1 (CLC Bio, Aarhus, Denmark) to generate variant calling files and coverage statistics. These, alongside a Roche chemistry BED file defining the sequenced regions, were utilized for the CNV caller function in VarSeq v2.2.0 (Golden Helix, Bozeman, MT). The internal reference group used for the CNV caller algorithm was a composite of 16 unrelated self-reported healthy individuals of European ancestry who had provided written consent for their DNA to be analyzed. Sequencing of the internal reference group was performed on the Illumina NovaSeq 6000 and average depth of coverage was 80x. Bioinformatic filters applied were consistent for cases and the internal reference group. The CNV caller algorithm compares the coverage for each region between a case and the mean internal reference group (n=16). Further, the algorithm computes the Z-score metric for depth of coverage, which measures the number of standard deviations from the mean internal reference group. A second metric is the ratio, which is the normalized mean of the AF case divided by the average normalized mean of the internal reference group. It is considered suspicious for a duplication if the ratio is >1.25 and a deletion if it is <0.75. The P-value is the probability that a given Z-score for a particular area would occur by chance in a diploid region. The Z-score threshold for CNV consideration was 2 (or -2); however, a lower threshold was accepted in the presence of a strong P-value ($P < 1x10^{-5}$).

2.4.8. CNV breakpoint analysis

PCR and Sanger sequencing based analysis was used to identify the exact nucleotide breakpoints of a CNV. Briefly, long-range PCR primers were used to narrow the approximate regions harboring the breakpoints and subsequent primer walking and sequencing were employed to determine their exact locations. The exact locations were confirmed by sequence alignment of the amplified product with a known reference sequence.

2.4.9. TTN CNV frequency in a control population

To identify any CNVs overlapping the detected *TTN* CNV region in a control population, we evaluated sequencing data from 4,958 unrelated individuals. Sequencing data were aligned to the GRCh38/hg38 reference genome using the Sentieon implementation of BWA-mem. The

average depth of coverage was 43.5x for the *TTN* CNV frequency controls (n=4,958). Bioinformatic filters applied to the *TTN* CNV controls were any CNVs >=1 kb in the overlapping region of the detected TTN deletion.

The pipeline used to generate alignments (CRAM files) and small variant calls (VCF files) for the MSSNG DB6 release is based on software by Sentieon. This pipeline was developed for maximum concordance with the Broad Institute's Best Practices workflows for Data pre-processing for variant discovery (conforming to the CCDG functional equivalence standard) and Germline SNPs and indels. For a detail description of the workflow: https://dockstore.org/workflows/github.com/DNAstack/mssng-db6-ccdg-alignment-and-calling:1.0.0?tab=info.

CNVs >=1 kb were detected using a previously described workflow (Trost et al., 2018) involving the algorithms ERDS and CNVnator. The "intersect" function of BEDTools was used to identify CNVs overlapping the detected *TTN* CNV location. Integrative Genomics Viewer was used to visually verify the correctness of any CNVs detected (Trost et al., 2018).

2.4.10. Statistical analysis

Continuous variables are presented as means ± standard deviation. Two-by-two contingency tables with chi-square analysis were used to calculate odds ratios (ORs) of rare LOF variant enrichment. Comparison of the *TTN* CNV frequency in AF cases versus 4,958 controls was performed using Fisher's exact test. Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC). Statistical significance was defined as $P < 0.05$.

2.5. Results

2.5.1. Clinical characteristics of AF cases

A total of 255 early-onset "lone" AF cases underwent WES; principal component analysis restricted the analysis to a total of 195 "lone" AF cases with European genetic ancestry. Their clinical characteristics are summarized in **Table 2.1**. The average age at AF diagnosis was 44.47 ± 9.75 years and 81% were male. A total of 173 (88.7%) individuals had paroxysmal AF at the time diagnosis, whereas the remaining 22 (11.3%) patients had persistent AF. Positive family history of AF was noted in 59 (30%), defined as at least one first-degree relative possessing the

arrhythmia. Mean body-mass index was 27.24 ± 3.80, and the mean left atrial diameter was 3.96 ± 0.55 cm.

Table 2.1 Clinical Characteristics of the "Lone" AF Cohort

Clinical Variable	"Lone" AF Cohort n = 195
Age at diagnosis (years)	44.47 ± 9.75
Sex (male)	157 (80.5%)
Body mass index (kg/m^2)*	27.23 ± 3.81
Type of AF at diagnosis	
Paroxysmal	173 (88.7%)
Persistent	22 (11.3%)
Family History of AF	59 (30.2%)
ECG Values	
PR-interval*	167.56 ± 26.50
QRS duration	94.82 ± 14.65
QTc	420.55 ± 27.05
Left atrial diameter (cm)*	3.97 ± 0.56

Data are n (%) or mean ± standard deviation.

AF = atrial fibrillation; * values for body mass index, PR-interval, and left atrial diameter were missing for 1, 2, and 1 cases, respectively.

2.5.2. LOF variants in DCM genes

Genetic analysis revealed that 3.1% (6/195) of individuals from the AF cohort possessed a LOF variant among the 43 screened DCM genes, including 4 novel variants and 2 that had been previously observed (**Table 2.2**). Half of the variants identified in the AF cohort were present in the *TTN* gene (3/6) and the remaining 3 variants were identified in the *NEXN*, *SDHA*, and *CRYAB* genes. Evaluation of the 1KG controls revealed that 0.79% (4/503) possessed heterozygous LOF variants in the *SYNE1*, *MYBPC3*, *ABCC9* and *MYH7* genes. The majority of the LOF variants in the AF cohort were stop-gain (4/6) and the remaining two were frameshift mutations, while the majority of variants in the controls impacted splice acceptor sites (3/4). The average CADD Phred score of the variants in all AF cases was 40.76 in comparison with 24.15 for controls. The odds ratio of rare LOF variants found in the AF cohort compared to the 1KG controls was 3.96 (95% CI: 1.10-14.2; P=0.033).

Restricting the analysis to the 12 highly penetrant DCM genetic culprits identified by Mazzarotto and colleagues revealed that 2.05% (4/195) of AF cases possessed a LOF variant (*TTN* (3/4) and *NEXN*)(Mazzarotto et al., 2020). In comparison, 0.20% (1/503) of 1KG controls possessed a heterozygous LOF variant (*MYH7)*. These findings corresponded to 10.51-fold (95% CI: 1.73-128.9; P=0.02) increased odds of a rare LOF variant being present in the AF cohort compared to the 1KG controls.

Table 2.2 Loss-of-Function and Copy Number Variants From Ventricular Cardiomyopathy Genes Identified in "Lone" Atrial Fibrillation Cases

AF Family	Gene	Chr:Position	Ref/Alt	Amino Acid Change	Variant Type	CADD Phred Score	rsID	gnomAD MAF
1	*TTN*	2:179459226	T/-	His19332Profs*18	Frameshift	57	rs397517633	-
2		2:179547542	A/C	Tyr10992Ter	Stop Gain	49	-	-
3		2:179642033	G/A	Gln1553Ter	Stop Gain	36	-	-
4	*NEXN*	1:78399009	C/T	Gln366Ter	Stop Gain	39	-	-
5	*SDHA*	5:223624	C/T	Arg31Ter	Stop Gain	36	rs142441643	0.000411
6	*CRYAB*	11:111779517	GC/-	Lys166Asnfs9_stoploss, 14stopgain	Frameshift	27.6	-	-
7	*TTN*	2:179517952-179526473	-	-	Heterozygous Deletion	-	-	-
8			-				-	-

Abbreviations: bp = base pairs; Chr = chromosome; Ref = reference; Alt = alternate; MAF = minor allele frequency from the Non-Finnish European gnomAD . Genomic coordinates to GRCh37 (hg19) reference genome

2.5.3. CNVs in AF cases and control cohort

CNV analysis with breakpoint confirmation revealed the presence of a novel heterozygous 8,522 base pair deletion in the *TTN* gene in two unrelated AF individuals among the 195 cases (**Table 2.2**). The heterozygous deletion encompasses 2 exons in the cardiac isoform (*N2BA*, NM_001256850) and 9 in the inferred complete isoform (*IC*, NM_001267550) of the I-band region and causes an in-frame deletion (**Figure 2.1, 2.2, 2.3**). The two deleted exons in the cardiac isoform encode PEVK domains, which are considered important for the spring activity of TTN. No CNVs overlapping the *TTN* deletion were detected in 4,958 control individuals compared with 2/195 AF cases (P=0.0014).

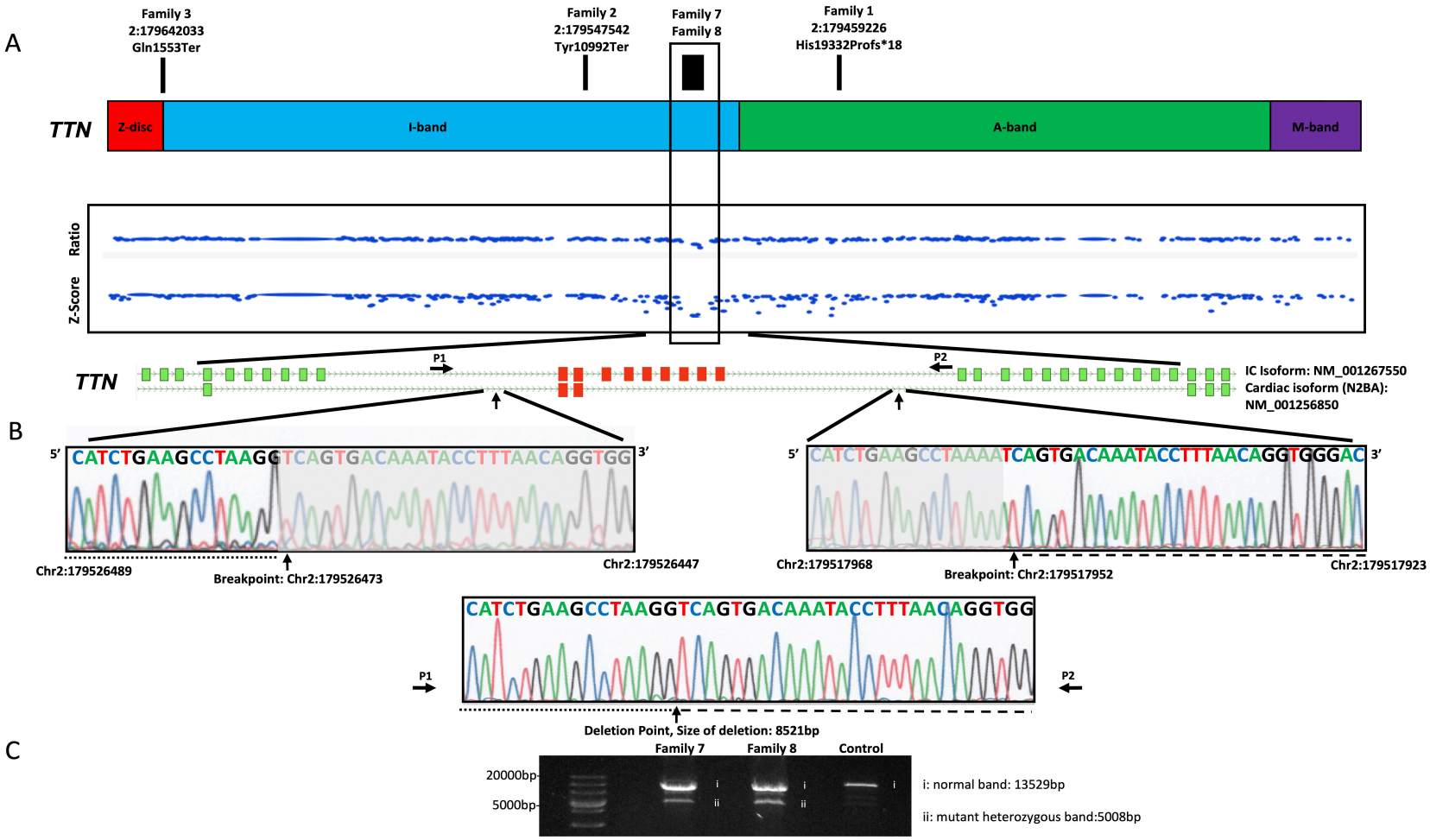

Figure 2.1 ***TTN*** **LOF Variants and a Novel CNV Identified in "Lone" AF Patients**

(**A**) *TTN* gene, with coloured bands to denote different regions, highlighting locations of *TTN* LOF variants and a novel CNV found in "lone" AF cases. (**B**) *TTN* heterozygous deletion identified using whole-exome sequencing ratio and Z-score values and Sanger sequencing tracing of breakpoints. (**C**) PCR detection of deletion with electrophoresis in 2 AF cases (Families 7 and 8). Genomic coordinates to GRCh37(hg19) reference genome. AF = atrial fibrillation, IC isoform = inferred complete isoform, Chr = chromosome, bp = base pairs

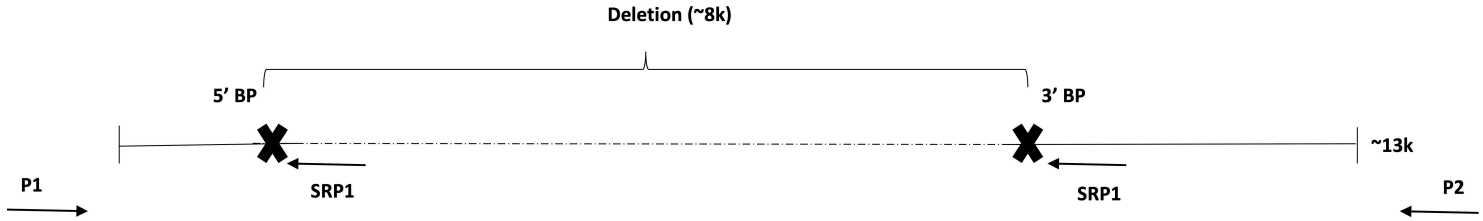

Figure 2.2 Region of the Heterozygous Deletion (8,581bp) and Location of the PCR and Sequencing Primers

P1 and P2 are the PCR primers that localized the zone of the deletion to a 13,529 bp region. SRP1 is the reverse sequence primer that binds to 2 sites in a normal subject (before the 5' and 3' breakpoints) due to sequence similarity, while in the two AF cases (Family 7 and 8) it only binds and sequences one region.

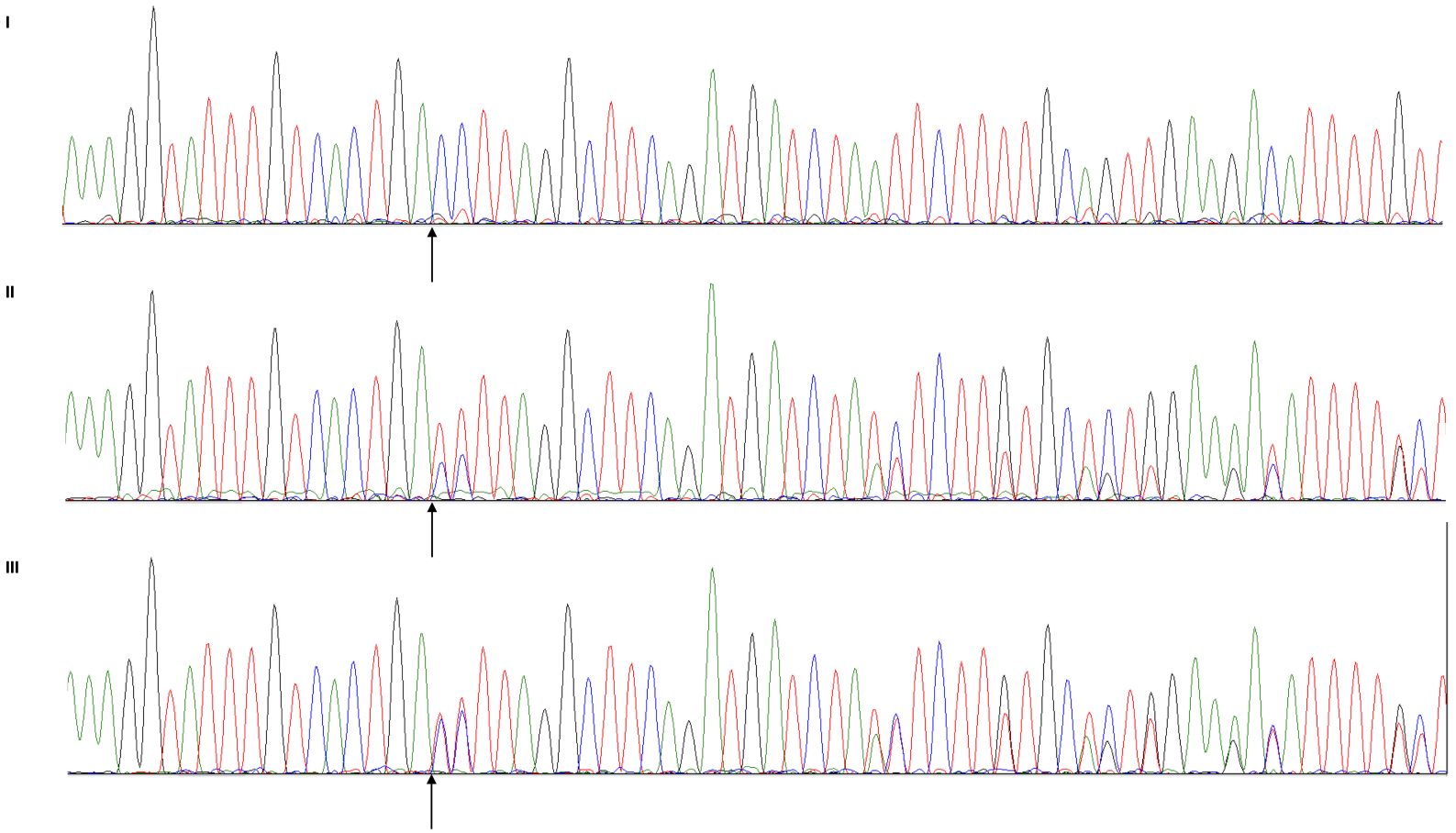

Figure 2.3 Reverse Sequence of Family 7 with the *TTN* CNV (I) and Two Controls (II, III)

Given the high degree of repeated sequence, our primers bind to 2 different regions as observed on the sequences for controls II and III. However, in both AF cases (Families 7 and 8) the primer binds in one area due to the deleted CNV, instead of two, displaying a single sequence. The same results have been observed multiple times and utilizing multiple different controls. This is additional proof that both patients carry a deletion in the *TTN* gene.

2.5.4. Genotype-phenotype segregation

Familial cascade screening was conducted in 5 of the 8 families possessing an LOF variant or CNV within a DCM gene; screening was either declined or not feasible in the remaining 3 families (**Figure 2.4 and 2.5**). Based on our definition for genotype-phenotype segregation requiring 2 or more genotype positive family members to possess either AF or a DCM phenotype, co-segregation was observed in 2 families (**Figure 2.4**). In Family 1, the female proband (TTN-p.His19332Profs*18) was diagnosed with AF at the age of 56 years. Evaluation of family members revealed that the brother of the proband, who possessed the familial variant, was diagnosed with AF and DCM at 66 years of age, while an unaffected 68-year old sister was genotype negative (**Figure 2.4 A**). Notably, the original presentation of the brother consisted of admission to hospital for congestive heart failure felt to be secondary to new onset AF with rapid ventricular response. His initial left ventricular ejection fraction was 20-25%; however, following treatment of his heart failure and electrical cardioversion restoring sinus rhythm, his ejection fraction normalized consistent with a tachycardia-induced cardiomyopathy. For Family 2 (TTN-p.Tyr10992Ter), the male proband was diagnosed with AF at 49 years. Cascade screening revealed that his son, diagnosed with AF at 23 years of age, possessed the variant, whereas his 65-year old brother was genotype negative and had a normal phenotype (**Figure 2.4 B**).

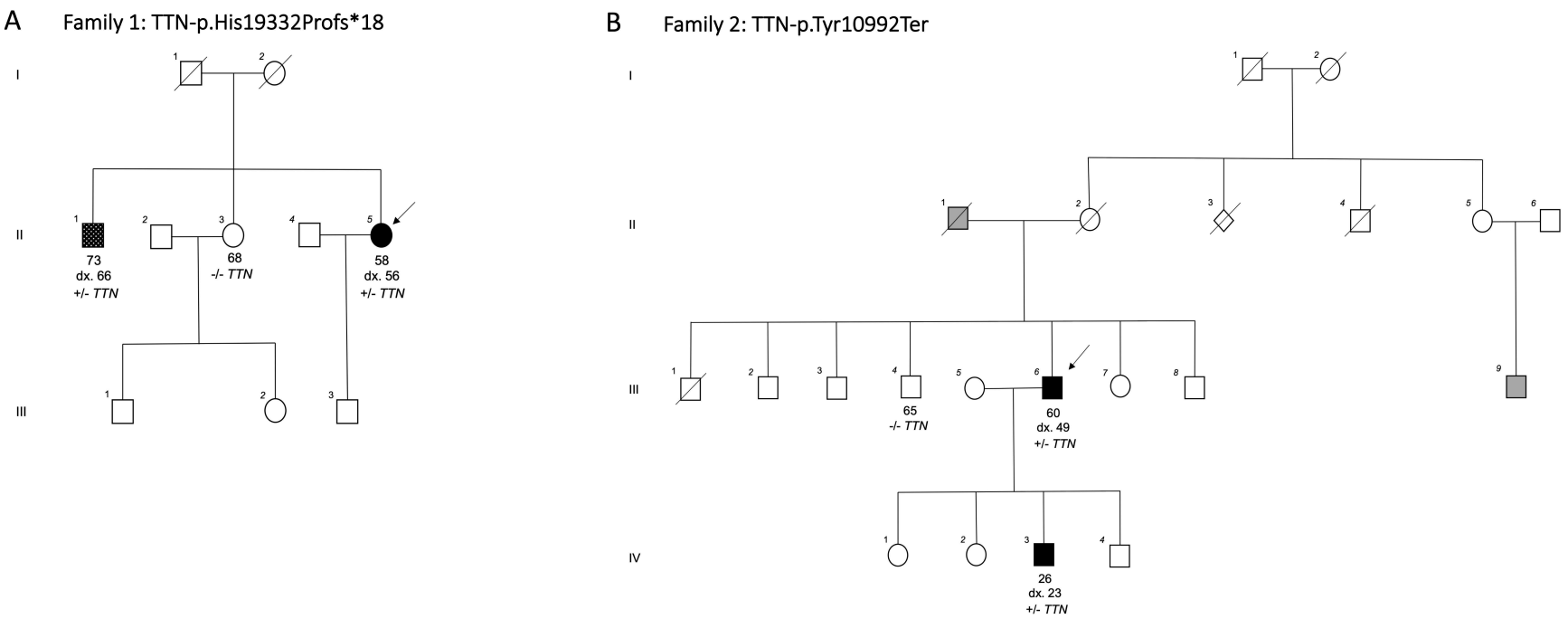

Figure 2.4 Pedigree of 2 Families with Rare LOF Variants in the *TTN* (A and B) Segregating with Phenotypes of AF and/or Atrial Flutter

Probands are identified with an arrow. Clinical status is defined as: black = AF; white dots = tachycardia-induced cardiomyopathy and AF; diagonal stripes = atrial flutter; grey = AF phenotype reported, but not clinically confirmed by our group; white = unaffected; slash = deceased. Genotype is indicated as + for variant present and – for no variant, followed by the gene name. Current age is listed first, followed by age at time of diagnosis, if applicable.

AF = atrial fibrillation, dx = age at diagnosis.

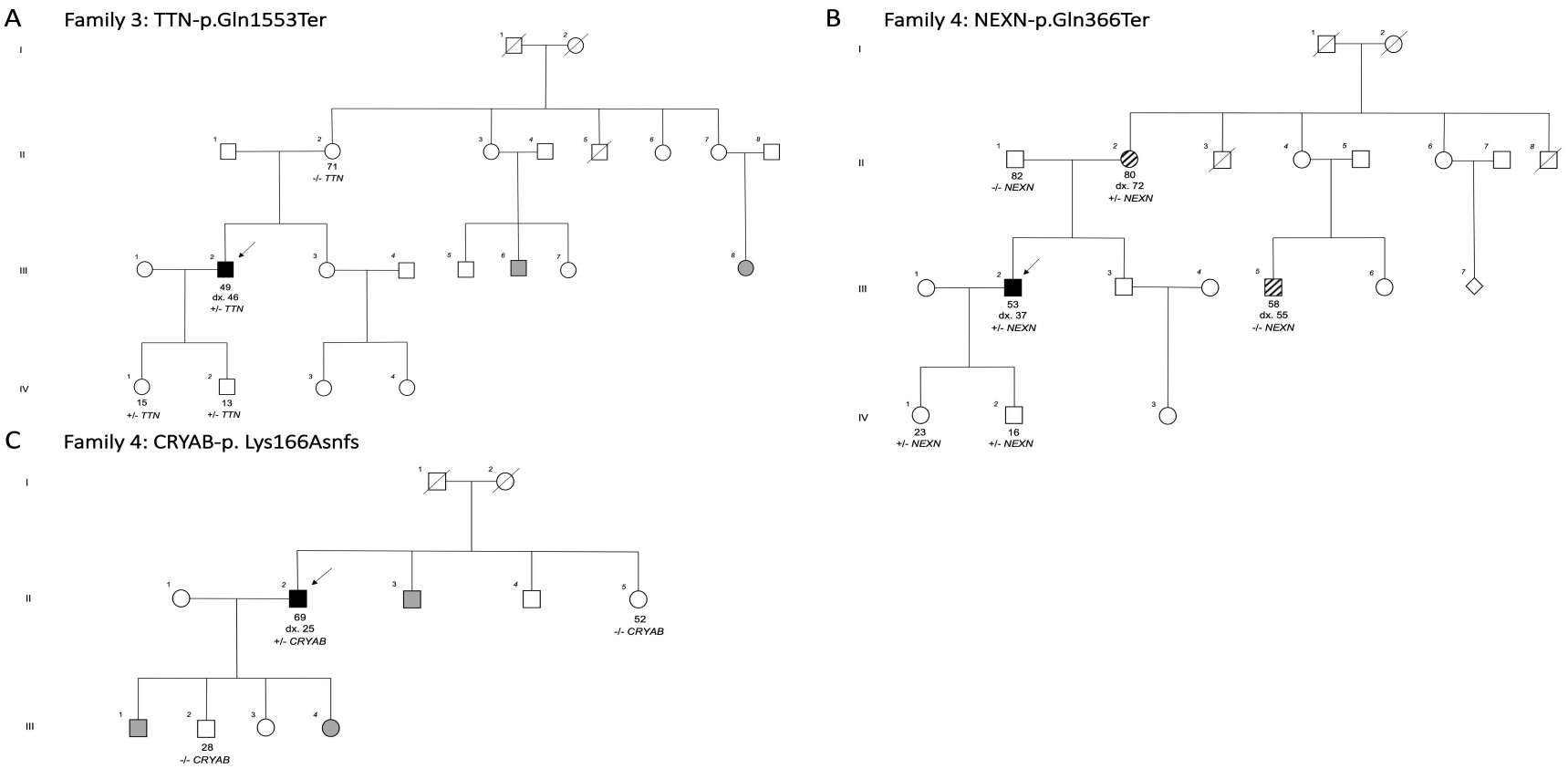

Figure 2.5 Pedigrees of 3 Families with Rare LOF Variants in the *TTN* (A), *NEXN* (B) and *CRYAB* (C) Genes

Probands are identified with an arrow. Clinical status is defined as: black = AF; grey = AF phenotype reported, but not clinically confirmed by our group; white = unaffected; slash = deceased. Genotype is indicated as + for variant present and – for no variant, followed by the gene name. Current age is listed first, followed by age at time of diagnosis, if applicable.

AF = atrial fibrillation, dx = age at diagnosis.

For Family 4, the male proband possessing the NEXN-p.Gln366Ter variant developed AF at 37 years of age. Cascade screening revealed that the mother of the proband possessed the *NEXN* variant and developed atrial flutter at 72 years of age, however given that atrial flutter is not uncommon in this age group, this should not be viewed as strong evidence for genotype-phenotype segregation (**Figure 2.5 B**). Both children of the proband also carried the variant and were phenotype negative, however their young age renders interpretation of their unaffected status unclear. A cousin diagnosed with atrial flutter at age 55 notably did not possess the *NEXN* variant. Cascade screening in Family 3 (TTN-p.Gln1553Ter) was limited and clarification of genetic carrier status of 2 cousins reported to be affected was not possible (**Figure 2.5 A)**. The CRYAB-p.Lys166Asnfs9_stoploss,14stopgain variant in Family 6 did not reveal evidence of familial segregation of the LOF variant with arrhythmic or cardiomyopathic phenotypes, though clinical and genetic evaluation of family members was again limited (**Figure 2.5 C)**. Cascade screening was either not feasible or was declined for Families 5 (SDHA-p.Arg31Ter), 7 (*TTN* CNV [deletion]), and 8 (*TTN* CNV [deletion]).

2.6. Discussion

Among a cohort of 195 "lone" AF cases of European ancestry, defined as development of the arrhythmia in the absence of a known predisposing clinical risk factor, we identified an enrichment in heterozygous LOF variants in DCM genes. Overall, 3.1% of the AF cohort had rare LOF variants compared to 0.79% in the 1KG cohort (OR: 3.96; 95% CI: 1.10-14.2; P=0.033). Restricting the analysis to 12 highly penetrant DCM genes revealed a heterozygous LOF variant in 2.05% of the AF cohort relative to 0.20% of the 1KG cohort (OR: 10.51; 95% CI: 1.73-128.9; P=0.02). Enrichment of cardiomyopathy variants in "lone" AF cases was primarily driven by findings within *TTN* in both analyses. Further, we identified two carriers of a novel heterozygous deletion in the *TTN* gene that was absent from a control set of 4,958 individuals (P= 0.0014).

Our findings align with recent genetic studies suggesting that atrial cardiomyopathy may represent an important arrhythmogenic substrate responsible for AF development (Choi et al., 2018a). Of particular importance in our study is the finding that pathogenic mutations within genes implicated in DCM are associated with a phenotype of AF in the absence of ventricular

cardiomyopathy. This observation alludes to the existence of differential chamber penetrance in the atria and ventricles that varies among individuals and highlights that patients possessing a pathogenic cardiomyopathy mutation may manifest an isolated ventricular cardiomyopathy, isolated AF, or a combined phenotype of ventricular cardiomyopathy and AF. Recognition of this concept may be particularly important when counseling patients on the potential clinical phenotypes that may manifest in the setting of pathogenic variant within one of these genes. Although the mediators of differential chamber penetrance among patients are unknown, we would hypothesize genomic background and environmental influences as potential candidates.

Our findings also allude to the notion that a genetic atrial myopathic substrate may be the primary driver of AF among individuals with genetic forms of ventricular cardiomyopathy. Increased atrial filling pressures in the setting of ventricular cardiomyopathy causing atrial stretch and dilation had previously been considered the primary culprit responsible for AF development; however, it is conceivable that their contribution is minor. Additionally, although also speculative, experts have hypothesized that AF that develops secondary to an underlying structural atrial cardiomyopathy may be associated with an increased stroke risk relative to primarily electrical forms of the arrhythmia (Darlington & McCauley, 2020). Should this be proven correct, insight into the underlying genetic etiology for AF within an individual may prove helpful for stroke risk stratification and the need for anti-coagulation.

Among 5 affected individuals, we identified 3 LOF variants and 1 CNV (identical in-frame deletion in 2 unrelated cases) in *TTN*, the largest gene in the human genome, whose function is to facilitate the longitudinal movement of the sarcomere. *TTN* truncating variants are estimated to have a prevalence of approximately 0.4% in the general population and have consistently been associated with various diseases, including ventricular cardiomyopathies (Pirruccello et al., 2020). Titin is comprised of 4 different regions, namely the Z-disk, the I-band, the A-band, and the M-line (**Figure 2.1**). Approximately 20% of patients with DCM possess a truncating *TTN* variant, the majority being localized to the A-band region of the protein (Ware et al., 2016). Mirroring the findings of Choi and colleagues, the distribution of the *TTN* LOF variants and the CNV were found in the Z-disc, I-band, and A-band regions (**Figure 2.1**) (Choi et al., 2018a).

Our investigation is the first, to our knowledge, to identify a rare recurrent *TTN* deletion in 2 unrelated "lone" AF patients. Previously, Tsai *et al* identified a common intronic duplication in *KCNIP1,* which encodes the potassium interacting channel 1 protein (Tsai et al., 2016). The intronic CNV was associated with increased levels of the *KCNIP1* transcript and its overexpression in zebrafish was shown to increase AF susceptibility. Unfortunately, evaluation for familial genotype-phenotype segregation was not possible for either "lone" AF case in our study possessing the large *TTN* deletion, precluding additional insight into its potential clinical relevance.

The remaining LOF variants were identified in the *NEXN* (nexilin F-actin binding protein), *SDHA* (succinate dehydrogenase complex flavoprotein subunit A) and *CRYAB* (crystallin alpha B) genes. *NEXN* rare variants have been documented to be enriched in DCM cases relative to controls in 2 separate studies and knockdown of its expression using an antisense oligonucleotide led to a DCM phenotype in a zebrafish model, which was suggested to be secondary to destabilization of the cardiac Z-disc region within the sarcomere (Hassel et al., 2009; Mazzarotto et al., 2020). *SDHA* has a critical role in mitochondrial function as an enzymatic constituent of the Krebs cycle and loss-of-function variants result in succinate dehydrogenase deficiency, which includes DCM as one of its phenotypic traits (Davili et al., 2007). Rare variants within the *CRYAB* gene, which acts as a molecular chaperone preventing protein aggregation, have been shown to be associated with DCM in the absence of skeletal myopathy, including a stop-loss variant that results in a similar protein elongation effect as observed in our AF case (van der Smagt et al., 2014).

A recent re-evaluation of the genetic architecture of DCM has suggested that only 12 genes are reflective of highly penetrant genetic culprits for the condition (Mazzarotto et al., 2020). Although this refined insight is critical for proper interpretation of genetic testing results in clinical practice, absence of a highly penetrant effect does not preclude the possibility that functionally relevant variants in the remaining genes may exert a more modest effect on the risk of DCM. As such, we felt it was still reasonable to proceed with our original hypothesis of evaluating 43 genes previously implicated in DCM, however also elected to conduct a separate analysis involving the 12 highly penetrant DCM genetic culprits. In both instances, "lone" AF cases were enriched in presumed damaging LOF variants relative to healthy controls. Notably,

in both analyses the statistically significant results observed were driven primarily by *TTN* variants. Although our size sample was modest, no other cardiomyopathy gene emerged as a prominent culprit for "lone" AF.

Findings from our study support the notion that atrial cardiomyopathy constitutes a genetic sub-phenotype of AF, however it is important to note that we are not concluding that each of the genes identified to possess an LOF variant among AF cases should now be considered a genetic culprit of the arrhythmia. As evidenced by damaging LOF variants also being identified in controls, it is almost certain that some LOF variants identified in AF cases were incidental findings. Although the LOF variants we identified using strict filtering criteria are predicted to be damaging and were statistically enriched, individual causality for the observed AF phenotype will require further study. Here, we built family pedigrees, where possible, to investigate co-segregation as additional evidence for causality and identified modest degrees of genotype-phenotype segregation in 2 families. It should also be noted that the different sequencing technologies utilized for the cases and controls may introduce bias in relation to variant ascertainment. Although an important limitation, the large sizes of the 1KG and CNV control cohorts may provide improved precision of allele and CNV frequencies in healthy control populations.

2.7. Conclusion

In a cohort of early onset "lone" AF, we found an enrichment of rare LOF variants and a novel deletion in a pre-specified set of DCM genes compared to controls, providing additional evidence that atrial cardiomyopathy may be a genetic sub-phenotype of AF. This enrichment was primarily driven by *TTN* variants and notably no other cardiomyopathy genes emerged as obvious contributors to the "lone" form of the arrhythmia. Our results also highlight that AF may develop in the context of these cardiomyopathy variants in the absence of a discernable ventricular cardiomyopathy.

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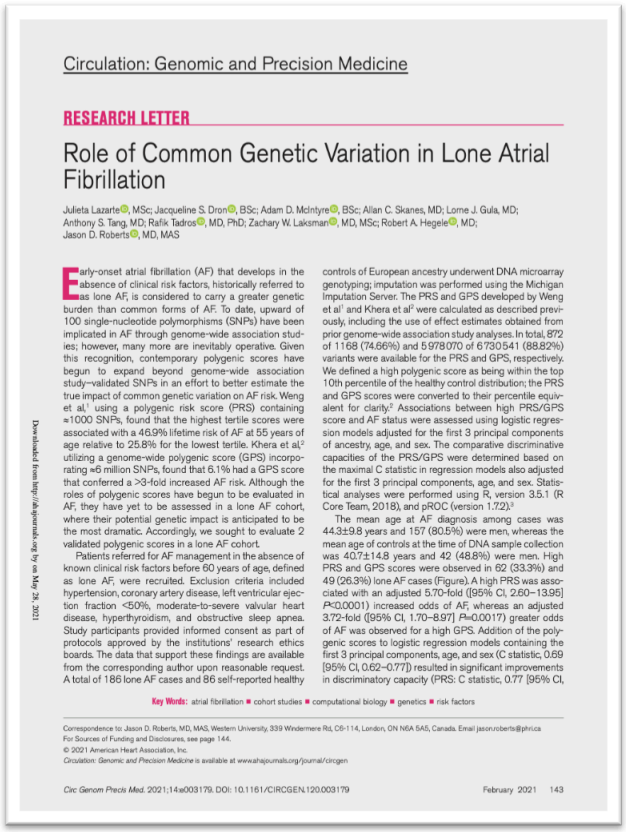
Chapter 3. The Role of Common Genetic Variation in "Lone" Atrial Fibrillation

The work contained in this Chapter has been edited from its original publication in *Circulation: Genomic and Precision Medicine* and *Canadian Journal of Cardiology Open* for brevity and consistency throughout this PhD Thesis.



Circulation: Genomic and Precision Medicine

RESEARCH LETTER

Role of Common Genetic Variation in Lone Atrial Fibrillation

Julieta Lazarte, MSc; Jacqueline S. Dron, BSc; Adam D. McIntyre, BSc; Allan C. Skanes, MD; Lorne J. Gula, MD; Anthony S. Tang, MD; Rafik Tadros, MD, PhD; Zachary W. Laksman, MD, MSc; Robert A. Hegele, MD; Jason D. Roberts, MD, MAS

Early-onset atrial fibrillation (AF) that develops in the absence of clinical risk factors, historically referred to as lone AF, is considered to carry a greater genetic burden than common forms of AF. To date, upward of 100 single-nucleotide polymorphisms (SNPs) have been implicated in AF through genome-wide association studies; however, many more are inevitably operative. Given this recognition, contemporary polygenic scores have begun to expand beyond genome-wide association study–validated SNPs in an effort to better estimate the true impact of common genetic variation on AF risk. Weng et al,[1] using a polygenic risk score (PRS) containing ≈1000 SNPs, found that the highest tertile scores were associated with a 46.9% lifetime risk of AF at 55 years of age relative to 25.8% for the lowest tertile. Khera et al,[2] utilizing a genome-wide polygenic score (GPS) incorporating ≈6 million SNPs, found that 6.1% had a GPS score that conferred a >3-fold increased AF risk. Although the roles of polygenic scores have begun to be evaluated in AF, they have yet to be assessed in a lone AF cohort, where their potential genetic impact is anticipated to be the most dramatic. Accordingly, we sought to evaluate 2 validated polygenic scores in a lone AF cohort.

Patients referred for AF management in the absence of known clinical risk factors before 60 years of age, defined as lone AF, were recruited. Exclusion criteria included hypertension, coronary artery disease, left ventricular ejection fraction <50%, moderate-to-severe valvular heart disease, hyperthyroidism, and obstructive sleep apnea. Study participants provided informed consent as part of protocols approved by the institutions' research ethics boards. The data that support these findings are available from the corresponding author upon reasonable request. A total of 186 lone AF cases and 86 self-reported healthy controls of European ancestry underwent DNA microarray genotyping; imputation was performed using the Michigan Imputation Server. The PRS and GPS developed by Weng et al[1] and Khera et al[2] were calculated as described previously, including the use of effect estimates obtained from prior genome-wide association study analyses. In total, 872 of 1168 (74.66%) and 5 978 070 of 6 730 541 (88.82%) variants were available for the PRS and GPS, respectively. We defined a high polygenic score as being within the top 10th percentile of the healthy control distribution; the PRS and GPS scores were converted to their percentile equivalent for clarity.[3] Associations between high PRS/GPS score and AF status were assessed using logistic regression models adjusted for the first 3 principal components of ancestry, age, and sex. The comparative discriminative capacities of the PRS/GPS were determined based on the maximal C statistic in regression models also adjusted for the first 3 principal components, age, and sex. Statistical analyses were performed using R, version 3.5.1 (R Core Team, 2018), and pROC (version 1.7.2).[3]

The mean age at AF diagnosis among cases was 44.3±9.8 years and 157 (80.5%) were men, whereas the mean age of controls at the time of DNA sample collection was 40.7±14.8 years and 42 (48.8%) were men. High PRS and GPS scores were observed in 62 (33.3%) and 49 (26.3%) lone AF cases (Figure). A high PRS was associated with an adjusted 5.70-fold ([95% CI, 2.60–13.95] $P<0.0001$) increased odds of AF, whereas an adjusted 3.72-fold ([95% CI, 1.70–8.97] $P=0.0017$) greater odds of AF was observed for a high GPS. Addition of the polygenic scores to logistic regression models containing the first 3 principal components, age, and sex (C statistic, 0.69 [95% CI, 0.62–0.77]) resulted in significant improvements in discriminatory capacity (PRS: C statistic, 0.77 [95% CI,

Key Words: atrial fibrillation ■ cohort studies ■ computational biology ■ genetics ■ risk factors

Correspondence to: Jason D. Roberts, MD, MAS, Western University, 339 Windermere Rd, C6-114, London, ON N6A 5A5, Canada. Email jason.roberts@phri.ca
For Sources of Funding and Disclosures, see page 144.

Circulation: Genomic and Precision Medicine is available at www.ahajournals.org/journal/circgen

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CJC Open ■ (2021) 1–7

Original Article

Evaluating Polygenic Risk Scores in "Lone" Atrial Fibrillation

Julieta Lazarte, MSc,[a] Jacqueline S. Dron, BSc,[a] Adam D. McIntyre, BSc,[a] Allan C. Skanes, MD,[b] Lorne J. Gula, MD,[b] Anthony S. Tang, MD,[b] Rafik Tadros, MD, PhD,[d] Zachary W. Laksman, MD, MSc,[c] Robert A. Hegele, MD,[a] and Jason D. Roberts, MD, MAS[b]

[a] Department of Medicine and Robarts Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada
[b] Section of Cardiac Electrophysiology, Division of Cardiology, Department of Medicine, Western University, London, Ontario, Canada
[c] Department of Medicine, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada
[d] Division of Cardiology, Montréal Heart Institute, University of Montréal, Montréal, Québec, Canada

ABSTRACT

Background: Polygenic scores incorporating varying numbers of single nucleotide polymorphisms (SNPs) have been demonstrated to exert a prominent role in atrial fibrillation (AF). We sought to compare the relative discriminatory capacities of 2 previously validated polygenic scores in "lone" AF.

Methods: A total of 186 lone AF cases of European ancestry underwent SNP genotyping. A genome-wide polygenic score (GPS) and polygenic risk score (PRS) involving 6,730,541 and 1168 SNPs, respectively, were calculated for 186 cases and 423 controls of

RÉSUMÉ

Contexte : Il a été démontré que des scores polygéniques intégrant un nombre variable de polymorphismes mononucléotidiques (PMN) jouent un rôle important en ce qui concerne la fibrillation auriculaire (FA). Nous avons comparé le potentiel discriminatoire relatif de deux scores polygéniques déjà validés dans la FA idiopathique.

Méthodologie : Au total, 186 sujets d'ascendance européenne atteints de FA idiopathique ont été soumis à un génotypage des PMN. Un score polygénique génomique (SPG) et un score de risque polygénique (SRP) comprenant respectivement 6 730 541 et 1 168 PMN ont été calculés

Rare and common genetic variants have been shown to affect the risk of developing atrial fibrillation (AF).[1] Notably, 22% of AF heritability has been suggested to be explained primarily by the cumulative effects of common variants or single nucleotide polymorphisms (SNPs), highlighting the significant polygenic burden of AF, with a minimal, yet important, contribution of rare variants with large effect size such as those found in the structural gene titin (TTN).[1,2] To date, genome-wide association studies (GWAS) have identified more than 100 SNPs that significantly associate with AF susceptibility.[4,5] In this context, polygenic scores that incorporate multiple small-effect SNPs and identify a proportion of subjects from the general population at an increased risk for AF, are viewed as harbouring great clinical utility, given the potential to enhance screening and prevention therapies.[6] Notably, however, three-quarters of AF genetic risk has been identified to be driven by common variants in regions that have yet to satisfy the stringent thresholds for GWAS statistical significance.[2] Recognizing that sizeable amounts of genomic data may not be adequately leveraged at present, contemporary genetic risk scores have begun to include loci that have yet to reach GWAS levels of significance, in an effort to better estimate AF susceptibility.[2,6]

"Lone" AF represents a subtype of the arrhythmia that develops in the absence of identifiable clinical risk factors.[7] Although previously criticized because of its heterogeneous definitions and our improved ability to identify predisposing clinical risk factors, such cases are considered to harbour a greater genetic contribution relative to more common forms of the arrhythmia that develop in the setting of structural heart disease or other established risk factors.[8] We previously explored the role of genetic risk scores in a lone AF cohort and a locally procured healthy control set.[10] We found that genetic risk scores developed by both Weng et al. (2018; an ~1000 SNP polygenic risk score [PRS]) and Khera et al. (2018; ~6 million genome-wide polygenic risk score [GPS]), identified a significant portion of patients with lone AF with an elevated polygenic score compared with healthy controls.[6,7,10] However, no difference was observed when comparing the

Received for publication January 10, 2021. Accepted February 1, 2021.

Ethics Statement: Participants provided informed written consent under protocols that were approved by the research ethics boards of Western University (#107249) and the University of British Columbia (# H15-02970). The study complies with the Declaration of Helsinki.

Corresponding author: Dr Jason D. Roberts, 339 Windermere Road, C6-114, London, Ontario N6A 5A5, Canada. Tel.: +1-519-663-3746, ext. 34526; fax: +1-519-663-3782.
E-mail: jason.roberts@hsc.on.ca
See page 6 for disclosure information.

https://doi.org/10.1016/j.cjco.2021.02.001

Lazarte, J., Dron, J.S., McIntyre, A.D., Skanes, A.C., Gula, L.J., Tang, A.S., Tadros, R., Laksman, Z.W., Hegele, R.A., and Roberts, J.D. (2021). Role of Common Genetic Variation in Lone Atrial Fibrillation. *Circ Genom Precis Med.* Advance online publication. (PMID: 33517663)

Lazarte, J., Dron, J.S., McIntyre, A.D., Skanes, A.C., Gula, L.J., Tang, A.S., Tadros, R., Laksman, Z.W., Hegele, R.A., and Roberts, J.D. (2021). Evaluating Polygenic Risk Scores in "Lone" Atrial Fibrillation. *CJC Open*. 3(6), 751–757.

3.1. Study rationale

The genetics underlying atrial fibrillation (AF) are slowly being unravelled; like other complex diseases, both rare and common variants have been implicated in AF (Choi et al., 2020). Further, the role of polygenic scores have begun to be evaluated in AF, but they have yet to be assessed in a "lone" AF cohort, where their potential genetic impact is anticipated to be the most dramatic. "Lone" AF is suspected to have a greater genetic burden than AF alone and as we have demonstrated in our previous study, a significant percentage of "lone" AF heritability (96%) was not explained by rare variants alone. Hence the exploration and comparison of two validated polygenic risk scores in a "lone" AF cohort, should unravel the role of common variants in "lone" AF susceptibility and further elucidate which polygenic score harbors the greatest predictive power, a concept that has yet to be evaluated in the AF literature.

3.2. Overview

Increasing numbers of single nucleotide polymorphisms (SNPs) have been implicated in AF, however small effect sizes limit their clinical utility in isolation. Polygenic scores incorporating large numbers of SNPs have suggested common genetic variation exerts a prominent role in AF but have yet to be evaluated in "lone" AF. We sought to evaluate 2 previously validated polygenic scores in "lone" AF. A total of 186 "lone" AF cases of European ancestry underwent SNP genotyping. A genome-wide polygenic score (GPS) and a polygenic risk score (PRS) involving 6,730,541 and 1,168 SNPs, respectively, were calculated for "lone" AF cases and 423 European 1000 Genomes (1KG) Project controls. A high polygenic score was defined as > 90^{th} percentile within controls. The discriminatory ability of polygenic scores was compared using receiver operating characteristic (ROC) curves. A total of 64 of 186 (34.4%) "lone" AF cases had a high GPS corresponding to 4.64-fold increased odds (95% confidence interval (CI) [2.99-7.18]; P<0.001) relative to controls. A high PRS was present in 49 of 186 (26.3%) individuals with "lone" AF and was associated with a 3.16-fold increased odds (95% CI [2.01-4.98]; P<0.001) of the arrhythmia. Comparison of C-statistics from ROC curves indicated improved discriminatory capacity of the GPS (0.76) relative to the PRS (0.70) (p=0.002). Our study evaluating 2 validated polygenic scores suggests that common genetic variation exerts a critical role in the pathogenesis of "lone" AF and reinforces their clinical potential.

3.3. Introduction

The genetics underlying atrial fibrillation (AF) are slowly being unraveled and, though significant progress has been made, there remains ongoing debate regarding the relative contributions of rare and common genetic variation to the arrhythmia (Choi et al., 2020; Khera et al., 2018). To date, rare variants in several genes encoding ion channels, a hormone, and cardiomyocyte structural proteins have been implicated in the arrhythmia and, although they appear to exert dramatic effect sizes, they have only been identified in a small minority of AF cases (Chen et al., 2003a; Choi et al., 2018a; Hodgson-Zingman et al., 2008). In contrast, the role of common genetic variation to AF susceptibility in the general population has been firmly established through genome wide association studies (GWAS), however the effect sizes of individual single nucleotide polymorphisms (SNPs) are minimal, which has led to questions regarding their clinical relevance and potential utility (Nielsen et al., 2018; Roselli et al., 2018).

Although a single SNP in isolation may have a trivial impact on AF risk, large numbers are potentially relevant to arrhythmogenesis within a given individual. The presumed cumulative impact of SNPs has led to the development of polygenic scores, which incorporate large numbers of small-effect common variants. To date, there have been upwards of 100 SNPs identified to be relevant in AF through GWAS, however many more are inevitably operative (Nielsen et al., 2018; Roselli et al., 2018). Given this recognition, contemporary polygenic scores have begun to involve larger numbers of SNPs, including those yet to satisfy stringent GWAS thresholds, in order to better estimate the true impact of common genetic variation on AF risk (Vilhjalmsson et al., 2015). Weng and colleagues, using a polygenic risk score (PRS) containing ~1000 SNPs, found that the highest tertile of polygenic risk was associated with a 46.9% lifetime risk of the arrhythmia at 55 years of age relative to a 25.8% risk for the lowest tertile (Weng et al., 2018). Khera and colleagues, utilizing a genome-wide polygenic score (GPS) incorporating ~ 6 million SNPs, found that 6.1% of a European population had a GPS score that conferred a greater than 3-fold increased risk of the arrhythmia (Khera et al., 2018).

AF that develops in the absence of identifiable clinical risk factors has been historically referred to as “lone” AF (Evans & Swann, 1954; Fuster et al., 2006). Although use of the term “lone” AF has been criticized secondary to heterogeneous definitions, the contribution of genetics appears most prominent in cases of the arrhythmia that develop in the absence of clinical risk factors

(Wyse et al., 2014b). Notably, prospective cohort studies have documented that the presence of a first degree relative possessing the arrhythmia increases the risk of developing AF by ~ 1.5 fold, whereas this risk increases to upwards of 3-fold when analyses are restricted to "lone" AF (Ellinor et al., 2005; Fox et al., 2004; Lubitz et al., 2010b; Marcus et al., 2008; Oyen et al., 2012).

Although the role of polygenic scores has begun to be evaluated in AF, they have yet to be assessed in a "lone" AF cohort, where their potential impact is anticipated to be most dramatic. Accordingly, we sought to evaluate two validated polygenic scores in a carefully phenotyped cohort of patients with early onset AF that had developed the arrhythmia in the absence of identifiable clinical risk factors.

3.4. Methods

3.4.1. AF study cohort

Patients referred for AF management at the London Health Sciences Centre, London, Ontario, Canada and St. Paul's Hospital, Vancouver, British Columbia, Canada with AF in the absence of known clinical risk factors prior to 60 years of age, defined as "lone" AF, were recruited to the study. All patients had at least one episode of electrocardiographically documented AF characterized by erratic atrial activity without distinct P waves and irregularly irregular QRS intervals lasting >30 seconds. Exclusion criteria consisted of known clinical risk factors for AF, including hypertension, coronary artery disease, left ventricular ejection fraction <50%, moderate to severe valvular heart disease, hyperthyroidism, obstructive sleep apnea, and presence of inherited cardiomyopathy. All patients underwent, at minimum, a clinical history, physical examination, 12-lead ECG, and echocardiogram. A positive family history of AF was defined as presence of the arrhythmia in a first- or second-degree relative. Participants provided informed written consent under protocols that were approved by the research ethics boards of Western University (#107249) and the University of British Columbia (# H15-02970). The study complies with the Declaration of Helsinki.

3.4.2. Control cohorts

Genetic ancestry based on principal component analysis was determined by utilizing population data from the publicly available 1000 Genomes Project (1KG), a multi-ancestry cohort of 1,756

individuals over the age of 18 that self-reported as healthy (Illumina Omni 2.5 M SNP data) (Genomes Project et al., 2015; Roslin NM, 2016).

For the polygenic score calculations and analyses, 2 reference cohorts were considered; one was 423 European (non-Finnish) individuals from 1KG Project (Illumina Omni 2.5 M SNP data)(Genomes Project et al., 2015; Roslin NM, 2016) and the second was 86 self-reported healthy individuals of European ancestry who had provided written consent for their DNA to be analyzed, hereafter referred to as "lab controls".

3.4.3. DNA preparation and microarray genotyping

Genomic DNA for “lone” AF cases and lab controls was isolated using the Puregene® DNA Blood Kit (Gentra Systems, Qiagen Inc., Mississauga, Ontario, Canada). Microarray analysis was performed with Infinium™ Global Screening Array-24 v2.0 (Illumina) at Genome Quebec, Montreal, Quebec, Canada. GenomeStudio software (Illumina) was utilized to retrieve and export the microarray data to SNP & Variant Suite (SVS) v8.8.3 (Golden Helix Inc, Bozeman, MT, USA). To improve genotyping accuracy, data points were filtered if they had a GenCall score cutoff < 0.15. Microarray data was further cleaned by filtering samples with a <95% rate of autosomal SNP calls over the total number of SNP calls in the dataset to avoid inappropriate results from faulty genotyping calls (n=2) (Miyagawa et al., 2008). Utilizing X chromosome heterozygosity, samples were removed if positive for sex discordance between clinical data and genotype information (n=4). Linkage disequilibrium (LD) pruning was applied to all autosomes to prepare the data for identity by descent estimation analysis. One father and son pair was detected and the father was removed from further analysis. Deviation from Hardy-Weinberg equilibrium was not used as a method to filter SNPs given that it was unclear if various assumptions were met for both the lone AF and 1KG cohorts, including random mating and sufficiently large population sizes (Roslin NM, 2016).

3.4.4. Principal component analysis on ancestry- European sub-group

Ancestry inference using principal component analysis was performed on SNP & Variant Suite (SVS) v8.8.3 (Golden Helix Inc, Bozeman, MT, USA) for the “lone” AF cases and lab controls (separately) post LD pruning using EIGENSTRAT (Price et al., 2006). Among 5 formulated eigenvalues, the top 3 explained the majority of the stratification (**Figure 3.1**). Around the 1KG

European (non-Finnish) population cluster, a centroid was mathematically identified and any case sample that fell outside the 1.5 inter-quartile range was excluded from the analysis as it was deemed outside the European (non-Finnish) population cluster. Among a total of 240 “lone” AF cases that had undergone DNA microarray analysis, 54 were excluded on this basis.

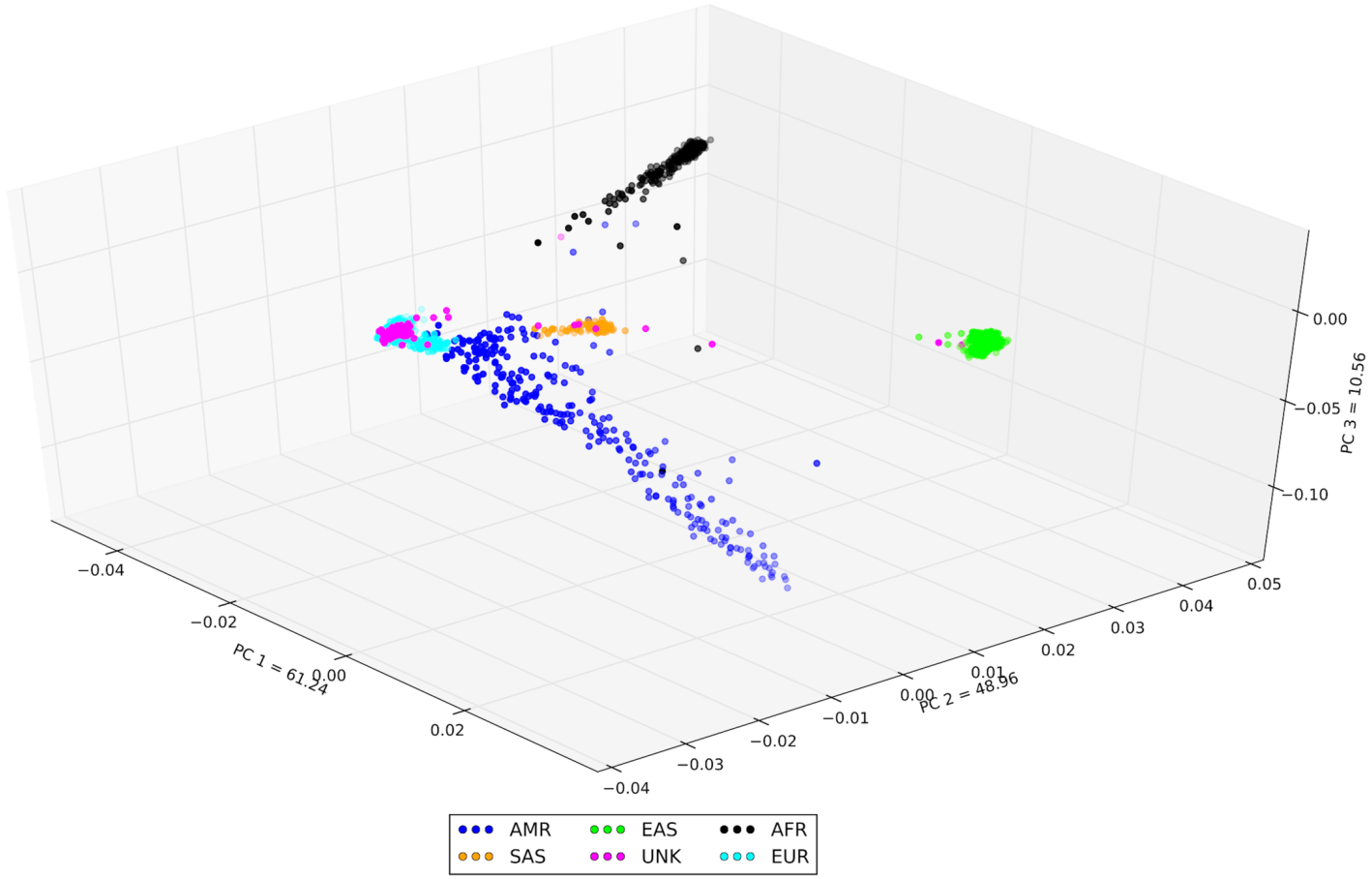

Figure 3.1 3-Dimension Scatter Plot of the First 3 Principal Components for the 1KG Control Super-Populations and "Lone" AF Cases of Unknown Ancestry

Super populations along with the lone AF cohort are shown as: AMR (Admixed American)= dark blue; EAS (East Asian)= green; AFR (African)= black; SAS (South Asian)= orange; EUR (European)= light blue and UNK (lone AF cases)= pink.

PC = principal components

3.4.5. Imputation

Genotype imputation of DNA microarray data was performed for all 3 cohorts to increase genomic coverage using the Michigan Imputation Server (Das et al., 2016). Imputation was performed using the Minimac4 1.2.4 imputation algorithm with the Haplotype Reference Consortium r1.1 (cases and lab controls jointly) and 1KG Phase 3 v5 (1KG controls) reference set. Only SNPs that were genotyped or imputed with high quality (r^2 >0.3) were utilized for score calculation.

3.4.6. Polygenic score calculation

Two previously derived and validated AF polygenic scores were applied: a GPS developed by Khera et al. and a PRS developed by Weng et al. (both in 2018), hereafter referred to as "GPS" and "PRS", respectively (Khera et al., 2018; Weng et al., 2018). The GPS was derived by Khera and colleagues using the LDPred algorithm and association data from a prior genome wide association study for AF, using separate testing and validation datasets from the UK Biobank (Christophersen et al., 2017a; Khera et al., 2018). The PRS developed by Weng and colleagues also used association data from the same AF genome wide association study, however used pruning and thresholding at various tuning parameters for its derivation (Christophersen et al., 2017a; Weng et al., 2018). A total of 30 candidate scores were developed and tested within the UK Biobank dataset and the optimal one was identified on the basis of its goodness-of-fit in accordance with the Akaike's Information Criterion (Weng et al., 2018). Individuals were scored by counting genetic dosages of imputed variants using the --score option in PLINK2.0 and Wrapper Python script (Khera et al., 2019). For each variant, the number of risk alleles present is multiplied by its respective weight and the products for each variant are added to generate the final score. In total, 5,978,070 of 6,730,541 variants (88.82%) were available for the GPS and 872 of 1,168 variants (74.66%) for the PRS in the 3 cohorts. We defined a high GPS/PRS as the top 10^{th} percentile of the 1KG control distribution.

3.4.7. Statistical analysis

GPS/PRS in all groups ("lone" AF cases, 1KG controls, and lab controls) were assessed for normality using the D'Agostino-Pearson omnibus K2 test. Odds ratios (ORs) were calculated by comparing proportions of individuals with high GPS/PRS (in the top 10 percentile) as well as top

5 and 1 percentile using 2-by-2 contingency tables with Fisher's exact test (correction for ancestry was done prior with principal component analysis). The performance of the GPS and PRS were assessed using receiver operating characteristic (ROC) curves with 1KG controls as the reference and compared in R version 3.5.1 (R Core Team, 2018) with *pROC* (version 1.7.2) (Robin et al., 2011). Impact of a high GPS/PRS score on age at diagnosis (divided into quartiles: <37, 37-45, 46-51, >51) among "lone" AF cases was assessed using the Chi-square test and the Chi-square test for trend. Evaluation for a different likelihood of possessing a high GPS/PRS score in "lone" AF cases by sex and by body mass index (BMI) > 30kg/m^2 was assessed using 2-by-2 contingency tables with Fisher's exact test. Unless otherwise stated, all statistical analyses were conducted using GraphPad Prism 8 for Windows (version 8.3.1; GraphPad Software, San Diego CA). Statistical significance was defined as $P < .05$.

3.4.7.1. Post-hoc analysis: AF cases vs. lab controls

Associations between high GPS/PRS score and AF status was assessed using logistic regression models adjusted for the first 3 principal components (PC) of ancestry, age and sex. The comparative discriminative capacities of the GPS/PRS were determined based on the maximal C-statistic in regression models also adjusted for the first 3 PCs, age and sex.

3.5. Results

3.5.1. Baseline characteristics

A total of 186 "lone" AF cases were included in the study; demographic and clinical characteristics are described in **Table 3.1**. The mean age at AF diagnosis was 44.5 ± 9.8 years and 157 (80.5%) cases were male. The mean age of the lab controls at the time of DNA sample collection was 40.7 ± 14.8 years and 42 (48.8%) were male. Twenty-two (11.3%) study participants had persistent AF at the time of diagnosis, whereas the remainder presented with paroxysmal AF. The mean left atrial diameter on echocardiography at the time of presentation was 4.0 ± 0.6 cm.

Table 3.1 Clinical Characteristics of the "Lone" AF Cohort

Clinical Variable	"Lone" AF Cohort n = 186
Age at diagnosis (years)	44.26 ± 9.85
Sex (male)	151 (81.2%)
Body mass index (kg/m^2)*	27.39 ± 3.92
Type of AF at diagnosis	
Paroxysmal	165 (89.7%)
Persistent	19 (10.3%)
Family History of AF*	57 (30.8%)
ECG Values	
PR-interval*	166.73 ± 25.95
QRS duration	95.27 ± 14.74
QTc	420.60 ± 26.70
Left atrial diameter (cm)*	3.96 ± 0.55

Data are n (%) or mean ± standard deviation.

AF = atrial fibrillation; * values for body mass index, family history of AF, PR-interval, and left atrial diameter were missing for 1, 1, 2, and 2 cases, respectively.

3.5.2. Polygenic risk scores

The distributions of the GPS (A) and PRS (B) across the three study cohorts are shown in **Figure 3.2**. The GPS had a Gaussian distribution across the three groups, whereas the PRS was skewed to higher polygenic scores in the 1KG controls failing the normality test (p=0.02).

A high GPS (top 90th percentile) was observed in 64 of 186 "lone" AF patients (34.4%), 43 of 423 1KG controls (10.2%) and 13 of 73 lab controls (15.1%) (**Figure 3.2 A, Table 3.2**). "Lone" AF patients had a 4.64-fold increased odds (95% confidence interval (CI) [2.99-7.18]; p<0.001) of having a high GPS compared with 1KG controls and a 2.95-fold greater odds (95% CI [1.52-5.72]; p<0.001) compared with lab controls (**Figure 3.3**). The odds of a score within the top 5% and 1% of GPS values were 4.22-fold (95% CI: 2.40-7.57; p<0.0001) and 3.76-fold (95% CI: 1.18-10.30, p=0.02) more likely among lone AF patients relative to 1KG controls, respectively (**Table 3.2)**. There was no difference in the odds of possessing a high GPS between the 1KG and lab controls (OR=0.64; 95% CI [0.32-1.27]; p=0.19).

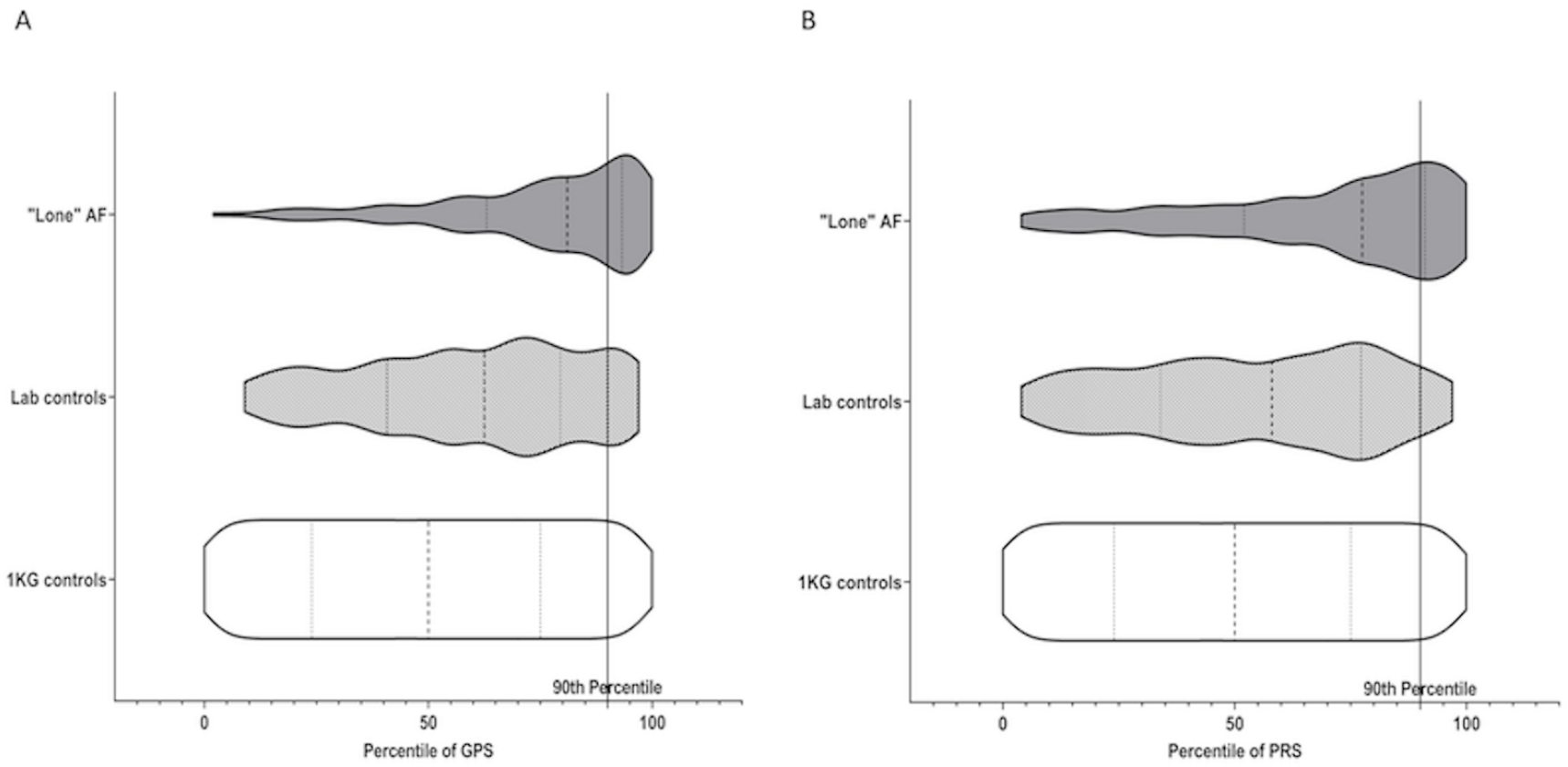

Figure 3.2 Distribution of the GPS (A) and PRS (B) Among "Lone" AF Cases and Controls (Lab controls and 1KG controls)

The median (dashed line), 25th and 75th percentiles (finely dotted lines) for each group are highlighted. The solid line across all 3 cohorts represents the 90th percentile score according to the 1KG control distribution (defined threshold for a high polygenic risk score).

AF = atrial fibrillation, GPS = genome wide polygenic risk score containing 5,978,070 single nucleotide polymorphisms, PRS = polygenic risk score containing 872 single nucleotide polymorphisms, 1KG= 1000 Genomes.

Table 3.2 Proportion of "Lone" AF Cases and Odds of Possessing a GPS/PRS in the Top 10, 5 and 1 Percentiles

High GPS Definition	"Lone" AF cases	1KG controls	Odds Ratio	95% CI	P-value
Top 10% of distribution	64 (34.4%)	43 (10.2%)	4.64	2.99 - 7.18	<0.0001
Top 5% of distribution	35 (18.8%)	22 (5.2%)	4.22	2.40 - 7.57	<0.0001
Top 1% of distribution	8 (4.3%)	5 (1.2%)	3.76	1.18 - 10.30	0.02
High PRS Definition					
Top 10% of distribution	49 (26.3%)	43 (10.2%)	3.16	2.00 - 4.96	<0.0001
Top 5% of distribution	27 (14.5%)	22 (5.2%)	3.10	1.71 - 5.49	0.0002
Top 1% of distribution	15 (8.1%)	5 (1.2%)	7.33	2.61 - 18.55	<0.0001

Data are n (%). *Percentile of the polygenic risk score corresponds to the distribution of the 1KG controls.

AF = atrial fibrillation, GPS = genome wide polygenic risk score containing 5,978,070 single nucleotide polymorphisms, PRS = polygenic risk score containing 872 single nucleotide polymorphisms, 1KG= 1000 Genomes, CI= confidence interval.

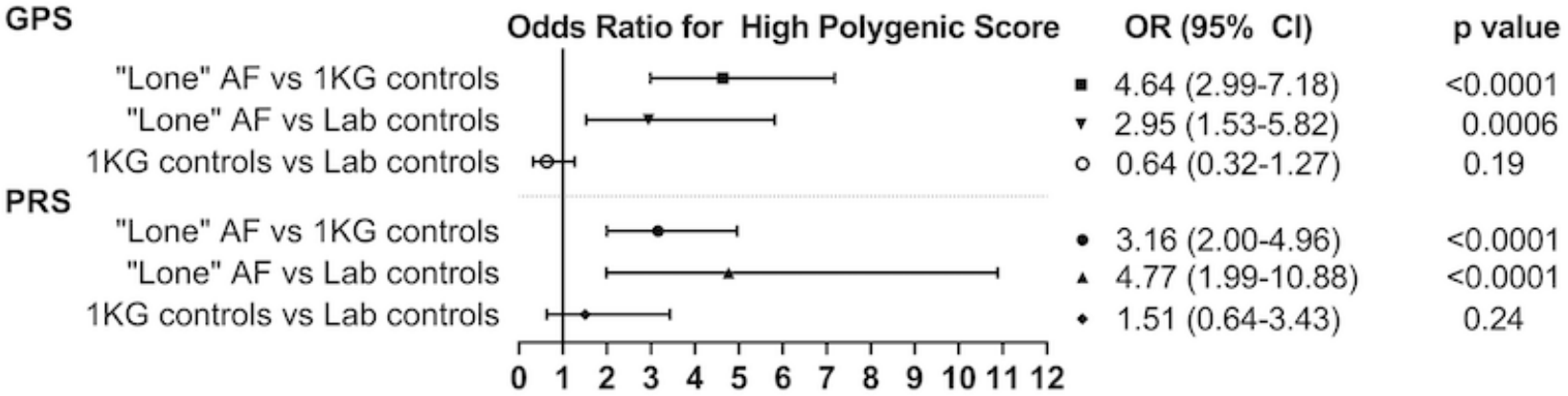

Figure 3.3 Odds Ratio for a High Polygenic Score (GPS/PRS) Between "Lone" AF Cases and Controls (Lab controls and 1KG controls)

A high polygenic risk score was defined as > 90th percentile within 1KG controls.

AF = atrial fibrillation, GPS = genome wide polygenic risk score containing 5,978,070 single nucleotide polymorphisms, PRS = polygenic risk score containing 872 single nucleotide polymorphisms, 1KG = 1000 Genomes., OR = odds ratio, CI = confidence interval.

A total of 49 of 186 "lone" AF patients (26.3%) had a high PRS (top 90th percentile) in comparison with 43 of 423 1KG controls (10.2%) and 6 of 86 lab controls (7.0%) (**Figure 3.2 B, Table 3.2**). Lone AF cases had 3.16-fold increased odds (95% CI [2.01-4.98]; p<0.001) of having a high PRS relative to 1KG controls and 4.77-fold increased odds (95% CI [1.96-11.63]; p<0.0001) compared with lab controls (**Figure 3.3**). Relative to 1KG controls, the odds of a PRS within the top 5% and 1% of PRS values were 3.10-fold (95% CI: 1.71-5.49; p=0.0002) and 7.33-fold (95% CI: 2.61-18.55; p<0.0001) more likely among lone AF cases, respectively (**Table 3.2)**. No statistically significant difference in the odds of possessing a high PRS was observed between 1KG controls and lab controls (OR=1.51; 95% CI [0.64-3.43]; p=0.24).

3.5.3. Discriminative capacity of GPS and PRS

The ability of a high polygenic score to differentiate between lone AF cases and 1KG controls was assessed using ROC curve analysis. The C-statistic for the GPS were 75.9 (95% CI: 71.9-79.9) and for the PRS were 70 (95% CI: 65.5-74.5). The GPS was noted to be superior relative to the PRS in discriminating lone AF cases versus 1KG controls (p=0.002) (**Figure 3.4**).

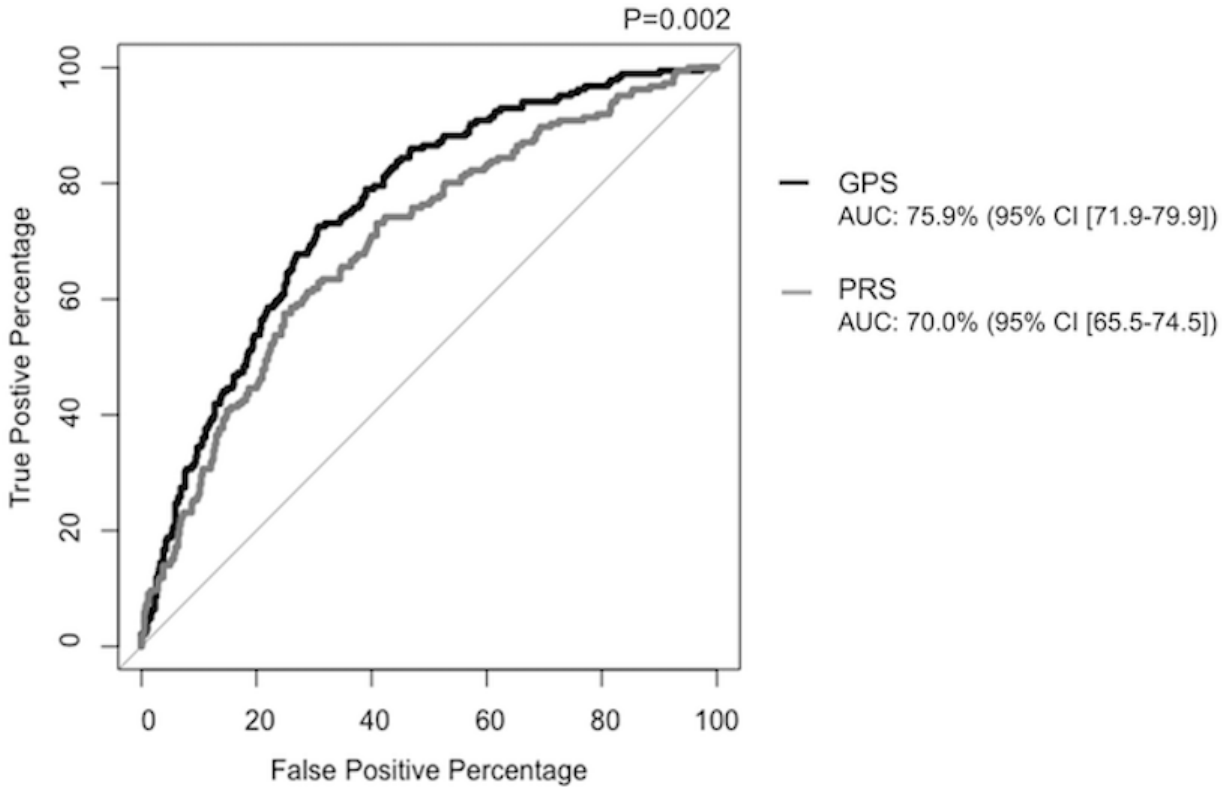

Figure 3.4 Receiver Operating Characteristic Curves for the GPS (black line) and PRS (grey line) with the 1KG Control Distribution as the Reference

The area under the curve for the GPS (75.9%, 95% CI 71.9-79.9) was consistent with improved discriminatory capacity relative to the PRS (70.0%, 95% CI 65.5-74.5, P=0.002).

GPS = genome wide polygenic risk score, PRS = polygenic risk score, AUC = area under the curve, CI = confidence intervals

3.5.3.1. Post-hoc analysis: lone AF vs lab controls

A high GPS was associated with an adjusted 3.72-fold (95% CI: 1.70-8.97; p=0.0017) increased odds of AF, whereas an adjusted 5.70-fold (95% CI [2.60-13.95]; p<0.0001) greater odds of AF was observed for a high PRS when comparing with lab controls only (**Figure 3.5**). Addition of the polygenic scores to logistic regression models containing the first 3 PCs, age and sex (C-statistic: 69.0, 95% CI 62.0-77.0) resulted in significant improvements in discriminatory capacity (PRS: C-statistic: 77.0, 95% CI 71.0-83.0; p=0.0005 and GPS: C-statistic: 0.79.0, 95% CI 73.0-84.0, p=0.0007). No difference was observed between the PRS and GPS models (p=0.40).

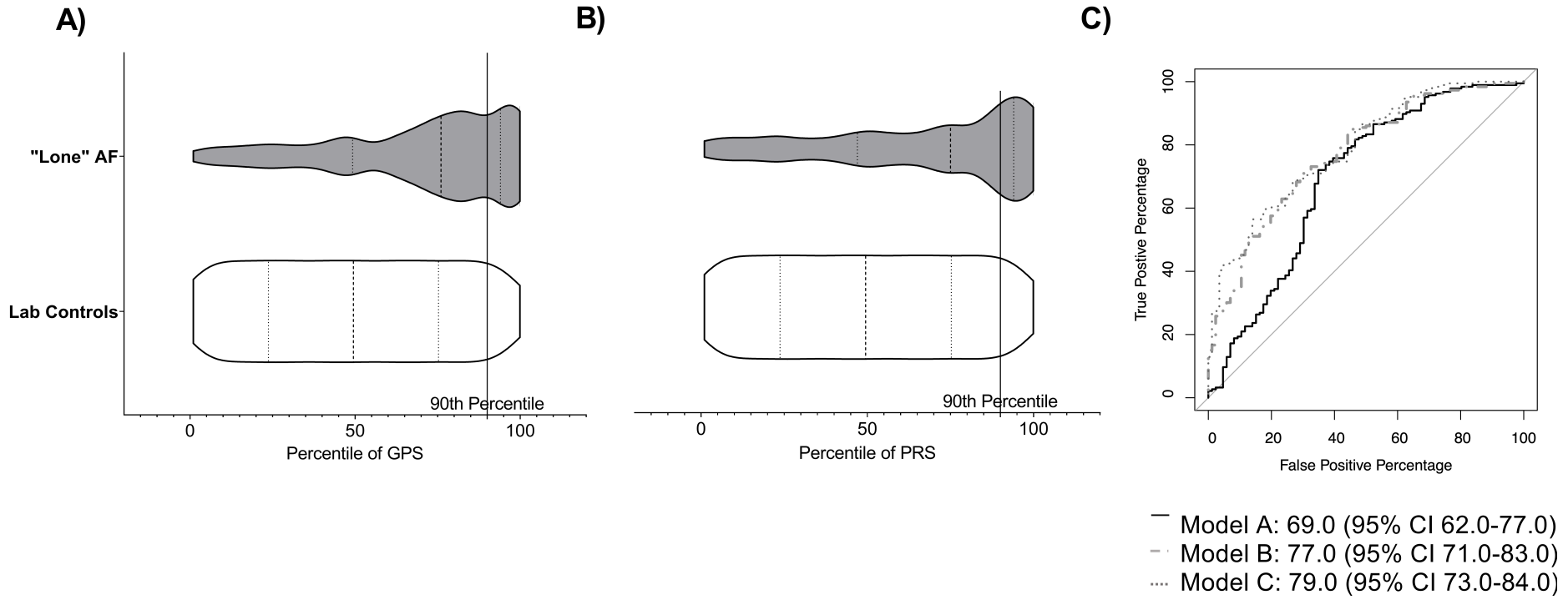

Figure 3.5 Distribution of the GPS (A) and PRS (B) among "Lone" AF Cases with the Lab Controls as the Reference

The median (dashed line), 25th and 75th percentiles (finely dotted lines) for each group are highlighted. The solid line across represents the 90th percentile score according to the control distribution (defined threshold for a high polygenic risk score). **(C)** Receiver Operating Characteristic Curves for Models A (3 PCs, age and sex – black solid line), B (PRS, 3 PCs, age and sex – dotdash light grey line), and C (GPS, 3 PCs, age and sex – dotted dark grey line). AUC: Model A (69.0, 95% CI 62.0-77.0), Model B (77.0, 95% CI 71.0-83.0) and Model C (79.0, 95% CI 73.0-84.0). Models B and C exhibited improved discriminatory capacities relative to Model A (p=0.0005, p=0.0007, respectively). No statistical difference was observed between Models B and C (p=0.40).

AF = atrial fibrillation, PRS = polygenic risk score, GPS = genome wide polygenic risk score, AUC = area under the curve, CI = confidence interval.

3.5.4. Impact of age and sex on GPS and PRS

The likelihood of having a high polygenic score did not differ on the basis of age (p=0.65 [GPS], p=0.98 [PRS]) or sex (p=0.17 [GPS], p=0.56 [PRS]) in the "lone" AF cohort and no statistical trend was identified for age (p=0.61 [GPS], p=0.71 [PRS]).

3.6. Discussion

Our findings indicate that common genetic variations play a critical role when AF develops in the absence of identifiable clinical risk factors. "Lone" AF cases had a strikingly significant 4.64-fold increased odds of possessing a high GPS relative to healthy 1KG controls, which corresponded to 34.4% of the "lone" AF cohort. Similar, though generally smaller magnitude effects were observed for the PRS. Further, the advanced discriminatory capacity of the GPS, additionally improved in a model including PCs, age and sex, was demonstrated on the ROC curve analysis, collectively highlighting the value of maximizing the depth of genetic detail incorporated into polygenic AF scores. Beyond highlighting the relevance of common genetic variation to "lone" AF, these findings also allude to the potential clinical utility of common genetic variation in the clinical setting.

Although AF-associated SNPs exert a minimal impact on arrhythmia risk in isolation, our study demonstrates for the first time their striking contribution to the "lone" AF phenotype when evaluated in a collective manner. The finding that 34.4% of "lone" AF cases have a high GPS serves as strong evidence that common genetic variation is an important contributor to arrhythmogenesis in a large proportion of "lone" AF cases. These findings differ markedly relative to the current yield associated with genetic screening for established highly penetrant rare genetic variants in AF, which are currently only observed in a small minority of cases (Choi et al., 2020).

That being said, even though our study does provide strong evidence for a prominent role for common genetic variation in "lone" AF, it should not be viewed as evidence that rare genetic variation is less relevant in AF. It is conceivable that rare genetic variation has an equally or perhaps even more prominent role and its currently perceived minimal impact is merely secondary to our limited insight into the overall contribution of rare genetic variation to the

arrhythmia (Ritchie et al., 2012). Establishing definitive evidence to implicate rare variants in AF is particularly challenging for multiple reasons, including the study size required to achieve adequate statistical power and the costs of deep sequencing relative to SNP genotyping Choi et al. (2018b).

Genome-wide scores, so called due to their attempt to capture relevant data from the entire genome, have previously been shown to provide a superior capacity to discern affected patients from healthy controls across various disease entities (Dron & Hegele, 2019). These findings are consistent with the incrementally improved performance of the ~ 6 million SNP GPS relative to the ~ 1,000 SNP PRS in our "lone" AF cohort. In our study, the GPS captured a greater proportion of individuals with a high polygenic score relative to the PRS (34.4% versus 26.3%) and also exhibited an improved discriminatory capacity on ROC curve analysis. Although these findings should appropriately lead to enthusiasm to favor genome-wide scores, it is important to acknowledge that specialized infrastructure is currently required for their calculation.

Insight into gene-environment interactions in AF remains limited, however prior interaction has been observed (Weng et al., 2017b). Evidence for gene-environment interactions at a genome wide level of significance was previously evaluated for 4 established clinical AF risk factors, namely increasing age, male sex, hypertension, and obesity (Benjamin et al., 1994; Christophersen et al., 2016). The rs6817105 SNP, residing within the locus containing *PITX2*, was found to differentially impact the risk for AF in individuals younger than 65 relative to older individuals, a finding that was subsequently replicated in a second cohort (Weng et al., 2017b). No other genome wide signification interactions were replicated for either increasing age or the remaining 3 clinical risk factors when SNPs were evaluated in isolation. Using polygenic scores in our "lone" AF cohort, no interactions for age or sex were observed, though the lack of signal should be interpreted with caution given our limited sample size.

In addition to providing insight into the genetic architecture of "lone" AF, the findings from our study also allude to the potential use of common genetic variation in the clinical setting. The rapidly expanding prevalence of AF has led to major impetus to try to curb incident cases. Although evidence for prevention of AF through upstream therapies has yet to be established with randomized trials, the use of Mendelian randomization studies has served to bolster the

probable causal role of certain AF risk factors, including body mass index and increased thyroid activity, suggesting that intervening on these factors may prevent AF (Chatterjee et al., 2017; Ellervik et al., 2019; Salem et al., 2019). The ability to identify individuals deemed particularly high risk on the basis of both genetic and clinical risk factors may enable targeted delivery of therapies to individuals at greatest likelihood to benefit. Additionally, AF is a prominent culprit for stroke, however due to its often paroxysmal and asymptomatic nature, it is often challenging to detect (Sanna et al., 2014). Use of polygenic scores could potentially be used as an additional tool by neurologists to decipher those individuals whose stroke was secondary to AF and require anti-coagulation to prevent a recurrent and potentially devastating cerebral embolic event.

3.6.1. Limitations

Although our study provides important insight into the role of common genetic variation in "lone" AF, it has several limitations. Our study was restricted to European ancestry, partially necessitated by the previously validated polygenic scores having been derived in this ancestry, and hence it is unclear if our findings are generalizable to other ancestries. Use of the 1KG dataset was primarily pursued to provide a larger control group, however we acknowledge potential biases introduced secondary to different genotyping methods and population substructures. Due to these concerns, we performed a second set of analyses using a second, albeit smaller, set of control samples derived from the same region as our cases and genotyped with the same technology. The consistent results from both analyses were reassuring and we believe bolster the validity of our overall findings. Although our study sample size was relatively modest for a contemporary investigation into the complex genetics of a common disease, the statistically significant findings for our primary hypotheses highlight that our statistical power was adequate, though insufficient power likely precluded meaningful assessment for interactions between clinical risk factors and the polygenic scores in relation to AF risk. Lastly, although all control participants reported as healthy and denied a history of arrhythmia, it is conceivable that some had undetected AF, however the likelihood of AF under ascertainment would not be anticipated to be affected by GPS/PRS values. The corresponding non-differential misclassification of the outcome among controls would only serve to reduce our statistical power secondary to bias towards the null, rather than resulting in spurious false positive associations.

3.7. Conclusions

Our study findings suggest a critical role for common genetic variation in the pathogenesis of the "lone" AF phenotype. In addition to providing important insight into the genetic substrate of "lone" AF, our findings further reinforce a potentially valuable future role for common genetic variation in clinical management of patients. Although common genetic variation appears to exert a prominent role in "lone" AF pathogenesis, our study does not exclude a critical role for rare variation, whose currently perceived minimal impact may be secondary to our understanding of its contribution to AF being in its relative infancy.

3.8. References

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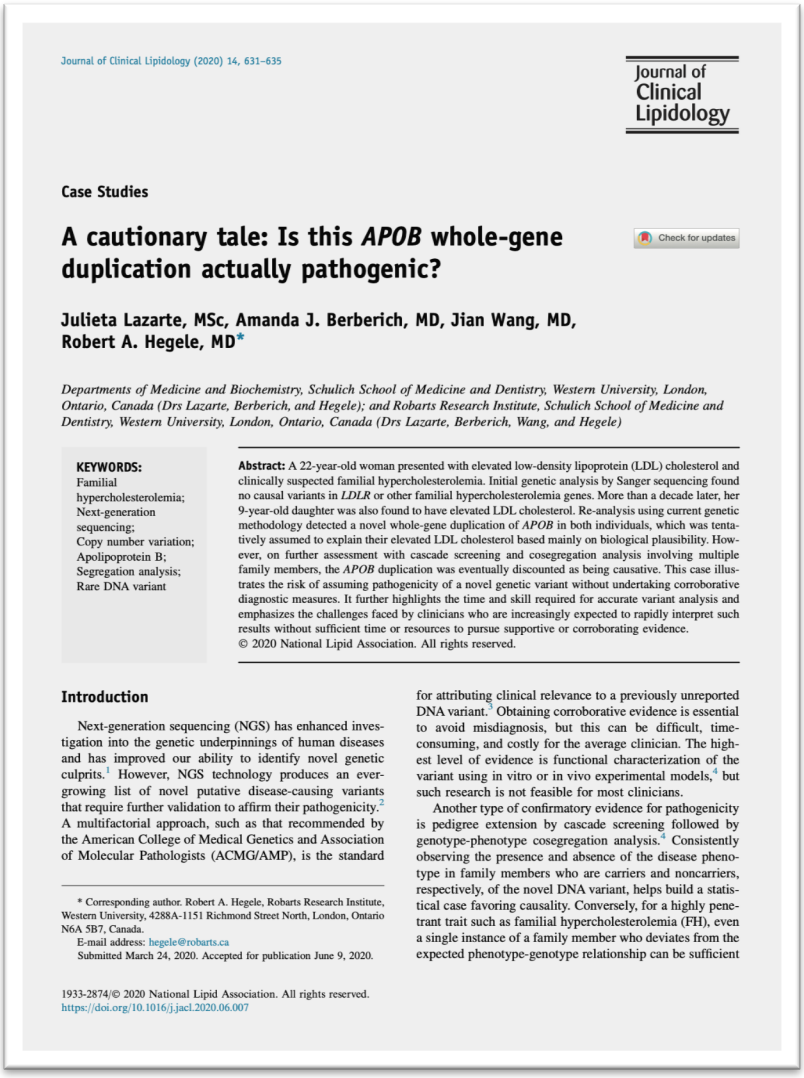
Chapter 4. Misattribution of pathogenicity to novel genetic variants

The work contained in this Chapter has been edited from its original publication in *Journal of Clinical Lipidology* for brevity and consistency throughout this PhD Thesis.



Journal of Clinical Lipidology (2020) 14, 631–635

Journal of Clinical Lipidology

Case Studies

A cautionary tale: Is this *APOB* whole-gene duplication actually pathogenic?

Julieta Lazarte, MSc, Amanda J. Berberich, MD, Jian Wang, MD, Robert A. Hegele, MD*

Departments of Medicine and Biochemistry, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada (Drs Lazarte, Berberich, and Hegele); and Robarts Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada (Drs Lazarte, Berberich, Wang, and Hegele)

KEYWORDS:
Familial hypercholesterolemia;
Next-generation sequencing;
Copy number variation;
Apolipoprotein B;
Segregation analysis;
Rare DNA variant

Abstract: A 22-year-old woman presented with elevated low-density lipoprotein (LDL) cholesterol and clinically suspected familial hypercholesterolemia. Initial genetic analysis by Sanger sequencing found no causal variants in *LDLR* or other familial hypercholesterolemia genes. More than a decade later, her 9-year-old daughter was also found to have elevated LDL cholesterol. Re-analysis using current genetic methodology detected a novel whole-gene duplication of *APOB* in both individuals, which was tentatively assumed to explain their elevated LDL cholesterol based mainly on biological plausibility. However, on further assessment with cascade screening and cosegregation analysis involving multiple family members, the *APOB* duplication was eventually discounted as being causative. This case illustrates the risk of assuming pathogenicity of a novel genetic variant without undertaking corroborative diagnostic measures. It further highlights the time and skill required for accurate variant analysis and emphasizes the challenges faced by clinicians who are increasingly expected to rapidly interpret such results without sufficient time or resources to pursue supportive or corroborating evidence.

Introduction

Next-generation sequencing (NGS) has enhanced investigation into the genetic underpinnings of human diseases and has improved our ability to identify novel genetic culprits.[1] However, NGS technology produces an ever-growing list of novel putative disease-causing variants that require further validation to affirm their pathogenicity.[2] A multifactorial approach, such as that recommended by the American College of Medical Genetics and Association of Molecular Pathologists (ACMG/AMP), is the standard for attributing clinical relevance to a previously unreported DNA variant.[3] Obtaining corroborative evidence is essential to avoid misdiagnosis, but this can be difficult, time-consuming, and costly for the average clinician. The highest level of evidence is functional characterization of the variant using in vitro or in vivo experimental models,[4] but such research is not feasible for most clinicians.

Another type of confirmatory evidence for pathogenicity is pedigree extension by cascade screening followed by genotype-phenotype cosegregation analysis.[4] Consistently observing the presence and absence of the disease phenotype in family members who are carriers and noncarriers, respectively, of the novel DNA variant, helps build a statistical case favoring causality. Conversely, for a highly penetrant trait such as familial hypercholesterolemia (FH), even a single instance of a family member who deviates from the expected phenotype-genotype relationship can be sufficient

* Corresponding author. Robert A. Hegele, Robarts Research Institute, Western University, 4288A-1151 Richmond Street North, London, Ontario N6A 5B7, Canada.
E-mail address: hegele@robarts.ca
Submitted March 24, 2020. Accepted for publication June 9, 2020.

1933-2874/
https://doi.org/10.1016/j.jacl.2020.06.007

J. Lazarte, A.J. Berberich, J. Wang, R.A. Hegele. (2020) "A cautionary tale: Is this APOB whole-gene duplication actually pathogenic?" *J Clin Lipidol*. 2020; 14:631-635. (PMID: 32654994)

4.1. Study rationale

Advances in NGS technology has allowed us to identify thousands of variants in a single patient, leaving us the challenge of sorting through what is important. For instance, a variant that is interpreted as being 'damaging', 'pathogenic' or 'causal' is often but not always clinically relevant. Further, with the growing availability of NGS technology, clinicians are increasingly expected to interpret genetic results for clinical decision-making. Fortunately, guidelines for variant interpretation have been proposed by the American College of Medical Genetics and Genomics and Association of Molecular Pathologists (ACMG/AMP). Once a novel variant is found, the ACMG has published guidelines (discussed in section 1.6) to attribute pathogenicity to previously unreported variants; however, this can be difficult, time consuming, and costly for the average clinician to pursue. Indeed, obtaining corroborative evidence is essential to avoid misdiagnosis and provide optimal diagnostic and treatment advice to patients. In this investigation, we demonstrated the impact of pursuing cascade screening and co-segregation analysis on a patient's diagnosis and how valuable this evidence can be in addition to other factors when determining the causality of a novel variant.

4.2. Overview

A 22 year-old woman presented with elevated LDL cholesterol and clinically suspected familial hypercholesterolemia (FH). Initial genetic analysis by Sanger sequencing found no causal variants in *LDLR* or other FH genes. More than a decade later, her 9 year-old daughter was also found to have elevated LDL cholesterol. Re-analysis using current genetic methodology detected a novel whole-gene duplication of *APOB* in both individuals, which was tentatively assumed to explain their elevated LDL cholesterol. However, upon further assessment with cascade screening and co-segregation analysis involving multiple family members, the *APOB* duplication was eventually discounted as being causative. This case illustrates the risk of assuming pathogenicity of a novel genetic variant without undertaking corroborative diagnostic measures. It further highlights the time and skill required for accurate variant analysis and emphasizes the challenges faced by clinicians who are increasingly expected to rapidly interpret such results without sufficient time or resources to pursue supportive or corroborating evidence.

4.3. Introduction

Next-generation sequencing (NGS) has enhanced investigation into the genetic underpinnings of human diseases and has improved our ability to identify novel genetic culprits (Chakravarti & Turner, 2016). However, NGS technology produces an ever-growing list of novel putative disease-causing variants that require further validation to affirm their pathogenicity (Sunyaev, 2012). A multifactorial approach, such as that recommended by the American College of Medical Genetics and Association of Molecular Pathologists (ACMG/AMP) is the standard for attributing clinical relevance to a previously unreported DNA variant (Richards et al., 2015). Obtaining corroborative evidence is essential to avoid misdiagnosis, but this can be difficult, time-consuming and costly for the average clinician. The highest level of evidence is functional characterization of the variant using in vitro or in vivo experimental models (Iacocca et al., 2018a), but such research is not feasible for most clinicians.

Another type of confirmatory evidence for pathogenicity is pedigree extension by cascade screening followed by genotype-phenotype co-segregation analysis (Iacocca et al., 2018a). Consistently observing the presence and absence of the disease phenotype in family members who are carriers and non-carriers, respectively, of the novel DNA variant, helps to build a statistical case favoring causality. Conversely, for a highly penetrant trait such as familial hypercholesterolemia (FH), even a single instance of a family member who deviates from the expected phenotype-genotype relationship can be sufficient to reject a causal role. But while sampling the extended kindred and performing co-segregation analysis are relatively more feasible and comprehensible for a clinician than laboratory experiments, this is still a time-consuming and resource intensive effort.

Heterozygous FH is a relatively common genetic disorder affecting ~1 in 250 individuals that presents with abnormally high levels of low-density lipoprotein (LDL) cholesterol (Defesche et al., 2017). Genetic variation in canonical lipoprotein metabolism genes such as *LDLR*, *APOB* and *PCSK9* has been well-established to cause FH (Berberich & Hegele, 2019a). Early diagnosis and treatment of heterozygous FH increases life expectancy by reducing risk of premature atherosclerotic cardiovascular disease (ASCVD) (Nordestgaard et al., 2013). DNA analysis has helped with FH diagnosis in many cases (Dron et al., 2020). Among patients referred to our lipid clinic with elevated LDL cholesterol and clinically suspected heterozygous FH, we have

identified either a monogenic or polygenic etiology in ~60% (Wang et al., 2016). Among monogenic etiologies, pathogenic copy number variants (CNVs) are found in 6-10% of patients, primarily affecting the *LDLR* gene (Wang et al., 2016), although we recently reported two families with severe hypercholesterolemia who have a CNV in the *PCSK9* gene, specifically a whole-gene duplication associated with extremely high circulating levels of proprotein convertase subtilisin kexin type 9 (PCSK9) (Iacocca et al., 2018b). Up to a third of referred patients with LDL cholesterol >95th percentile have no identifiable genetic cause (Wang et al., 2016); furthermore CNVs in other causative genes for FH such as *APOB* have not been reported.

We now describe a young woman with elevated LDL cholesterol who was found by NGS to carry a novel whole-gene *APOB* duplication. Initially, we assumed that this was a likely causal mutation. However, after extensive studies to confirm pathogenicity, specifically family cascade screening and co-segregation analysis, we ruled out a direct causal role for this variant, which had significant ramifications for the patient and family members.

4.4. Case

A 22 year-old Canadian female of Italian ancestry (**Figure 4.1 A,** subject III-2) presented to our clinic for assessment of an abnormally high LDL cholesterol discovered incidentally through her participation in a weight loss program (**Table 4.1**). Her past medical history was remarkable only for pernicious anemia. Past surgical history included tonsillectomy and adenectomy, and nasal surgery. She was taking vitamin B12 injections, chewable fiber pills and a multivitamin. On physical examination, her blood pressure was 90/70 mm Hg, with a regular heart rate of 54 beats per minute. Her body mass index was 22.9 kg/m^2. She had no xanthomas, xanthelasmas, arcus cornealis, or any signs of premature vascular disease. Her total and LDL cholesterol were both elevated at 7.51 and 5.41 mmol/L, respectively, while her apolipoprotein (apo) B was elevated at 1.84 g/L. There were no secondary causes of hypercholesterol

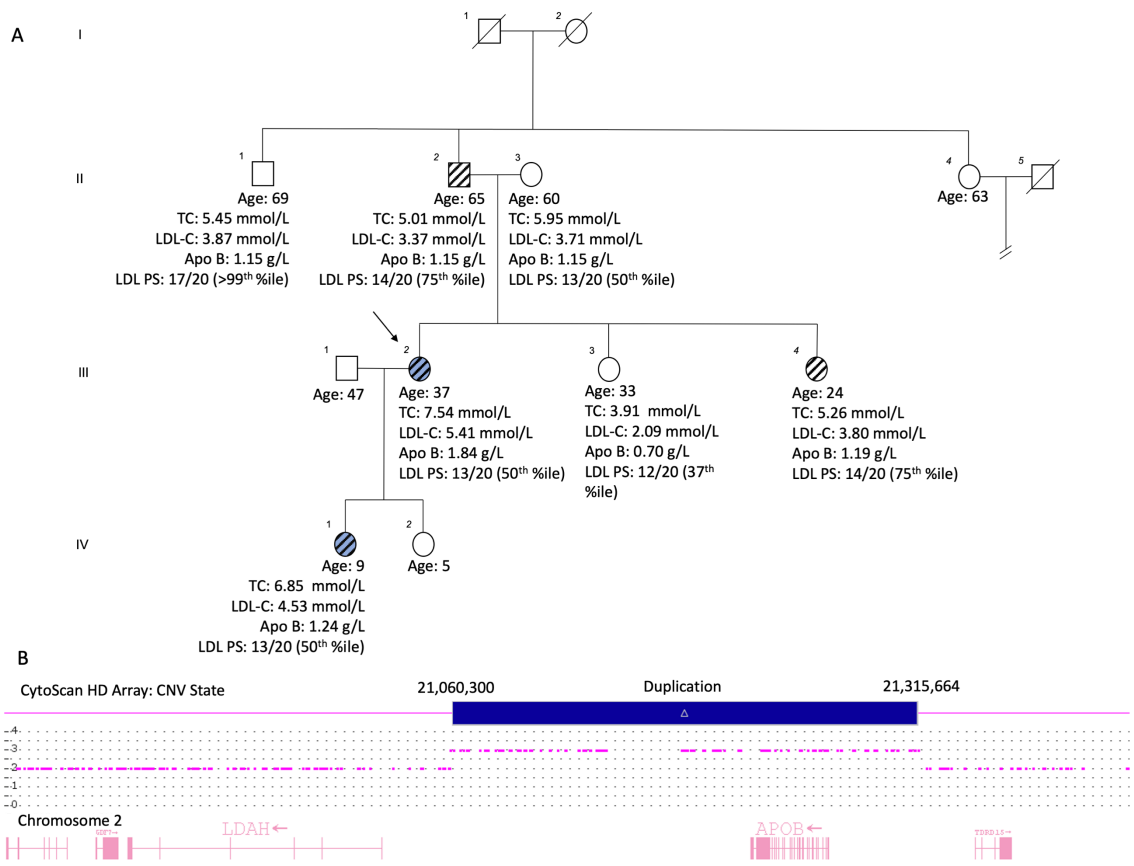


Figure 4.1 Family Pedigree Diagram and Cytoscan HD Confirmation of an *APOB* CNV in Index Case III-2

A) Proband is identified with the arrow. Clinical status is defined as: blue: FH, white: unaffected, and slash: deceased. Genotype is indicated as diagonal stripes for *APOB* duplication carriers. Ages are current or at time of death.

B) Duplicated region includes all 29 exons of the *APOB* gene, spanning chromosome 2 nucleotide bases 21,060,300 to 21,315,664.

TC = total cholesterol, LDL-C = low-density lipoprotein cholesterol, apo B = apolipoprotein B, LDL PS = low-density lipoprotein polygenic score, %ile = percentile.

Table 4.1 Baseline Laboratory Results for Proband

Laboratory test	Patient's result	Normal range
Total cholesterol	7.54 mmol/L	<=4.60 mmol/L
Triglycerides	1.28 mmol/L	<=2.30 mmol/L
HDL cholesterol	1.55 mmol/L	0.90 – 2.07 mmol/L
LDL cholesterol	5.41 mmol/L	<=3.50 mmol/L
Apolipoprotein B	1.84 g/L	0.8 – 1.2 g/L
Apolipoprotein A1	1.65 g/L	1.2 – 1.4 g/L
Lipoprotein(a)	<9.9 mg/dL	<=35.0 mg/dL
Hemoglobin A1c	4.6%	<6.0%

At her initial presentation, genetic analysis was performed using Sanger sequencing of *LDLR, APOB* and *PCSK9* genes: no putative causal genetic variant was found. She was intermittently adherent to low intensity statin treatment (rosuvastatin 10 mg daily) and ezetimibe: her lowest total and LDL cholesterol levels on treatment were 5.33 and 3.68 mmol/L, respectively. She remained stable and asymptomatic. More than 10 years later, improved technology using the LipidSeq NGS platform with ClinVar software (Dron et al., 2020) was able to detect a novel *APOB* CNV. This *APOB* gene duplication was 255 megabases in size, spanning all 29 exons, which resulted in the proband having three full copies of the *APOB* gene (**Figure 4.1 B**); its presence was confirmed by whole exome sequencing and microarray hybridization (Cytoscan HD, ThermoFisher, London ON). This CNV had not been reported in any human genetic variation database. Her polygenic score for LDL cholesterol was at the 50th percentile (Dron et al., 2020). Given the causal

relationship between certain *APOB* mutations and FH, this novel CNV was a strong candidate to explain her hypercholesterolemia.

Following identification of the *APOB* CNV, the proband and her immediate family members were evaluated using LipidSeq. The pedigree, lipid profiles and *APOB* genotypes for all participating family members are shown in **Figure 4.1 A**. The proband's 9 year-old daughter (**Figure 4.1 A**, subject IV-1) had elevated LDL cholesterol and apo B at 4.53 mmol/L and 1.24 g/L, respectively. She also carried the *APOB* duplication, strengthening our initial assumption that this CNV was causal for hypercholesterolemia. Family cascade screening found that the proband's father (**Figure 4.1 A**, subject II-2) also carried the *APOB* CNV duplication, although his LDL cholesterol and apo B were not elevated. His polygenic score for LDL cholesterol was at the 50th percentile. He had a diagnosis of systemic lupus erythematosus and had been taking hydroxychloroquine for >10 years. Once the *APOB* CNV was established in the paternal line, further investigations were pursued in siblings and paternal relatives. One of the proband's sisters (**Figure 4.1 A**, subject III-4) carried the *APOB* CNV variant but had neither elevated LDL cholesterol nor apo B. Her polygenic score for LDL cholesterol was at the 75th percentile. The proband's paternal uncle (**Figure 4.1 A,** subject II-1) and sister (**Figure 4.1 A,** subject III-3) were each non-carriers of the *APOB* CNV; LDL cholesterol and apo B were not elevated in either individual. Phenotype-genotype analysis failed to show segregation concordance across generations, with two clear cases, leading to the inescapable conclusion that the *APOB* CNV was not causal for hypercholesterolemia in this family.

4.5. Discussion

We present a young female with elevated LDL cholesterol, a large novel CNV in the *APOB* gene and no other identifiable genetic cause of hyperlipidemia. At first glance, the whole-gene duplication of *APOB* seemed to be the cause for her hypercholesterolemia. We previously reported a similar CNV causing a whole-gene duplication of *PCSK9* that fully co-segregated with hypercholesterolemia in several members of two unrelated families (Iacocca et al., 2018b). We initially speculated that an analogous mechanism might increase apo B expression in affected members of the present family. Indeed,

overproduction of apo B, the key structural protein of LDL particles, increases circulating levels of atherogenic lipoproteins including LDL (Elovson et al., 1988). Additionally, this *APOB* duplication has never been reported in thousands of FH individuals studied molecularly or in public databases, which provided another criterion for causality according to the ACMG/AMP. Family screening revealed that the proband's 9 year-old daughter had both abnormally high LDL cholesterol levels and the *APOB* duplication, sustaining our initial suspicion of its potential pathogenic role. However, the proband's normolipidemic father and sister also both carried the *APOB* duplication. While her father had a diagnosis of systemic lupus erythematosus and had been taking hydroxychloroquine, the predicted effect of this treatment on LDL cholesterol would only be modest; we estimate a ~0.20 mmol/L reduction in LDL cholesterol based on previous literature reports (Morris et al., 2011).

The normolipidemia observed in both the proband's father and sister indicated that the *APOB* CNV was either not sufficient for expression of hyperlipidemia or not involved in the clinical phenotype. It remains possible that in particular patients, the *APOB* CNV may contribute indirectly to the elevated LDL cholesterol phenotype by acting in combination with other uncharacterized genetic or genomic factors, or with unidentified environmental factors.

This case highlights the importance of pursuing additional confirmatory data before assigning pathogenicity to a novel, previously unreported variant found by NGS. Pedigree tracing and segregation analysis is helpful evidence that is potentially applicable in the clinic setting. Here, after several months of effort, extending the kindred showed that this rare novel *APOB* CNV could not explain the dyslipidemia. Building an extended pedigree and assessing co-segregation should always be considered for corroborating a new variant's causality (Mohammadi et al., 2009). However, this may not be practical or feasible in many clinical situations.

Clinical genetic testing in dyslipidemias is becoming more accessible, and clinicians are increasingly being called upon to expeditiously interpret raw or processed genetic data. While valuable guidance for variant interpretation is found in the ACMG/AMP

guidelines (Richards et al., 2015), most lipidologists have not been recently trained in the complexities of contemporary genetic analysis and may be insufficiently equipped to provide such assessments. Here in the initial stages of assessment, it seemed obvious that the novel *APOB* CNV could explain the clinical phenotype in the proband and daughter. However, while making a molecular diagnosis appears straightforward, this case illustrates the risk of rushing to judgement by assuming that a rare novel variant with a plausible link to the disease is necessarily causal.

Most variants found by NGS for any condition are neutral or benign (Dron et al., 2020). Over-diagnosis (i.e. false positive) is possible without applying due diligence to confirm pathogenicity before communicating results to patients, which may have significant ramifications. For example, a variant that is erroneously interpreted as pathogenic during pre-natal testing could affect a decision for pregnancy termination. Similarly, communicating false positive results to an adult patient may result in inappropriate diagnostic labelling, stigmatization and potentially unsuitable management. Here, we informed the proband and her daughter that while we were unable to molecularly diagnose monogenic heterozygous FH, it was still appropriate to manage their elevated LDL cholesterol according to expert recommendations (Anderson et al., 2016).

4.6. Conclusion

This unique case emphasizes the potential hazards involved in interpreting novel genetic findings, specifically in making a diagnosis based on a single piece of genetic data without orthogonal supporting or corroborating evidence. While performing functional molecular biological laboratory experiments to prove a mutation's pathogenicity is beyond the scope of feasibility for most clinicians, alternative clinic-friendly approaches, including pedigree extension and assessing for co-segregation of variant and phenotype could be helpful. Although this will consume time and resources, its importance is justified if it enables a more definitive diagnosis, which could have potentially lifelong consequences for the patient and their family members.

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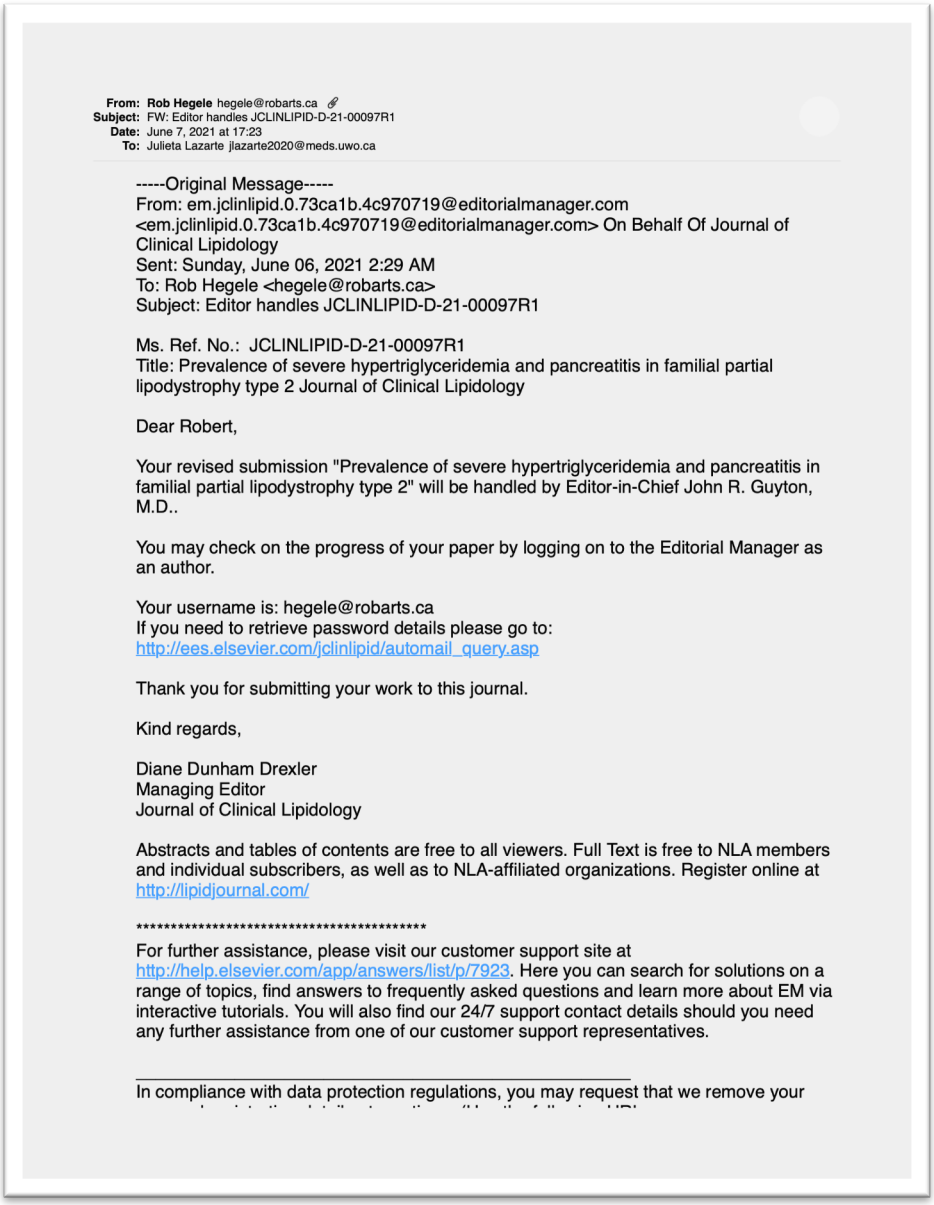
Chapter 5. Prevalence of severe hypertriglyceridemia and pancreatitis in familial partial lipodystrophy type 2

The work contained in this Chapter has been edited from the revised submission to the *Journal of Clinical Lipidology* for brevity and consistency throughout this PhD Thesis.



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5.1. Study rationale

Despite the increasing complexity of the genetics field, in this Chapter we demonstrate how the simple application of a genetics diagnosis can elucidate clinically relevant insights of a rare monogenic disease. Familial partial lipodystrophy (FPLD) is a rare heterogenous disorder with a prevalence of less than 1 in 10 million individuals with the Dunnigan subtype (Garg, 2004). Given its rarity, it is quite difficult to collect enough individuals to have statistical power to discern the natural history of the disorder. Often enough, the various subtypes are studied together under the lipodystrophy umbrella. However, given the differences in presentation of the various subtypes, it was logical to study them separately. In this investigation, we demonstrated the impact of an accurate genetic diagnosis in determining the risk of pancreatitis in a well-genotyped and phenotype cohort.

5.2. Overview

Familial partial lipodystrophy (FPLD) is a rare Mendelian condition listed in the differential diagnosis of severe hypertriglyceridemia (HTG) and pancreatitis. Here we determined the prevalence of severe HTG and pancreatitis among a cohort of 74 FPLD patients assessed in a lipid clinic. We studied lipid profiles from individuals with either of the two most common pathogenic monoallelic variants in *LMNA*, namely p.R482Q (N= 51) and p.R482W (N= 23). In total, 28 (37.8%) patients with a mean age of 41.8 ± 14.8 years had diabetes, while 46 (62.2%) patients with a mean age of 35.4 ± 19.4 years had no diabetes. Among patients with and without diabetes, mean TG levels (interquartile range) were 2.73 (4.78) and 1.86 (1.66) mmol/L (242 [423] and 165 [147] mg/dL), respectively. Overall, 4 subjects (5.4%) had triglyceride levels > 10 mmol/L (> 875 mg/dL), of whom 3 (4.1%) had a history of hospitalization for acute pancreatitis. All 4 patients with severe HTG had diabetes, i.e. 14.3% of those with diabetes. In contrast, FPLD2 patients without diabetes had only mild HTG, with no instances of severe HTG or pancreatitis. Thus, among this selected lipid clinic cohort with lipodystrophy, severe HTG and pancreatitis in FPLD2 are relatively common when diabetes is present.

5.3. Introduction

Lipodystrophy refers to a heterogenous group of rare congenital or acquired metabolic disorders characterized by a complete or partial loss of adipose tissue (Brown et al., 2016). Familial partial lipodystrophy (FPLD) is an inherited syndrome of body fat loss that is limited to certain regions (Brown et al., 2016). The most common subtype, i.e. Dunnigan-type or FPLD2 (OMIM 151660), is caused by autosomal dominant pathogenic rare variants in the *LMNA* gene encoding lamin A/C (Hegele et al., 2007). The FPLD2 clinical phenotype develops around puberty, with noticeable loss of subcutaneous fat in the extremities, trunk and gluteal region and a potential accumulation of fat in the face, neck, back, abdomen and labia majora. In some cases, acanthosis nigricans, hirsutism, menstrual abnormalities, dyslipidemia, hepatosteatosis and polycystic ovarian disease (PCOS) can also occur. Later in life, many patients develop severe insulin resistance that can progress to diabetes mellitus termed "lipoatrophic diabetes", which increases the risk for microvascular and macrovascular complications. In some rare instances an overlap with arrhythmias or cardiomyopathy has been observed (Brown et al., 2016; Schmidt, 2002).

The main dyslipidemia associated with FPLD2 is hypertriglyceridemia (HTG), defined as fasting plasma triglyceride (TG) $\geq$ 95th percentile for age and sex, which is observed in many FPLD2 patients (Jackson et al., 1997). High density lipoprotein (HDL) cholesterol is also often depressed (Hegele et al., 2007). HTG is typically mild-to-moderate in severity in FPLD2, and likely plays a role in premature atherosclerotic cardiovascular disease (ASCVD) experienced by some patients (Hegele, 2001). However, experienced clinicians have also encountered rare FPLD2 patients with severe refractory HTG (TG > 10 mmol/L or > 885 mg/dL) who present with acute pancreatitis (Haque et al., 2002). There are several dramatic case reports describing HTG-related pancreatitis in FPLD2 patients, as well as among patients with more severe congenital generalized lipodystrophy for which a pancreatitis prevalence of 15 to 20% has been reported.(Lima et al., 2018) Indeed, lipodystrophy syndromes, including partial lipodystrophy, have been proposed both as inclusion criteria for clinical trials and as possible clinical indications for new potent biological treatments for severe HTG, such as volanesorsen

(Esan & Wierzbicki, 2020). However, the prevalence of severe HTG and pancreatitis among FPLD2 patients is unclear. Thus, in this observational study our overarching aim was to determine in a Canadian FPLD2 cohort the prevalence of severe HTG and pancreatitis.

5.4. Methods

This single-center, retrospective chart review included patients who were either: 1) referred to the Lipid Genetics Clinic, London Health Sciences Centre, University Hospital (London, ON) with a presumptive clinical diagnosis of lipodystrophy; or 2) identified following cascade screening of a genetically diagnosed individual with FPLD2. Biochemical assessment included lipid profile and hemoglobin A1c. Imaging studies of abdomen, adipose tissue or ovaries were not routinely performed. Our targeted next-generation sequencing gene panel, LipidSeq, was used for all genetic analyses (Johansen et al., 2014). LipidSeq includes reagents to screen all coding regions plus 150 base pairs at intron-exon boundaries of the 16 known genes that are causative for all forms of inherited lipodystrophy. Of 130 patients who were assessed, we limited eligible cases to those with heterozygous *LMNA* p.R482Q and p.R482W mutations, since these are classical unequivocal causal variants for FPLD2, in contrast to other rare variants in the *LMNA* gene, which can be causative for up to 16 diverse disorders referred to as laminopathies (Hegele, 2005). In total, 74 FPLD2 patients were included in the analysis. Our protocol was approved by the Western University Research Ethics Board (# 0379), and all individuals provided informed consent.

5.4.1. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 7 for Windows (version 7.04; GraphPad Software, La Jolla CA). Differences between patients with and without diabetes were assessed using a Mann-Whitney test for nonparametric data, while for parametric data, unpaired Student's t-tests were applied. Statistical significance was nominally set at $P < 0.05$.

5.5. Results

5.5.1. Clinical and biochemical characteristics

Clinical and biochemical characteristics according to diabetes status are summarized in **Table 5.1**. Overall, the average age at diagnosis was 38 ± 18 years and 77% of the total cohort was female. Included individuals were of self-identified Caucasian ancestry. The heterozygous *LMNA* p.R482Q variant was seen in 51 patients (68.9%) while the p.R482W variant was seen in 23 patients (31.1%). A total of 28 (37.8%) individuals had a diabetes diagnosis at the time of assessment; subsequent analyses were stratified by diabetes status. Twenty individuals with diabetes were taking oral hypoglycemic agents, and of these 10 were also on insulin. Hemoglobin A1c was significantly higher in individuals with diabetes (P = 0.00001). A greater proportion of patients with diabetes was female compared to those without diabetes (89.3% vs. 67.4%; P = 0.049). HDL cholesterol levels were significantly lower in patients with diabetes compared to those without diabetes (P = 0.0001). A higher proportion of patients with diabetes were taking statins compared to those without diabetes (39.3% versus 15.2%). Five patients (three with diabetes, two without) had acanthosis nigricans. Three female patients (two with diabetes, one without) reported a diagnosis of PCOS. No patient had cardiomyopathy, skeletal myopathy or neuropathy.

Table 5.1 Clinical and Biochemical Characteristics

	Diabetes present	***No diabetes***	***P-value***
Number of subjects	28	46	
Age (years)	41.8 ± 14.8	35.4 ± 19.4	NS
Sex (females %)	25 (89.3%)	31 (67.4%)	0.049
Body mass index	24.5 (4.5) kg/m^2	24.5 (5.0) kg/m^2	NS
Total cholesterol	5.08 (2.43) mmol/L 196 (94) mg/dL	4.94 (1.15) mmol/L 191 (45) mg/dL	NS
Triglyceride	2.73 (4.78) mmol/L 242 (423) mg/dL	1.86 (1.66) mmol/L 165 (147) mg/dL	0.0057
HDL cholesterol	0.83 ± 0.29 mmol/L 32 ± 11 mg/dL	1.10 ± 0.25 mmol/L 43 ± 10 mg/dL	0.0001
LDL cholesterol	2.75 ± 0.98 mmol/L 106 ± 38 mg/dL	2.97 ± 1.08 mmol/L 115 ± 42 mg/dL	NS
Hemoglobin A1c	8.42 ± 0.90%	6.35 ± 0.73%	0.00001
LMNA p.R482Q mutation (%)	17 (60.7%)	34 (73.9%)	NS
Number (%) with triglyceride > 10 mmol/L (> 885 mg/dL)	4 (14.3%)	0 (0%)	0.018
Number (%) with pancreatitis history	3 (10.7%)	0 (0%)	0.051

Values shown are mean ± standard deviation or median (interquartile range). Lipid values are P-values are two-tailed.

*Incomplete data set: for HDL cholesterol, LDL cholesterol and hemoglobin A1c, in the no diabetes category, 5, 6 and 3 data points are missing; and in the diabetes present category, 3, 9 and 1 data points are missing, respectively.

HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant.

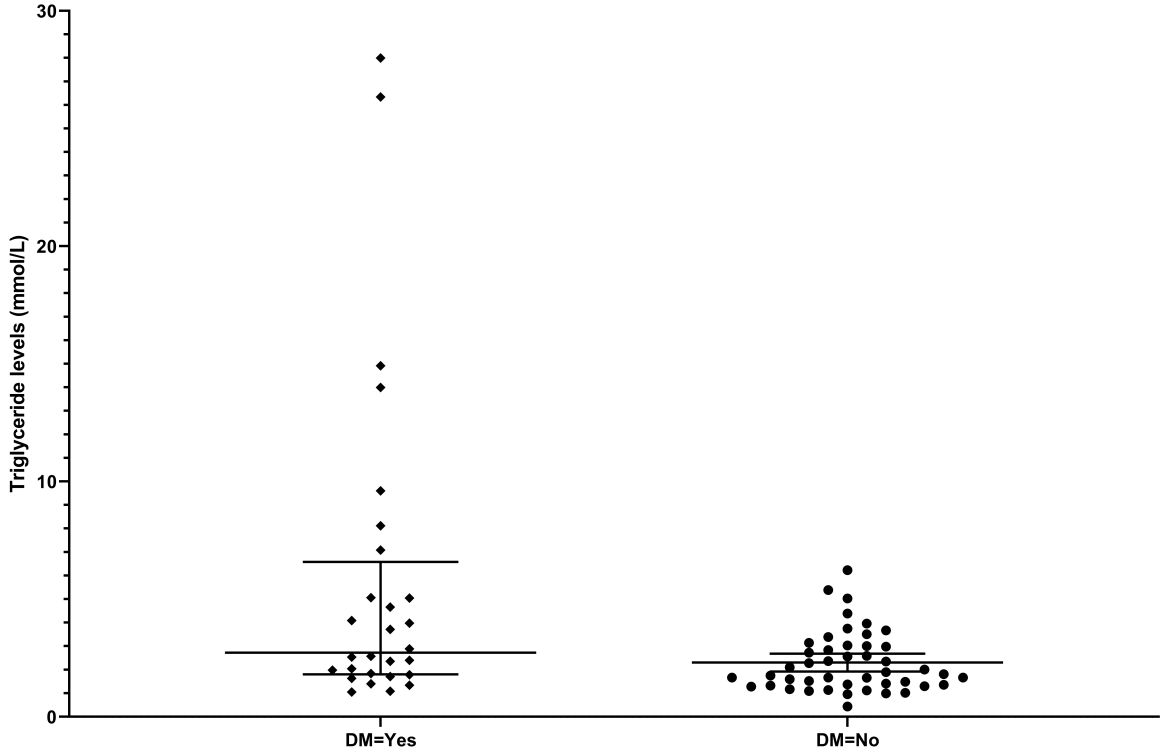

Figure 5.1 Distribution of Plasma Triglyceride Levels in FPLD2 Subjects Stratified by Diabetes Status

Median and interquartile ranges are depicted with the horizontal lines.

DM = diabetes mellitus.

5.5.2. Prevalence of HTG and pancreatitis

Overall, 16.2% (12/74), 5.4% (4/74) and 4.1% (3/74) of subjects had TG levels > 5 and > 10 mmol/L (> 438 and > 875 mg/dL), and a history of hospitalization for pancreatitis, respectively. All three subjects with a pancreatitis history had TG > 10 mmol/L. Among patients with and without diabetes, mean TG levels (interquartile range) were 2.73 (4.78) and 1.86 (1.66) mmol/L (242 [423] and 165 [147] mg/dL), respectively (**Figure 5.1**). Only one patient without diabetes had TG > 5 mmol/L (> 445 mg/dL) and patients without diabetes had neither TG > 10 mmol/L (> 885 mg/dL) nor a history of pancreatitis. Among FPLD2 patients with diabetes (28/74), 14.3% (4/28) and 10.7% (3/28) had TG > 10 mmol/L and a pancreatitis history, respectively.

5.6. Discussion

In a Canadian cohort of FPLD2 patients the overall prevalence of severe HTG and hospitalization for acute pancreatitis was 5.4% and 4.1%, respectively. There was no FPLD2 patient without diabetes who had either of these complications. Among FPLD2 patients with diabetes and the prevalence of severe HTG (> 10 mmol/L or > 885 mg/dL) and pancreatitis were 14.3% and 10.7% compared to no cases of either in patients FPLD2 without diabetes, respectively. This suggests that the risk of severe HTG and pancreatitis in FPLD2 depends on the concurrent presence of diabetes.

Our findings help to quantify the prevalence of severe HTG and pancreatitis in patients with partial lipodystrophy, which would otherwise be challenging based solely on anecdotal case reports and small case series. In our FPLD2 cohort, the 95% confidence interval (CI) for the observed overall prevalence of severe HTG of 5.4% ranges between 1.5 and 13.3%. Furthermore, in the FPLD2 subgroup with diabetes, the 95% CI for the observed overall prevalence of severe HTG of 14.3% ranges between 4.0 and 32.7%. These CI upper bounds suggest that the prevalence of severe HTG in FPLD2 might be substantial, particularly for the subgroup with diabetes. On the other hand, the prevalence of these complications is extremely low in the subgroup without diabetes.

As previously reported in FPLD2 patients, metabolic decline characterized by development of diabetes is a key determinant of the onset of life-threatening ASCVD

(Hegele, 2001). The current study shows that, as with ASCVD, the risk of HTG-associated acute pancreatitis in FPLD2 appears to be negligible in the absence of diabetes but is substantial if diabetes is present.

Our findings indicate that FPLD2 patients need to be closely monitored for lipids and glycemic status both prior to, and especially after the onset of diabetes. Adequate glycemic control with glucose-lowering medications would very likely mitigate development of severe HTG in lipoatrophic diabetes as in other forms of diabetes. Patients in this study received statins inconsistently and were not previously receiving specific TG-lowering therapies such as fibrates or prescription omega-3 preparations, which were not available in Canada until recently. Such treatment might be beneficial in the situation of severe HTG related to diabetes in FPLD2. Furthermore, these patients might benefit from new treatments for severe HTG such as volanesorsen, an antisense RNA targeting apolipoprotein C-III. Volanesorsen dramatically reduces TG levels in patients with severe HTG and familial chylomicronemia syndrome (Laufs et al., 2020). Furthermore, a randomized, double-blind, placebo-controlled, open-labeled trial investigating volanesorsen in FPLD patients should report soon (www.clinicaltrials.gov NCT02527343). Also, subcutaneous leptin markedly reverses metabolic abnormalities in patients with generalized lipodystrophy and somewhat less effectively in patients with FPLD (Ajluni et al., 2017), although leptin is not currently available in Canada.

This study advances our understanding of the risk of severe HTG and pancreatitis in patients with FPLD2. In a previous report of a family with FPLD2 due to the *LMNA* p.R482W variant, only a single family member – the one with diabetes - had TG > 10 mmol/L and pancreatitis (Vantyghem et al., 2004). Also, a previous study that included 149 partial lipodystrophy patients, of whom 68 were clinically defined FPLD2 subjects, reported an age-adjusted prevalence of pancreatitis of 11.9% in the entire group (Akinci et al., 2019). However, there was no description or correlation between the genetic subtype of FPLD and pancreatitis. In a report of several families with FPLD2, but without a description of the *LMNA* genotype, the prevalence of pancreatitis events was 30% in females compared to 0% in males (Garg, 2000). Similarly, in the current study the diabetic subgroup had 89% female patients, which was borderline significantly higher

compared with the non-diabetic subgroup that had 67% females (P = 0.049). Consistent with prior literature, females seem to show more severe clinical and biochemical features. In another study overall pancreatitis prevalence was found in a cohort of 23 lipodystrophy patients (22 with FPLD and 1 generalized lipodystrophy), in which 7 patients had recurrent pancreatitis (Ajluni et al., 2017). Further research is warranted to determine whether the pancreatitis prevalence differs in other FPLD subtypes (for instance, FPLD3) or is being driven by other undetermined genetic or environmental causes.

Factors known to aggravate HTG are likely to be deleterious for FPLD2 patients with diabetes. For instance, there are two case reports of young females who presented to emergency department with pancreatitis after having been prescribed combined oral contraceptive treatment for PCOS and hirsutism; the diagnosis of FPLD was made in retrospect in both cases (Haque et al., 2002; Vatier et al., 2019). In another case report, a woman with a history of several acute pancreatitis episodes that began at age 20, was diagnosed while pregnant with FPLD2 due to the *LMNA* p.R482Q variant (Belo et al., 2015). These examples demonstrate that secondary factors for HTG need to be considered in all FPLD2 patients (Laufs et al., 2020).

A limitation of this cross-sectional study is that the plasma TG levels are from a single time point, i.e. at the patient's first clinic visit. It is likely that some patients who had mild-to- moderate TG elevations followed longitudinally might in the future deteriorate metabolically and develop severe HTG. A prospective study might help quantify this disease evolution.

5.7. Conclusion

FPLD2 patients with diabetes on average had moderate HTG, while about one in seven had severe HTG and one in ten had a pancreatitis history. In contrast, FPLD patients without diabetes had mild HTG and none had severe HTG. This suggests that the risk of severe HTG and pancreatitis in FPLD depends on the concurrent presence of diabetes.

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Chapter 6. Discussion

6.1. Overview

The projects described in this PhD Thesis represent my collective efforts over the past 3 years. The unifying theme has been the application of technologies, methods and analytical approaches made possible by the human genome project in order to fill gaps in our knowledge of human diseases. The long-term goal of this work is to provide a fact-based substrate for improving diagnosis and intervention, in addition to expanding our understanding of the underlying molecular biology and genetics of these conditions. The disorders that I studied – atrial fibrillation (AF), familial hypercholesterolemia (FH) and familial partial lipodystrophy (FPLD) - each have distinctive clinical presentations, but all are linked by involvement of the cardiovascular system in affected individuals. The genetic component that I studied in each of these conditions encompasses to varying degrees pathogenic rare DNA variants and/or common small effect variants. Furthermore, there is a range of phenotypic severity that is related to both underlying genetic etiologies but also concurrently to non-genetic or environmental factors.

The centerpiece of my thesis work was related to elucidating the genetic contribution underlying "lone" AF and demonstrating the prominent genetic underpinnings for this condition (Chapters 2 and 3). Specifically, I applied a comprehensive approach and investigated rare single-nucleotide variants (SNVs), copy-number variants (CNVs), and common single-nucleotide polymorphisms (SNPs) utilizing next-generation sequencing (NGS) data (whole-exome) and DNA microarray data. I published that all these types of DNA variations contribute to susceptibility to "lone" AF, which as a result of my thesis work must now be considered to have a much more prominent and diverse genetic basis than was previously appreciated. The lessons learned from describing the genetic foundation of "lone" AF can likely be more widely applied to the genetics of many other disorders within adult internal medicine.

The secondary projects in my thesis work – namely the study of a putative new *APOB* CNV in FH and the range of clinical phenotypes in FPLD2 carriers of heterozygous pathogenic *LMNA* variants (Chapters 4 and 5), provided limited incremental new

knowledge. However, these projects were carried out at the interface of clinical medicine and next-generation DNA analysis, and each provided an important clinical lesson about application of modern genetics in the clinic and on the medical ward. In the case of the *APOB* CNV, the lesson was that even when a newly discovered rare variant appears by all accounts to be pathogenic, you cannot assume this without complete due diligence of acquiring confirmatory evidence, which in the case of the family I studied involved contacting family members and extending the pedigree to obtain confirmatory information. This avoided a potentially incorrect diagnosis in the family. A concern that this demonstrates is that most clinicians who interpret genetic results such as exome sequencing might not have time to perform such additional careful work, and might leap to a premature conclusion about causation resulting in a misdiagnosis that could have lifelong implications for the patient and family. In the case of the FPLD2 *LMNA* variant carriers, my work clarified that the greatest risk of serious complications was among the subgroup of individuals who had already developed diabetes, with the clinical lesson that this subgroup needs to be extremely carefully monitored and managed preventively.

6.2. Summary of research findings

6.2.1. "Lone" atrial fibrillation

The results described in Chapters 2 and 3 are the summation of a comprehensive assessment of the contribution of rare cardiomyopathy SNVs and CNVs, and the first assessment of common variant accumulation in individuals with "lone" AF. An overview of the genetic factors contributing to "lone" AF is depicted in **Figure 6.1.**

We investigated the genetic factors of a cohort of 186 "lone" AF patients from two Canadian centres, London Health Sciences Centre, London, Ontario and St. Paul's Hospital, Vancouver, British Columbia. Utilizing NGS data, we interrogated for rare loss-of-function (LOF) SNVs and CNVs within 43 cardiomyopathy candidate genes and confirmed the variants with Sanger sequencing. We then pursued cascade screening in family members to determine if the variant and disease co-segregated.

Collectively 4% of the "lone" AF cohort had a rare LOF SNVs in a cardiomyopathy gene (**Figure 6.1**). The majority of the LOF SNVs were identified in the *TTN* gene; co-

segregation of *TTN* variants with “lone” AF was demonstrated in 2 multigenerational pedigrees. Additionally, one novel deletion in *TTN* was identified across two individuals. This is the first reported instance of two unrelated “lone” AF patients carrying the same CNVs in the *TTN* gene, as the presumed main genetic determinant for their phenotype (Lazarte et al., 2021d).

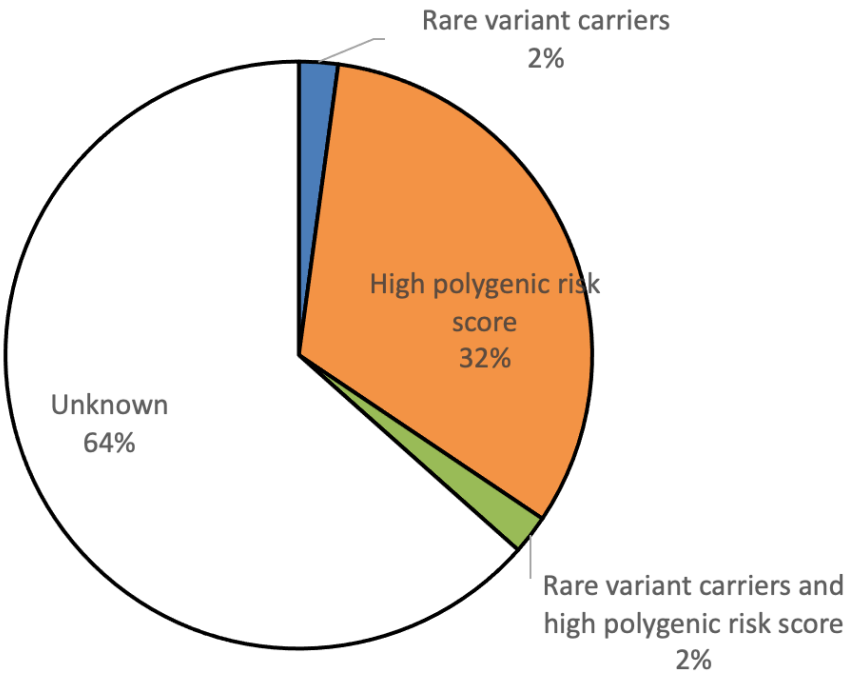

Figure 6.1 The updated genetic contribution underlying the "lone" atrial fibrillation

Carriers of rare loss-of-function SNVs (N= 6) and CNVs (N= 2) in cardiomyopathy genes and individuals with a high genome-wide polygenic score (N= 64), developed by Khera et al. (2018), are depicted in this pie chart. The total cohort size is 186 from Chapter 3 analysis.

With the majority of patients lacking a rare LOF variant (96%), we sought to determine whether there was an accumulation of common variant alleles contributing towards the "lone" AF phenotype. Leveraging imputed DNA microarray data, we calculated two previously published polygenic risk scores, a GPS (Khera et al., 2018) and PRS (Weng et al., 2018), compared distribution and discriminatory performance of each score among "lone" AF cases and two sets of controls.

"Lone" AF patients had a significant 4.64-fold increased odds of possessing an extremely high GPS relative to healthy controls, which corresponded to 34.4% of the "lone" AF cohort (**Figure 6.1**). Similar, though generally smaller magnitude effects were observed for the PRS. The first reported comparison of two polygenic risk scores determined that the GPS with roughly 6.6 million SNPs had an improved discriminatory capacity compared to the PRS with roughly 1,000 SNPs (Lazarte et al., 2021a, 2021c).

In a Canadian cohort of "lone" AF patients, we were able to identify a genetic culprit in 36% of the cohort by investigating for rare SNVs and CNVs in cardiomyopathy gene as well as for high polygenic burden (**Figure 6.1**). The remaining (64%) of individuals without an identifiable genetic culprit remain part of our missing heritability conundrum and signify the gaps and challenges we continue to have to decipher the genetic contribution of diseases. Together, the assessment of rare LOF SNVs and CNVs in cardiomyopathy genes and polygenic risk scores allowed for a comprehensive investigation of the genetic determinants underlying "lone" AF. Our findings provide additional evidence that atrial cardiomyopathy may be a genetic sub-phenotype as well as highlighted the substantial polygenic component of this phenotype. Overall, the findings from the three publications on this topic move the field forward first by providing strong evidence that "lone" AF has a genetic component, second by emphasizing the qualitatively and quantitatively diverse nature of the genetic component, and third by identifying underlying mechanistic links that could serve to direct development of new, more rational interventions.

6.2.2. Implications of genetic on clinical medicine

Collectively the findings described in Chapters 4 and 5 are the summation of clinically relevant lessons learned from the application of genetics on a family with familial hypercholesterolemia (FH) and on a cohort of familial partial lipodystrophy - Dunnigan variety (FPLD2).

All patients with a potential FH diagnosis are screened for genetic culprits using our targeted NGS panel, LipidSeq. In Chapter 4, we described a novel whole-gene duplication on *APOB* found in an FH patient with no other identifiable genetic factor. Although there are published guidelines on the assessment of pathogenicity to previously unreported variants, it is not always possible for a clinician to gather and interpret all the evidence. Hence, we performed a corroborative assessment feasible for a clinician to undertake in the clinic setting, to increase the evidence required for the pathogenicity assessment of the novel variant. We carried out cascade screening and co-segregation analysis on multiple family members. When we extended the pedigree and compared the presence of phenotypic hypercholesterolemia with the heterozygous *APOB* gene duplication, we observed several instances in which the genotype and putative linked phenotype did not co-segregate, suggesting that the hypercholesterolemia in this family occurred independent of the *APOB* CNV. This study highlighted how important it is to pursue corroborative assessments, such as co-segregation analysis, in the extended family before determining causality. It also provides a cautionary note against jumping to conclusions about attributing pathogenicity to an apparent causal variant newly revealed by modern DNA analysis without first seeking strong confirmatory evidence. Our experience tells us however that most clinicians have neither the time, resources or inclination to perform additional confirmatory work, and might sometimes communicate the incorrect diagnosis that emerged from the DNA analysis, with potential lifelong implications for the patient and family.

Lipodystrophy patients have been referred to the Lipid Genetics Clinic for over 25 years; all patients with a presumptive diagnosis of lipodystrophy are screened with LipidSeq. In Chapter 5, we described our investigation on the prevalence of high plasma triglycerides (TG) levels (>10mmol/L) and pancreatitis in a Canadian FPLD2 cohort (composed

exclusively of heterozygous carriers of *LMNA* variants). Our findings demonstrated that TG levels (>10mmol/L) and hospitalization for acute pancreatitis occurred in 5.4% and 4.1% of the cohort, respectively. Given that diabetes is a common comorbidity due to the profound insulin resistance that these patients suffer, we divided the group by concurrent diabetes. We found that 14.3% and 10.7% of FPLD2 patients with diabetes had severely elevated TG (>10 mmol/L) and a history of acute pancreatitis compared to FPLD2 without diabetes, respectively (P = 0.0057). This suggests that the risk of severe hypertriglyceridemia (HTG, with TG >10 mmol/L) and pancreatitis in FPLD2 depends on the concurrent presence of diabetes. The take-home point for the clinician is that once diabetes develops, carriers of the identical gene variant display a markedly amplified risk of severe HTG and potentially fatal acute pancreatitis, underscoring the need to monitor this subgroup of patients frequently and carefully in the outpatient clinic.

As genetic technologies improve and our understanding of the genetic contribution of diseases advances, genetic will have an increasingly prominent role in clinical care and management of patients. Together in Chapter 4 and 5, we highlight relevant lessons for the clinician such as the importance of diligence and thoroughness when interpreting novel genetic variants and a reminder of the role genetics continues to have in helping us identify subgroups with variable risk differences that can improve care.

6.3. Strengths, caveats, and limitations

In Chapters 2-5 of this PhD Thesis, the strengths and limitations specific to each study were described in the Discussion section of each chapter. Here, I will describe the overarching considerations—specifically related to study design and technological resources—that apply to my collective research efforts.

6.3.1. Strengths

In Chapters 2 and 3, we were able to comprehensively study rare SNVs and CNVs, and common variants in the entire "lone" AF cohort primarily because of the comprehensive study design that included the sequencing and genotyping of cases and some controls. For SNVs and CNVs, we had access to whole-exome sequencing (WES) and Sanger

sequencing, and for common variants we had DNA microarray data. The study design was fundamental for us to be able to interrogate for genetic culprits across the variant spectrum.

Further, we were able to access various control groups with the corresponding methodologies and in some instances, the same sequencing platforms, to appropriately compare the occurrence of the genetic factors in non-diseased cohorts. We were able to access WES and microarray data from the 1000 Genomes control group as well as WES data run with the same sequencing platform as our cases for the internal control group (Illumina NovaSeq 6000) and microarray data genotyped with the same platform as our cases for our lab controls (Infinium™ Global Screening Array-24 v2.0). In all instances possible we aimed to match the sequencing platforms, bioinformatic filters and coverage between cases and controls to decrease ascertainment biases that can occur when comparing data from different sequencing platforms.

In Chapter 4 and 5, the genetic data originated from LipidSeq (a targeted gene panel developed in the Hegele lab) the accuracy, resolution, and confidence of the LipidSeq generated data is a strength for both analyses. Briefly, this targeted gene panel was created to capture both monogenic and polygenic factors associated with dyslipidemias and metabolic phenotypes (Dron et al., 2020), as discussed in section 1.7.2.1. LipidSeq has many assets, including i) the ability to instantaneously compare results with over 3,000 individuals, ii) a high read-depth coverage (300x), iii) the identification of novel SNVs with high resolution and iv) the concurrent investigation for CNVs with high precision and confidence. Unique to this panel, the opportunity to investigate for CNVs at a large-scale has allowed for the identification of novel CNVs in a variety of dyslipidemias, including the whole-gene *APOB* duplication described in Chapter 4 (Iacocca et al., 2017; Iacocca et al., 2018b; Lazarte et al., 2020).

The study population and overall size were additional strengths of my research. In Chapter 2 and 3, we performed the investigation in “lone” AF patients. Although there remains significant debate as to whether “lone” AF patients have true “lone” AF (discussed in section 6.3.2), what we do know is that in individuals with extreme

phenotypes there is a higher genetic contribution and we exploited this difference to increase the likelihood to detect novel variants without necessarily increasing cohort size (MacArthur et al., 2014; Wyse et al., 2014a). Indeed, individuals that develop AF at an early age (<60 years of age) and without any other obvious risk factor, can be deemed at the extreme of the phenotype and this improves the chances to detect rare genetic culprits. In Chapter 4 and 5, the accuracy and confidence to detect a novel CNV and ability to risk profile a monogenic dyslipidemia was also in part due to the large number of dyslipidemia samples sequenced with the LipidSeq gene panel. For over 25 years, Dr. Hegele's laboratory has collected and sequenced over 3,000 dyslipidemic patients. The ability to compare raw sequencing data with over 3,000 individuals significantly elevates the accuracy of the genetic calls, especially for CNVs call. Additionally, we reported on one of the largest single centre study on FPLD2 patients (N=76) and for context, the population prevalence of FPLD2 is estimated to be less than 1 in 10 million individuals (Garg, 2004). Lastly, provided that about half of sequenced patients are from the Lipid Genetics Clinic, we had the opportunity to access the clinic chart for more information or contact the patients to pursue cascade screening.

6.3.2. Caveats

Certain caveats should be considered for the appropriate interpretation of the conclusions and implications of the data presented in this PhD Thesis.

In Chapters 2 and 3, we studied a less common subtype of AF, refer to as “lone” AF, and defined as the development of arrhythmia prior to 60 years of age, in the absence of known clinical risk factors such as hypertension, coronary artery disease, left ventricular ejection fraction <50%, moderate to severe valvular heart disease, hyperthyroidism, and obstructive sleep apnea. All patients included underwent, at minimum, a clinical history, physical examination, 12-lead ECG, and echocardiogram. For many clinicians, “lone” AF is synonymous of idiopathic AF with an age of onset cut-of (<60 years of age) (Potpara & Lip, 2014). However, the “lone” AF definition has long been problematic because it is inconsistently defined among various studies which causes the results of each study to not be comparable (Wyse et al., 2014a). Further, it is primarily a diagnosis of exclusion with no consensus definition. “Lone” AF prevalence previously accounted

for 30% of AF patients, now estimates of its prevalence are around 3% in part due to the advances in our understanding and detection ability of new heart disease forms that cause AF (Wyse et al., 2014a). Additionally, a common argument is that the lack of detection of concomitant heart disease does not mean that there is no heart disease, because it may just be below the threshold for detection; this is based on the concept of progressive (atrial) remodeling (Wyse et al., 2014a). Indeed, in a 12-year follow-up study of patients with a "lone" AF diagnosis, some patients remained with true "lone" AF while others developed cardiovascular comorbidities over time; demonstrating that true "lone" AF does exist (Potpara et al., 2012). Interestingly, despite the many studies on "lone" AF patients, not a clear mechanism that differentiates "lone" AF from regular AF has been identified, suggesting that "lone" AF may not be too different from AF after all. Regardless of the problematic definition and diagnosis, what we do know is that "lone" AF aggregates within families showing high heritability and has a distinct clinical presentation of early disease onset (Ellinor et al., 2005). Indeed, variant and gene discovery for complex traits, such as AF, is facilitated when studying a well-defined and carefully phenotyped cohort in the absence of clear environmental precipitant.

Various control cohorts were employed for Chapter 2 and 3: for the principal component analysis, rare, and common variant analysis, our control cohorts came from the 1000 Genomes Project (1KG) (Genomes Project et al., 2015; Roslin NM, 2016); for the CNV frequency analysis the control cohort was 4,958 unaffected parents from an autism study (Yuen et al., 2017); for the internal CNV analysis we used 16 unrelated self-reported healthy individuals of European ancestry; and lastly, for the PRS analysis, our second control ("lab controls") were 86 self-reported healthy individuals of European ancestry. Although all control cohorts by definition only included "healthy" samples, none had undergone specific AF screening. Hence it is possible that participants with AF or undiagnosed AF were part of the normal controls, which this would have increased the likelihood of false negative. Indeed, the average age of the 1000 Genomes project controls is not available, but we do know that for the CNV frequency analysis the participants were < 50 years of age. For the internal and lab control groups, we have more confidence of their non-disease status given that the individuals have worked in the laboratory or Research Institute at some point. Indeed, the prevalence of AF is age-

dependent with a 2-3% estimate in the general population that drops to <1% among individuals under 50 years of age, but is upwards of 10% in octogenarians (Zoni-Berisso et al., 2014). Despite the possibility of AF in the controls, the percentage of "lone" AF cases with rare variants or extreme scores relative to the 1KG or lab control was statistically higher.

6.3.3. Limitations

A major limitation in my PhD Thesis was the virtually exclusive study of participants of European ancestry. This was related to practical issues primarily of initial sample ascertainment, based on the demographics of the clinical cohorts, and secondarily to experimental design issues in which it became necessary to objectively stratify by ancestry-related principal components in order to reduce statistical noise and the chance of spurious findings. Specific to Chapters 2 and 3, we identified that the majority of the "lone" AF patients were of European ancestry with a minority from various ancestries. This was in part due to the populations that the clinics served (London, Ontario and Vancouver, British Columbia). However, ancestry matters in genetics, as discussed in section 1.2.4 and 1.5.1.2, rare and common variant frequencies are ancestry specific and ancestry is a confounding factor that can easily inflate type 1 error (MacArthur et al., 2014; Mathieson & McVean, 2012). Hence, our rare and common variant analysis became European-specific and by extension, our control cohorts had to be limited to European ancestry as well.

The lack of diversity is a major limitation in the field of AF genetics. For instance, the majority of genetic analysis on AF have been performed in individuals of European descent (Low et al., 2017). Indeed, there is a clear predominance of European-participants in AF GWAS as illustrated by Roselli et al. (2020) (Christophersen et al., 2017b; Ellinor et al., 2012; Nielsen et al., 2018). When genetic studies don't represent the world's diversity, we limit our understanding of disease etiology. For instance, thanks to a large Japanese AF GWAS, we know that 85% of the top GWAS hits overlapped with those from a European GWAS (Lubitz et al., 2014). However, the same cannot be concluded for other ancestries due to the lack of investigations and evidence. In terms of our investigation, we could not determine whether the *TTN* signal identified in our "lone"

AF cohort would be consistent across ancestries or unique to the European ancestral group. However, we now know that *TTN* variants have been reported in individuals of African American and Hispanic/Latinx ancestry, in keeping with our and other group's findings that *TTN* has a significant role in AF pathophysiology (Chalazan et al., 2021). Another limitation of genetic studies that are not diverse, is the restricted application of the findings to other populations. For instance, polygenic scores are meant to predict and identify individuals at a higher risk for disease; however, if they are only based on European ancestry individuals they won't accurately identify at risk individuals from other ancestral groups further exacerbating health care inequalities specially in countries with diverse populations like Canada (Duncan et al., 2019). Fortunately, significant efforts are being devoted to increase the inclusion of non-European participants in GWAS studies which will in turn advance the development of polygenic risk prediction tools for different ancestral groups (Roselli et al., 2020). For instance, programs such as All of Us (https://www. allofus.nih.gov), Million Veteran Program (https://www.mvp.va.gov), and others are focused in recruiting diverse populations.

There were some additional technical and methodological limitations that need to be address. With respect to the identification of CNVs in the "lone" AF cohort, unlike the detection of the CNV in the FH family, the whole-exome sequencing data of the "lone" AF cohort had on average a moderate read-depth (80x) and only 186 plus 16 additional controls run on the same chemistry and platform. Briefly with the explosions of NGS technology, computational algorithms like VarSeq-CNV® caller algorithm (Golden Helix, Inc., Bozeman MT, USA) have been developed to identify structural variants like CNVs from the raw sequencing data (Iacocca et al., 2019). Using VarSeq-CNV® caller algorithm, the accuracy of the CNV call is ultimately dependent on the read-depth and to a minor extent the availability of reference samples run on the same chemistry and platform. Hence although the lab has been able to identify with high confidence various CNVs, including the whole-gene *APOB* duplication in Chapter 4, it was quite difficult to translate that confidence and accuracy to the whole-exome sequencing data of the "lone" AF cases (Lazarte et al., 2021d). We adjusted by lowering the cut-off for the algorithm metrics to accommodate for the different sequencing data (whole-exome sequencing versus gene panel sequencing) and decided to focus in breakpoint analysis for the CNV

calls that met the metrics cut-off. From the many CNVs calls that were generated, we were able to successfully locate the breakpoint of one, the *TTN* CNV. However, we noticed a diminished resolution to detect CNVs when using whole-exome sequencing data and a high rate of miscalls. This is in keeping with the findings from a comprehensive evaluation of CNV detection algorithms that use NGS data, in which it found that it was challenging to identify all types of structural variants, there was significant detection variability between algorithms and that they all suffer from high rates of miscalling due to errors in variant call, alignment, or de novo assembly, and challenges in repetitive regions (Kosugi et al., 2019).

In Chapter 2 and 3, we incorporated publicly available cohorts because of the improved precision estimates of allele and CNVs frequencies that large cohorts like the 1KG dataset and the CNV control cohorts may provide. Collecting and sequencing/genotyping large healthy control cohorts is not possible for individual laboratories, like ours. However, this led to sequencing differences between our cases and controls which invariably introduced ascertainment bias. This is indeed a limitation of any study that leverages publicly available exome and genome data. We did maintain chemistry and technology consistent across the cohorts that we had access to and even compared, in Chapter 3 analysis, the results between our controls and the 1KG European controls finding no difference between them.

Lastly, despite the excellent design of the LipidSeq panel, one limitation of any gene panel like LipidSeq is the inability to do novel gene discovery. The reason being that a gene panel sequences a predefined set of genes which is based on the imperfect understanding of disease pathophysiology at that time. In Chapter 4, once we ruled out the *APOB* CNV as the culprit for the hypercholesterolemia in the family, we did not have any other identifiable genetic cause within the realms of the gene panel. In this case, whole-exome sequencing would have provided the opportunity to discover whether a novel gene with previously unknown associations with LDL cholesterol was the culprit for the hypercholesterolemia in this family.

6.4. Applications and future directions

Throughout this PhD Thesis, my primary focus was directed towards exploration of the genetic underpinnings of "lone" AF to identify valuable insights about the genetic architecture of AF. My work succeeded in advancing our understanding of the genetic basis of AF. Additionally, as a clinician-scientist trainee, I explored the application of genetic concepts in medicine to enhance the care of patients, providing some important clinical lessons that would overall advance our understanding of how to interact with an manage patients with genetic diseases. In the following paragraphs, I will discuss future direction in the field of genetics, link it back to our findings and conclude with the implications for medicine and patient care.

6.4.1. Missing heritability

In this PhD Thesis, only 36% of "lone" AF patients were carriers of an identifiable genetic factor that presumably explained their phenotype, leaving the remainder 64% without a genetic basis. This is referred to as missing heritability, a central concept in genetics, which alludes to the portion of variability that remains unexplained (Manolio et al., 2009). To decipher the missing heritability conundrum current strategies focus on systematically investigating the wide spectrum of genetic variants; from rare to common variants, whether it is a single nucleotide change to structural variants with no prioritization. Indeed, deciphering the genetic architecture of complex diseases, like AF, is a challenging endeavour. Further, despite the concepts and techniques being transferable, the missing heritability of one disease is different from another (Pritchard & Cox, 2002). The recognition of this concept fueled our efforts to holistically investigate the genetic underpinnings of "lone" AF, from rare to common variants, and prior to that influenced the design of the LipidSeq panel. The central purpose of the monumental effort to determine the genetic contribution of each disease is the clinical benefits that this knowledge bestow, such as, advances in detection, diagnosis, and treatment opportunities. In the following sections, I will describe different directions of the research efforts to tackle the missing heritability and provide clinical implications from our findings.

6.4.1.1. Re-emphasize in rare variants

After the explosion of GWAS much of the speculation on missing heritability has been focus on rare variants (Manolio et al., 2009). Rare variant investigation, as discussed in section 1.5.1.2, has become a feasible endeavour with the development of NGS technologies and the subsequent decrease in cost to sequence large amount of samples. Rare variants with large effect sizes that contribute to complex diseases have been discovered with linkage analysis, targeted sequencing studies and case-control studies, as we have shown in this PhD Thesis (Flannick et al., 2019; Lazarte et al., 2021d), however the majority of these studies are limited by small sample sizes. In fact, that has been a consistent problem of rare variants analysis. Novel methodological approaches of pooling rare variants have been developed to lower the sample size requirements and increase statistical power (Lee et al., 2012). However, the game changer has been the access to large-scale population-based sequencing data, like the UK Biobank database, which allows for the dissection of rare variants across populations and diseases, that would have otherwise not been possible. This is where the field of genetics is moving towards and tremendous amount of discoveries are expected to originate from the access to these large-scale sequencing efforts as it is already happening (Jurgens et al., 2020).

From our rare variant investigation in Chapter 2, we implicated dilated cardiomyopathy genes (in particular *TTN*) to "lone" AF, in the absence of ventricular cardiomyopathy. This work provided evidence that AF, developed in the context of atrial cardiomyopathy. Atrial cardiomyopathy as a culprit for atrial hypocontractibility and subsequent cause for unaccounted stroke in the setting of AF has become an emerging concept in the field of AF (Darlington & McCauley, 2020). The recognition of this concept, additionally corroborated by multiple studies, has the potential to impact clinical care (Ahlberg et al., 2018; Chalazan et al., 2021; Choi et al., 2020; Choi et al., 2018a). For instance, when counseling patients about stroke risk, given that there is an association between atrial cardiomyopathy and an increased stroke risk relative to primarily electrical forms of the arrhythmia (Darlington & McCauley, 2020; Goette et al., 2016; Guichard & Nattel, 2017). Should this be proven correct, insight into the underlying genetic etiology for AF

within an individual may prove helpful for stroke risk stratification and the need for anti-coagulation.

6.4.1.2. Don't forget structural variants

Structural variants can impact disease development, with the potential for a larger than expected effect on the function of genes, discussed in section 1.2.3. Thus far, several structural variants have been associated with various diseases such as FH, hypertriglyceridemia, maturity-onset diabetes of the young, Crohn disease, rheumatoid arthritis, and diabetes mellitus (Berberich et al., 2019; Iacocca et al., 2019; Wellcome Trust Case Control et al., 2010). However, despite advancements in screening for structural variants from the raw sequencing data, identification remains sparsely adopted, with most of the research efforts on SNVs (Iacocca et al., 2019). Additionally, detection remains challenging, compared to SNVs, and there remains a lot of variability in the detection capabilities of different algorithms (Kosugi et al., 2019). Regardless, as scientist continue to resolve the missing heritability, it will become imperative to systematically assess for structural variants; we can anticipate that novel mechanisms will be uncover from future investigations specially in clear genetic cases where no SNV culprit was found.

Based on our experience with the early adoption of large-scale CNV screening from the LipidSeq data, we have successfully identified the important contribution of CNVs in many metabolic disorders (Berberich et al., 2019; Dron et al., 2020; Iacocca et al., 2019). We have demonstrated how structural variants, in this case CNVs, have little overlap with SNVs and contribute to a portion of disease heritability. In addition, we are not only screening for them but, we are also able to assess for their pathogenicity; for instance, in Chapter 4 we assessed the pathogenicity of a novel *APOB* duplication. This is a perfect example a CNV that is potentially benign as it is estimated that 5% of the total genomic sequence in healthy individuals is composed of CNVs (Conrad et al., 2010). Our analysis in Chapter 4 also demonstrated the significant value of the additional evidence when assessing the novel variant and ultimately to provide an accurate diagnosis.

In Chapter 2, as part of our rare variant screening in the "lone" AF cohort, we identified and characterized a novel CNV deletion disrupting *TTN* in two individuals. Indeed, the *TTN* CNV, along a previously identified common intronic *KCNIP1* duplication in a Taiwanese population, are the only reported CNVs associated with AF (Tsai et al., 2016). Briefly, the intronic CNV was associated with increased levels of the *KCNIP1,* which encodes the potassium interacting channel 1 protein, and its overexpression in zebrafish was shown to increase AF susceptibility (Tsai et al., 2016). Although further support for pathogenicity will be necessary to clarify the role of the *TTN* CNV identified in our study (Lazarte et al., 2021d), collectively these findings highlight the large need to screen for large deletions and duplications that may be associated with AF. Structural variants remain a relatively unexplored field in most complex diseases, including AF (Roselli et al., 2020). However, we know from our large-scale CNVs screening with the LipidSeq panel, that CNVs detection increased FH genetic diagnosis by 10% (Iacocca et al., 2019). Thus, with the growth in NGS data, it is a matter of time before the full potential of this sequencing technology is seized. We anticipate that the field of genetics will begin to systematically assess for structural variants with the purpose to identify novel genetic mechanisms for complex diseases and comprehensively tackle the missing heritability problem.

6.4.1.3. Expansion of GWAS and genome-wide scores

Provided the continuous feasibility, low cost, and success with GWAS, they will continue to be an integral part in facilitating the discovery of novel disease genes and in the derivation of polygenic scores that can stratify disease risk in the general population. Concerted efforts are now dedicated to increase the sample sizes of GWAS and the inclusion of a broad range of ancestries (Roselli et al., 2020). Indeed, as GWAS sample size increased the validity of the results improved and the number of significant loci increased slowly encompassing the full genetic basis of diseases (Dron & Hegele, 2019; Roselli et al., 2020). For instance, the first AF GWAS on 550 AF cases remarkably identified one significant loci in the *PITX2* gene, compared to the most recent AF GWAS with 65,000 AF cases that identified over 100 significant loci (Gudbjartsson et al., 2007; Roselli et al., 2018). Despite the great discoveries, Weng et al. (2017a) identified that

known AF loci accounted for only one fourth of the AF heritability, indicating that additional susceptible genetic variants remain to be discovered and this is a common theme across complex diseases (Dron & Hegele, 2019). Recognizing the preponderance of European individuals across GWAS and the limitations that this causes (discussed in section 6.3.3), several large-scale sequencing projects are currently dedicated to biobank and sequence non-European individuals (Roselli et al., 2020).

Just like GWAS, polygenic scores have morphed over time and increased in the number of common variants that they include (Dron & Hegele, 2019). For instance, the first AF polygenic score included 12 SNPs while the one applied in our "lone" AF study incorporated over 6.6 million SNPs (Khera et al., 2018; Lubitz et al., 2014). It became evident that to accurately model the genetic contribution of common variants to a complex disease, many more variants, including those not yet at GWAS significance, would need to be accounted for (Boyle et al., 2017). Polygenic scores that include millions of SNPs are defined as "genome-wide scores", due to their attempt to capture relevant data from the entire genome. Genome-wide scores have been developed for a number of complex diseases such as CAD, AF, severe hypercholesterolemia among others (Khera et al., 2018; Natarajan et al., 2017). In Chapter 3, as part of our common variant investigation in the "lone" AF cohort, we determined the improved performance of the ~ 6.6 million SNPs score relative to the ~ 1,000 SNPs score. Consistent findings have been reported in a head-to-head comparison of a CAD polygenic scores (Khera et al., 2018). The logical expansion to genome-wide scores is an obvious future direction in the field as well as development of polygenic scores in non-European populations to broaden the clinical application of the scores (Roselli et al., 2020).

6.4.1.4. Combining rare and common variants

As we deepen our understanding of the genetic underpinnings of complex diseases, it is a matter of time before the genetic contribution to a disease is collectively assessed. At a smaller scale, the LipidSeq panel already achieves that comprehensive assessment for dyslipidemic patients by investigating rare and common variants (Dron et al., 2020). Further, several studies have demonstrated the interaction between rare variants and the accumulation of common variants, and this area is of extreme interest in the field of

genetics (Choi et al., 2020; Craig et al., 2020). For instance, Choi et al. (2020) identified that the penetrance of *TTN* variants was higher among AF individuals with an increased polygenic risk. Hence in the near future, the collective assessment of genetic variants may be the most accurate and predictive measure of the genetic contribution to a disease.

6.4.1.5. Implications of genetics in medicine

It is becoming increasingly evident that genetics is playing a growing role in clinical medicine, from providing a diagnosis to improving management and disease prevention strategies, and advancing the overall understanding of the etiology of complex diseases. However, medical trainees are only allotted a couple of hours at most of their medical school training to the basics of genetics, resulting in clinicians who are largely unequipped to handle the complexities and nuances of genetics in twenty-first century medicine. While detecting and reporting causative variants is technically straightforward, increasingly done by private laboratories, it is considerably more challenging to attribute a causal role for a suspected pathogenic variant within the context of patient's entire clinical situation. It is even challenging for experts in the field, hence the ACMG has published guidelines for variant interpretation (discussed in section 1.6) (Richards et al., 2015). But even with these guidelines, there is a surprisingly high rate of discordant diagnostic calls between clinicians an even between agnostic "objective" bioinformatic algorithms.

A genetic diagnosis can have a lifelong impact on a patient and its family; by providing a tangible molecular basis or taxonomy for the disease, initiating cascade screening to identify affected family member pre-symptomatically, in some cases in helping to obtain access to novel or specialized therapies, and by potentially motivating better adherence to medication use. Hence, genetic results should be carefully interpreted by an individual or team that has expertise in both genetics and the disease in question before assigning it to a patient. Indeed, in Chapter 4, we explored additional supporting data that a clinician can obtain relatively easy to facilitate a variant's pathogenicity assessment. Our findings from the cascade screening and co-segregation analysis proved to be essential in the assessment of this novel variant that was eventually rejected as causal.

As I have thoroughly described throughout my Thesis, genetic contribution also frequently encompasses polygenic factors. Indeed, as polygenic risk scores continue to be refined, their potential in the clinical setting is promising, although issues such as standardization and ethnic-group specific scores need to be carefully considered. As alluded to in the final sentence of our manuscript, regardless of the underlying mechanisms accounting for their association with AF, they will enable us to identify individuals from the general population that are at substantially increased risk of the arrhythmia prior to its onset (Lazarte et al., 2021b). For these reasons, we recommend that most clinicians without specific expertise in genetics should consult with an individual who is experienced in the theory and practice of genetics, such as a genetic counsellor or certified medical geneticist, to help interpret the genetic findings for patients and their families because the potential benefit for an accurate genetic diagnosis are immense.

Finally in Chapter 5, we investigated the natural history of familial partial lipodystrophy individuals by utilizing their genetic diagnosis to single out those that belong to the FPLD2 subtype. Our findings highlighted the improved diseases understanding that can be achieved when analyzing a well-genotyped and phenotype group. The findings also provided more direct and objective quantification of the risk of severe HTG and pancreatitis among heterozygotes for pathogenic variants in the *LMNA* gene at codon 482. The practical benefit of this information is that this prevalence was not previously known, since many clinicians had mistakenly assumed that the risk of this complication was uniform and fully expressed in these mutation carriers. Instead, we have found that the risk is restricted to those mutation carriers who have already developed diabetes, emphasizing the need to carefully monitor this at-risk subgroup of patients.

6.5. Conclusion

A comprehensive investigation of the wide array of genetic factors that determine a disease will undoubtedly improve the current missing heritability conundrum that characterizes most complex human diseases and that limits our current application of genetics in medicine. In this PhD Thesis, utilizing NGS and DNA microarray technologies, I describe my research efforts aimed at elucidating the genetic contribution

underlying complex diseases and the implications of genetics in clinical medicine. I investigated how rare and common genetic variants associate with "lone" atrial fibrillation patients (Chapters 2 and 3); assessed the pathogenicity of a novel variant and its impact on a genetic diagnosis (Chapter 4); and identified a subgroup at a higher risk for pancreatitis in a well-genotyped and phenotype cohort of familial partial lipodystrophy patients (Chapter 5). By better understanding the genetic architecture of "lone" atrial fibrillation, we now have additional evidence that atrial cardiomyopathy is a genetic sub-phenotype and that there is a significant contribution of polygenic factors increasing the disease susceptibility. Further, we have demonstrated the value of thoroughly assessing novel variants for providing the best care possible and the continuous role of genetics in elucidating rare diseases like familial partial lipodystrophy. As described multiple times in this Thesis, the comprehensive assessment of genetics factors that contribute to a disease will advance our understanding of disease etiology and by extension advance medical care from improved screening, to confirming diagnosis, and developing novel therapies, the benefits are potentially immense. But we also keep in mind the constant need for ongoing vigilance and caution when interpreting and translating powerful and illuminating genetic information.

6.6. References

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Appendices

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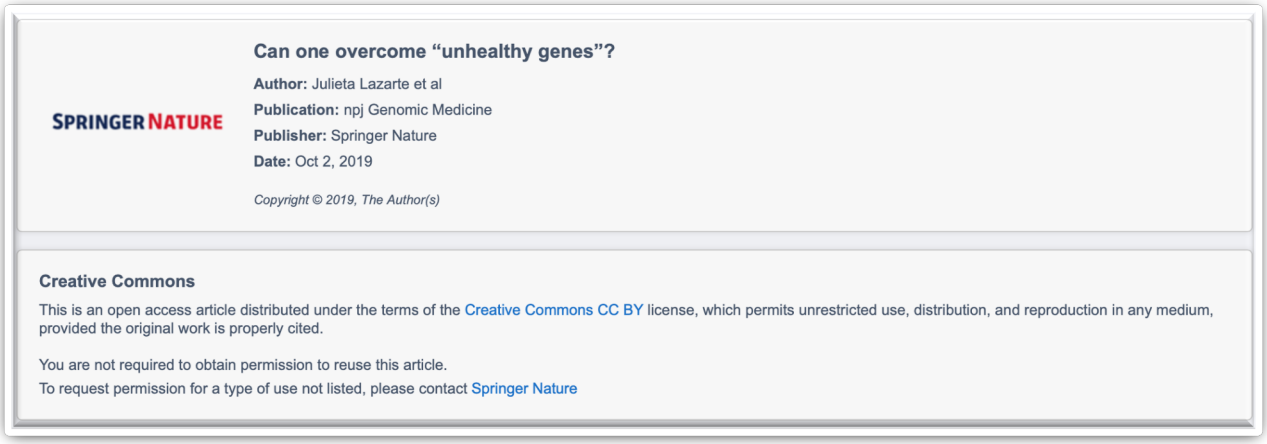
Lazarte, J., Hegele R.A. (2019) "Can one overcome “unhealthy genes”?. *npj Genom Med*. 4: 24. (PMID: 31602315)



SPRINGER NATURE

Can one overcome “unhealthy genes”?

Author: Julieta Lazarte et al
Publication: npj Genomic Medicine
Publisher: Springer Nature
Date: Oct 2, 2019

Lazarte, J., and Hegele, R.A. (2020). DNA sequencing in familial hypercholesterolaemia: the next generation. *Eur J Prev Cardiol*. Advance online publication. (PMID: 33623969)

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Lazarte, J., Hegele R.A. (2020) Can genetic testing help in the management of dyslipidaemias? *Curr Opin Lipidol*. 31(4): 187-193. (PMID: 32520779)

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Expected presentation date	Jul 2021
Portions	Section: SUGGESTED INDICATIONS FOR GENETIC TESTING Paragraph 2
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Lazarte, J., and Hegele, R.A. (2021). Editorial comment: hazards of interpreting genetic reports. *Curr Opin Lipidol* 32, 81-82. (PMID: 33606402)

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Evaluating Polygenic Risk Scores in "Lone" Atrial Fibrillation

Author:
Julieta Lazarte,Jacqueline S. Dron,Adam D. McIntyre,Allan C. Skanes,Lorne J. Gula,Anthony S. Tang,Rafik Tadros,Zachary W. Laksman,Robert A. Hegele,Jason D. Roberts

Publication: CJC Open

Publisher: Elsevier

Date: Available online 6 February 2021

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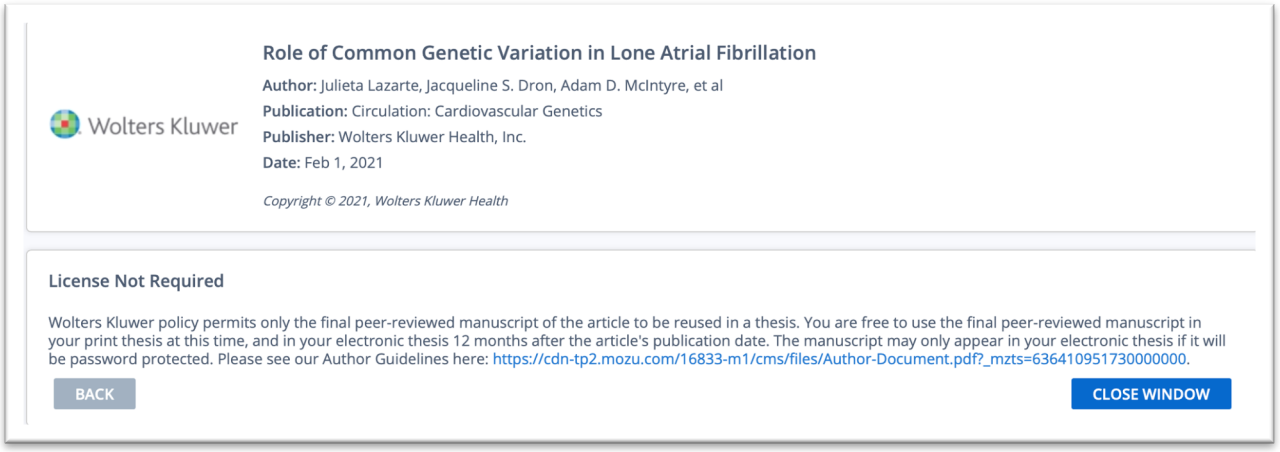

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Role of Common Genetic Variation in Lone Atrial Fibrillation

Author: Julieta Lazarte, Jacqueline S. Dron, Adam D. McIntyre, et al
Publication: Circulation: Cardiovascular Genetics
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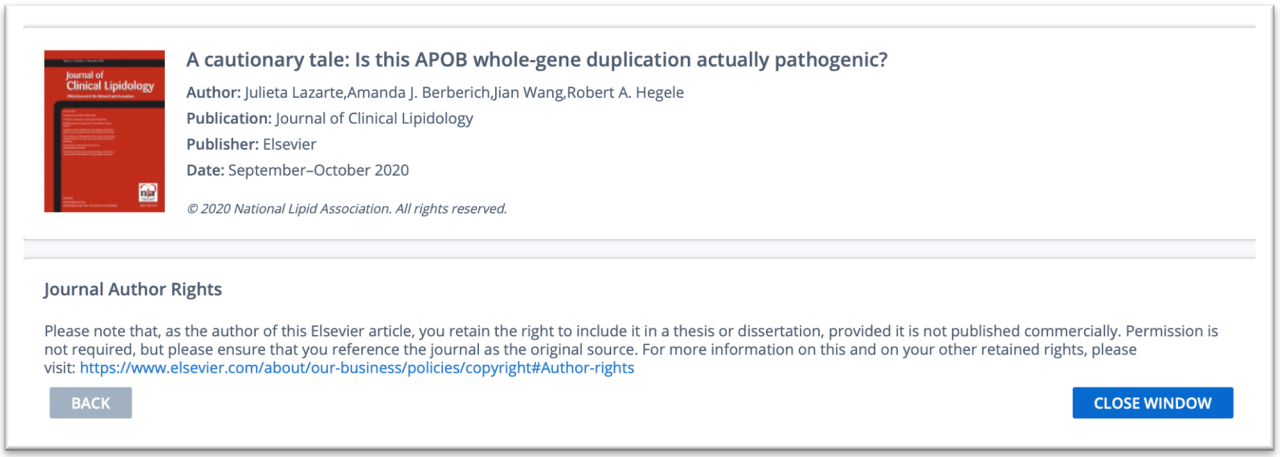

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Author: Julieta Lazarte,Amanda J. Berberich,Jian Wang,Robert A. Hegele
Publication: Journal of Clinical Lipidology
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Appendix B. University of Western Ontario - Ethics Approval 1

Western Research

Date: 3 January 2020

To: Jason Roberts

Project ID: 107249

Study Title: The Genetics of Atrial Fibrillation

Application Type: Continuing Ethics Review (CER) Form

Review Type: Delegated

REB Meeting Date: 14/Jan/2020

Date Approval Issued: 03/Jan/2020

REB Approval Expiry Date: 29/Jan/2021

Dear Jason Roberts,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

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Sincerely,

Daniel Wyzynski, Research Ethics Coordinator, on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Appendix C. University of Western Ontario - Ethics Approval 2

Western Research

Date: 28 January 2021

To: Dr. Robert Hegele

Project ID: 0379

Study Title: Candidate gene sequencing, genetic and genomic analysis for identification of new genetic determinants of intermediate traits of atherosclerosis, dyslipidemia, diabetes, obesity, hypertension, lipodystrophy and other rare metabolic or cardiovascular disorders in the human population. 07920E

Reference Number/ID: Not applicable

Application Type: HSREB Amendment Form

Review Type: Delegated

Full Board Reporting Date: 09/Feb/2021

Date Approval Issued: 28/Jan/2021 15:26

REB Approval Expiry Date: 03/Nov/2021

Dear Dr. Robert Hegele ,

The Western University Health Sciences Research Ethics Board (HSREB) has reviewed and approved the WREM application form for the amendment, as of the date noted above.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
18 Jan 2021, REB #379, Delegated HSREB Protcol Form,addition of study personnel to protocol, for submission	Other Materials	18/Jan/2021	18Jan 2021

Documents Acknowledged:

Document Name	Document Type	Document Date	Document Version
18 Jan 2021, Summary Letter of Amendment to REB 379, Addition of Study Team Members	Summary of Changes	18/Jan/2021	18 Jan 2021
18 Jan 2021, REB #379, Delegated HSREB Protcol Form,addition of study personnel to protocol, tracked changes	Summary of Changes	18/Jan/2021	18 Jan 2021

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Ms. Nicola Geoghegan-Morphet , Ethics Officer on behalf of Dr. Philip Jones, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Page 1 of 1

Curriculum Vitae
Julieta Lazarte, MSc.

Education

2016/09 – 2023/04	MD/PhD Candidate, Schulich School of Medicine and Dentistry PhD Supervisor: Dr. Hegele
2013/09 – 2015/11	MSc, Institute of Medical Science, University of Toronto. Supervisors: Dr. Rao & Dr. Delgado.
2013/09 – 2015/11	Certificate in Cardiovascular Sciences, The Collaborative Program in Cardiovascular Sciences, University of Toronto
2008/09 – 2012/06	HBSc, Life Sciences, University of Toronto.

Honours and career awards

2020/09 – 2021/09	Cobban Student Award in Heart and Stroke Research, $1,500 Schulich School of Medicine and Dentistry, University of Western Ontario
2020/05 – 2023/05	Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award, $30,000 Canadian Institutes of Health Research
2019/10 – 2019/10	CANet HQP Association for Trainees Travel Award, $1,500 Cardiac Arrhythmia Network of Canada
2019/10 – 2019/10	Abstract selected to compete at Trainee Research Award competition: Clinical Science category Canadian Cardiovascular Society
2019/09 – 2020/09	Cobban Student Award in Heart and Stroke Research, $1,500 Schulich School of Medicine and Dentistry, University of Western Ontario
2019/05 – 2020/05	Ontario Graduate Scholarship, $15,000 Government of Ontario and Ontario Universities
2018/09 – 2019/94	Schulich Travel Award, $1,500

	Schulich School of Medicine and Dentistry, University of Western Ontario
2018/04 – 2018/04	Abstract Selected to Compete at Philip K. Caves Award International Society for Heart and Lung Transplantation
2018/04 – 2018/04	ISHLT Travel Grant, $1,000 International Society for Heart and Lung Transplantation
2017/09 – 2018/04	Jack Banham Hargreaves/Jessie Louisa Florence Hargreaves MD Award, $10,000 Schulich School of Medicine, Western University
2016/09 – 2017/04	Jack Banham Hargreaves/Jessie Louisa Florence Hargreaves MD Award, $10,000 Schulich School of Medicine, Western University
2013/09 – 2015/09	Runner-up - Category: Trainee Research Award, $250 Canadian Cardiovascular Society
2015/03 – 2015/09	SGS Conference Grant, $1,000 University of Toronto
2011/09 – 2012/06	Dean’s Honour List 2012 University of Toronto
2009/09 – 2010/06	Dean’s Honour List 2010 University of Toronto
2007/09 – 2008/06	Queen Elizabeth II Aiming for the Top Scholarship 2008, $2,971 Government of Ontario

Employment

2015/09 – 2016/09	Research Assistant, Ted Rogers Centre for Heart Research Supervisor: Dr. Delgado/ Dr. Juvet
2011/06 – 2013/11	Research Assistant, Centre for Sleep and Chronobiology, Toronto Supervisor: Dr. I. Ferreira.
2012/09 – 2013/09	Research Assistant, Microbiology, Mount Sinai Hospital, Toronto Supervisor: Dr. P. Lam
2011/06 – 2012/06	Lab Assistant, University of Toronto

	Supervisor: Dr. B. Stewart
2010/09 – 2011/04	Work-study, University of Toronto
	Supervisor: Dr. M. Sokolowski

Teaching experience

2020/05 – 2020/08	Graduate Teaching Assistant, 2280A Biochemistry and Molecular Biology
	Department of Biochemistry, Western University
2019/05 – 2019/08	Graduate Teaching Assistant, 2280A Biochemistry and Molecular Biology
	Department of Biochemistry, Western University

Mentoring experience

1. Undergraduate Thesis Students, Western University [1]
2. Undergraduate Medical Students, Western University [4]

Community involvement

2020/01 – 2020/12	Mentorship Chair
	Clinician Investigator Trainee Association of Canada (CITAC)
2017/09 – Present	Director, Mentorship Committee
	Schulich School of Medicine, Western University
2017/09 – Present	Club Exec, MD/PhD Seminars
	Schulich School of Medicine, Western University
2017/09 – Present	Club Exec, C.P.R Seminar Series
	Schulich School of Medicine, Western University
2019/09 – 2019/10	Volunteer
	The Green Party of Canada
2019/03 – 2019/03	Volunteer - Discovery Robarts Community Presentation Series
	Robarts Research Institute
2019/01 – 2020/01	Annual General Meeting Co-Chair
	Clinician Investigator Trainee Association of Canada (CITAC)

2017/09 – Present	Director, Mentorship Committee Schulich School of Medicine, Western University
2017/09 – Present	Club Exec, MD/PhD Seminars Schulich School of Medicine, Western University
2017/09 – Present	Club Exec, C.P.R Seminar Series Schulich School of Medicine, Western University
2017/09 – 2018/09	Course Instructor, Medical Student Longitudinal Research Experience Schulich School of Medicine, Western University
2017/09 – 2018/09	Club Exec, Translational Research Club Schulich School of Medicine, Western University
2017/01 – 2017/04	Team member, Schulich Field Hockey
2016/09 – 2017/01	Team member, Schulich Ultimate Frisbee
2015/05 – 2016/09	Director, Furniture for Hope Organization Brampton, ON
2015/01 – 2016/09	Co-Director, IMS Mentorship Program Institute of Medical Science, University of Toronto
2014/10 – 2015/03	Team member, IMSSA Volleyball team
2014/09 – 2015/09	Committee member – IMS Belonging Committee Institute of Medical Science, University of Toronto
2014/09 – 2015/09	Committee member– IMS Recruitment Committee Institute of Medical Science, University of Toronto
2014/03 – 2014/04	Committee member, CSCP Student Research Day Cardiovascular Sciences Collaborative Program Student Association, University of Toronto
2014/01 – 2016/04	Volunteer, HeartLinks Group University Health Network, Toronto
2014/01 – 2016/09	Team member, Team Transplant Dragon Boat Team
2013/09 – 2015/09	MaRS Site Director, IMS Student Association Institute of Medical Science, University of Toronto

2013/09 – 2014/09	Vice President, CSCP Student Association Cardiovascular Sciences Collaborative Program Student Association, University of Toronto
2013/01 – 2014/05	Team Leader, Models of Human Diseases Consortium University of Toronto
2012/10 – 2013/11	Volunteer, Honeychurch Family Life Resource Centre Brampton, ON

Peer-review publications

1. **Lazarte, J**., Kanagalingam, T., and Hegele, R.A. (2021). Lipid effects of sodium-glucose cotransporter 2 inhibitors. *Curr Opin Lipidol* 32, 183-190. (PMID: 33870930)

2. **Lazarte, J**., and Hegele, R.A. (2021). Editorial comment: hazards of interpreting genetic reports. *Curr Opin Lipidol* 32, 81-82. (PMID: 33606402)

3. **Lazarte, J**., Dron, J.S., McIntyre, A.D., Skanes, A.C., Gula, L.J., Tang, A.S., Tadros, R., Laksman, Z.W., Hegele, R.A., and Roberts, J.D. (2021). Evaluating Polygenic Risk Scores in "Lone" Atrial Fibrillation. *CJC Open*. 3(6), 751–757.

4. **Lazarte, J**., Laksman, Z.W., Wang, J., Robinson, J.F., Dron, J.S., Leach, E., Liew, J., McIntyre, A.D., Skanes, A.C., Gula, L.J., et al. (2021). Enrichment of loss-of-function and copy number variants in ventricular cardiomyopathy genes in 'lone' atrial fibrillation. *Europace*. Advance online publication. (PMID: 33682005)

5. **Lazarte, J.,** Dron, J.S., McIntyre, A.D., Skanes, A.C., Gula, L.J., Tang, A.S., Tadros, R., Laksman, Z.W., Hegele, R.A., and Roberts, J.D. (2021). Role of Common Genetic Variation in Lone Atrial Fibrillation. *Circ Genom Precis Med.* Advance online publication. (PMID: 33517663)

6. Sun, B., Yao, J., Ni, M., Wei, J., Zhong, X., Guo, W., Zhang, L., Wang, R., Belke, D., Chen, Y.X., Lieve, K. V. V., Broendberg, A. K. , Roston, T. M., Blankoff, I., Kammeraad, J. A., von Alvensleben, J. C., **Lazarte, J.**, Vallmitjana, A., Bohne, L. J., Rose, R. A., Benitez, R., Hove-Madsen, L., Napolitano, C., Hegele, R. A., Fill, M., Sanatani, S., Wilde, A. A. M., Roberts, J. D., Priori, S. G., Jensen, H. K., Chen, S. R. W. (2021). Cardiac ryanodine receptor calcium release deficiency syndrome. *Sci Transl Med 13.* Advance online publication. (PMID: 33536282)

7. **Lazarte, J**., and Hegele, R.A. (2020). DNA sequencing in familial hypercholesterolaemia: the next generation. *Eur J Prev Cardiol*. Advance online publication. (PMID: 33623969)

8. Castanov, V., Zhou, T. E., Balestrini, C. S., Lefebvre, C., Cook, E. K., Whittaker, H. T., Macklin, J., Briard, J. N., **Lazarte, J.**, Trinder, M., Ware, M. A., Hu, S., Pietrobon, A., Marvasti, T. B., & Sivakami, M. (2020). Overview of the Canadian Clinician Investigator Trainees' research presented at the 2019 CSCI-CITAC Joint Meeting. *Clinical and investigative medicine. Medecine clinique et experimentale*, *43*(3), E5–E14. (PMID: 32971579)

9. Walsh, R., Lahrouchi, N., Tadros, R., Kyndt, F., Glinge, C., Postema, P. G., Amin, A. S., Nannenberg, E. A., Ware, J. S., Whiffin, N., Mazzarotto, F., Škorić-Milosavljević, D., Krijger, C., Arbelo, E., Babuty, D., Barajas-Martinez, H., Beckmann, B. M., Bézieau, S., Bos, J. M., Breckpot, J., Campuzano, O., Castelletti, S., Celen, C., Clauss, S., Corveleyn, A., Crotti, L., Dagradi, F., de Asmundis, C., Denjoy, I., Dittmann, S., Ellinor, P.T., Ortuño, C.G., Giustetto, C., Gourraud, J.B., Hazeki, D., Horie, M., Ishikawa, T., Itoh, H., Kaneko, Y., Kanters, J.K., Kimoto, H., Kotta, M.C., Krapels, IPC, Kurabayashi M, **Lazarte J.**, Leenhardt A, Loeys BL, Lundin C, Makiyama T, Mansourati, J., Martins, R.P., Mazzanti, A., Mörner, S., Napolitano, C., Ohkubo, K., Papadakis, M., Rudic, B., Molina, M.S., Sacher, F., Sahin, H., Sarquella-Brugada, G., Sebastiano, R., Sharma, S., Sheppard, M.N., Shimamoto, K., Shoemaker, M.B., Stallmeyer, B., Steinfurt, J., Tanaka, Y., Tester, D.J., Usuda, K., van der Zwaag, P.A., Van Dooren, S., Van Laer, L., Winbo, A., Winkel, B.G., Yamagata, K., Zumhagen, S., Volders, P.G.A., Lubitz, S.A., Antzelevitch, C., Platonov, P.G., Odening, K.E., Roden, D.M., Roberts, J.D., Skinner, J.R., Tfelt-Hansen, J., van den Berg, M.P., Olesen, M.S., Lambiase, P.D., Borggrefe, M., Hayashi, K., Rydberg, A., Nakajima, T., Yoshinaga, M., Saenen, JB, Kääb, S., Brugada, P., Robyns, T., Giachino, D.F., Ackerman, M.J., Brugada, R., Brugada, J., Gimeno, J.R., Hasdemir, C., Guicheney, P., Priori, S.G., Schulze-Bahr, E., Makita, N., Schwartz, P.J., Shimizu W, Aiba, T., Schott, J.J., Redon, R., Ohno, S., Probst, V.; Nantes Referral Center for inherited cardiac arrhythmia, Behr, E.R., Barc, J., Bezzina, C.R. (2020). Enhancing rare variant interpretation in inherited arrhythmias through quantitative analysis of consortium disease cohorts and population controls. *Genetics in medicine: official journal of the American College of Medical Genetics*, 10.1038/s41436-020-00946-5. 23(1):47-58. (PMID: 32893267)

10. Kanagalingam, T., **Lazarte, J.**, Wong, D.K.H., Hegele R.A. (2020). Liver injury associated with ezetimibe monotherapy. *CJC Open*. 3(2):195-197. (PMID: 33644733)

11. **Lazarte, J.**, Hegele R.A. (2020). A cautionary tale: Is this APOB whole-gene duplication actually pathogenic? *J Clin Lipidol*. 2020; 14:631-635. (PMID: 32654994)

12. **Lazarte, J.**, Hegele R.A. (2020) Can genetic testing help in the management of dyslipidaemias? *Curr Opin Lipidol*. 31(4): 187-193. (PMID: 32520779)

13. Adamson, M.B., Ribeiro, R.V.P., Yu, F., **Lazarte, J.,** Runeckles, K., Manlhiot, C., Rao, V., Delgado, D.H. (2020) Human Leukocyte Antigen G Donor-Recipient Matching Of The 14-Bp Polymorphism Protects Against Cancer Following Heart Transplant. *J Heart Lung Transplant*. 39(7): 686-694. (PMID: 32317137)

14. **Lazarte, J.**, Hegele R.A. (2020) Pediatric dyslipidemia - beyond familial hypercholesterolemia. *Can J Cardiol*. 36(9):1362-1371. (PMID: 32640212)

15. Adamson, M.B., Di Giovanni, B., Ribeiro, R.V.P., Yu, F., **Lazarte, J.,** Rao, V., Delgado, D.H. (2020) HLA-G +3196 polymorphism as a risk factor for cell mediated rejection following heart transplant. *Hum Immunol*. 81(4): 134–140. (PMID: 31928922)

16. **Lazarte, J.**, Hegele R.A. (2020) Regression of Xanthelasmas With Statin Treatment in a Normolipidemic Patient. *Ann Intern Med*. 172(10): 701-702. (PMID: 32066141)

17. **Lazarte, J.**, Hegele R.A. (2020) Dyslipidemia Management in Adults With Diabetes. *Can J Diabetes*. 44(1): 53–60. (PMID: 31521544)

18. **Lazarte, J.**, Hegele R.A. (2019) Can one overcome "unhealthy genes?. *npj Genom Med*. 4: 24. (PMID: 31602315)

19. **Lazarte, J.**, Ma, J., Martinu, T., Levy, L., Klement, W., White, M., Pelling, J., Guan, Z., Azad, S., Tikkanen, J., Rao, V., Tomlinson, G., Delgado, D., Keshavjee, S., Juvet, S. (2019) Donor HLA-G Single Nucleotide Polymorphisms Predict Post-Lung Transplant Mortality. *European Respiratory Journal*. 54(2):1802126. (PMID: 31164432)

20. **Lazarte, J.**, Hegele R.A. (2019) Cannabis Effects on Lipoproteins. *Curr Opin Lipidol*. 30(2):140-146. (PMID: 30649023)

21. Dron, J.S., **Lazarte, J.**, Hegele R.A. (2019) Recent Highlights of ATVB – Lipoproteins. *Arterioscler Thromb Vasc Biol*. 38(11): e185–e197. (PMID: 30354244)

22. **Lazarte, J.**, Adamson, M.B., Tumiati, L., Delgado, D. (2018) 10-Year Experience with HLA-G in Heart Transplantation. *Hum Immunol*. (PMID: 29859206)

23. **Lazarte, J.**, Goldraich, L., Manlhiot, C., Billia, F., Ross, H., Rao, V., Delgado, D. (2016) Human Leukocyte Antigen-G Single Nucleotide Polymorphism -201 (CC-CC) Donor-Recipient Genotype Matching as A Predictor of Severe Cardiac

Allograft Vasculopathy. *J Heart Lung Transplant*. 2016. Sep; 35(9):1101-7. (PMID: 27287629)

24. **Lazarte, J.**, Goldraich, L., Manlhiot, C., Kozuszko, S., Rao, V., Delgado, D. Human Leukocyte Antigen-G Polymorphisms Association With Cancer Post-Heart Transplantation. *Hum Immunol*. 2016. Sep; 77(9):805-11. (PMID: 26776459)

25. **Lazarte, J.**, Tumiati, L., Rao, V., Delgado, D. New Developments in HLA-G in Cardiac Transplantation. *Hum Immunol*. 2016. Sept; 77(9):740-5. (PMID: 26707934)

26. Grosman-Rimon, L., Jacobs, I., Tumiati, L., McDonald, M.A., Pollock Bar-Ziv, S., Kawajiri, H., **Lazarte, J.**, Ghashghai, A., Shogilev, D., Cherney, D.Z., Rao, V. Longitudinal Assessment of Inflammation in Recipients of CF-LVADs. *Can J Cardiol*. 2015 Mar; 31(3): 348-56. (PMID: 25746024)

Presentations and published abstracts

1. J. Lazarte, J. Wang, J.F. Robinson, J.S. Dron, A.D. McIntyre, H. Cao, Z. Laksman, R.A. Hegele, J.D. Roberts. Rare Loss-of-Function Variant Analysis in Lone Atrial Fibrillation. Canadian Congress of Cardiology, Montreal, QC. (Talk- 2019 Trainee Research Award Competition)

2. M. Adamson, R.V.P. Ribeiro, F. Yu, J. Lazarte, K. Runeckles, C. Manlhiot, V. Rao, D.H. Delgado. HLA-G 14BP Polymorphism Donor/Recipient Matching Protects Against Development Of Post-Transplant Malignancy Following Heart Transplantation. Canadian Congress of Cardiology, Montreal, QC. (Talk- 2019 Trainee Research Award Competition)

3. J. Lazarte, J. Wang, J.F. Robinson, J.S. Dron, A.D. McIntyre, H. Cao, Z. Laksman, R.A. Hegele, J.D. Roberts. Rare Loss-of-Function Variant Analysis in Lone Atrial Fibrillation. American Society of Human Genetics, Houston, TX. (Poster)

4. J. Lazarte, J. Wang, J.F. Robinson, J.S. Dron, A.D. McIntyre, H. Cao, Z. Laksman, R.A. Hegele, J.D. Roberts. Rare Loss-of-Function Variant Analysis in Lone Atrial Fibrillation. 8th Annual Canadian Human and Statistical Genetics Meeting and GE3LS HSPR Conference, Montebello, QC. (Talk)

5. J. Lazarte, J. Wang, J.F. Robinson, J.S. Dron, A.D. McIntyre, H. Cao, Z. Laksman, R.A. Hegele, J.D. Roberts. Rare Loss-of-Function Variant Analysis in Lone Atrial Fibrillation. London Health Research Day 2019, London, ON. (Poster)

6. D.C. Birriel, A. Ulahannan, J. Ma, J. Lazarte, T. Martinu, L. Singer, V. Rao, S. Keshavjee, D. Delgado, J. Tikkanen, S. Juvet. Recipient HLA-G Single Nucleotide Polymorphisms Predict Post-Lung Development of Donor Specific Antibodies. International Society of Heart and Lung Transplant 2019, Orlando, FL. (Talk)

7. M.B. Adamson, R.V. Ribeiro, F. Yu, J. Lazarte, K. Runeckles, C. Manlhiot, V. Rao, D. Delgado. HLA-G 14BP Polymorphism Matching Protects against Development of Post-Transplant Malignancy Following Heart Transplantation. International Society of Heart and Lung Transplant 2019, Orlando, FL. (Talk)

8. J. Lazarte, J. Ma, T. Martinu, L. Levy, W. Klement, M. White, J. Pelling, Z. Guan, S. Azad, J. Tikkanen, V. Rao, G. Tomlinson, D. Delgado, S. Keshavjee, S. Juvet. Donor HLA-G Single Nucleotide Polymorphisms Predict Post-lung Transplant Mortality. International Society of Heart and Lung Transplant 2018, Nice, France (Talk- 2018 Philip K. Caves Award Competition).

9. J. Lazarte, L. Goldraich, C. Manlhiot, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, V. Rao, D. Delgado. Human Leukocyte Antigen-G Polymorphisms Association with Post Heart Transplant Donor Specific Antibodies. International Society of Heart and Lung Transplant 2016, Washington, DC. (Talk)

10. J. Lazarte, L. Goldraich, C. Manlhiot, V. Rao, D. Delgado. Post Heart Transplant Cancer Diagnosis Association with Human Leukocyte Antigen-G Genotypes. International Society of Heart and Lung Transplant 2016, Washington, DC. (Poster)

11. J. Lazarte, L. Goldraich, C. Manlhiot, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, V. Rao, D. Delgado. Human Leukocyte Antigen-G Polymorphisms Association to Post Heart Transplant Donor Specific Antibodies. American Heart Association 2015, Orlando, FL. (Poster)

12. J. Lazarte, L. Goldraich, C. Manlhiot, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, V. Rao, D. Delgado. Human Leukocyte Antigen-G Polymorphisms Association to Post Heart Transplant Cardiac Allograft Vasculopathy. American Heart Association 2015, Orlando, FL. (Poster)

13. H. Kawajiri, A. Ghashghai, L. Tumiati, J. Lazarte, L. Grosman-Rimon, V. R Luengas, M. Badiwala, J. Butany, R. Li, F. Billia, V. Rao. Nrf2 Inhibits NF-κB Activation During Ischemia Reperfusion Injury and Suppresses Subsequent Development of Cardiac Allograft Vasculopathy in Murine Heart Transplantation. American Heart Association 2015, Orlando, FL. (Poster)

14. J. Lazarte, L. Goldraich, C. Manlhiot, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, H. Ross, V. Rao, D. Delgado. Human Leukocyte Antigen-G polymorphisms are predictors of post transplant cardiac allograft vasculopathy. Canadian Cardiovascular Society Congress 2015, Toronto, Ontario, Canada. (Talk- 2015 Trainee Research Award Winner)

15. H. Kawajiri, L. Goldraich, F. Foroutan, J. Lazarte, A. Ghashghai, L. Grosman-Rimon, W. Stansfield, H. Ross, F. Billia, V. Rao. Outcomes of 100 Continuous-flow Left

Ventricular Assist Devices: The Toronto General Hospital Experience. Canadian Cardiovascular Society Congress 2015, Toronto, Ontario, Canada. (Poster)

16. H. Kawajiri, L. Goldraich, F. Foroutan, J. Lazarte, A. Ghashghai, L. Grosman-Rimon, W. Stansfield, M. Badiwala, R. J. Cusimano, T. Yau, H. Ross, F. Billia, V. Rao. Requiring high pump speed may be a risk predictor for long-term mortality after CF-VAD insertion. Canadian Cardiovascular Society Congress 2015, Toronto, Ontario, Canada. (Poster)

17. H. Kawajiri, A. Ghashghai, J. Lazarte, L. Grosman-Rimon, L. Tumiati, M. Badiwala, J. Butany, R. Li, F. Billia, V. Rao. Nrf2 protects against ischemia reperfusion injury via inhibition of NF-κB activation and suppresses subsequent development of cardiac allograft vasculopathy in murine heart transplantation. Canadian Cardiovascular Society Congress 2015, Toronto, Ontario, Canada. (Poster)

18. J. Lazarte, L. Goldraich, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, V. Rao, D. Delgado. Human Leukocyte Antigen-G Polymorphisms as Predictors of Cardiac Allograft Vasculopathy. 7th International Conference on HLA-G 2015, Paris, France. (Talk)

19. J. Lazarte, L. Tumiati, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, V. Rao, D. Delgado. Association of Soluble HLA-G Levels and Immunosuppressive Treatment. 7th International Conference on HLA-G 2015, Paris, France. (Poster)

20. J. Lazarte, L. Goldraich, H. Kawajiri, A. Ghashghai, L. Grosman-Rimon, V. Rao, D. Delgado. Human Leukocyte Antigen-G Polymorphisms as Predictors of Early Cardiac Allograft Vasculopathy. International Society of Heart and Lung Transplant 2015, Nice, France. (Talk)

21. H. Kawajiri, L. Tumiati, A. Ghashghai, J. Lazarte, L. Grosman- Rimon, F. Billia, R. Li, M. Badiwala, V. Rao. Nrf2 Inhibits NF-kB Activation and Attenuates Ischemia-Reperfusion Injury in Heart Transplantation. International Society of Heart and Lung Transplant 2015, Nice, France. (Poster)

22. Ghashghai, H Kawajiri, L.C. Tumiati, J. Lazarte, S. Heximer, S.S. Bolz, T. Lindsay, V. Rao. Tert-Butylhydroquinone Rescues Cyclosporine-A Mediated Impairment in Vascular Function via Augmenting Phosphorylation of the Transcription Factor Nrf2. Canadian Cardiovascular Society Congress 2014, Vancouver, British Columbia, Canada. (Poster)

23. H. Kawajiri, A. Ghashghai, L. Tumiati, J. Lazarte, L. Grossman-Rimon, F. Billia, R. Li, V. Rao. Protective Effect of Nrf2 on Ischemia-reperfusion Injury in Murine Heart Transplantation. American Heart Association 2014, Orlando, FL. (Poster)

24. J. Lazarte, I. Ferreira. The effects of OSA in patients with COPD and asthma. 6th Conference of the Candian Sleep Society 2014, Halifax, Canada. (Poster)