RCPAQAP
The Royal College of Pathologists of Australasia
Quality Assurance Programs

RCPAQAP Molecular Genetics

Quality Use of Pathology Program

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Title

The development of a quality assurance pilot program for proficiency testing in cardiovascular disease

Final Report

A project funded under the Australian Government's Quality Use of Pathology Program

Contents

List of abbreviations:

CVD	Cardiovascular disease
DIN	DNA Integrity Number
EQA	External quality assurance
FH	Familial hypercholesterolemia
LDL	Low-density lipoproteins
NGS	Next generation sequencing
RCPAQAP	Royal College of Pathologists of Australasia Quality Assurance Programs
RPAH	Royal Prince Alfred Hospital
SNP	Single nucleotide polymorphism

1. Executive Summary

Cardiovascular disease (CVD) refers to a spectrum of diseases and conditions involving the heart and blood vessels. The main types of CVD in Australia are coronary heart disease, stroke, and heart failure/cardiomyopathy. One of the most common forms of CVD is familial hypercholesterolemia (FH) and is estimated to occur in 1/200 people (Benn et al., 2012; Sjouke et al., 2014). FH is an inherited disorder in which the ability of the liver to remove low-density lipoproteins (LDL) (commonly known as bad cholesterol) from the blood is impaired and results in high levels of blood cholesterol. Early diagnosis and treatment of FH are essential. Otherwise, cholesterol will build up in the arteries and increase an individual's risk of premature coronary heart disease and early death. The initial diagnosis process involves the genetic testing of three essential FH-associated genes (*APOB, LDLR, PCSK9*). However, accumulating evidence from global laboratories using next-generation sequencing platforms have indicated that there are DNA variations in other genes that are in addition to the three clinically relevant FH-associated gene mutations (Johansen et al., 2014; Iacocca et al., 2017; Dron et al., 2020). Also, patients with the same DNA gene variation in the FH-associated genes can express different forms of FH (i.e., have differences in phenotype) (Di Taranto et al., 2019). As such, the diagnostic evaluation of a small number of DNA gene variants alone may not be sufficient to explain the full disease process (Bertolini et al., 2004; Gaspar and Gaspar, 2019).

The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP), in association with the RCPAQAP Molecular Genetics Advisory Committee, needs to keep up-to-date with current diagnostic strategies so that EQA programs can be developed/modified to meet clinical needs. As such, Commonwealth funding is essential since this allows the RCPAQAP to develop new EQA programs that reflect discoveries relating to disease progression so that proficiency testing can be performed on laboratories adopting to new testing strategies.

The purpose of this study was to therefore assess a small number of facilities performing FH genetic testing so that the RCPAQAP could capture information relating to the technique and number of genes being tested and to proficiency test the clinical information provided by each facility. As such, the underlying principle for this pilot study was to identify in the short-term critical areas of problems associated with broadening the number of genes being sequenced for the early detection of FH. The data from this pilot can then be used to formulate a medium- to long-term strategy for the development of an external quality assurance (EQA) program for the genetic testing of numerous genes that become associated with FH.

There were three objectives of this study, (i) to help raise awareness of the total number of DNA variants that exist in FH genotypes, (ii) to identify a laboratory consensus set of genes that are under investigation by FH testing laboratories, and (iii) to assess the ability of FH testing laboratories to identify and interpret FH associated gene variants correctly. The methodology used for this RCPAQAP trial study specifically included, (i) performing whole genome sequencing on three FH patient DNA samples, (ii) distribution of these samples to five DNA testing facilities, and (iii) analysis of all laboratory returned data. Each EQA reference sample contained an FH case scenario, and laboratories were requested to test for DNA variants in the three core clinically accepted FH associated genes (*APOB, LDLR, PCSK9*). However, not all FH patients have DNA variants in the three core genes and laboratories are starting to extend DNA analysis to encompass other genes likely to be clinically relevant to FH. For proficiency testing any or all genes tested (in addition to the core genes), the RCPAQAP needed to perform whole genome sequencing on each of the three DNA reference samples. This allowed for the identification of every DNA variant in each sample. By using this strategy, it was then possible to proficiency test laboratories performing DNA analysis on any DNA region that was of clinical interest to FH. In addition, laboratories were also requested to clinically interpret all the DNA data generated.

The whole genome sequencing data was intriguing as it identified ~4.8 million DNA mutations in each of the three patient reference testing samples. This represents a sequence variant complexity that was previously unappreciated for FH development and progression. However, FH is part of the CVD spectrum of diseases and all represent complex disorders. The whole genome sequence data generated from this study further highlight this complexity at the DNA level. It nonetheless remains unknown as to how many of these DNA mutations are pathogenic for the FH disease. As such, these data provide a huge challenge to clinicians as interpretation of these variants remains impossible at this time. Further research will need to be performed in the future to identify such clinically relevant gene variant associations.

The data from this study identified that six DNA variants were consistently analysed. These variants, therefore, represented the consensus data that were used for assessing each laboratory. In total, only two laboratories were concordant for both correct DNA identification and clinical interpretation. The other three laboratories had differing styles for reporting data which was problematic and impacted on their assessment. For example, one laboratory's procedure is to not report DNA variants (in the

key FH associated genes) if these are outside of the variants of current clinical interest. Although this is acceptable, these variants are equally likely to impact on protein function and clinical explanations should be still be provided and references cited as these could still impact on patient management. One laboratory provided clinical interpretations that were not relevant to the EQA case scenarios, and one laboratory refused to test a sample in the absence of family genotype data. These reporting styles could have detrimental impacts for the clinical management of patients since key data would be missing or wrong. However, the identification of such issues is essential information to relay back to laboratories since it allows for the laboratory to troubleshoot their testing and reporting pipeline so that data analysis and performance can be improved. These data further demonstrate the importance of participation in an EQA program.

In the short term, the data from this EQA pilot can help laboratories modify their reporting styles so that a consistent level of clinical reporting is reached. For the intermediate term, the consistency of each laboratory report can be monitored, and improvements suggested for areas found to be of clinical concern. For the long term, it is hoped that the whole genome sequence data will help raise awareness into the complexity of FH DNA diagnostics that will guide further work into establishing other DNA regions that are likely to be of clinical relevance for future diagnoses. As such, the RCPAQAP will play an essential role in not only proficiency testing currently accepted gene regions for diagnostic analysis but also in helping identify other relevant regions for future testing.

The current Commonwealth funded project has allowed the RCPAQAP to perform whole genome sequencing to identify all DNA variants in three reference DNA samples isolated from three FH patients. Laboratory testing issues were encountered and areas for improvements identified. Using this EQA strategy in the future will enable laboratories to perform DNA sequencing on DNA regions in addition to the traditional core FH testing regions for proficiency testing. As such, the RCPAQAP are in a position, to offer a full EQA program for the proficiency testing of any gene region relating to FH diagnostics. However, clinical guidelines will also need to be updated as new DNA regions of interest get added to FH diagnostic testing. The proficiency testing approach used for this program will allow the RCPAQAP to develop a long-term strategy for building other EQA programs for all other cardiovascular spectrum diseases.

2. Aims and Outcome of the Study

(*What is the aim/purpose of the project?*)

2.1 Aims

The original aims of this study were to (1) develop an EQA program for cardiovascular disease testing; and (2) develop a scoring system designed to assess the ability of laboratories to correctly identify and clinically interpret specific DNA gene variants. Critically, cardiovascular disease represents at least 68 different subtypes with familial hypercholesterolemia being the most common. The development of an EQA that encompasses whole genome sequencing of the reference testing material for just one cardiovascular disease was therefore deemed applicable as the methodology used would be appropriate to all other cardiovascular diseases. New follow-on EQA programs can then be modified in the future to suit each specific cardiovascular disease that gain more clinical interest for DNA testing.

The RCPAQAP, in association with all discipline Advisory Committees, has since updated its proficiency testing procedure and has determined that an EQA scoring system applicable to all disciplines was not feasible. This is because the RCPAQAP wants to harmonise all discipline reports so that they are reflective of a common RCPAQAP reporting style. As such, a single scoring system could not accommodate all disciplines due to their different measuring strategies and testing protocols. Therefore, this part of our initial aim had to be removed and modified as it would not be reflective of the updated RCPAQAP proficiency testing practise. Proficiency testing was therefore based on the laboratory consensus data.

The modified aims were therefore to develop an EQA program that encompasses genetic testing of the whole genome, which can be additionally adapted to cover the entire spectrum of cardiovascular diseases. To do this, it was first necessary to target one cardiovascular disease so that DNA testing and clinical interpretation issues can be identified and solved. As such, this study focused on the most common cardiovascular disease (familial hypercholesterolemia). There were three key aims of this study, (i) to help raise awareness of the total number of DNA variants that exist in FH genotypes, (ii) to identify a laboratory consensus set of genes that are under investigation by FH testing laboratories, and (iii) to assess the ability of FH testing laboratories to identify and interpret FH associated gene variants correctly.

2.2 Outcome

The whole genome sequence data from the study identified that at least 4.8 million mutations exist in FH patient DNA. However, it remains unknown as to how many of these mutations impact on FH, but these data nonetheless highlight an underlying disease complexity that was previously unappreciated. Current genetic testing tends to focus on three core genes (*APOB, LDLR, PCSK9*) for FH diagnostic analysis. However, not all patients have DNA mutations in these three core genes which indicates that variants in other genes are likely to be involved with FH development. This is further reflected by the whole genome sequence data.

In total, the laboratory data from this EQA pilot study identified a consensus of six different DNA variants in four genes (*APOB, LDLR, HFE, TGFB3*). These were therefore used for proficiency testing each laboratory. Two laboratories were concordant for the genotypes tested and for clinical interpretation. Three laboratories were inconsistent for their reporting of data and need to improve on their reporting style for efficient FH testing. This study therefore achieved the three objectives for raising awareness of the total number of DNA mutations in FH samples, identifying consensus DNA variants, and in assessing laboratories for FH testing performance.

3. Background

(*What is the overview of the project and its importance for disease diagnostics?*)

Identifying abnormal cellular function in response to genetic DNA variation, microbial infection, or as a consequence of another underlying disease pathology is key to understanding the human disease process. Inherited disease, complex disease, and age-related diseases all require an assessment of patient DNA so that DNA sequence alterations in key genes can be identified and a disease-causing status assigned. However, assigning a specific gene variant as disease-causing can be difficult since the human genome is estimated to contain approximately 21,000 protein encoding genes (Willyard, 2018), and data from the 1000 genomes project identified that 1000s of DNA variants exist in these genes in normal healthy individuals (1000 genomes project consortium, 2015). Importantly, some of these same DNA variants are also present in disease pathologies, which makes interpreting specific DNA gene mutations as disease-causing challenging to clarify. An additional complexity is that gene mutations previously characterised as disease-causing have also been found in centenarians who are disease-free (Freudenberg-Hua et al., 2014; Zhang et al., 2019). Given this level of complexity, key organisations are now aiming to sequence the entire genome in disease individuals to fully identify multiple DNA gene variants associated with specific genetic disease. In particular, Genomics England is currently performing a 100,000 genomes study designed to map the entire human genome in 85,000 individuals (and their family members) with rare disease and individuals with cancer (https://www.genomicsengland.co.uk/about-genomics-england/the-100000-genomes-project/). Completion of the 100,000 genomes project is expected to enhance our genetic understanding of rare diseases which will provide a foundation for future developments of diagnostic, therapeutic, and preventive medical applications in other complex and age-related diseases. Clinical adoption of whole genome next generation sequencing may occur in the near future as sequencing costs reduce. However, the data will be complex and clinical guidelines on all diseases will need to be updated.

The current study focused on the cardiovascular disorder of FH, which represents 1 of at least 69 different cardiovascular associated diseases (Australian Institute of Health and Welfare, 2011). Cardiovascular disease is classified as a leading cause of death and disease burden in Australia (Australian Burden of Disease Study, 2011). As a consequence, multiple laboratories are now providing a service for genetic testing of different cardiovascular-associated diseases. However, this is of growing concern since EQA programs are unavailable for most of the cardiovascular disorders, and no EQA program offers proficiency testing for all DNA regions characterised using the technique

of whole genome DNA sequencing. The RCPAQAP strategy was to perform whole genome next generation sequencing on three patient FH DNA samples to identify all DNA variations. These samples served as the reference testing material for the program. The reference samples were then distributed to five participating laboratories to perform their routine screening for FH diagnostics. Laboratories were further required to comment and interpret all gene variants identified. The development of an FH EQA program that covers the whole genome will allow laboratories to enrol for proficiency testing of any identified DNA region that becomes clinically relevant in the future.

4. Addressing essential needs

(*What need/s will this project address?*)

Cardiovascular disease represents a complex disease. In 2015 and 2016, 89,355 Australian deaths were recorded as a direct consequence of CVD (Australian Bureau of Statistics 2016, 2017). This represents 28% of all total deaths reported in Australia during this time. Currently, there are no EQA programs available for proficiency testing all gene regions in any of the CVD spectrum of disorders. As such, there is an essential need to develop a first CVD EQA program that will encompass the proficiency testing of all DNA regions that are currently clinically relevant. The development of such a program can then be adapted to cover other cardiovascular disorders and to include DNA regions that will become clinically relevant in the future. This is important since more laboratories are adopting next generation DNA sequencing platforms for DNA diagnostics to sequence more significant parts of the human disease genome.

5. Benefits

(*What benefit will the project be to consumers of pathology services?*)

This project will help increase the performance of laboratories performing CVD gene diagnostics since all gene regions that become clinically relevant can be proficiency tested. This will, therefore, directly benefit patients and family members since test results will be produced with high sensitivity and reproducibility, leading to accurate data production and allow the clinician to make better-informed decisions on patient management. Medical practitioners who request genetic tests will further benefit from a higher quality diagnostic service. Clinical geneticists and genetic counselors who counsel patients and family members will have more confidence in the accuracy of the results obtained.

6. Reference samples

(*What were the samples used in the project*)

The reference testing samples used for this project were provided by the Royal Prince Alfred Hospital (RPAH). Genomic DNA was extracted from three consenting FH patients (one male and two female). All three DNA samples were distributed to laboratories for FH diagnostic gene testing.

7. Methods

(*Technical background of the study*)

7.1 Laboratories

A total of five facilities participated in the RCPAQAP familial hypercholesterolemia proficiency testing program. These facilities were based in Australia (three laboratories), South Korea (one laboratory), and Turkey (one laboratory).

7.2 Reference testing of samples

Reference testing of each FH case sample was performed by the RPAH, where next generation sequencing was performed on the three clinically relevant FH-associated genes (*LDLR, APOB, PCSK9*). Laboratory consensus data were additionally used to support the reference data.

7.3 Homogeneity and stability of the reference DNA

Homogeneity and stability testing was performed using a DNA quality testing analyser. DNA was assayed on a DNA TapeStation 4200 (Agilent Technologies) to determine the homogeneity quality and overall stability of the DNA. The TapeStation is a microfluidic platform that assesses the quantity and integrity of genomic DNA in the sizing range of 200 to >60000 DNA base pairs (bp). A software algorithm generates both a virtual gel image and a DNA Integrity Number (DIN) that are representative of whole genomic DNA integrity. The DIN value ranges from 0 (highly degraded and poor-quality DNA) to 10 (highly intact and high-quality DNA).

7.4 Whole genome DNA sequencing

RCPAQAP whole genome DNA sequencing of the three reference testing samples allows laboratories to perform DNA sequencing on additional gene regions that are deemed or putatively deemed to be clinically relevant for FH testing. As such, laboratories could be proficiency tested for more than the current three clinically relevant genes of *LDLR, APOB, PCSK9*.

7.5 Clinical data

Clinical case scenarios were devised by the RPAH and supplied with each FH test sample.

Case 1

70-year-old female. Known always to have had moderately high cholesterol since testing of her cholesterol began 20 years ago. LDL level became more markedly elevated (5.8 mmol/L) after menopause. Commenced on atorvastatin and ezetimibe with a moderate response before the addition of Repatha with a marked reduction in LDL. LDL of 1.6 mmol/L on current lipid-lowering treatment No previous history of stroke or ischaemic heart disease but two paternal uncles with bypasses in their 50s. Examination unremarkable. High lipoprotein (a) level (560 mg/L) and coronary calcium score >95% percentile for age.

Case 2

29-year-old male. Had had cholesterol tested one-month before appointment after father's cholesterol was noted to be markedly elevated post-AMI evaluation. The patient's LDL at that time was 6.9 mmol/L. Commenced on statin treatment (rosuvastatin 40 mg daily) and LDL cholesterol on review one month later on this treatment was 5.1 mmol/L. No other relevant past medical history or medications. No arcus senilis but possible tendon xanthomas noted on examination. No other biochemical abnormalities noted on bloods. No cardiac investigations had been undertaken prior to review.

Case 3

41-year-old female. Brother a known carrier of FH mutation. LDL 6.7 on presentation. Marked tendon xanthomas and arcus senilis. No known cardiac history. Previously subclinical hypothyroidism (now resolved and off treatment). No other relevant medical history. No regular medications. No previous cardiac investigations. Other biochemistry unremarkable. Wishing to have a second child (cholesterol status of first child unknown).

7.6 Proficiency testing

For proficiency testing, laboratories were assessed directly against the reference testing laboratory data and the consensus data for correctly identifying all gene variants. Also, laboratories were assessed for providing a clinical interpretation of their gene variant data. Concordance or not assessed was awarded for correct or not reported analysis, respectively. Discordance was not assigned to any laboratory given that this was a trial EQA study.

8. Results and Discussion

(*Results and understandings from the study*)

8.1 Laboratories

All five testing laboratories returned data for proficiency testing.

8.2 Reference testing of samples

Reference testing of each DNA FH sample was performed by the RPAH using next generation DNA sequencing. The following clinically relevant gene variants were identified and confirmed:

Case 1

Gene: *LDLR*

cDNA variant: c.2479G>A

Protein variant: p.Val827Ile

Case 2

Gene: *LDLR*

cDNA variant: c.1871_1873delTCA

Protein variant: p.Ile624del

Case 3

Gene: *LDLR*

cDNA variant: c.2312-3C>A

Protein variant: p.Ala771_796del

8.3 Homogeneity and stability

The three FH test cases were analysed on a DNA TapeStation. Each DNA sample was homogenous and produced a whole genomic DNA band of ~50,000bp with no DNA fragmentation pattern (Figure 1). The high DIN values confirmed the integrity of each DNA sample. The combination of gel image and DIN confirmed that the reference testing DNA samples were of good quality for the EQA program. The laboratory DNA sequencing consensus data additionally confirmed the stability of each supplied DNA sample.

8.4 Whole genome DNA sequencing

Whole genome sequencing identified a total of 4.8 million DNA variants in each of the FH reference testing samples (Table 1). These data are very complex and provide a perspective on the underlying DNA mechanisms involved with FH. In addition, the data also highlights the challenges faced by clinicians when trying to interpret and make sense of such overwhelming DNA sequence data.

8.5 Proficiency testing data

The number of genes screened, and the number of coding region variants reported by each laboratory are listed in Table 2. A total of six DNA variants were consistently reported, and these served as the laboratory consensus data (Tables 3 – 7). Concordance was awarded for laboratories identifying and reporting on each consensus variant for each case tested. No laboratory was found to be discordant. Laboratories that do not traditionally report on variants that are deemed to be non-pathogenic were classified as "Not assessed" (Table 8). In total, 60% (3/5) of laboratories were concordant for case 1, 100% (5/5) of laboratories were concordant for case 2, and 60% (3/5) of laboratories were concordant for case 3 for all genotyping analyses (Table 8). For clinical interpretations, Laboratory 1 were not assessed since no clinical interpretation of the genotype data were supplied for any of the test cases (Table 8). Laboratory 2 were not evaluated since the provided interpretations were not based on the known FH associated gene variants and were therefore not clinically relevant to the patient case scenario. Laboratories 3 and 4 were fully concordant for each test case. Laboratory 5 does not list or provide interpretations of variants that are deemed to be non-pathogenic (Case 1). In addition, Laboratory 5 would not perform analyses on Case 3 since the referring clinician would not supply the known family variant mentioned in the clinical case scenario (Table 8).

These data highlight different levels of reporting by individual laboratories. This further emphasizes the importance of EQA programs for helping raise awareness of such reporting differences that may have the potential to impact on the appropriate clinical management of patients.

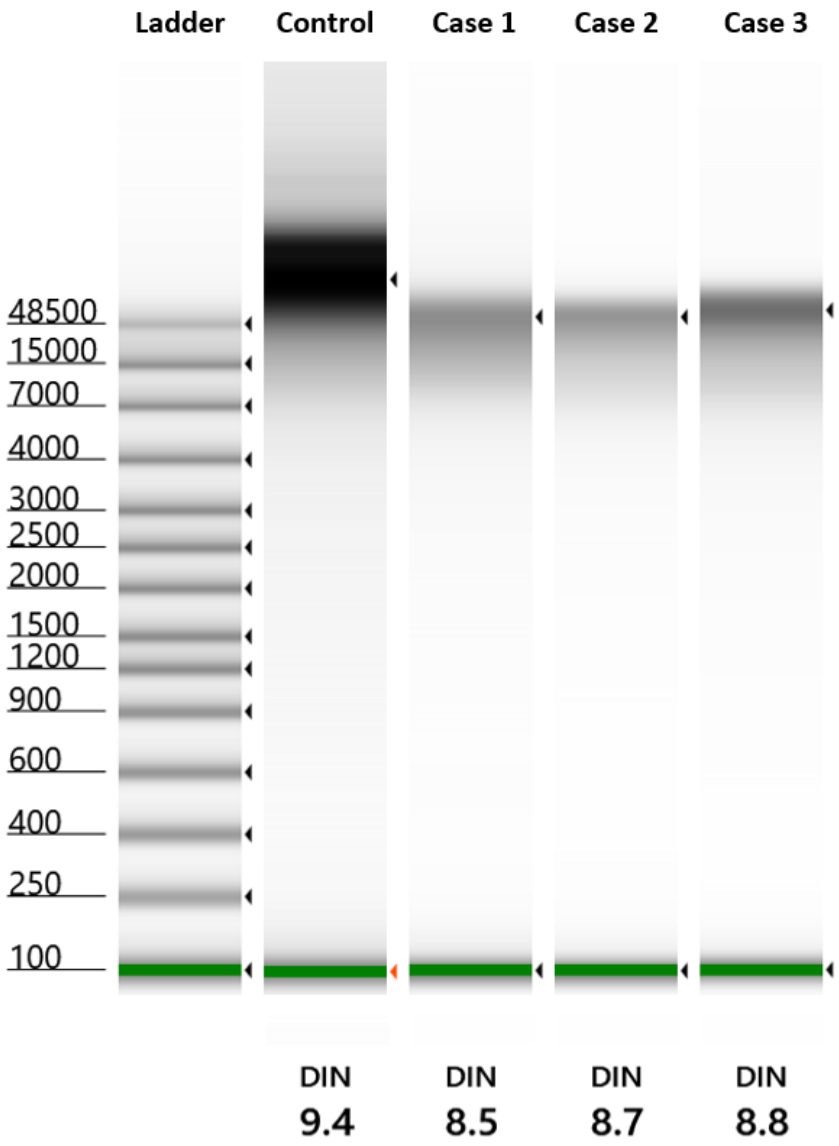

Figure 1. DNA gel image and DNA integrity number (DIN) values of control DNA and FH Cases 1, 2, and 3. High DIN values and lack of DNA fragmentation confirm the stability and quality of DNA. The ladder bands are sized DNA fragments in base pairs (bp). The 100bp fragment is an internal marker of known concentration that is used to calculate the DIN.

Table 1. Whole genome next generation sequencing data on the three reference familial hypercholesterolemia cases.

Case	SNPs	Small insertions	Small deletions	Synonymous variants	Non-synonymous variants	Splicing variants	Stop gained	Stop lost	Frame shift	Total variants
Case 1	3,864,171	457,538	464,599	11,845	11,135	249	96	23	176	4,809,832
Case 2	3,881,123	457,897	465,134	11,979	11,288	263	97	20	157	4,827,958
Case 3	3,841,476	457,259	464,204	11,789	11,053	278	103	24	167	4,786,353

Legend:

SNPs – single nucleotide polymorphisms (a single base change in a DNA region)

Small insertions – a small number of bases that have been inserted into a DNA region

Small deletions – a small number of bases that have been deleted from a DNA region

Synonymous variants – a coding region DNA base change that does not alter an amino acid in a protein sequence

Non-synonymous variants – a coding region DNA base change that alters an amino acid in a protein sequence

Splicing variants – a DNA base change that can cause exon skipping altering a protein sequence

Stop gained – a DNA sequence change that produces a new stop codon signal producing a smaller protein

Stop lost – a DNA sequence change that removes a normal stop codon signal producing a larger protein

Frame shift – a DNA insertion or deletion that alters the normal protein coding sequence to produce a different protein

Table 2. Number of genes screened, and total number of coding region variants reported in the EQA study.

Laboratory	No. genes screened	Total coding region variants reported			Tested genes	Laboratory status
		Case 1	Case 2	Case 3		
Lab 1	~21,000	11679	11825	11625	WHOLE GENOME	Commercial
Lab 2	128	77	74	75	128 genes (see appendix)	Commercial
Lab 3	4	2	1	1	LDLR, APOB, PCSK9, LDLRAP1	Clinical
Lab 4	3	1	1	1	LDLR, APOB, PCSK9	Clinical
Lab 5	8	0	1	Not tested	LDLR, APOB, PCSK9, APOE, LDLRAP1, LIPA, ABCG5, ABCG8	Clinical

Table 3. Laboratory 1 data compared against the reference and consensus data.

Sample	Reference genotype	Consensus genotypes reported	*Lab 1 Reported genotype	Lab 1 Clinical interpretation
Case 1	LDLR c.2479G>A Heterozygous	LDLR c.2479G>A Heterozygous APOB c.4072C>T Heterozygous	LDLR c.2479G>A Heterozygous APOB c.4072C>T Heterozygous	Interpretation not provided
Case 2	LDLR c.1871_1873delTCA Heterozygous	LDLR c.1871_1873delTCA Heterozygous HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	LDLR c.1871_1873delTCA Heterozygous HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	Interpretation not provided
Case 3	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	Interpretation not provided

* Laboratory 1 screened the whole genome. As such, there are too many variants to list, so the variants shown here are only those that match the consensus data.

Table 4. Laboratory 2 data compared against the reference and consensus data.

Sample	Reference genotype	Consensus genotypes reported	Lab 2 Reported genotype	Lab 2 Clinical interpretation
Case 1	LDLR c.2479G>A Heterozygous	LDLR c.2479G>A Heterozygous APOB c.4072C>T Heterozygous	None	No pathogenic/likely pathogenic or of unknown significance variants are detected
Case 2	LDLR c.1871_1873delTCA Heterozygous	LDLR c.1871_1873delTCA Heterozygous HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	No pathogenic/likely pathogenic variants were detected, related to the phenotype investigated. A pathogenic variant in the *HFE* gene and a variant of unknown significance in *TGFB3* were detected, as incidental findings. A heterozygous variant was detected in the HFE gene; c.187C>G or p.(His63Asp). This variant is recorded as pathogenic in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/variation/10/) and LOVD databases (https://grenada.lumc.nl/LSDB_list/lsdbs/ HFE). Mutations in the HFE gene are related to haemochromatosis (https://omim.org/entry/613609). A heterozygous variant was detected in the TGFB3 gene; c.197C>T or p.(Pro66Leu). No entries were found in the databases searched. According to the ACMG criteria PM2 and PP3, this is a rare variant predicted to be damaging from at least 3 in silico analyses (Mutation Taster, POLYPHEN2 and SIFT) and therefore, it is classified as of unknown clinical significance. Mutations in the TGFB3 gene are related to Arrhythmogenic right ventricular dysplasia 1 (https://omim.org/entry/190230).
Case 3	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	NEBL c.535C>T Heterozygous TNNT2	No pathogenic/likely pathogenic variants were detected, related to the phenotype investigated.

			c.571-1G>A Heterozygous	Variants of unknown significance, as incidental findings, were detected in the following genes: *NEBL, TNNT2* A heterozygous variant was detected in the NEBL gene; c.535C>T or p.(Arg179*). This variant is recorded as of uncertain significance in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/47687546/). According to the ACMG criteria PVS1, PM2 and PP3, this is a rare mutation, leading to a stop codon, with pathogenic in silico predictions and therefore classified as pathogenic. In the absence of evidence of haploinsufficiency for NEBL, even though it might be pathogenic, we also classify this mutation as of unknown significance. Mutations in the NEBL gene are related to Endocardial Fibroelastosis and Familial Isolated Dilated Cardiomyopathy (https://www.genecards.org/cgi-bin/carddisp.pl?gene=NEBL). A heterozygous variant was detected in the TNNT2 gene; c.571-1G>A. This variant is recorded as of uncertain significance in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/variation/132940/). According to the ACMG criteria PVS1, PM2 and PP3, this is a rare mutation, expected to disrupt RNA splicing, with pathogenic in silico predictions and therefore classified as pathogenic. Although it is expected to disrupt RNA splicing and likely results in an absent or disrupted protein product, the current clinical and genetic evidence is not sufficient to establish whether loss-of-function variants in TNNT2 cause disease. In the absence of further pathogenicity documentation, even though it might be pathogenic, we also classify this mutation as of unknown significance. Mutations in the TNNT2 gene are related to different types of Cardiomyopathy (https://omim.org/entry/191045).

Table 5. Laboratory 3 data compared against the reference and consensus data.

Sample	Reference genotype	Consensus genotypes reported	Lab 3 Reported genotype	Lab 3 Clinical interpretation
Case 1	LDLR c.2479G>A Heterozygous	LDLR c.2479G>A Heterozygous APOB c.4072C>T Heterozygous	LDLR c.2479G>A APOB c.4072C>T	No Pathogenic or Likely Pathogenic variants were detected in the genes analysed in this assay. Two variants of unknown significance were identified, one variant in the LDLR gene and one variant in the APOB gene (see comments). Clinically actionable variants were not detected in the genes tested. Diagnosis and management of this patient should be based on current clinical and cardiovascular risk reduction guidelines. Cascade screening based on lipid testing is recommended for at-risk relatives. Comments: LDLR - NM_000527.4: c.2479G>A (p.Val827Ile): this variant has been published multiple times in association with FH. However, there are conflicting reports concerning its pathogenicity, suggesting that V827I alone is not sufficient to cause the full FH phenotype. APOB - NM_000384.2:c.4072C>T (p.Leu1358Phe): This is a rare variant not previously reported in patients with FH and not previously identified in population databases. There is therefore insufficient information available to classify this variant. Genetic counselling to discuss this result may be helpful. Refer to current CSANZ guidelines for further management advice.
Case 2	LDLR c.1871_1873delTCA Heterozygous	LDLR c.1871_1873delTCA Heterozygous HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	LDLR c.1871_1873delTCA Heterozygous	Likely Pathogenic variant DETECTED in the LDLR gene LRG_274t1(LDLR):c.1871_1873delTCA p.(Ile624del). A heterozygous likely pathogenic variant was detected in the LDLR gene. This result confirms a diagnosis of familial hypercholesterolemia. Specialist consultation and genetic counselling is recommended. Cascade genetic testing can be offered to at-risk family members. Comments: The p.(Ile624del) variant is located in β-propeller region and leads to an in-frame deletion of an amino acid in the LDLR gene. It has already been described in the literature, detected in patients with familial hypercholesterolemia, and associated

				with elevated cholesterol and LDL-C levels. It has been found to result in a defective protein by functional studies, reducing LDL-LDLR binding to 5-10%, and LDL-LDLR uptake and cell surface LDL to <5%. (Hum Mutat. 2015 Jan; 36(1):129-41). Refer to current CSANZ guidelines for further management of heterozygous individuals.
Case 3	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	Pathogenic variant DETECTED in the LDLR gene: LRG_274t1(LDLR):c.2312-3C>A p(.?) A heterozygous **pathogenic variant** was detected in the LDLR gene. This result confirms a diagnosis of familial hypercholesterolemia. Specialist consultation and genetic counselling is recommended. Cascade genetic testing can be offered to at-risk family members. Comments: The c.2312-3C>A variant leads to a change in the splice acceptor in intron 15 of the LDLR gene. It has already been described in the literature, detected in patients with familial hypercholesterolemia, and associated with elevated cholesterol and LDL-C levels. In in vitro models, the mutation leads to almost complete loss of LDL receptor activity. cDNA sequencing shows skipping of exon 16, predicted to result in p.(Ala771_Ile796del). It has been reported as one of the common pathogenic variants that causes FH. This variant has previously been identified in 18 FH patients (10 unrelated families). See PMID21865347 and 11317362. This result should be correlated with the FH genetic test result for the brother (not provided). Refer to current CSANZ guidelines for further management of heterozygous individuals.

Table 6. Laboratory 4 data compared against the reference and consensus data.

Sample	Reference genotype	Consensus genotypes reported	Lab 4 Reported genotype	Lab 4 Clinical interpretation
Case 1	LDLR c.2479G>A Heterozygous	LDLR c.2479G>A Heterozygous APOB c.4072C>T Heterozygous	LDLR c.2479G>A Heterozygous	Interpretation Genomic coordinates: Chr19 (GRCh37):g.11240278G>A Position in LDLR gene: exon 17 of 18 Evidence for LDLR variant c.2479G>A being classified as VUS - suggesting pathogenic Recommendations If not already undertaken, clinical review of this patient by a cardiologist and/or at a specialist lipid clinic is recommended. Appropriate genetic counselling is also recommended. The LDLR:c.2479G>A variant IS NOT appropriate for predictive DNA testing in asymptomatic relatives. Family studies may also help define the pathogenicity of this *LDLR* gene variant. We recommend all DNA tests are confirmed by duplicate testing on a second independently collected sample.
Case 2	LDLR c.1871_1873delTCA Heterozygous	LDLR c.1871_1873delTCA Heterozygous HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	LDLR c.1871_1873delTCA Heterozygous	Interpretation Genomic coordinates: Chr19 (GRCh37):g.11230793_11230795del Position in LDLR gene: exon 13 of 18 Evidence for LDLR variant c.1871_1873delTCA being classified as likely pathogenic. Recommendations If not already undertaken, clinical review of this patient by a cardiologist and/or at a specialist lipid clinic is recommended. Appropriate genetic counselling and testing of other at-risk relatives is recommended. Family studies may also help define the pathogenicity of this *LDLR* gene variant. We recommend all DNA tests are confirmed by duplicate testing on a second independently collected sample.
Case 3	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	Interpretation Genomic coordinates: Chr19 (GRCh37):g.11238681C>A Position in LDLR gene: intron 15 Evidence for LDLR variant c.2312-3>A being classified as likely pathogenic Recommendations

				If not already undertaken, clinical review of this patient by a cardiologist or at a specialist lipid clinic is recommended. Appropriate genetic counselling for this family is recommended, to discuss reproductive options, and should include ascertaining the FH mutation detected in a first degree relative – to exclude the rare convergence of two different *LDLR* variants in this family. Testing of other at-risk relatives, including the patient's offspring, is recommended. Family studies may also help define the pathogenicity of this *LDLR* gene variant. We recommend all DNA tests are confirmed by duplicate testing on a second independently collected sample.

Table 7. Laboratory 5 data compared against the reference and consensus data.

Sample	Reference genotype	Consensus genotypes reported	Lab 5 Reported genotype	Lab 5 Clinical interpretation
Case 1	LDLR c.2479G>A Heterozygous	LDLR c.2479G>A Heterozygous APOB c.4072C>T Heterozygous	None	Result: Multiple rare sequence variants were detected in the hypercholesterolaemia genes analysed, but none were considered pathogenic for familial hypercholesterolaemia. Conclusion: A monogenic cause of hypercholesterolaemia was not identified. The diagnosis of FH cannot be excluded. A pathogenic variant is not detected in 10% or more of patients classed as 'definite FH' by the Dutch Lipid Clinic criteria.
Case 2	LDLR c.1871_1873delTCA Heterozygous	LDLR c.1871_1873delTCA Heterozygous HFE c.187C>G Heterozygous TGFB3 c.187C>G Heterozygous	LDLR c.1871_1873delTCA Heterozygous	Interpretation: This heterozygous 3 bp in-frame deletion in *LDLR* exon 13 would result in the loss of isoleucine at position 624 and is pathogenic for familial hypercholesterolaemia (FH). The *LDLR* p.Ile624del variant is absent from population databases (1000 Genomes, gnomAD) and has previously been identified in multiple cohorts of FH patients worldwide (1). *In vitro* cell transfection studies demonstrated that the p.Ile624del variant is retained within the endoplasmic reticulum, resulting in minimal expression (~5% of normal) of LDL-receptors on the cell surface (2). References 1. ClinVar. NM_001195798.2(LDLR):c.1868_1870TCA[1] (p.Ile624del) https://www.ncbi.nlm.nih.gov/clinvar/variation/252097/ 2. Etxebarria A, et al. Functional characterization and classification of frequent low-density lipoprotein receptor variants. Hum Mutat 2015;36(1):129-41. Conclusion: The finding of a heterozygous pathogenic *LDLR* variant is consistent with familial hypercholesterolaemia. Recommendation: Family cascade screening is strongly recommended. The risk of FH in first degree relatives is 50%.
Case 3	LDLR c.2312-3C>A Heterozygous	LDLR c.2312-3C>A Heterozygous	None	-

Table 8. Assessment outcome.

Laboratory	Sample	Genotype	Clinical Interpretation	Assessment Comments
Lab 1	Case 1	Concordant	Not assessed	Interpretation not provided.
	Case 2	Concordant	Not assessed	Interpretation not provided.
	Case 3	Concordant	Not assessed	Interpretation not provided.
Lab 2	Case 1	Not assessed	Not assessed	Clinically relevant genes for FH not tested.
	Case 2	Concordant	Not assessed	Clinically relevant genes for FH not tested.
	Case 3	Not assessed	Not assessed	Clinically relevant genes for FH not tested.
Lab 3	Case 1	Concordant	Concordant	Acceptable (zygosity not reported)
	Case 2	Concordant	Concordant	Acceptable
	Case 3	Concordant	Concordant	Acceptable
Lab 4	Case 1	Concordant	Concordant	Acceptable
	Case 2	Concordant	Concordant	Acceptable
	Case 3	Concordant	Concordant	Acceptable
Lab 5	Case 1	Not assessed	Not assessed	No variants reported for this case.
	Case 2	Concordant	Concordant	Acceptable
	Case 3	Not assessed	Not assessed	Lab 5 did not test Case 3 due to the reference clinical case scenario being insufficient in relation to genotype data.

9. Project findings

The 2019 – 2020 QUPP Commonwealth funding allowed the RCPAQAP to develop a novel EQA program for the cardiovascular disease of familial hypercholesterolemia. The program was designed to enable participating laboratories to perform DNA sequencing on any gene region that was of clinical relevance. To achieve this, the RCPAQAP needed to perform whole genome sequencing to identify all DNA variants present in each reference testing sample. Importantly, this strategy allows the RCPAQAP to proficiency test all DNA regions that become clinically relevant in the future without the need to change or update the EQA program.

The whole genome sequencing data was vast and highlighted a DNA sequence variant complexity that was previously unappreciated for FH development and progression. However, it remains unknown as to how many of these DNA variants are pathogenic for the FH disease. Further research will need to be performed in the future to identify such clinically relevant gene variant associations.

The laboratory reporting styles varied, and these could potentially be problematic for full FH clinical diagnostics. For example, laboratories not reporting all variants tested, assuming some variants are non-pathogenic without explanation, reporting clinical interpretations that are not relevant to the case scenarios, and refusal to test in the absence of family genotype data could have detrimental impacts for the clinical management of patients. However, the identification of such issues is essential information to relay back to laboratories since it allows for the laboratory to troubleshoot their testing and reporting pipeline so that data analysis and performance can be improved. These data further demonstrate the importance of participation in an EQA program. This study, therefore, achieved its aim in identifying putative new gene regions for FH diagnoses and highlighting areas of concern relating to laboratory testing and reporting.

10. Problems encountered

There were three key issues encountered in this study. The first issue was in getting FH patients to agree to volunteer DNA for EQA purposes. This took longer than expected and caused a hold-up of the study in relation to providing data for the progress reports to the QUPP. The second issue related to the time required by laboratories to perform analysis on the reference testing material. Laboratories insisted on taking between 8 to 12 weeks to perform analysis. The reason for this length of time is that each laboratory wanted to collect enough DNA samples so that cost-effective batch next generation sequencing could be performed. Again, this caused an issue for providing updated information in the form of progress reports to the QUPP. The third issue related to the time required for our clinical reviewers to review all laboratory data. This was severely impacted due to the COVID-19 pandemic since our reviewers needed to focus on their patients and this took priority over reviewing the laboratory data. As such, the EQA Survey Reports took longer to generate.

Over the 12-month period, the delay in obtaining patient samples and laboratories requiring extended periods of time for analysis did not impact on the overall data received. These delays only impacted on providing data for the QUPP progress reports but ultimately had no impact on the final report for the whole study. However, the COVID-19 pandemic was unforeseen. As such, clinical review of the laboratory data could not proceed until the clinicians had enough time to spare during the ongoing pandemic. We therefore had to adapt to this ongoing situation as best we can and supply reports to the laboratories when they were ready. All laboratories understood this as it was out of our control.

11. Future diagnostics

(*Does the proposed project complement other similar services, activities and resources?*)

The data from this project directly complements two previously funded QUPP projects awarded in 2017. The first funded project was for developing different EQA programs for proficiency testing (i) DNA extraction, (ii) circulating free DNA for cancer-associated biomarkers and for non-invasive prenatal testing, and (iii) leukaemia-associated DNA variants (Agreement id: 4-4YYPT91). The second funded project was for developing a mass spectrometry EQA program for proficiency testing human disease-associated biomarker proteins (Agreement id: 4-4Z3AWAN). As such, the familial hypercholesterolemia data directly complement these awards by continually developing new EQA programs related to disease biomarkers. Also, future diagnostic protocols are likely to include analyses of genomic DNA and circulating levels of key DNA and protein biomarkers that are associated with different human diseases (Cohen et al 2017). Such analyses would be far more informative for the clinician and may help identify disease development early so that appropriate clinical management can be initiated.

The development of new EQA programs is an essential process for the RCPAQAP and is reflective of the clinical demand. Genetic testing is growing as a clinical practice and is being adopted by multiple disciplines, including chemical pathology, anatomical pathology, microbiology, immunology, cytopathology, haematology, and biosecurity. It is, therefore, critical to keep pace with multi-discipline clinical findings so that the RCPAQAP can develop new EQA programs that are clinically relevant to new disease understandings. This is particularly relevant in the current climate for the COVID-19 pandemic, where the RCPAQAP was the first EQA provider to develop an EQA program for coronavirus testing.

12. Project assessment

12.1 Short term

In the short term, the data from this EQA pilot will help laboratories modify their reporting styles so that a consistent harmonised level of clinical reporting is reached. This is important for the ongoing clinical management of FH patients.

12.2 Intermediate Term

For the intermediate term, the consistency of each laboratory produced report can be monitored, and improvements suggested for areas found to be of clinical concern that may impact on effective patient care.

12.3 Long term

For the long term, it is hoped that the whole genome sequence data will help raise awareness into the complexity of FH DNA diagnostics that will guide further work into establishing other DNA regions that are likely to be of clinical relevance for future diagnoses. As such, the RCPAQAP will play an essential role in not only proficiency testing currently accepted gene regions for diagnostic analysis but also in helping identify other relevant regions for future testing.

The rapid growth in molecular testing is now allowing the detection of multiple DNA sequence variations associated with either inherited (genetic) or acquired (cancer) diseases. Next generation sequencing (NGS) technology can detect DNA variation in the entire human genome in a single assay. In this study, a total of 4.8 million DNA variants in each test sample were detected using NGS technology. These data provide insight into DNA variation in the cardiovascular disease of familial hypercholesterolemia. They can be used to identify other potential regions likely to be of clinical relevance for future diagnostic testing. The long-term outcome of this EQA program is to encourage laboratories to adapt to whole genome NGS for diagnosing cardiovascular disease as the derived data are much more informative.

12.3.1 Economy

Proficiency assessments of new developing technologies allow laboratories to benchmark their overall performance and to lower costs by reducing the time required to optimise a key diagnostic test. The availability of an EQA for assessments of clinical diagnostics will ensure that all clinicians

are working to the same standard for diagnoses. The cost of healthcare and treatment plans can, therefore, be significantly reduced and implemented earlier. This will increase patient care by allowing earlier diagnoses, which will benefit patient management strategies and thus increase cost-effectiveness.

12.3.2 Efficiency

Diagnostic applications incorporating new strategies using emerging technology (i.e., NGS) is rapidly growing. As such, new understandings of disease processes combined with modern technology, allows for increased levels of diagnostic sensitivity, high throughput capacity, reduced patient invasiveness, and increased efficiency in terms of time and cost. Diagnostic laboratories now have the ability to sequence the whole genome in a single assay. This increases the detection rate of DNA variation in a much shorter time period with high accuracy and sensitivity. EQA proficiency testing allows for aberrant testing issues to be identified and solved so that improvements in a testing technology can be continually recommended.

12.3.3 Effectiveness

The development of new EQA programs will additionally allow the establishment of cross-functional RCPAQAP inter-discipline collaboration. For example, the RCPAQAP Anatomical Pathology, Biosecurity, Chemical Pathology, Cytopathology, Haematology, Immunology, Serology and Microbiology disciplines will benefit from new understandings of disease-associated DNA biomarkers since new disease processes can be linked in to other disciplines for improved proficiency testing and clinical interpretation of data. This combined discipline approach will result in the production of highly-developed quality assurance programs for future efficiency testing and cost-effective analyses across the RCPAQAP.

13. Project sustainability

The current FH EQA program will be offered to laboratories for proficiency testing any or all genes that are of clinical interest once our Advisory Committee are in agreement in relation to the reported DNA data and associated clinical interpretation. This will then be the first EQA program to offer quality assurance testing on the whole genome for a cardiovascular disease. We now intend to take this further by developing other EQA programs relating to different cardiovascular diseases as the testing strategy will be exactly the same.

Cardiovascular disease gene testing is growing owing to NGS data confirming the presence of multiple DNA variants in patient samples and the cost of NGS becoming cheaper. Linking multiple DNA variants with clinical outcomes is however, challenging. EQA programs can assist in developing clinical guidelines for genetic diagnostics and clinical characterisation. As such, interest in familial hypercholesterolemia will grow owing to the increased usage of NGS technology and the identification of more clinically relevant DNA regions. This will make the current program sustainable for the future.

14. Appendix

List of cardiovascular disease associated genes tested by Laboratory 2.

ABCC9	DSP	KCNJ5	RBM20
ACTA1	DTNA	KCNJ8	RYR2
ACTC1	EMD	KCNQ1	SCN10A
ACTN2	EYA4	LAMA4	SCN1B
AKAP9	FGF12	LAMP2	SCN2B
ALPK3	FHL1	LDB3	SCN3B
ANK2	FHL2	LMNA	SCN4B
ANKRD1	FKTN	MYBPC3	SCN5A
APOA1	FLNC	MYH6	SCO2
ATP2A2	GAA	MYH7	SGCD
BAG3	GATA4	MYL2	SLC8A1
CACNA1C	GATA6	MYL3	SLMAP
CACNA1D	GATAD1	MYLK2	SNTA1
CACNA2D1	GJA1	MYOM1	STRN
CACNB2	GJA5	MYOZ2	SURF1
CALM1	GJC1	MYPN	TAZ
CALM2	GLA	NEBL	TBX20
CALM3	GPD1L	NEXN	TBX5
CALR3	HCN4	NKX2-5	TCAP
CASQ2	HEY2	NOS1AP	TGFB3
CAV3	HFE	NPPA	TMEM43
CHRM2	JPH2	NUP155	TMPO
CRYAB	JUP	PDLIM3	TNNC1
CSRP3	KCNA5	PKP2	TNNI3
CTF1	KCNAB2	PLN	TNNT2
CTNNA3	KCND3	PRDM16	TPM1
DES	KCNE1	PRKAG2	TRDN
DMD	KCNE2	PSEN1	TRPM4
DOLK	KCNE3	PSEN2	TRPM7
DPP6	KCNE5	PTPN11	TTN
DSC2	KCNH2	RAF1	TTR
DSG2	KCNJ2	RANGRF	VCL

15. References

1000 Genomes Project Consortium, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR (2015) A global reference for human genetic variation. Nature. 526:68-74.

Australian Burden of Disease Study: impact and causes of illness and death in Australia 2011. Study series number 3.

Australian Bureau of Statistics. Causes of death 2015 (3303.0). September 2016.

Australian Bureau of Statistics. Causes of death 2016 (3303.0). September 2017.

Australian Institute of Health and Welfare 2011. Cardiovascular disease: Australian facts 2011. Cardiovascular disease series. Cat. no. CVD 53. Canberra: AIHW.

Benn M, Watts GF, Tybjaerg-Hansen A, et al. Familial hypercholesterolemia in the Danish general population: prevalence, coronary artery disease, and cholesterol-lowering medication. J Clin Endocrinol Metab. 2012 Nov;97(11):3956–64.

Bertolini S, Pisciotta L, Di Scala L, Langheim S, Bellocchio A, Masturzo P, Cantafora A, Martini S, Averna M, Pes G, Stefanutti C, Calandra S. Genetic polymorphisms affecting the phenotypic expression of familial hypercholesterolemia. Atherosclerosis. 2004 May;174(1):57-65.

Cohen JD et al., (2017) Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. Proc Natl Acad Sci U S A. 114:10202-10207.

Di Taranto MD, Giacobbe C, Fortunato G. Familial hypercholesterolemia: A complex genetic disease with variable phenotypes. Eur J Med Genet. 2019 Dec 25:103831. doi: 10.1016/j.ejmg.2019.103831.

Dron JS, Wang J, McIntyre AD, Iacocca MA, Robinson JF, Ban MR, Cao H, Hegele RA. Six years' experience with LipidSeq: clinical and research learnings from a hybrid, targeted sequencing panel for dyslipidemias. BMC Med Genomics. 2020 Feb 10;13(1):23.

Freudenberg-Hua Y, Freudenberg J, Vacic V, Abhyankar A, Emde AK, Ben-Avraham D, Barzilai N, Oschwald D, Christen E, Koppel J, Greenwald B, Darnell RB, Germer S, Atzmon G, Davies P (2014) Disease variants in genomes of 44 centenarians. Mol Genet Genomic Med. 2:438-450.

Gaspar IM, Gaspar A. Variable expression and penetrance in Portuguese families with Familial Hypercholesterolemia with mild phenotype. Atheroscler Suppl. 2019 Mar;36:28-30.

Iacocca MA, Wang J, Dron JS, Robinson JF, McIntyre AD, Cao H, Hegele RA. Use of next-generation sequencing to detect LDLR gene copy number variation in familial hypercholesterolemia. J Lipid Res. 2017 Nov;58(11):2202-2209.

Johansen CT, Dubé JB, Loyzer MN, MacDonald A, Carter DE, McIntyre AD, Cao H, Wang J, Robinson JF, Hegele RA. LipidSeq: a next-generation clinical resequencing panel for monogenic dyslipidemias. J Lipid Res. 2014 Apr;55(4):765-72.

Sjouke B, Kusters D, Kindt I, et al. Homozygous autosomal dominant hypercholesterolaemia in the Netherlands: prevalence, genotype–phenotype relationship, and clinical outcome. Eur Heart J. 2014 Feb 28; doi: 10.1093/eurheartj/ehu058.

Willyard C. New human gene tally reignites debate. Nature. 2018; 558:354-55.

Zhang L, Dong X, Lee M, Maslov AY, Wang T, Vijg J (2019) Single-cell whole-genome sequencing reveals the functional landscape of somatic mutations in B lymphocytes across the human lifespan. Proc Natl Acad Sci U S A.116:9014-9019.