Development of a novel pre-screen algorithm for cardio-metabolic risk management using a genomics database resource

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DECLARATION

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SUMMARY

The timely assessment and treatment of dyslipidaemia is an important component of cardiovascular risk screening and intervention. The *apolipoprotein E* (*APOE*) ε-2/ε-3/ε-4 polymorphism associated with impaired lipid homeostasis provides a genetic link between cardiovascular disease (CVD) and late-onset Alzheimer's disease (AD). Realization that the phenotypic expression of the risk associated *APOE*ε-2 and ε-4 alleles may be dependent on non-genetic factors supports the inclusion of *APOE* genotyping in chronic disease screening programs. The lack of well-defined selection criteria for *APOE* genotyping, however, limits the use of this biomarker in clinical practice.

The aim of the present study was to develop a pre-screen algorithm for identification of a target population most likely to benefit from *APOE* genotyping, performed in conjunction with a clinical and lifestyle assessment. Towards this goal, comprehensive patient data were evaluated from a total of 580 unrelated Caucasians enrolled in a chronic disease screening program over a five-year period (2010-2015), using an ethically approved study questionnaire. Biochemical tests performed according to standard laboratory protocols were extracted from the research database. All study participants were genotyped for the *APOE* ε-2/ε-3/ε-4 polymorphism.

APOE genotype distribution differed significantly ($p<0.05$) between study participants with and without a family history of AD. A positive association between dietary fat intake and low-density lipoprotein (LDL) cholesterol ($p=0.001$), as well as an inverse association with high-density lipoprotein (HDL) cholesterol ($p=0.002$), were observed in patients with a family history of AD. Body mass index (BMI) was positively associated with LDL cholesterol and inversely associated with HDL cholesterol levels ($p<0.001$), irrespectively of an AD family history. Smoking was associated with higher triglycerides ($p<0.001$) and lower HDL cholesterol levels ($p=0.004$) in the total study group. Alcohol intake was positively associated

with BMI (p=0.008) as well as triglyceride levels (p=0.021) in patients with a positive family history of AD. The clinical expression of a hypercholesterolaemic phenotype in *APOE* ε-4 allele carriers, as well as apparent mitigation by regular physical activity, were dependent on the interaction between a family history of AD and *APOE* genotype (p<0.001). *APOE* ε-2 carriers without an AD family history showed a significant increase in triglyceride levels (p=0.014). The modulating influence of *APOE* ε-4 on the relationship between alcohol intake and BMI as well as total cholesterol levels was also dependent on the presence or absence of AD family history (p<0.05).

This study resulted in the addition of a family history of AD as a novel component to the pre-screen algorithm developed for selection of at-risk individuals prior to *APOE* genotyping performed as part of a chronic disease screening program. The lifestyle questionnaire used in this study furthermore facilitated interpretation of the clinical relevance of variation detected in the *APOE* gene. This is important to prioritize the use of lipid-lowering medication towards patients with severe subtypes of dyslipidaemia such as familial hypercholesterolaemia (FH), which remains largely undiagnosed and untreated in the high-risk South African population. Incorporating the research findings into clinical practice would suggest that physical activity may be the most effective risk reduction strategy in carriers of the *APOE*ε-4 allele, as supported by international studies.

OPSOMMING

Die tydige opsporing en behandeling van dislipidemie is 'n belangrike komponent van kardiovaskulêre risikobepaling en intervensie. Die *apolipoproteïn E* (*APOE*) ε-2/ε-3/ε-4 polimorfisme wat geassosieer is met abnormale lipied metabolisme toon ‘n genetiese verband tussen kardiovaskulêre siekte (KVS) en laat- aanvang Alzheimer se siekte (AD). Die bevinding dat die fenotipiese uitdrukking van die risiko geassosieerde *APOE* ε-2 en ε-4 allele afhanklik mag wees van nie-genetiese faktore ondersteun die insluiting van *APOE* genotipering in kroniese siekte siftingsprogramme. Die gebrek aan goed-gedefinieerde seleksie kriteria vir *APOE* genotipering beperk egter die gebruik van hierdie biomerker in kliniese praktyk.

Die doel van hierdie studie was om 'n pre-toets algoritme te ontwikkel vir identifikasie van ‘n teiken populasie waar *APOE* genotipering waarde kan byvoeg, tesame met die evaluering van kliniese en leefstyl risikofaktore. Om hierdie doel te bereik is pasiëntdata geanaliseer van ‘n totaal van 580 nie-verwante Koukasiërs wat deelneem aan ‘n kroniese siekte siftingsprogram oor ‘n vyf-jaar periode (2010-2015), met gebruik van ‘n eties goedgekeurde studievraelys. Biochemiese toetse wat uitgevoer is volgens standaard laboratorium protokolle is geëekstraheer uit die navorsingsdatabasis. Genotipering van alle studiedeelnemers is uitgevoer vir die *APOE* ε-2/ε-3/ε-4 polimorfisme.

APOE genotipe verspreiding het betekenisvol verskil ($p<0.05$) tussen studiedeelnemers met en sonder ‘n familiegeskiedenis van AS. ‘n Positiewe assosiasie is waargeneem tussen vet-inname in die dieet en lae-digtheid lipoproteïen (LDL) cholesterol ($p=0.001$), terwyl ‘n omgekeerde assosiasie met hoë-digtheid lipoproteïen (HDL) cholesterol ($p=0.002$) waargeneem is in deelnemers met ‘n familiegeskiedenis van AS. Liggaamsmassa-indeks (BMI) was positief geassosieer met LDL cholesterol en omgekeerd geassosieer met HDL

cholesterolvlakke (p<0.001), ongeag van AS familiegeskiedenis. Rook was geassosieer met verhoogde trigliserides (p<0.001) and laer HDL cholesterolvlakke (p=0.004) in die totale studiegroep. Alkohol inname was positief geassosieer met BMI (p=0.008) asook trigliseriedvlakke (p=0.021) in deelnemers met ‘n positiewe familiegeskiedenis van AS. Die kliniese uitdrukking van ‘n hipercholesterolemiese fenotipe in *APOE* ε-4 alleel draers, asook die verlaagde cholesterolvlakke met gereëlde fisiese aktiwiteit, was afhankik van die interaksie tussen AS familiegeskiedenis en *APOE* genotipe (p<0.001). *APOE* ε-2 draers sonder ‘n AS familiegeskiedenis het ‘n betekenisvolle verhoging in trigliseriedvlakke getoon (p=0.014). Die modulerende invloed van *APOE* ε-4 op die verhouding tussen alkohol inname en BMI asook totale cholesterolvlakke is ook bepaal deur die teenwoordigheid al dan nie van ‘n AS familiegeskiedenis (p<0.05).

Hierdie studie het gelei tot die byvoeging van ‘n familiegeskiedenis van AS as ‘n nuwe komponent tot die pre-toets algoritme wat ontwikkel is om hoë-risiko individue te selekteer voor *APOE* genotipering as deel van n kroniese risiko bestuur program. Die leefstyl vraelys wat gebruik is vergemaklik die interpretasie van die kliniese relevansie van die genotipe resultate. Dit is belangrik sodat die noodsaaklikheid van lipied-verlagende farmakoterapie geprioritiseer kan word in deelnemers met erge subtipes van dislipidemie soos familiële hipercholesterolaemie (FH), wat meestal ongediagnoseer en onder-behandel bly in die hoë-risiko Suid-Afrikaanse populasie. Inkorporering van die navorsingsresultate in kliniese praktyk behels die aanbeveling van fisiese aktiwiteit as die mees effektiewe risikoverlagende strategie in draers van die *APOE* ε-4 alleel, soos ook ondersteun word deur intenasionale studies.

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LIST OF ABBREVIATIONS

A

A-β: amyloid beta

AChEI: acetylcholine esterase inhibitors

AD: Alzheimer's disease

ANCOVA: analysis of covariance

APOE: apolipoprotein E

B

BMI: body mass index

BNP: brain natriuretic peptide

C

CAA: cerebral amyloid angiopathy

CRP: C-reactive protein

CVD: cardiovascular disease

CYP2D6: cytochrome P450

D

DIOS: dysmetabolic iron overload syndrome

DTC: direct to consumer

F

FH: familial hypercholesterolemia

G

GWAS: genome-wide association studies

H

HCP: healthcare professional

HDL: high-density lipoprotein

HER2: human epidermal growth factor 2

HFE: hemochromatosis

HREC: health and research ethics committee

I

IL-6: interleukin-6

IQR: interquartile range

L

LDL: low-density lipoprotein

LDLR: low-density lipoprotein receptor

LOAD: late-onset Alzheimer's disease

M

MDR: multifactor-dimensionality regression

N

NCD: non-communicable disease

NFT: neurofibrillary tangle

P

PSGT: pathology-supported genetic testing

R

RCT: randomized controlled trial

RXR: retinoid x receptor

S

SD: standard deviation

W

WHO: world health organization

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INTRODUCTION

The coordinated interaction between the low-density lipoprotein receptor (LDLR) underlying familial hypercholesterolaemia (FH) and apolipoprotein E (APOE) as the primary ligand of the LDLR forms the basis for the metabolic regulation of cholesterol. Determination of cholesterol levels forms an integral part of population-based chronic disease / wellness screening programs aimed at prevention of cardiovascular disease (CVD) and other non-communicable diseases (NCDs). This is of particular relevance in South Africa, where the prevalence of FH is increased 5-10 times compared to most other populations in the world due to a founder effect. This led to the development of a cost-effective test for FH based on a limited number of founder mutations responsible for the disease in the majority of affected patients in South Africa. The finding that polymorphic variation in the *APOE* gene contributes more to fluctuation in lipid levels in the general population (Siest et al. 1995) than any other gene identified to date, highlights the potential role of genetic testing for identification of dyslipidaemia subtypes with different treatment requirements.

The *APOE* ε-2/ε-3/ε-4 polymorphism is one of the most extensively studied genetic variations underlying CVD in the general population. The cholesterol-raising APOE ε-4 allele was furthermore identified as the most important genetic risk factor for late-onset Alzheimer's disease (AD), which shows striking pathogenic overlap with CVD. In this context, a growing body of evidence supports the role of the *APOE* ε-2/ε-3/ε-4 polymorphism as an important target for risk reduction intervention in chronic disease screening programs.

The lack of well-defined eligibility criteria for identification of a subgroup of the population set to derive optimal benefit from *APOE* genotyping limits the use of this biomarker in clinical practice. In addition, clinicians remain hesitant to refer patients for *APOE* genotyping due to the association between the ε-4 allele and increased risk for AD, considered an incurable disease. *APOE* genotype also has limited individual utility as a diagnostic, predictive and prognostic marker in the assessment of cardiovascular risk. This could be explained by the fact that genetic risk conferred by the *APOE* ε-2/ε-3/ε-4 polymorphism is context-dependent,

with its deleterious effects expressed only in a high-risk environment. This knowledge also provides an opportunity for risk reduction at the gene-environment level.

Family history is routinely used to determine eligibility for high-penetrance mutation screening used to confirm a suspected diagnosis of Mendelian disorders such as FH. However, the clinical usefulness of a family history for selection of high-risk individuals most likely to benefit from a chronic disease screening program including APOE genotyping, remains unclear. In this context, we investigated whether a family history of AD could serve as the basis for a pre-screen algorithm that can be applied prior to *APOE* genotyping in order to facilitate the development of tailored treatment paths in a subgroup of dyslipidaemic patients at increased risk of CVD and/or AD. Towards this goal, a retrospective evaluation of prospectively collected data of nearly 600 study participants was performed with specific emphasis on the clinically actionable *APOE* polymorphism. Data was extracted from a secure centrally maintained research database was assessed with the use of a preliminary statistical tool to determine the value of *APOE* genotyping as a component of chronic disease screening in the South African population. Clinical information in this database was corroborated with data contained in patient files, including verification of appropriate patient consent.

The thesis is presented in four chapters, starting with a review of the literature (Chapter 1), relevant to the purpose of the study. Data from two sub-studies written as original articles published in scientific journals are subsequently presented. In the first sub-study (Chapter 2) we present data which supports the clinical relevance of inquiry concerning AD family history as part of a pre-screen selection step for *APOE* genotyping. In the second sub-study (Chapter 3) the value of a questionnaire-based lifestyle assessment is evaluated to allow for appropriate clinical interpretation of *APOE* genotyping results. Conclusions from this research project are presented in Chapter 4, followed by the Appendix including supporting material.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Genomic medicine is based on the assumption that risk for the onset and pathogenic progression of most clinical disorders is modified or determined by genetic factors. Recent technological developments have advanced our understanding of the genetic basis underlying the pathogenesis of chronic multifactorial non-communicable diseases (NCDs) considered a primary contributor to global morbidity and mortality (Murray and Lopez 2013). Increasing appreciation for this shared genetic architecture underpinning the etiopathogenic basis of most chronic multifactorial disorders, provides a strong incentive for the development of a unifying health model allowing for the evaluation of current and future disease risk to promote wellness throughout the course of a person's lifespan (Kotze et al. 2013; Li et al. 2013).

The successful integration of genetic testing as a routine component of patient care has the capacity to advance a paradigm shift away from "reactive medicine" in favour of a new healthcare model which resonates with the principles and ideals of personalized medicine (Hood 2013). A number of limitations however impede the adoption of genomics in general medical practice and continue to widen the gap between the generation of increasingly complex genetic data and the reality of its application to improve long-term health outcomes (Khoury et al. 2007). It is imperative that these factors be addressed in order to enable the integration of genomics as a routine component of patient care.

In this literature overview, we discuss existing limitations which impede the advancement of a genomics-based approach to chronic disease screening, in addition to those inherent to a

genetics-only approach to risk management and intervention. A pathology-supported genetic testing (PSGT) model (Kotze et al. 2015) is presented as a means of incorporating screening into existing risk classification models as part of a multidisciplinary approach to patient management. In conclusion, the value of using a pathways-based approach to validate the appropriateness of genetic testing related to dyslipidaemia is considered with specific emphasis on a functional polymorphism (ε-2/ε-3/ε-4) in the *APOE* gene.

1.2. Limitations impeding the widespread clinical adoption of genetic testing

The general public is becoming increasingly aware of the potential harms associated with inappropriate genetic testing, including loss of privacy, breach of confidentiality, stigmatization and employment as well as insurability concerns. To ensure its appropriate use, a code of conduct has been compiled for the insurance industry in South Africa (reviewed by Kotze et al. 2004). The code aims to promote the fair, equitable, confidential and evidence-based handling of genetic information.

The most efficient and practical means to integrate, interpret and communicate genetic data to inform clinical decision making remain imprecisely defined (Guttmacher et al. 2007). A lack of sufficient pre- and post-clinical training and experience in genomics contributes towards a lack of expertise and confidence among healthcare practitioners (HCPs) in handling certain genetic services by themselves within the framework of existing management strategies to improve patient outcomes. Clinician education should ideally include basic knowledge of a variety of areas of clinical relevance including the principles and indications for genetic testing, the role of genetic counsellors, the clinical value of currently available tests as well as interpretation of genetic information. Haspel et al. (2010) suggested that the existing curriculum for pathology training in North America should include a greater emphasis on genomics education and personalized medicine. Similarly, the National Human Genome Research Institute appealed for improved strategies to enhance the confidence of HCPs to refer for genetic testing (Feero and Green 2011).

In order to affirm the demand for greater education and training in genomics, existing perceptions and knowledge of HCPs regarding the utility of genetic testing, particularly in the development of tailored therapeutic algorithms to optimize patient care, needs to be established. Eisinger et al. (2008) used a questionnaire-based survey to assess the opinions of 600 general practitioners and 1504 members of the public regarding screening tests for cancer. The authors found surprising disagreement between evidence-based recommendations for genetic testing and actual clinical practices. Clinicians tended to overestimate the negative psycho-dynamic impact of testing results on patients, whilst failing to appreciate the importance of their role in testing initiation and result interpretation. Klitzman et al. (2013) recently confirmed the need for further HCP training regarding the indications for genetic testing, patient counselling, interpretation of genetic information and maintenance of confidentiality and privacy. This study further found that patient inquiry regarding genetic testing was more strongly associated with the requesting of such tests rather than being prompted by patient-care objectives. These findings were echoed in an earlier study by Sifri et al. (2003) in which the authors found that the application of genetic screening tests for cancer were primarily affected by patient inquiry, and lastly by the practice environment of the provider. In summary, a solely education-based focus concerning the principles underlying genetics is insufficient in accomplishing these goals. Unless current attitudes towards genetic testing are also addressed, and the reasons behind clinical hesitancy towards its integration defined, genomics will remain an underutilized resource in healthcare, despite substantial government and private investments into research and training of medical scientists.

1.3. Limitations imposed by a genomics-only approach to NCD risk screening

An ongoing shift in research focus from the limited scope of high-penetrance Mendelian disorders to polygenic NCDs requires that the analytical validity and clinical utility of common single-nucleotide polymorphisms (SNPs) be established. This issue is further complicated by the lack of a clear distinction between genetic variants considered as “causative mutations”,

"genetic risk modifiers" and "susceptibility variants" (Donahue et al. 2006; Kotze et al. 2013). It is therefore imperative to determine whether personalized genomic testing performed as part of comprehensive NCD risk assessment is able to accurately predict adverse clinical or therapeutic outcomes at the individual level. Establishing whether a given genomic test with sufficient predictive accuracy as supported by clinical evidence brings about a change in patient management is considered an important further milestone towards the confirmation of clinical utility as a prerequisite for the routine clinical implementation of emerging genomic applications (Janssens and van Duijn 2008).

Genome-wide association studies (GWAS) have identified thousands of common susceptibility variants as putative genetic risk modifiers across a broad spectrum of chronic NCDs (Cluett and Melzer 2009). These risk-associated variants however fail to reach significance in population-based studies and have limited utility as individual risk predictors in complex phenotypic traits (Janssens and van Duijn 2008; Pharoah et al. 2008). It has been proposed that genotype risk scores incorporating a sufficient number of susceptibility variants functioning in known pathogenic pathways could reach statistical significance to the extent that a substantial proportion of phenotypic variance for a particular trait is explained (Janssens et al. 2006). For a polygenic risk prediction model to be developed and validated in such a way that it accurately identifies people at increased NCD risk, the research study needs to be performed using a sufficient and often very large sample size for the number of susceptibility variants considered, which is not always practical or logistically feasible in resource-limited environments (Janssens et al. 2006; Chatterjee et al. 2013).

The majority of genotype risk scores developed to date have failed to reach sufficient discriminatory power required to accurately predict individual risk for etiologically complex NCDs such as type II diabetes mellitus (Meigs et al. 2008; Liu and Song 2010). The predictive value of such polygenic risk models is further limited by the prevalence and degree of heritability for the NCD of interest: as such, a genotype-only approach to NCD risk

prediction could equate but likely not supersede existing clinical assessment schemes, with the exception of perhaps a limited number of restricted applications (Janssens and van Duijn 2008). It does however appear that the addition of a simple genomics component comprising a limited number of susceptibility variants to existing clinical risk prediction algorithms significantly improves their performance in predicting individual risk for the development or progression of NCDs including coronary artery disease (Humphries et al. 2007) and malignant melanoma (Cust et al. 2014). This may be compared to the use of limited mono-variant testing such as hemochromatosis (HFE) genotyping used as a viable alternative to invasive liver biopsy in diagnosing patients with type I hereditary hemochromatosis (HH), as previously reviewed by Kotze et al. (2009) in relation to other causes of iron dysregulation. While a number of metabolic causes for hepatic iron overload exist, there is increasing interest in the dysmetabolic iron overload syndrome (DIOS) as the most common differential diagnosis for type I genetic HH in obese patients. Increased serum ferritin as the only reason to refer patients to HFE genotyping may lead to the erroneous diagnostic confirmation of genetic HH in patients for whom DIOS is rather more likely based on a compatible clinico-biochemical profile despite incidental HFE mutation carriage (Riva et al. 2008).

A notable advantage of polygenic risk models over single-variant analysis is their ability to reflect the additive effects of multiple susceptibility variants on NCD risk or clinical outcomes. These genotype risk scores are often based solely on allelic summation and mostly fail to consider the importance of synergistic interactions between the risk variants of interest as a major determinant of functional outcomes (Onay et al. 2006; Machiela et al. 2011). This shortcoming is intrinsically related to the limited capabilities of parametric statistical methods to characterize the impact of risk genotypes on phenotypic expression patterns when they are to an extent dependent on epistatic interactions with other susceptibility variants (Templeton 2000). Not only are case-control studies not ideal for evaluating the long-term impact of genetic testing on clinical decision-making and patient outcomes, but different statistical methods vary in their ability to model the influence of risk-associated genetic

variants on NCD risk. For example, while logistic regression is a statistical classification method often used to determine how accurately a set of putative risk genotypes can predict a binary clinical outcome, this approach is considered impractical for modelling higher-order variant-variant interactions: owing to the high frequency of empty contingency-based cells, data sparseness can lead to 1) the standard error of the regression coefficient being very large, increasing the probability of a type II error occurring, or 2) failure of model convergence, resulting in the regression coefficients derived there from being of limited value (Hosmer and Lemeshow 2000). Model-free statistical methods including multifactor-dimensionality reduction (MDR) have been proposed as a more useful alternative for effectively modelling higher-order epistatic effects, although this technique is itself limited insofar as it is computationally intensive given the incorporation of a large number of risk variants (Ritchie et al. 2001).

It can therefore be argued that it is simply no longer appropriate to consider a genotype-only approach to NCD risk management as the ideal conceptualization of how low-penetrance genomic testing should be applied to predict the onset or clinical progression of multifactorial polygenic conditions or adverse long-term clinical and therapeutic outcomes. It is imperative that the correct study design be used as well as the correct statistical tools to factor in the importance of environmental and epistatic factors as modifiers of phenotypic expression associated with carriage of a particular risk variant. The limited utility of isolated genetic testing as the sole basis for clinical risk prediction in chronic NCDs is further complicated by the significant variability in particular platforms and predictive algorithms used, as well as functional polymorphisms selected for inclusion in specific multi-gene testing assays. This has yielded significant diversity in risk estimation even for the same individual (Imai et al. 2011; Kalf et al. 2014). As most multifactorial conditions are considered complex phenotypic traits, isolated allelic variations are largely insufficient in accounting for clinical emergence, which is rather dependent on multiple environmental and epistatic drivers of phenotypic expression acting upon a polygenic susceptibility background. Summarily, considering

genetic testing results in isolation most likely profoundly limits the capacity for accurate risk assessment and stratification (Donahue et al. 2006; Kotze et al. 2013).

1.4. Population-based genetic susceptibility screening for common adult-onset NCDs

In South Africa, population-based screening for genetic diseases has historically been restricted to screening asymptomatic high-risk individuals for monogenic disorders with increased prevalence in certain local population groups due to a so-called “founder effect” (Kotze et al. 2015). Recent advances in genomic understanding have sparked a renewed interest in population-based screening for genetic susceptibility towards common adult-onset NCDs. Genetic susceptibility screening in a specific target population or subgroup will enable clinicians to facilitate the timely implementation of tailored lifestyle-based intervention strategies (“primary prevention”) as well as provide targeted pharmacotherapy to those already affected (“secondary prevention”).

These ideals echo the PSGT pathways-based approach to the genetic characterization of distinct NCD subtypes aimed at informing clinical decision making and guiding the selection of appropriate treatment (Kotze et al. 2015). In this context, “pathways” is referred to the in the context of dysfunctional regulation of cholesterol/folate/iron metabolism as well as thrombophilia implicated in CVD and related comorbidities associated with the metabolic syndrome. Wilson and Jungner (1968) initially proposed a set of screening criteria (Table 1) adopted by the World Health Organization (WHO) and long considered the gold standard for determining whether population-based screening is applicable to a particular medical condition. Effective evidence-based genetic screening has significant potential for advancing the clinical translation of emerging genomic insights into practical healthcare benefits at the population level. There is however a general consensus among public health experts that a number of important limiting factors inherent to genetics-based screening first need to be addressed before population-based testing can be recommended as routine, as previously discussed.

Table 1. World Health Organization (WHO) population-based screening criteria [Wilson and Jungner 1968].

1	Medical condition is identified as an important health concern
2	Treatment for medical condition in question is available
3	Facilities for disease diagnosis and treatment should be available and functional
4	The clinical course of the medical condition including its latent phase should be understood
5	A suitable test or examination should be available
6	Test or examination in question should be relevant to the target population
7	Natural history of disease should be adequately understood
8	Policy concerning eligibility/indications for treatment should be in place
9	Total cost attributed to finding a case economically balanced in relation to total medical expenditure (cost-effective)
10	Case finding should be viewed as an ongoing continuous process

1.5. Clinical research translation: from novel genomic test to practical healthcare implications

The successful introduction of a novel genomic test as a routine component of patient care necessitates a framework which supports a “continuum” of clinical translation from original genotype-phenotype data to powerful new genomic tools capable of improving long-term health outcomes. The PSGT model enabled by an open-innovation platform linking routine genetic testing to the generation of a secure centrally maintained online genomics database resource provides just such a framework (Kotze et al. 2015).

The “ideal” approach to genomics-based chronic disease risk assessment should be accompanied by adherence to a core set of principles which govern population-based screening approaches to common adult-onset conditions. These principles were first outlined by Wilson and Jungner in 1968 for the World Health Organization (Table 1) and later

modified by several authors and regulatory bodies (Table 2) as reviewed by Andermann et al. (2008). In this context, it is becoming accepted that a logistically feasible, cost-effective genetic screening program should respond to a particular healthcare concern identified for the target population of interest. In addition, the genetic test and corresponding intervention should be provided according to clearly defined objectives and supported by a substantial body of high-quality evidence regardless of outcome. These cardinal principles (Table 2) could provide a useful framework for the development of public healthcare policies to ensure that the necessary legislative frameworks and regulatory bodies are put in place to oversee ethical and transparent research conduct. This could minimize the potential for public apprehension and anxiety concerning loss of privacy and confidentiality, data sharing and genetic discrimination (Khoury et al. 2007).

Table 2. Adapted set of core principles governing population-specific genetic susceptibility testing for common adult-onset NCDs (adopted from review by Andermann et al. 2008).

Category	Core principle
Positioning of genomic test in relation to population-based healthcare concern	Genomic test positioned as response to an important healthcare issue which imposes significant morbidity and mortality in the target population of interest
	Frequency and phenotypic associations of risk variant known for target population
	Efficacy of corresponding intervention in target population supported by high-quality clinical evidence
Quality assurance and logistic feasibility of genomic test	Analytical validation of novel genomic test against existing laboratory standards
	Centrally maintenance of secure data storage resource
	Cost-effectiveness of genomic test in relation to corresponding intervention in target population
Ethico-legal dilemmas and cost issues	Potential for psychological harm / risk-benefit ratio
	Impact on insurability of information used for actuarial underwriting
	Willingness to pay in relation to test reimbursement

1.6. Pathology-supported genetic testing as a novel approach to personalized genomics

An effective genomics-based approach to chronic disease risk screening should reflect the limited capacity for clinical application imposed by single-gene assessment inherent to direct-to-consumer (DTC) testing in complex multifactorial disorders, which limits the

capacity for clinical translation of genomic research (Donahue et al. 2006; Imai et al. 2011; Kotze et al. 2013; Kalf et al. 2014). The consideration of genetic testing results in relation to phenotypically enriched patient data could however allow for comprehensive chronic disease risk assessment aimed at promoting the optimal use of personalized genomics to guide patient management (Kotze et al. 2013; Grant et al. 2013). The integration of molecular genomics into multidisciplinary clinical framework has proven both complex and challenging. Its correct application however has tremendous potential for advancing disease prevention and providing individualized treatment modalities targeting existing metabolic abnormalities and shared pathogenic pathways rather than merely the symptomatic manifestations of disease.

The value of genetic testing in South Africa was highlighted in a series of local articles published between 2005-2006 in the “South African Family Practice” to communicate the benefits and limitations of a range of genetic tests available to HCPs at that time. This was based on extensive studies performed in the local population over more than a decade. These studies focused on illustrating the benefits of genetic testing in well-defined clinical domains, including the prevention of adverse and potentially lethal drug reactions in patients with breast cancer (Kotze et al. 2005a). This involved the integration of genetic knowledge with existing clinical and lifestyle management strategies also for cardiovascular disease (CVD) and thrombophilia, associated with an increased risk of Alzheimer’s disease (Kotze and Badenhorst 2005d; Kotze et al. 2006) and unexplained recurrent pregnancy loss (Kotze et al. 2005a) respectively. These and subsequent studies provided a strong scientific basis for the development and implementation of a clinically integrative NCD risk management strategy in Southern Africa. The aim is to advance the clinical translation of genomic research as part of a combined open-innovation research and service delivery platform. It is against this background, based on extensive genetic research conducted in the South African setting over more than 20 years, that a novel approach to the clinical integration of

personalized genomics, termed pathology-supported genetic testing (PSGT) was developed (Kotze et al. 2015).

The PSGT model aims to overcome the limitations inherent to direct-to-consumer (DTC) genetic testing by advocating that testing results be considered within a multidisciplinary clinician-mediated framework assisted by comprehensive patient data gleamed from diverse fields of healthcare linked via the golden thread of pathology. Following informed consent from the patient, a questionnaire is used to capture data on personal and family medical conditions, medication use and side effects, lifestyle factors and pathology test results relevant to the genetic analysis performed (Kotze et al. 2013). Relevant pathological and biochemical investigation guided by pre-screening clinical assessment may provide valuable insight into existing metabolic abnormalities and underlying disease processes, applicable to single-gene testing as well as multi-locus analysis. Using the aforementioned PSGT approach, comprehensive patient data is utilized to provide an individualized report to the referring clinician, containing clinically actionable information used to guide clinical and therapeutic management. A suitable structure for the interpretation of genetic information is therefore provided for the purpose of stratifying patients into meaningful prognostic subgroups based on the identification of high-risk treatable NCD subtypes requiring a tailored approach to therapeutic intervention.

PSGT acknowledges the limitations inherent to single-variant testing to account for the phenotypic expression of a disease-associated gene. A multidisciplinary approach is used to facilitate the characterisation of treatable NCD subtypes based on the assessment of inter-related metabolic pathways and associated dysfunctions implicated in a wide range of NCDs (Figure 1). This model described by Kotze et al. (2015) allows for the development and timely implementation of tailored lifestyle-based intervention strategies which target underlying core biological dysfunctions to decrease cumulative disease risk and prevent disease progression in affected patients, in addition to affording an opportunity for pre-

clinical therapeutic mediation to prevent initial development in unaffected individuals. The PSGT approach further assists clinicians in the development of tailored pharmacogenomics algorithms aimed at predicting treatment failure and limiting exposure to hazardous and potentially lethal drug side-effects with the ultimate goal of improving patient compliance.

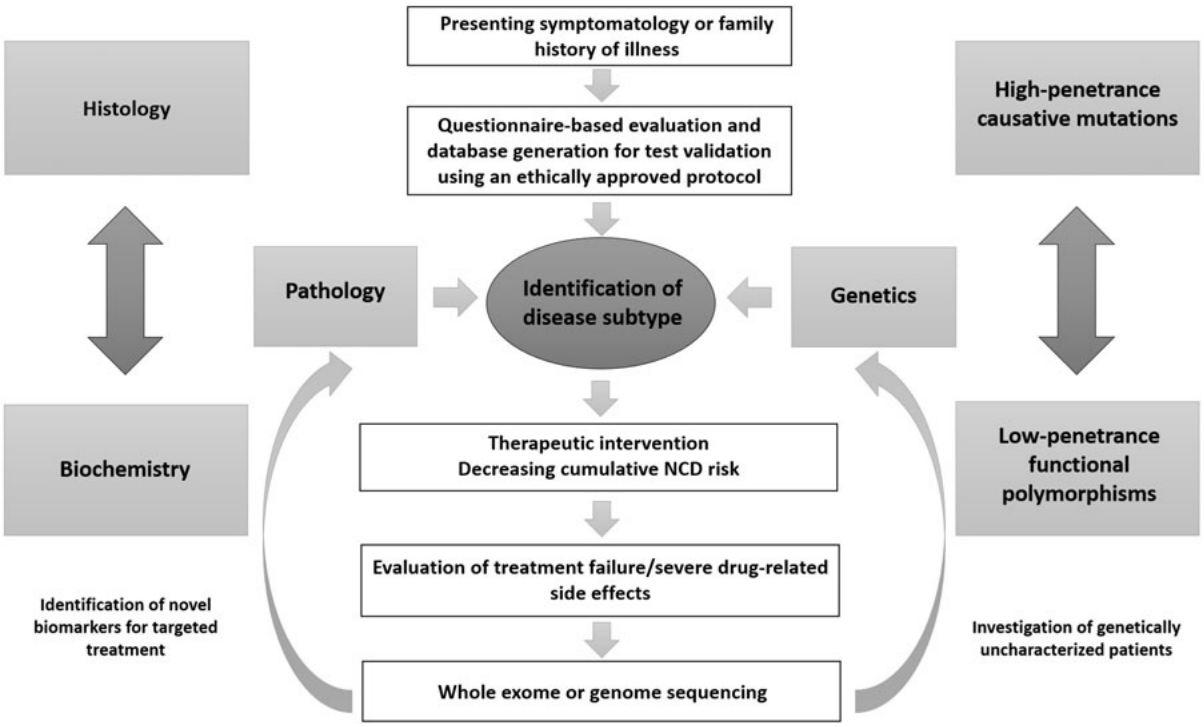

Figure 1. Description of a pathology-supported genetic testing (PSGT) strategy used to characterize treatable chronic disease subtypes and assess current as well as future risk with the goal of informing clinical and therapeutic decision making.

1.7. Use of patient databases as a resource for clinical research translation

The clinically integrative PSGT model makes use of a combined open-innovation platform linking service delivery to the generation of a secure centrally maintained research database (accessible to registered users at www.gknowmix.org). PSGT research databases for breast cancer and other chronic diseases linked via the common thread of pathology were developed to facilitate research translation at the interface between the laboratory bench and the bedside. A number of recent reviews and original articles have discussed the value of

this resource for local validation studies aimed at developing pre-screen algorithms for a range of genomic applications as well as accompanying treatment pathways. In the context of breast cancer leading the way in the application or personalised genomic medicine, the following examples of implementation contributed to the development of the genomics database resource:

1) Referral guidelines for cytochrome P450 (CYP2D6) genotyping in breast cancer patients with comorbid depression treated with a combination of tamoxifen and antidepressants (van der Merwe et al. 2012)

2) A pre-screen algorithm and corresponding reimbursement policy for gene expression profiling using the 70-gene microarray-based MammaPrint assay, shown to decrease the need for adjuvant chemotherapy in South African patients with early-stage breast cancer (Grant et al. 2013)

3) Use of microarray-based determination of human epidermal growth factor 2 (HER2) tumour status in patients with early-stage breast cancer led to extension of the MammaPrint pre-screen algorithm to also include equivocal and borderline-positive HER2-positive breast cancer patients (Grant et al. 2015).

1.8. Developing referral guidelines for genomic risk screening

The development of standardised referral guidelines for inclusion in genomics-based chronic disease screening programs is an important step towards the application of genetic testing in a goal-directed and cost-effective manner. In this context, eligibility criteria for multi-gene risk assessment could ensure that the benefits derived from extended genetic testing outweigh the potential for associated harm. Selection criteria for high-penetrance mutation screening used increase diagnostic reliability for certain Mendelian disorders and high-risk NCD subtypes are already in place based on existing diagnostic guidelines (Kotze et al. 1993;

Kotze et al. 2005a-d; Schoeman et al. 2013). In the context of impaired cholesterol homeostasis, diagnostic algorithms for FH as a severe monogenic subtype of dyslipidaemia found at an increased prevalence in certain South African population groups have been developed. This is based on family history, age of disease onset, hypercholesterolaemia from birth, and clinical features such as xanthomas characteristic of FH. Due to the clinical variability of FH, DNA testing is the method of choice for accurate diagnosis and family screening to identify high-risk individuals requiring long-term statin treatment (Kotze et al. 1994).

There is an ongoing need to develop and validate standardized referral guidelines for inclusion of eligible patients in NCD screening programs incorporating a genomics component, based on high-quality clinical evidence. The implementation of a pre-screen algorithm could serve to identify patients most likely to benefit from genetic testing, in addition to preventing unnecessary testing in cases where it is not indicated (Kotze et al. 2015). Referral guidelines for genomics-based NCD screening would conceivably be more complex than existing eligibility criteria for monogenic disorders, and may take the form of a multi-step diagnostic algorithm. Clinical factors such as a positive family history of illness and early symptom onset/clinical presentation, which play a well-established role in determining eligibility for high-penetrance mutation screening, could also prove useful in the development of referral guidelines for NCD screening incorporating both mono-variant analysis and multi-gene risk assays. This notion is supported by a growing body of evidence indicating that 1) early-onset subtypes of complex multifactorial NCDs such as major depressive disorder (MDD) have a strong genetic basis (Delport et al. 2014), and that 2) the majority of familial aggregation for conditions such as breast cancer can also be largely explained by common low- to moderate-penetrance susceptibility variants (Gracia-Aznarez et al. 2013). These and other observations provide the scientific rationale for ongoing research aimed at determining the clinical importance of clinical inquiry concerning family history beyond a purely diagnostic scope.

While randomized controlled clinical trials (RCTs) are considered the gold standard for developing new therapeutic interventions, it remains unclear whether RCTs are always indicated for confirming the clinical utility of novel genomic applications as well. RCTs require very large sample sizes to conduct, are costly and time-consuming to perform, and may therefore not be considered logistically feasible in resource-limited settings. In many cases, results from these trials may only become available once the genomic test in question is already considered obsolete. A number of alternatives have been proposed, although there is currently no consensus on what constitutes the ideal means of overcoming the limitations inherent to RCTs. The retrospective evaluation of randomized and prospectively collected patient data ("surrogate trials"), which could allow for the conditional approval of novel genomic applications under the condition that established eligibility criteria for testing formulated from sufficient high-quality evidence supporting the performance and utility of an ever-increasing number of genomic tests should already be in place. In this context, an open-innovation research and service delivery platform as described in Figure 2 may be considered ideally positioned. It allows the development of a preliminary framework aimed at promoting the successful clinical implementation of personalized genomic testing performed in a defined target population as part of novel multidisciplinary approach to NCD risk screening. Ongoing development and improvement of the PSGT research database could furthermore facilitate the development and validation of much-needed standardized referral guidelines for selection of patients most likely to benefit from genomics-based chronic disease screening programs as facilitated by the application of a pathways-based approach to the genetic characterization of complex multifactorial NCDs.

1.9. A pathways-based approach to genomics-based cardio-metabolic risk screening

The clinical implementation of a simple and cost-effective screening strategy used to assess cardio-metabolic risk could assist in predicting the onset of overt cardiovascular disease during the clinically quiescent pre-symptomatic stage (Gilstrap and Wang 2012). Existing clinical risk stratification schemes however underestimate risk for cardiovascular disease and adverse ischemic events in patients with the metabolic syndrome and/or coexisting hepatosteatosis (Dekker et al. 2005; Wannamethee et al. 2005; Khanna et al. 2013). Concerns have also been raised over the accuracy of clinical risk stratification schemes such as the Framingham risk score in predicting long-term adverse cardiac outcomes, as well as their applicability in younger patients. Increasing recognition of these important limitations has created interest in the incorporation of emerging biochemical and functional genomic markers with the goal of improving the performance of existing risk classification models. Since existing cardiovascular risk stratification methods are population-based and therefore not necessarily applicable or reach significance at the individual level (Lloyd-Jones 2010), further adaptation could prove useful in optimizing cardiovascular risk assessment in certain patient subgroups including those with the metabolic syndrome.

The importance of identifying biochemical and functional genomic markers which could add value to cardio-metabolic risk assessment and screening lies in the fact that 1) the majority of patients with established cardiovascular disease have a limited number of clinical risk factors and, 2) vascular disease and its associated comorbidities are characterized by a quiescent subclinical stage prior to the symptomatic stage. The routine implementation of a novel cardiovascular biomarker would depend on whether it is 1) accurate and reproducible, 2) easily interpretable, 3) highly sensitive or specific based on the desired outcome, 4) explains a reasonable proportion of variation for a particular trait, and 5) to what extent high-quality clinical evidence supports these assumptions. Hlatky et al. (2009) also emphasised that an ideal cardiovascular biomarker should show an incremental increase in predictive value over conventional clinical risk markers, with its assessment altering pre-test risk to

bring about a change in patient management. In this context, a specific tailored therapeutic intervention should also change the individual-specific levels of the biomarker in question, which should be equated to a reduction in absolute risk for a particular condition of interest. In addition, the incorporation of a novel biomarker as a routine component of patient care is predicated on its ability to predict cardio-metabolic risk independently and more accurately than conventional risk classification schemes.

There is significant and ongoing interest in the identification and validation of emerging biochemical risk markers for cardiovascular disease and its associated ischemic complications. In a comprehensive review of 214 meta-analyses, van Holten et al. (2013) concluded that the optimal biomarkers for new-onset CVD include those implicated in impaired lipid homeostasis and chronic inflammation, with C-reactive protein (CRP) identified as the most promising candidate risk marker. In contrast to these findings, several studies have failed to show that the addition of CRP to existing clinical risk stratification schemes, such as the Framingham risk score, significantly improves their discriminatory power and predictive capabilities in the context of cardiovascular risk assessment (Folsom et al. 2006; Shah et al. 2009). Therefore, despite the potential for certain biomarkers to predict future risk for adverse cardiac events, added value beyond conventional clinical risk markers is minimal, with this approach failing to benefit risk reclassification in the majority of patients (Melander et al. 2009). Elevated levels of CRP and interleukin-6 (IL-6) reflect a core pathogenic process (i.e. chronic inflammation) implicated in the pathogenesis of vascular disease and its associated comorbidities. In contrast, other circulating biomarkers such as B-type natriuretic peptide (BNP) reflect a consequence of cardiac injury, and have been shown to outperform CRP in the prediction of future ischemic events and cardiovascular mortality.

A multi-marker approach has been proposed as a means of overcoming the limitations inherent to the assessment of individual biochemical markers of cardio-metabolic risk (Wang et al. 2006; de Ruijter et al. 2009). Several studies have shown that the assessment of

multiple cardiovascular risk markers improves clinical risk prediction and facilitates accurate risk reclassification (Zethelius et al. 2008; Blankenberg et al. 2010). These studies have however been criticised due to the inclusion of patients with existing cardiovascular disease as well as the restriction of the study population to a specific sex and age group. In contrast, conflicting studies have shown that, despite the significant association between certain biomarkers such as brain natriuretic peptide (BNP) and the urinary albumin-to-creatinine ratio, these markers fail to improve the diagnostic capabilities of existing clinical risk stratification (Wang et al. 2006; Melander et al. 2009). It seems intuitive that the assessment of an increasing number of biomarkers would add additional value to clinical risk classification. A limited number of biomarkers (<10) may, however, be sufficient in cases where there is little or no correlation between these variables. This notion needs to be validated in the routine clinical arena prior to its justification as a sufficient and validated approach to cardio-metabolic risk assessment (Wang 2011).

In comparison to biochemical markers, the assessment of functional genomic markers as a component of cardiovascular risk screening offers distinct advantages as well as important limitations. For example, the fact that a certain genetic risk profile is static infers the ability to assess individual susceptibility long before disease onset is clinically overt. However, an associated disadvantage is that the assessment of functional genomic markers does not provide sufficient information on whether subclinical vascular disease has progressed over time. In this context, certain biomarkers may reflect dysfunction in metabolic pathways early on in the disease process, while others provide greater insight into aberrant biological activity as clinical risk progresses. Despite the fact that many genetic risk markers are strongly associated with the onset and progression of cardiovascular disease, effect sizes are often small, with the inclusion of such biomarkers as a component of existing models failing to significantly improve their predictive capabilities and diagnostic accuracy. It is therefore imperative to establish whether the assessment of functional genomic variants in relation to context-dependent determinants of clinical expression adds value to existing

clinical risk assessment in order to determine whether genetic testing truly adds independent value to patient care (Humphries et al. 2004).

A number of variants in genes such as *APOE* have been identified as putative susceptibility markers for cardiovascular disease, adverse ischemic events and cardiac mortality. In particular, a number of case-control and retrospective studies performed since 2007 have provided convincing evidence supporting an association between the 9p21 locus and increased risk for coronary artery disease due its primary association with an “atherosclerotic phenotype”. The association between the 9p21 locus and increased cardiovascular disease is independent of other metabolic risk traits including obesity, hypertension and dyslipidaemia, with its assessment shown to improve clinical risk prediction in the context of coronary artery disease (Holdt and Teupser 2012; Chan et al. 2013). In a genome-wide analysis, Surakka et al. (2015) demonstrated that polymorphic variants in genes such as *APOE* known to be implicated in impaired lipid homeostasis explain a sufficient extent of inter-individual variance in quantitative metabolic phenotypes associated with cardiovascular disease. The assessment of candidate low-frequency variants in genes such as *CD300LG* and *TM6SF2* also explained a sufficient additional degree of variance in lipid-related traits. The authors concluded that the imputation of uncommon risk variants could offer a more cost-effective approach to resequencing using genomic applications including whole exome sequencing (WES). Next-generation sequencing has been proposed as a means of validating the added benefits of existing genetic risk markers identified in previous genome-studies, as well as allowing for the identification of novel causative mutations of large clinical effect.

1.9.1. The role of *APOE* genotyping in the assessment of cumulative risk for cardiovascular disease and dementia

It has been estimated that up to 50% of inter-individual variation in serum lipid profiles is inherited. In this context, polymorphic variation in the *APOE* gene is considered an important

determinant of altered cholesterol levels associated with increased risk for premature cardiovascular disease (Pablos-Mendez et al. 1997). Three different *APOE* alleles (ε-2/e-3/ε-4) encode the three corresponding protein isoforms which differ in their binding affinity for cholesterol (E4>E3>E2) (Johnson et al. 2014). In accordance with the physiological effects of APOE on hepatic lipid metabolism and clearance, the cholesterol-raising ε-4 allele has been identified as an important genetic risk factor for metabolic syndrome, ischemic heart disease, stroke and Alzheimer's disease (AD) (Song et al. 2004; Sima et al. 2007; Verghese et al. 2011). *APOE* ε-4 is considered a major genetic risk factor for dementia as well as a determinant of inter-patient heterogeneity in clinical presentation, prognosis and therapeutic outcomes in AD due to its pleiotropic effects on disease risk, including an association with impaired lipid homeostasis and dyslipidaemia (Figure 2). These observations are in accordance with existing evidence which suggests that CVD and AD share a common pathogenic basis. As illustrated in Figure 3, a number of mechanisms, including chronic inflammation and impaired lipid homeostasis, are shared by both AD and CVD.

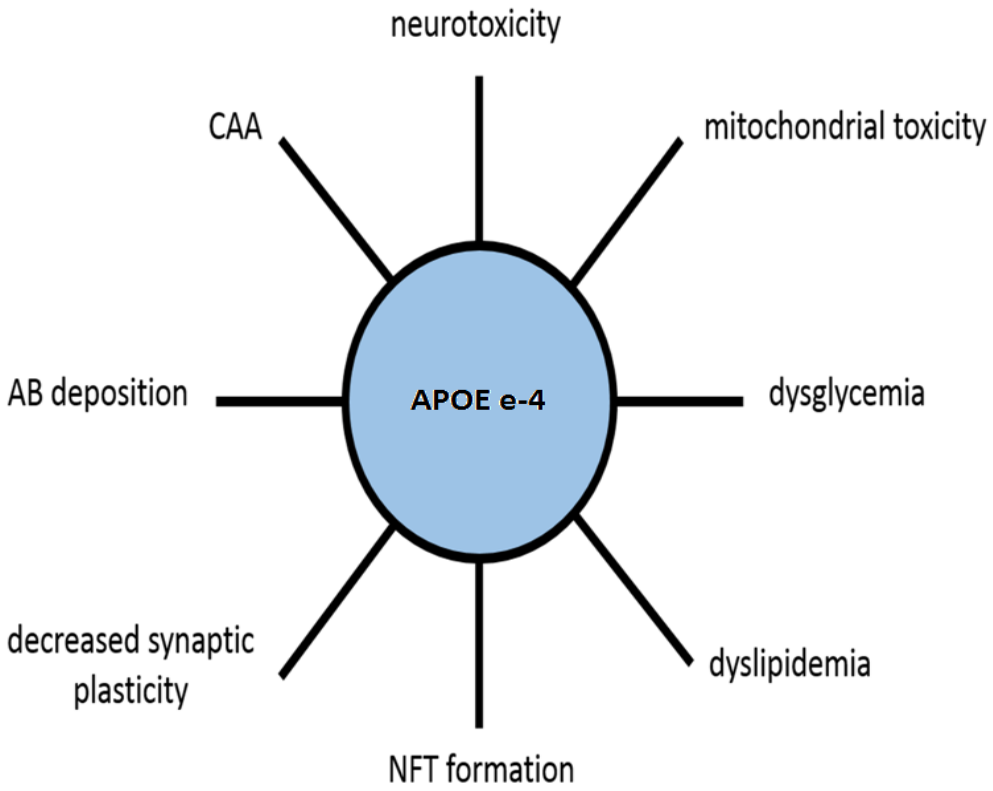

Figure 2. Relationship between *APOE* ε-4 and pathogenic mechanisms implicated in Alzheimer's disease (AD).

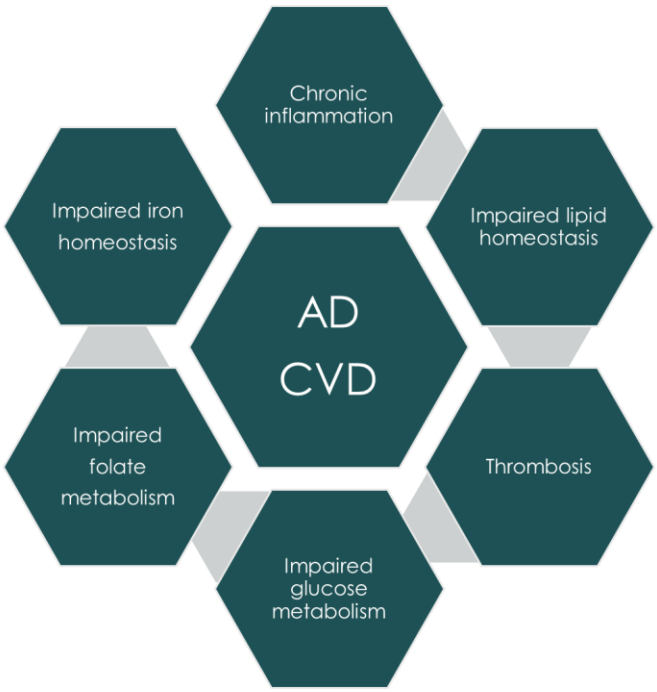

Figure 3. Common pathogenic mechanisms shared by Alzheimer's disease (AD) and cardiovascular disease (CVD).

Dyslipidaemia is considered an important modifiable risk factor for a number of age-related multifactorial conditions including CVD and AD. It has been suggested that *APOE* genotype modifies the association between dyslipidaemia and AD risk (Dufouil et al. 2005). *APOE* ε-4 is also an important determinant of clinical heterogeneity in patients with dementia, being associated with a primarily amnestic subtype and conversion from mild cognitive impairment to AD. It is interesting to note that although the *APOE* ε-4 allele accelerates disease progression in AD, patients who present atypically or at an earlier age seldom carry the risk-associated allele (Khachaturian et al. 2004; Lam et al. 2013). In patients with AD, *APOE* ε-4 allele carriage is strongly correlated with amyloid beta deposition in the leptomeningeal arteries resulting in cerebral amyloid arteriopathy (CAA). This common (80%–90%) neuropathological characteristic of AD is closely related to structural white matter abnormalities, arising secondary to endothelial dysfunction, basement membrane thickening, lobar haemorrhages, and vessel stenosis (McCarron and Nicoll 2000). The presence of CAA in *APOE* ε-4 allele carriers may play a synergistic role in disease progression (Love 2004). Conflicting evidence however suggests that, while *APOE* genotype is associated with the development of CAA, it is primarily a determinant of cerebral localization, which contributes towards focal pathology (Hirono et al. 2000; Kljajevic et al. 2014). Clinical heterogeneity in AD is partly a reflection of localized cortical abnormalities, including atrophy and hypoperfusion. Executive dysfunction and visuospatial symptoms are associated with synucleinopathy and microvascular pathology, focal temporal atrophy with an amnestic picture, and parietal pathology with disordered visuospatial processing. Given the reciprocal influence of *APOE* genotype and CAA on parenchymal as opposed to vascular Aβ aggregation, further investigation of whether the combined presence of these factors influences clinical and therapeutic heterogeneity in AD is also warranted (Liu et al. 2013).

The assessment of *APOE* genotype could also prove useful in identifying a subgroup of patients at increased risk for adverse cardiac events (Peña et al. 2001). In this context, the *APOE* ε-4 allele has been associated with the development of hypertension, coronary artery

disease and ischemic vascular complications in patients at increased cardio-metabolic risk (Dzimiri et al. 1999). Khan et al. (2013) also demonstrated a dose-responsive positive correlation between the *APOE* ε-4 genotype and increased low-density lipoprotein (LDL) cholesterol levels as well as carotid intima media thickness. The authors however concluded that the relationship between the ε-2 allele and ischemic stroke required further investigation. In a recent study of six candidate variants implicated in increased cardio-metabolic risk, only the *APOE* ε-4 allele was independently associated with dyslipidaemia, with homozygotes exhibiting a more than three-fold increased risk for this quantitative trait compared to non-carriers (Smolková et al. 2015). The aforementioned findings support the role of *APOE* genotype as a determinant of inter-individual variation in lipids profile and dyslipidaemia risk.

Differentiating between hypercholesterolaemic patients with monogenic dyslipidaemias including FH and those carrying the modifier *APOE* ε-4 allele is important, as treatment strategies would differ. A relatively high incidence of mild cognitive impairment has recently been reported in FH patients, possibly due to early exposure to elevated cholesterol or low-density lipoprotein dysfunction (Zambón et al. 2010). The importance of the availability of a DNA test for diagnosis of FH as a high-risk cardiovascular subtype (Kotze et al. 2003) is emphasized by the clinical variability of this condition, which complicates clinical diagnosis (Kotze et al. 1993, 1994). Patients with FH require long-term treatment with lipid-lowering medications to reduce their risk of ischemic vascular disease. In contrast, *APOE* ε-4 carriers are less responsive to statin therapy (Baptista et al. 2011). Future investigation is required to elucidate the effect of statin therapy in ε-4 allele carriers with AD who are normocholesterolemic (Caballero and Nahata 2004). The beneficial role of statins in lowering dementia risk appears to be independent of APOE genotype, whereas the association between hyperlipidemia and increased dementia prevalence is only evident in patients without AD and in ε-4 allele non-carriers. Clinicians should therefore be aware of the genetic tests available which could assist in differentiating between FH and other forms of

dyslipidaemia where *APOE* polymorphisms could play a contributory role in disease pathogenesis (Table 3).

Table 3. Comparison between high-penetrance diagnostic testing for familial hypercholesterolemia and a combined approach to cardiovascular risk screening and management based on the assessment of clinically actionable low- to moderate penetrance variants implicated in vascular disease including those associated with dyslipidaemia.

Test	Indications for testing	Clinical application
Familial hypercholesterolemia test (LDLR gene mutations)	Increased pre-treatment total and low-density lipoprotein cholesterol levels Family history of early-onset cardiovascular disease Clinical signs of familial hypercholesterolemia: tendon xanthomas, xanthelasma, corneal arci etc.	Used to confirm a suspected diagnosis of familial hypercholesterolemia
Chronic disease risk screening utilizing a CVD multi-gene risk assay	Normal or abnormal lipid levels which can be accompanied by impaired glucose tolerance or other biochemical abnormalities (increased homocysteine levels, elevated C-reactive protein, etc.) Increased metabolic risk including obesity, hypertension and dysglycaemia	Used to facilitate disease prevention through cumulative risk reduction for cardiovascular disease and associated comorbidities guided partly from a genetic background
Familial hypercholesterolemia test and CVD test	Combination of the abovementioned indications	Used to characterize treatable dyslipidaemia subtypes and improve case finding of familial hypercholesterolemia in patients for whom dyslipidaemia cannot be accounted for by clinically actionable variants incorporated into the multi-gene CVD risk assay

APOE ε-4 carriers appear to be more vulnerable to the deleterious effects of excessive alcohol consumption, cigarette smoking, a sedentary lifestyle, and high dietary intake of saturated fat, suggesting interventions targeting these risks may be beneficial in decreasing risk aimed at AD prevention (Kivipelto et al. 2008; Valenti et al. 2014). Lowering of cumulative risk aimed at AD prevention is very important in *APOE* ε-4 allele carriers, as the benefits associated with many lifestyle-orientated interventions, including regular physical exercise and supplementation with omega-3 fatty acids, may be less pronounced or absent in AD patients carrying the *APOE* ε-4 allele (Table 4). Early detection of a genetic predisposition for AD is therefore considered an important added benefit of *APOE* genotyping when applied in the context of CVD risk. The clinical application of *APOE* genotyping as part of a multidisciplinary approach to chronic disease risk screening could assist clinicians in the implementation of tailored therapeutic interventions in dyslipidaemic patients. Given the relatively high frequency of the ε-4 allele in the general population globally (30-40%), *APOE* genotyping could have significant implications for population-based risk screening to inform public healthcare policies (Eisenberg et al. 2010).

Table 4. Summary of findings from studies which investigated whether *APOE* genotype affects the benefits of lifestyle-based interventions in relation to Alzheimer's disease risk.

Study design	Lifestyle intervention	Clinical interpretation	References
Prospective cohort study	Dietary antioxidant intake	Higher dietary antioxidant intake decreases risk of Alzheimer's disease irrespective of *APOE* genotype	Engelhart et al. 2002
Prospective study	Physical activity	Physical activity attenuates the effects of genetic risk on episodic memory in individuals free of dementia at baseline	Ferencz et al. 2014
Prospective study	Fish high in omega 3 fatty acids more than two times a week	Regular consumption of foods rich in omega 3 fatty acids reduces Alzheimer's disease risk in *APOE* ε-4 non-carriers	Huang et al. 2005
Prospective study	Dietary antioxidant intake (vitamin E)	Adequate dietary intake of vitamin E may reduce Alzheimer's disease risk in *APOE* ε-4 non-carriers without dementia at baseline	Morris et al. 2002
Prospective study	Physical activity	Physical activity normalizes serum lipid profiles and related metabolic abnormalities in *APOE* e-4 allele carriers as well as mitigating the deleterious effects of advancing age on multiple biochemical disturbances associated with dementia risk	Okonkwo et al. 2014
Randomized control trial	Omega 3 fatty acid supplementation	No effect on clinical outcomes in patients with mild to moderate Alzheimer's disease	Quinn et al. 2010

Up to 80% of interpatient variation in the therapeutic response to medications commonly used to treat AD may be explained by genotype (Cacabelos 2008). Multiple studies have demonstrated that AD patients who carry the *APOE* ε-4 allele show a differential response to conventional pharmacotherapy. *APOE* genotype is known to modulate the therapeutic effects of acetylcholine esterase inhibitors (AChEI) in patients with AD (Bizzarro et al. 2005; Hanson et al. 2015). In this context, findings from several studies suggest that ε-4 allele carriers respond more favourably to donepezil (Choi et al. 2008) and rivastigmine (Han et al. 2012). Evidence concerning the role of *APOE* genotype as a determinant of the efficacy of drugs such as galantamine is however inconclusive (Babić et al. 2004; Suh et al. 2006).

APOE is also considered an emerging therapeutic target in the management of vascular disease and dementia. For example, novel treatment modalities such as retinoid X receptor (RXR) agonists could pose therapeutic benefit in hypercholesterolaemic patients at risk for or diagnosed with AD by modulating APOE lipidation and secretion (Boehm-Cagan and Michaelson 2014; Koldamova et al. 2014; Zhao et al. 2014). The observation that RXR-receptor activation induces APOE up-regulation supports the notion that aberrant retinoid metabolism is reciprocally related to impaired cholesterol homeostasis implicated in the pathogenesis of AD (Akram et al. 2010). The putative role of APOE in amyloid-beta (Aβ) metabolism also infers that retinoids could reduce intra-cerebral peptide deposition and neuritic plaque formation (Cao et al. 2007). Retinoids are also known to exert beneficial anti-inflammatory effects in patients with or at risk for AD by reducing glial production of certain cytokines and chemokines (Sodhi and Singh 2014). Findings from phase III RCTs also show that treatment with the targeted antibody bapineuzumab prevents amyloid beta deposition and decreases levels of phosphorylated tau protein in *APOE* ε-4 allele carriers but not non-carriers. These findings suggest that isoform-dependent *APOE* variation influences the efficacy of tailored pharmacotherapy in patients with AD. Side-effects related to bapineuzumab, including vasogenic cerebral oedema and micro-haemorrhages, occur more frequently in *APOE* ε-4 carriers. Collectively, these observations support the use of *APOE*

genotyping to guide the selection of appropriate existing and emerging therapeutic modalities in patients affected by AD.

2.10. Rationale and aims of study

There is increasing appreciation for the limitations inherent to single-gene testing in the context of genomics-based risk assessment. This is due to the context-dependent nature of clinical expression evident for most common low-moderate penetrance susceptibility variants. This has led to the development and clinical application of multi-gene panels used to guide risk reduction intervention of chronic multifactorial NCDs. The use of multi-gene risk assays in conjunction with relevant clinical and biochemical assessments could assist clinicians in the identification of treatable NCD subtypes, with the goal of stratifying patients into meaningful prognostic subgroups. The ultimate goal is to help inform clinical decision making and facilitate the timely implementation of tailored lifestyle-based intervention strategies. In order to promote the successful implementation of personalized genomic testing in general medical practice, there is however a pressing need to develop standardized referral guidelines or pre-screen algorithms for participation in NCD screening programs. These criteria could serve to stratify patients based on individual perceived benefit, in addition to limiting unnecessary initial referral in cases where it is not indicated.

The amount of information which can be derived from the assessment of cardiovascular risk markers is dependent on where they fit in along the spectrum of pathological processes underlying disease progression from a subclinical to overt symptomatic stage. The genotypic approach to the assessment of the *APOE* polymorphism offers several advantages compared to phenotypic screening, as noted by Eichner et al. (2002). Despite the well-established associations between the *APOE* polymorphism and genetic risk for both CVD and AD, there is a pressing need to develop well-defined eligibility criteria used to identify a target population set to derive optimal benefit from *APOE* genotyping. The *APOE* polymorphism (ε-2/ε-3/ε-4) which provides a genetic link between CVD and AD was

identified as an important clinical endpoint in cardio-metabolic risk management. Realization that the gene effect may be exacerbated by lifestyle risk factors such as smoking and alcohol consumption, while the risk of disease development or progression could be reduced by regular exercise and maintaining a healthy body weight, supports the inclusion of *APOE* genotyping in chronic disease screening programs. However, the lack of a well-defined target population set to derive the greatest benefit from *APOE* genotyping, as well as clear treatment paths based on early detection of a subgroup of dyslipidaemics at increased risk of AD, have limited the widespread use of this biomarker in clinical practice.

In this context, the aim of the study was to determine whether the clinical application of a study questionnaire could prove useful in assisting clinicians to identify patients eligible for *APOE* genotyping, as well as how results gathered from such testing could be used to optimize patient care. Insight gathered as a result of this study may provide the rationale for development of a novel pre-screen algorithm, which may lead to conditional approval by local medical aid schemes for reimbursement of chronic disease screening including *APOE* genotyping. Implementation of the PSGT approach to *APOE* genotyping could assist clinicians in optimizing cardiovascular risk management in a treatable subgroup of dyslipidaemic patients at increased risk of CVD and AD to facilitate cumulative risk reduction and improve clinical and therapeutic outcomes.

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CHAPTER 2

SUBSTUDY 1

Title: Clinical relevance of apolipoprotein E genotyping based on a family history of Alzheimer's disease

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Status: Reproduced with approval

Contribution of the Candidate:

The first author wrote the article after selecting relevant data pertaining to eligible study participants who were genotyped for the *APOE* polymorphism. Ethics approval forms signed by the participants and laboratory results available in the files were checked for accuracy during transfer to the research database. Statistical analysis was performed by the candidate based on a course using R Studio and checked by a qualified statistician. Lifestyle, pathology and genetic data were interpreted and discussed in a clinical context in keeping with the research aims and hypothesis.

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Clinical Relevance of *Apolipoprotein E* Genotyping Based on a Family History of Alzheimer's Disease

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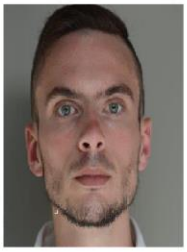

H. K. Lückhoff

Abstract: Having a family history of Alzheimer's disease (AD) may potentiate cumulative risk associated with phenotypic expression of the ε-4 allele of the *apolipoprotein E* (*APOE*) gene. In this study, we compared the genotype distribution and allele frequencies of *APOE* ε-2 (rs7412) and ε -4 (rs429358) in 537 South African individuals participating in a chronic disease screening program, in order to establish whether AD family history modulates the expression of their dyslipidemic effects. Significant differences in the genotype distribution for *APOE* ε-2 (p=0.034) as well as *APOE* ε-4 (p=0.038) were found between study participants with (n=67) and without (n=470) a family history of AD. LDL cholesterol levels were inversely associated with physical activity in the study group with a positive family history of AD (p<0.001) but not in those with a negative family history of AD (p=0.257). Similar to its existing use in the diagnosis of monogenic dyslipidemias such as familial hypercholesterolemia, clinical inquiry regarding family history was identified as an important determinant of eligibility for *APOE* genotyping performed in the context of chronic disease risk management. To our knowledge, this is the first study to demonstrate the modulating influence of AD family history on expression of a dyslipidemic phenotype associated with the *APOE* ε-4 allele. Our findings provide the scientific rationale supporting a novel clinical application for *APOE* genotyping as a means of identifying a genetic subgroup of dyslipidemic patients set to derive the greatest benefit from early lifestyle-based interventions aimed at decreasing cumulative risk for cardiovascular disease and prevention of AD later in life.

Keywords: Alzheimer's disease, apolipoprotein E, dyslipidemia, family history, genotype-phenotype association, personalized genomics.

INTRODUCTION

Both a positive family history and the ε-4 allele of the *apolipoprotein E* (*APOE*) gene are well-established risk factors for sporadic late-onset Alzheimer's disease (AD) [1, 2]. Recent evidence suggests that having a family history of AD contributes to cumulative risk associated with phenotypic expression of the *APOE* ε-4 allele in an additive or synergistic manner [3]. A multidisciplinary approach to risk management is currently indicated in light of the significant heterogeneity which characterizes genetic susceptibility towards AD.

While a maternal family history of AD as well as inheritance of the *APOE* ε-4 allele have been associated with regional cerebral atrophy, grey matter pathology observed in cognitively normal individuals with a first-degree family history has also been demonstrated independent of *APOE* genotype [4-6]. Okonkwo *et al.* [7] further showed that, although neither of these risk factors contributed independently to the development of atrophy, an interactive effect was apparent in specific AD-vulnerable cerebral regions. Functional magnetic resonance imaging (fMRI) studies have also demonstrated independent as well as additive effects on neurocognitive functioning [8-11]. Similarly, while diffusion tensor imaging (DTI) studies do not support the independent contribution of *APOE* genotype towards impaired microstructural white matter integrity, evidence for an interactive effect with AD family history has emerged [12-14]. Although the *APOE* ε-4 allele is associated with neurofibrillary tangle formation, Mosconi *et al.* [15] reported increased fibrillary beta-amyloid burden in cognitively normal individuals with a family history of AD independent of *APOE* genotype. These findings collectively suggest that the pathophysiological implications of the interaction between AD family history and *APOE* genotype are context-dependent and modified by non-genetic factors which remain incompletely understood.

Impaired cholesterol homeostasis associated with the *APOE* ε-4 allele promotes atherogenesis and contributes towards the development of cardiovascular disease (CVD), ischemic stroke and AD [16, 17]. It has recently been proposed that the *APOE* ε-2 allele may be neuroprotective and

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decreases incident risk for AD by increasing beta-amyloid clearance [18]. However, its association with hypertriglyceridemia and related features of the metabolic syndrome as risk factors for cognitive decline and dementia suggests a deleterious effect [19, 20]. When performed within a multidisciplinary clinically orientated framework, *APOE* genotyping may be useful to identify a high-risk genetic subgroup of dyslipidemic patients with a known family history of AD set to derive the greatest benefit from the timely implementation of individually tailored lifestyle-based intervention strategies aimed at decreasing cumulative cardiovascular risk to prevent the development of AD later in life [21, 22]. The clinical relevance of inquiry concerning family history to determine eligibility for diagnostic testing aimed at identifying high-penetrance causative mutations implicated in monogenic disorders such as familial hypercholesterolemia (FH), a severe subtype of dyslipidemia found at a 5-10 times increased prevalence in the Afrikaner population, is well-established in South Africa [21]. A diagnosis of FH necessitates the early initiation of pharmacotherapy to effectively combat disease risk, while *APOE* ε-4 carrier status by itself does not automatically imply that patients require lipid-lowering medication. Notably, a relatively high incidence of mild cognitive impairment has been reported in patients with FH [23]. Although selection criteria for genetic testing of FH already exist based on established diagnostic algorithms [24], similar referral guidelines for inclusion in chronic disease screening programs which incorporate testing for risk-associated functional polymorphisms in low-penetrance genes such as *APOE* are currently lacking [25]. A greater understanding of where additional genetic information could be applied within the context of existing diagnostic and treatment algorithms may ensure that the benefits derived from testing outweigh potential risks as well as limit unnecessary genetic testing.

Growing evidence supports the notion that having a family history of AD promotes the progression of characteristic neuropathology associated with the *APOE* ε-4 allele. However, to what extent the expression of a dyslipidemic phenotype in ε-4 allele carriers is similarly influenced by AD family history remains uncertain. In this study, the individual and combined genotype as well as allele frequency distributions for *APOE* ε-2 (rs7412) and ε-4 (rs429358) were compared between study participants with and without a family history of AD. We further investigated whether expression of the well-established dyslipidemic effects of these polymorphic variants was modified or influenced by an AD family history. Insights gained from the present study may facilitate the development of referral guidelines for *APOE* genotyping in order to limit unnecessary testing, as well as provide this option to those set to derive the greatest benefit when performed as part of chronic disease risk management.

METHODS

Ethical approval for the study was granted by the Health and Research Ethics Committee (HREC) of Stellenbosch University under the project number N09/08/224. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Written informed consent for participation in the research component of a chronic disease screening program was obtained from all study participants.

Selection of Study Participants

The records of 579 South African patients prospectively enrolled in a chronic disease risk management program following referral by a private practicing clinician or attendance of Wellness Days at the workplace or other venues over a five year period (2009-2014) were available in a centrally maintained database (accessed at www.gknowmix.org), linked to the research component of a pathology-supported genetic testing service [21, 25]. The data set included 537 Caucasians of European descent, 27 Coloured individuals of Mixed Ancestry, and 15 Black Africans. from the diverse South African population. To exclude ancestral background as a potential confounder, and given the small number of non-Caucasian individuals who provided informed consent for research, comparative analyses were performed only in the Caucasian study group, based on the presence (67) or absence (470) of a known family history of AD. Full lipid profiles were determined for a subgroup of 276 (51%) study participants.

Questionnaire-Based Clinical and Lifestyle Assessment

Prior to enrollment in the chronic disease screening program, all prospective study participants were asked to complete the ethically approved study questionnaire (available at www.gknowmix.com) developed in collaboration with a registered dietician and approved by the HREC at Stellenbosch University. This questionnaire was used to document socio-demographic information, clinical data such as body mass index (BMI), lifestyle factors (current smoking status, physical activity level, alcohol consumption and dietary fat intake) as well as medication use (statins). In patients who reported a positive family history of AD, the relation to the affected individual was also documented in addition to age of onset. Alcohol intake was differentiated into abstain, occasionally (1-2 units/week), 1-13 units/week, 14-21 units/week and ≥22 units/week. Physical activity was categorized as none/occasionally, low (once/week), moderate (2-3 times/week) or high (≥4 times per week). Study participants were also asked how frequently they ate certain foods rich in saturated or trans-fats over the course of the preceding three months. This was used to derive a fat score, considered to be moderate when ranging between 22 and 26, low between 16-21, very low if <16, and high if ≥27.

APOE Genotyping

Genomic DNA was extracted from whole blood (QIAamp® DNA Midi-kit) or saliva samples (Oragene® reagents). Conventional polymerase chain reaction (PCR) followed by direct DNA sequencing was performed for detection of the *APOE* ε-2 and ε-4 alleles in five internal control DNA samples (K1-K5). Direct sequencing of the PCR fragments resulted in the identification of the wild type, heterozygous and homozygous genotypes for the polymorphic variants of interest. These samples were used for analytical validation of high-throughput genotyping in our laboratory using TaqMan® SNP Genotyping Assays (Applied Biosys-

tems, Werterstadt, Germany) performed on the Corbett Rotor-Gene™ 6000 series Multiplexing System.

Statistical Analysis

All statistical analyses were performed using the R Studio software and R package *genetics*, freely available from http://www.r-project.org. The baseline characteristics of the study population were described and compared between study participants with and without a family history of AD. Qualitative characteristics were described using cross-tabulation and frequency tables and compared between study groups using the Pearson's Chi-squared or Fisher's exact test as appropriate. Quantitative phenotypes were described as the means along with standard deviations (SD) and compared between study groups using a Student's t-test assuming normality of distribution. In cases where quantitative outcomes showed a non-symmetrical distribution, such variables were log-transformed for analyses. If the best-fitting log-transformed model still showed a non-normal distribution, these were described as the median with interquartile range (IQR) and compared between study groups using the Wilcoxon signed rank test. Individual and combined genotype as well as allele frequency distributions for *APOE* ε-2 and ε -4 were estimated from allele counts and Hardy-Weinberg equilibrium (HWE) assessed using an the exact test. *APOE* ε-2 as well as ε -4 homozygotes were initially grouped together with heterozygotes for subsequent analyses, owing to the small sample sizes for the former genotype group. Before including genetic information in the statistical models used for genotype-phenotype association studies, potential confounders were identified by comparing the distribution of these variables between genotype groups considering both *APOE* ε-2 and ε-4 genotypes. Age, sex, BMI, alcohol intake, current smoking status, physical activity levels and statin use did not differ between these groups for either genotype, and were thus not adjusted for in subsequent analyses. Linear regression analysis was performed in order to assess the influence of *APOE* genotype and AD family history on lipid profiles in both study groups. A p-value of <0.05 was seen as statistically significant with non-significant trend associations noted where applicable.

RESULTS

Four of the 27 (15%) study participants of Mixed Ancestry (aged 26-66) reported a family history of AD, while none of the 15 patients of African ancestry (aged 29-54) noted a positive family history. The clinical characteristics of Caucasian study participants selected for comparative analyses are shown in (Table **1**), stratified based on the presence or absence of a family history of AD. No significant difference in socio-demographic and clinical characteristics, lifestyle and dietary habits, lipid profiles or statin use were noted between the two study groups. This also held true when comparing the subgroup of study participants for whom full lipid profiles were determined (n=276) with those where biochemical

Table 1. Comparison of clinical characteristics between study participants with (n=67) and without (n=470) a family history of AD.

Characteristics	Positive AD Family History (n=67)	Negative AD Family History (n=470)	Unadjusted *P*-value
Age (years)	44.62 (12.14)	46.61 (12.00)	0.21
Sex (male/female)	15/52	140/330	0.25
Body Mass Index (kg/m²)	26.77 (5.91)	26.96 (5.82)	0.78
Alcohol intake			
Abstain	16 (24%)	108 (24%)	0.70
1-2 units occasionally	29 (43%)	182 (40%)	
1-13 units/week	20 (30%)	139 (31%)	
14-21 units week	2 (3%)	22 (4%)	
≥22 units/week	0 (0%)	1 (1%)	
Physical activity			
None/occasionally	21 (31%)	139 (30%)	0.76
Low (once/week)	8 (12%)	37 (8%)	
Moderate (2-3 times/week)	21 (31%)	174 (38%)	
High (≥4 times/week)	17 (25%)	113 (24%)	
Fat score (units)	16.55 (7.87)	16.29 (8.15)	0.80
Current smoker (yes/no)	6/61	51/401	0.20
Statin use (yes/no)	2/65	30/440	0.41
Total cholesterol (mmol/L)	5.56 (1.06)	5.43 (1.05)	0.45
HDL cholesterol (mmol/L)	1.49 (0.49)	1.43 (0.43)	0.48
LDL cholesterol (mmol/L)	3.51 (0.83)	3.42 (0.94)	0.58
Triglycerides (mmol/L)	1.15 (0.75)	1.26 (0.92)	0.46

analysis was either not performed or cases where only partial lipid profiles were determined (n=261).

Relationship Between BMI, Lifestyle Habits and Lipid Profiles

BMI was positively correlated with triglyceride ($p<0.001$) as well as low-density lipoprotein (LDL) cholesterol levels ($p=0.004$) and negatively correlated with high-density lipoprotein (HDL) cholesterol levels ($p<0.001$) in 34 study participants with a family history of AD. In 247 study participants without a family history of AD, a similar positive relationship between BMI and triglyceride ($p=0.001$) as well as LDL cholesterol levels ($p=0.039$) and negative relationship with HDL cholesterol levels ($p<0.001$) was noted. Dietary intake of saturated and trans-fats was positively correlated with LDL cholesterol ($p=0.001$) and inversely related to HDL cholesterol levels ($p=0.002$) only in study participants with a positive family history of AD. Regular physical activity was associated with a significant decrease in serum triglyceride levels in both study groups ($p=0.01$). A significant inverse association between LDL cholesterol and physical activity was similarly noted, for study participants with a positive family history of AD ($p<0.001$) but not those with a negative family history of AD ($p=0.257$) (Fig. **1**).

Genotype Distribution and Allele Frequencies for *APOE* ε-2 and ε-4

Genotype distribution and allele frequencies for *APOE* ε-2 and ε-4 together with unadjusted HWE p-values are shown in (Table **2**). A significant difference in the genotype distribution was noted for *APOE* ε-2 ($p=0.034$) as well as *APOE* ε-4 ($p=0.038$) between study participants with and without a family history of AD. The genotype distribution for *APOE* ε-2/ε-4 also differed significantly between the two study groups ($p=0.012$). No significant difference in the allele frequencies for *APOE* ε-2 ($p=0.097$) and *APOE* ε-4 ($p=0.164$) were noted between study participants with and without a family history of AD.

Influence of AD Family History on the Dyslipidemic Effects of *APOE* ε-2 and ε-4

The *APOE* ε-4 allele was associated with elevated total cholesterol levels in study participants with a positive family history of AD ($p=0.050$) but not those with a negative family history ($p=0.93$). Similarly, a marginal positive association was noted between the ε-4 allele and increased LDL cholesterol levels in study participants with a positive family history ($p=0.070$) but not those with a negative family history of AD ($p=0.583$). The *APOE* ε-2 allele was further associated with a significant elevation in triglyceride levels in study participants without a family history of AD ($p=0.014$) but not those with a positive family history ($p=0.410$). HDL cholesterol levels did not differ significantly based on APOE genotype for either study group ($p>0.05$).

DISCUSSION

APOE genotyping performed as part of chronic disease risk assessment is generally not recommended, since specific

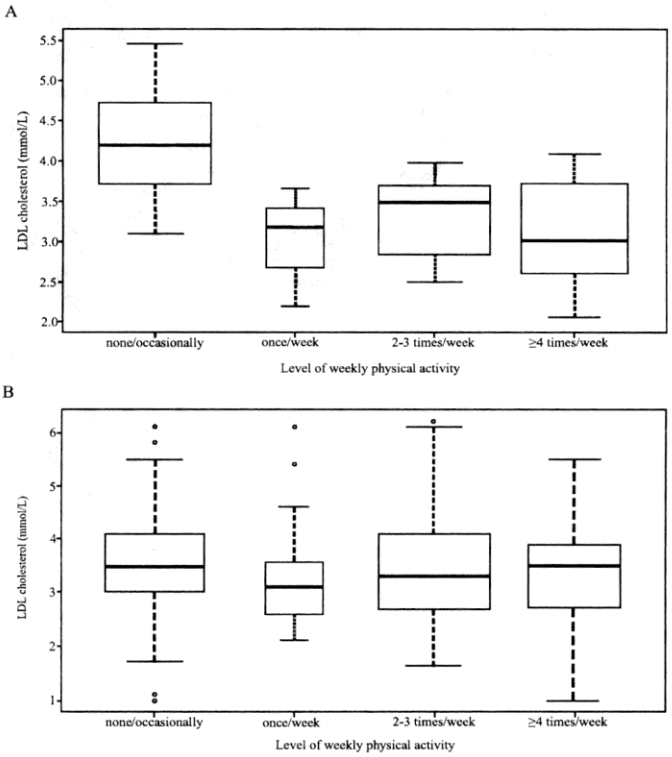

Fig. (1). Box-and-whisker plot illustrating a significant decrease in mean LDL cholesterol associated with regular physical activity (once or more/week) noted for study participants with a positive family history of AD ($p<0.001$) (**A**) but not those with a negative family history ($p=0.257$) (**B**).

Table 2. Individual and combined genotype distributions and allele frequencies for *APOE* ε-4 and ε-2 compared between patients with (n=67) and without (n=470) a family history of AD.

	Positive Family History of AD	Negative Family History of AD	Unadjusted *P*-value
Typed	**67**	**470**	
***APOE* ε-4**			
Wild type (WT) (ε-3/ ε-3)	0.54	0.67	
ε-4 allele heterozygote (HET)	0.40	0.31	**0.038**
ε-4 allele homozygote (HOM)	0.06	0.02	
Allele frequency			
ε-4 allele	0.26	0.17	0.164
HWE* p-value	0.72	0.16	
***APOE* ε-2**			
Wild type (WT) (ε-3/ ε-3)	0.96	0.84	
ε-2 allele heterozygote (HET)	0.04	0.15	**0.034**
ε-2 allele homozygote (HOM)	0.00	0.01	
Allele frequency			
ε-2 allele	0.02	0.08	0.097
HWE* p-value	0.85	0.31	
***APOE* ε-4/ε-2**			
ε-4 WT/ε-2 WT	0.52	0.54	
ε-4 WT/ε-2 HET	0.02	0.13	
ε-4 WT/ε-2 HOM	0.00	0.01	
ε-4 HET/ε-2 WT	0.37	0.29	
ε-4 HET/ε-2 HET	0.03	0.02	**0.012**
ε-4 HET/ε-2 HOM	0.00	0.00	
ε-4 HOM/ε-2 WT	0.06	0.02	
ε-4 HOM/ε-2 HET	0.00	0.00	
ε-4 HOM/ε-2 HOM	0.00	0.00	

*HWE=Hardy-Weinberg equilibrium

referral guidelines or directed therapies are not currently available. However, this view is expected to change as differences in treatment efficacy are demonstrated between individuals with different *APOE* genotypes [17]. As there is considerable public interest in such a service which poses no apparent psychological harm [26], it is expected that clinicians will increasingly be required to be sufficiently knowledgeable concerning the indications for genotyping as well as the interpretation and reporting of testing results to their patients. To facilitate integration of genetic susceptibility testing as part of AD risk management, it is imperative that misconceptions about the etiopathogenesis and clinical course of this illness and its associated modifiable risk factors be appropriately addressed [27].

Towards this aim, the genotype distribution and allele frequencies for *APOE* ε-2 and ε-4 were compared between South African individuals with and without a family history of AD. We further investigated whether expression of the established dyslipidemic effects of the *APOE* ε-2/ε-4 alleles was influenced by AD family history. Significant differences in the genotype distribution for *APOE* ε-2 and ε-4 were demonstrated between study participants with and without a family history of AD. These findings support the well-known association between the *APOE* ε-4 allele and a genetic predisposition towards sporadic late-onset AD. We propose that the apparent protective effect evident for the *APOE* ε-2 allele may be a reflection of a selection against the ε-4 genotype, since their inheritance is co-dependent. Our

observation that the association between the *APOE* ε-2 allele and elevated serum triglycerides in this study restricted to family members with a negative family history of AD further confirms that hypercholesterolemia is the primary dyslipidemic determinant of AD risk.

It was also shown that expression of the hypercholesterolemic effects of the ε-4 allele was mediated by the interaction between *APOE* genotype and AD family history, as evidenced by its association with an elevation in total and LDL cholesterol levels being restricted to study participants with a positive family history of AD. Evidence supporting the clinical relevance of the *APOE* ε-4 allele as a genetic risk factor for dyslipidemia in the South African population was reported in an earlier study conducted by Kotze *et al.* [28]. The authors demonstrated that, while *APOE* ε-4 is associated with hypercholesterolemia in the general population, carriage of this allele does not contribute towards a synergistic increase in total cholesterol in FH patients. A "founder effect" is known to account for the high prevalence of FH observed in the genetically homogeneous Afrikaner population, consistent with the underrepresentation of ε-4 noted in this particular study [28], as well as its dissociation from a hypercholesterolemic phenotype, suggesting that the effects of polymorphic variation in *APOE* are overridden by high-penetrance mutations in the *LDL-R* gene in patients with FH. In the present study, participants were selected from a chronic disease screening program aimed primarily at managing risk related to metabolic phenotypes implicated in CVD, without a specific emphasis on screening for monogenic hypercholesterolemia, minimizing pre-selection for the FH phenotype.

In addition to the hypercholesterolemic effects of the *APOE* ε-4 allele, the cholesterol-lowering benefits attributable to regular physical exercise [29] were limited to participants who reported a family history of AD in our study. Aerobic exercise is known to improve cognition partly due to its favourable effects on lipid profiles [29] and several studies have shown that the beneficial role of physical activity in reducing dementia risk may be more pronounced in genetically susceptible individuals [30, 31]. Not only physical inactivity, but also dietary fat intake, alcohol consumption and smoking are associated with the risk of dementia and AD, particularly in *APOE* ε-4 carriers. This supports the finding that the activity of promoter polymorphisms known to regulate *APOE* expression are themselves also modulated by lifestyle and dietary factors [32]. In accordance with previous studies which suggest that regular physical activity may attenuate the dyslipidemic effects of the *APOE* ε-4 allele [31, 33], findings from the present study suggest that these benefits might be greatest in hypercholesterolemic risk-allele carriers with a positive family history of AD.

Findings from the present study may provide the scientific basis for the development of reimbursement policies for *APOE* genotyping as part of health coverage by insurance companies. Public anxiety that the use of genetic information for actuarial underwriting may negatively impact upon their insurability has led to a general unease surrounding the relationship between genetics and the insurance industry. However, genetic testing performed in the context of chronic disease risk management should not negatively influence insurability when used to identify clinically actionable genetic risk factors that are not deterministic of the disease state [34]. Taylor *et al.* [35] emphasize the beneficial scenarios arising from access to genetic data, but it remains contested whether patients would be willing to pay for *APOE* genotyping performed in the context of chronic disease risk management. To clarify this question, Kopits *et al.* [36] investigated to what extent patients were willing to pay for genetic tests performed in this context. The majority of study participants (71%) reported that they would request such a service if covered by insurance, with 60% of participants being willing to pay for this service themselves even if not reimbursed by health insurers. It was concluded that many patients are willing to pay for a test which provides personal utility and value, although this was negatively affected by a fear of discrimination or actuarial unfairness. The majority of patients who undergo *APOE* genotyping nevertheless report that the benefits associated with testing outweigh potential risks [37]. The use of *APOE* genotyping in the context of AD risk assessment and management may thus be indicated, provided that clear indications for referral and reimbursement policies are put in place. Findings from our study therefore reinforce the importance of considering genetic information within a multidisciplinary clinical framework in order to maximize patient benefit [21].

The relatively small number of participants with a family history of AD was a limiting factor necessitating replication in a larger study population to clarify to what extent AD family history influences expression of the dyslipidemic effect of the *APOE* ε-4 allele. Another important shortcoming was the restriction of statistical comparisons to the Caucasian study participants, due to the limited number of individuals from other population groups enrolled in the screening program with informed consent for further research. Preliminary data showed that three of the four Coloured individuals of Mixed Ancestry who reported a positive AD family history were *APOE* ε-4 carriers, with the allelic frequency noted being ~1.5-fold higher compared to patients without a family history of AD (0.38 vs. 0.26). While it has previously been reported that Caucasian admixture accounts for the high prevalence of FH observed in the Coloured population [40], the significance of variation in the *APOE* gene as a determinant of impaired cholesterol homeostasis and AD risk in this population group warrants further consideration. Previous studies have noted that risk for cognitive decline and AD attributable to African ancestry is not synergistically increased by carriage of the *APOE* ε-4 allele [38, 39]. Although several methodological issues are known to complicate the assessment of ancestral background as a modifier of the relationship between genetic factors and disease phenotypes, extended analysis in patients from underrepresented African population groups is an important focus of ongoing research aimed at clarifying to what extent ethnic background modifies a gene effect [41].

The strengths of the present study lie in the demonstration that family history modulates the expression of a particular phenotypic trait reflecting the combined effect of gene-environment interactions in a specific population group, which has not yet been studied either locally or abroad in the context of AD. To the best of our current knowledge, this is the first study to demonstrate the modulat-

ing influence of AD family history on the expression of a dyslipidemic phenotype associated with the *APOE* ε-4 allele. This interactive effect provides the scientific rationale for assessment of AD family history, which likely embodies multiple other genetic susceptibility factors, prior to *APOE* genotyping aimed at guiding the therapeutic management of dyslipidemia. Also, in contrast to the controlled research environment used in clinical trials, the use of data obtained from patients seen in the real-world clinical domain limits the potential for study bias. Perhaps most importantly, our study provides evidence in support of a novel clinical application for *APOE* genotyping in the context of chronic disease risk management in asymptomatic individuals.

Currently, the clinical use of *APOE* genotyping is largely restricted to increasing the diagnostic accuracy for AD in symptomatic patients who present at a younger age or with an atypical clinical picture [42]. Complementary to this role, we advocate the use of *APOE* genotyping as a means of identifying a genetic subgroup of dyslipidemic patients set to derive the greatest therapeutic benefit from the timely implementation of lifestyle-based intervention strategies targeting known cardio-metabolic risk determinants to decrease cumulative cardiovascular risk and ultimately help prevent the onset of AD [21, 25]. This approach to patient management, guided partly from a genetic background, resonates with the relationship between metabolic phenotypes implicated in CVD and increased risk for AD being mediated by the promotion of atherosclerosis and subclinical cerebrovascular pathology reciprocally related to *APOE* ε-4 carriage [43, 44]. In addition, it is supported by the known long-term health implications evident for early therapeutic intervention aimed at targeting these shared pathogenic mechanisms [17, 21]. As proposed by Donnelly and colleagues [45], *APOE* may therefore be considered a valuable biomarker as ancillary to standard biochemical assessment. This could help identify a genetic subgroup of patients who are most likely to benefit from health-promoting lifestyle and dietary interventions.

Inquiry regarding family history plays an established role in determining eligibility for genetic testing considering high-penetrance mutations implicated in certain monogenic disorders. Results from our study suggest this approach might also be clinically useful in identifying dyslipidemic patients set to derive the greatest benefit from *APOE* genotyping performed in the context of chronic disease risk management, while simultaneously serving to limit unnecessary and expensive further testing where it is not indicated. Although current knowledge posits that there is still insufficient evidence supporting the clinical utility of many low-penetrance genetic tests, *APOE* may be considered a noteworthy exception due to its moderate penetrance and semi-dominant pattern of inheritance [46].

In conclusion, we report significant differences in the genotype distribution for *APOE* ε-4 and ε-2 between South African individuals with and without a family history of AD. Family history was shown to modulate the expression of the known hypercholesterolemic effects of the *APOE* ε-4 allele, while the cholesterol-lowering benefits of regular physical exercise were limited to participants with a positive family history of AD. Results from our study support the novel clinical application of *APOE* genotyping as a means of identifying a high-risk genetic subgroup of asymptomatic dyslipidemic patients with a family history of AD, set to derive the greatest therapeutic benefit from targeted lifestyle-based intervention strategies aimed at decreasing cumulative cardiovascular risk and preventing the development of AD later in life. The consideration of such testing performed in a multidisciplinary context may therefore address current health concerns to prevent future disease development, thereby promoting long-term health and wellbeing. Towards this goal, we implemented a pathology-supported genetic testing service [21, 25] linked to the generation of a research database for health outcome studies over time.

CONFLICT OF INTEREST

Prof Kotze is a director and shareholder of Gknowmix (Pty) Ltd. that has developed a database tool for research translation under the auspices of the Innovation Centre of the South African Medical Research Council. The other authors declared no conflict of interest and no writing assistance was obtained in the preparation of this manuscript.

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CHAPTER 3

SUBSTUDY 2

Title: *Apolipoprotein E* genotyping and questionnaire-based assessment of lifestyle risk factors in dyslipidemic patients with a family history of Alzheimer's disease: test development for clinical application

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Status: Reproduced with approval

Contribution of the Candidate:

The first author wrote the article after selecting relevant data pertaining to eligible study participants who were genotyped for the APOE polymorphism. Ethics approval forms signed by the participants and laboratory results available in the files were checked for accuracy during transfer to the research database. Statistical analysis was performed by the candidate based on a course using R Studio and checked by a qualified statistician. Lifestyle, pathology and genetic data were interpreted and discussed in a clinical context in keeping with the research aims and hypothesis.

ABSTRACT

Introduction: The cholesterol-raising properties of the apolipoprotein E (*APOE*) epsilon-4 (ε-4) allele has been validated in the South African population. Mounting evidence supports the added value of *APOE* genotyping for the evaluation of cardiovascular risk in dyslipidaemic patients beyond its established role in the diagnosis of late-onset Alzheimer's disease (AD).

Aim: The aim of this study was to determine the potential benefits of combining AD family history with questionnaire-based lifestyle assessment to facilitate the clinical interpretation of *APOE* genotyping results.

Methods: A total of 580 unrelated South African individuals prospectively enrolled in a chronic disease screening program incorporating a genetic component (2010-2015) was selected for inclusion in this study based on the presence (75) or absence (505) of AD family history. Biochemical assessment of their lipid profiles was performed according to standard laboratory protocols. All study participants were genotyped for the *APOE* ε-2/ε-3/ε-4 alleles using allele-specific real-time polymerase chain reaction technology.

Results: In patients without a family history of AD, *APOE* genotype modified the relationship between alcohol intake and body mass index (p=0.026), with a significant positive correlation noted between these parameters being limited to ε-4 allele carriers. *APOE* genotype also modified the association between alcohol intake and total serum cholesterol in patients with a positive family history of AD (p=0.026).

Conclusions: We demonstrated the benefits of a questionnaire-based approach for assessment of lifestyle risk factors to facilitate clinical interpretation of *APOE* genotyping results for targeted intervention in a genetic subgroup of dyslipidaemic patients at increased risk for AD.

INTRODUCTION

Alzheimer's disease (AD) and cardiovascular disease (CVD) share common risk factors and overlapping pathogenic mechanisms (Cechetto et al. 2008; Kalaria 2010; O'Brien and Markus 2014). Recognition that the *apolipoprotein E* (*APOE*) gene provides a genetic link between vascular disease and dementia creates interest in the value of genetic testing for early intervention aimed at decreasing cumulative cardio-metabolic risk to prevent the development of late-onset AD with advancing age (Kivipelto and Solomon 2008; Kotze and van Rensburg 2012). The addition of a pre-clinical stage to the diagnostic criteria for late-onset AD (Sperling et al. 2011) offers a critical window of opportunity for the management of vascular risk factors at the interface between genes and the environment (Imtiaz et al. 2014). Lifestyle-based preventive measures aimed at delaying disease onset by at least one year could result in 9.2 million people being spared this devastating diagnosis by the year 2050 (Brookmeyer et al. 2007). The lack of a well-defined target population set to derive the greatest benefit from *APOE* genotyping used to optimize vascular risk management in a subgroup of dyslipidaemic patients at increased risk for AD however constitutes an important factor limiting the clinical application of genetic testing in general medical practice.

The *APOE* gene has emerged as a useful biomarker which could improve the performance of existing risk stratification tools for targeted therapeutic intervention in a high-risk genetic patient subgroup (Gauthier et al. 2012; Valenti et al. 2014). *APOE* offers an aetiological bridge between vascular risk factors and cognitive decline, owing to its multifunctional role in cholesterol transport and metabolism, amyloid-beta (Aβ) processing, oxidative stress and chronic inflammation (Weisgraber 1994). The *APOE* gene is highly polymorphic, with three apolipoprotein isoforms corresponding to the ε-2 (rs7412), ε-3 and ε-4 (rs429358) alleles which differ in their binding affinity to the low-density lipoprotein receptor (LDLR) (E4>E3>E2). APOE is the primary ligand of the LDLR underlying familial hypercholesterolaemia (FH). Isoform-dependent variation in systemic cholesterol metabolism and clearance accounts for an extent of inter-patient variation in serum lipid

profiles (Johnson et al. 2014). *APOE* ε-3 considered as neutral is the most common allele carried by the general population (de Knijff et al. 1994). The cholesterol-raising effects of *APOE* ε-4 promote atherosclerosis (Davignon 2005; Khan et al. 2013; Zende et al. 2013) conferring independent or additive risk for AD (Duron and Hannon 2008). This mechanism is mediated by vascular dysfunction attributable to ischemic macrovasculopathy (Kalaria 2000) and cerebral amyloid arteriopathy (Love 2004; McCarron and Nicoll 2010), which are in turn associated with reduced regional cerebral perfusion known to precede clinically overt symptomatic onset in dementia (Bu et al. 2013).

APOE ε-4 is the most important genetic risk factor for sporadic late-onset AD (Bertram et al. 2007; Kim 2009; Verghese et al. 2011) and a major determinant of inter-patient heterogeneity in clinical presentation, prognosis and therapeutic outcomes (Risacher et al. 2013; Dixon et al. 2014; Kotze et al 2015a). The *APOE* ε-4 allele is also associated with significant risk for the metabolic syndrome (Sima et al. 2007) and vascular disease (McCarron et al. 1999; Song et al. 2004), in addition to mediating the pathogenic relationship between these inter-related clinical entities (Teixeira et al. 2014). In keeping with the dissociation between *APOE* ε-2 and hypercholesterolemia (Bazzaz et al. 2010), it has been suggested that this allele protects against the onset of ischemic stroke and dementia. Recent studies have also proposed that *APOE* ε-2 is associated with a decreased risk for AD due to its modulating effect on cerebral Aβ processing and clearance (Wang et al. 2009; Conejero-Goldberg et al. 2014; Serrano-Pozo et al. 2015). The association between *APOE* ε-2 and type III dysbetalipoproteinemia (Blom et al. 2002) as well as the metabolic syndrome (van Velden et al. 2011) in South African patients however suggests a deleterious rather than beneficial role in the pathogenesis of vascular disease.

Contradictory findings may be partly explained by the fact that genetic risk conferred by these *APOE* polymorphisms is dependent on a conducive metabolic milieu and influenced by environmental as well as epistatic modulators of phenotypic expression (Teixeira et al. 2014). This observation explains the limited individual utility of APOE ε-2/ε-3/ε-4 as

diagnostic and predictive markers in the assessment of vascular risk (Villeneuve et al. 2014). The limitations of a genomics-only approach to cardiovascular risk screening can however be overcome by including *APOE* genotyping as part of a multidisciplinary framework incorporating diverse patient data gleamed from a variety of sources (Kotze et al. 2015b; Lückhoff et al. 2015). The assessment of modifiable dietary habits in this context is supported by the well-evidenced association between high saturated as well as trans-fat intake and increased risk for AD (Kalmijn et al. 1997; Puglielli et al. 2001; Morris et al. 2003; Morris and Tangney 2014). In addition to a direct correlation with increased cerebral Aβ burden, a high-fat diet is associated with the metabolic syndrome as an important risk factor for cognitive impairment and dementia (Elias et al. 2003; Nguyen et al. 2014). Lifestyle habits such as smoking and alcohol intake are also known to exert a dose-dependent effect on risk for CVD and AD (Luchsinger et al. 2004; Harwood et al. 2010). In this context, findings from a recent South African cross-over alcohol intervention study (Kotze et al. 2014) support the role of *APOE* genotype as a modifier of the association between red wine consumption and inter-individual variation in the serum lipid profile.

The association between *APOE* ε-4 and increased serum cholesterol levels in patients without FH has previously been validated in the South African population by Kotze et al. (1993). Despite its potential role in the differential diagnosis of FH, concerns over the added value of *APOE* genotyping in improving clinical outcomes in dyslipidaemic patients were initially met with hesitance as to the routine implementation of such testing. This is of particular clinical importance given that FH remains vastly underdiagnosed and untreated in general medical practice, despite the 5-10 times increased prevalence of this severe form of monogenic dyslipidaemia in certain South African population groups (Kotze et al. 1993). In South African patients with FH, *APOE* ε-2 allele carriage is associated with hypertriglyceridemia uncharacteristic of the disease state, with second-hit pathologies such as obesity and hypothyroidism known to contribute towards the genetic disorder III dysbetalipoproteinemia (Blom et al. 2002).

The development of a central database resource for translational research linked to a genomics-based chronic disease screening program incorporating *APOE* genotyping provided an ideal platform to facilitate the development of referral guidelines for such testing in the South African healthcare setting (Kotze et al. 2015b). This database facilitated recent local research aimed at determining whether the assessment of non-genetic information could assist in identifying patients set to derive optimal benefit from *APOE* genotyping performed in this context. Using this resource, Lückhoff et al. (2015) indeed demonstrated that the genotype distribution for *APOE* ε-2/ε-4 differs between South African individuals with and without a family history of AD. In this particular study, the clinical expression of a hypercholesterolaemic phenotype in ε-4 allele carriers, as well as its mitigation by regular physical activity, was shown to be dependent on the interaction between *APOE* genotype and a family history of AD. The known association between *APOE* ε-2 and increased triglyceride levels was also demonstrated in study participants without a family history of AD. In addition, the relationship between the dietary fat score, body mass index (BMI) and serum lipid profiles was also dependent on the presence or absence of a self-reported family history of AD (Lückhoff et al. 2015). Findings from this study collectively support the role of clinical inquiry concerning AD family history alongside the assessment of serum lipid profiles as part of a novel pre-screen algorithm used to determine eligibility for *APOE* genotyping performed as an integral component of cardiovascular risk screening and management. The added value of *APOE* genotyping used to optimize vascular risk management in a subgroup of dyslipidemic patients is positioned as ancillary to its existing role to improve diagnostic reliability for late-onset AD (Sun et al. 2012).

As an extension of the abovementioned findings, we sought to determine the potential benefits of combining family history of AD with non-genetic information for clinical interpretation of *APOE* genotyping results. In particular, we sought to replicate the modifying influence of *APOE* genotype on the association between lifestyle habits and cardio-metabolic risk traits as demonstrated in previous international studies (Lindsay et al. 2002;

Ruitenberg et al. 2002; Mukamal et al. 2003; Harwood et al. 2010) in relation to a self-reported family history of AD. Insight gathered as a result of this study could provide the scientific rationale supporting a multidisciplinary approach to cardiovascular risk screening, incorporating *APOE* genotyping to inform clinical and therapeutic decision making in the context of FH case finding, cardio-metabolic risk reduction and AD prevention.

MATERIALS AND METHODS

Ethical approval for this cross-sectional genotype-phenotype association study was granted by the Health and Research Ethics Committee (HREC) of Stellenbosch University under project number N09/08/224. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Written informed consent for participation in the research component of a chronic diseases screening program was obtained from all study participants.

Selection of Study Participants

Data of 580 Caucasian individuals of European Ancestry (404 females, 176 males, mean age 46±12 years) were prospectively enrolled in a chronic disease screening program incorporating a genomics component. This group included all 537 participants (93%) enrolled in our previous study (Lückhoff et al. 2015) as well as an additional 43 eligible individuals referred for inclusion in the abovementioned screening program since April 2014. Full lipid profiles were determined for a subgroup of 291 patients (50%) enrolled in this study following referral by a private practicing clinician or attendance of Wellness Days at the workplace or other venues over a five year period (2010-2015) were available in a centrally maintained database (accessed at www.gknowmix.org). To exclude ethnicity as a potential confounder, and given the small number of non-Caucasian individuals who provided informed consent for participation in research linked to a pathology-supported genetic testing service, comparative analyses were performed only in the Caucasian study group, who were selected based on the presence (75) or absence (505) of a family history of AD.

Questionnaire-Based Clinical and Lifestyle Assessment

Prior to enrolment in the chronic disease screening program, all prospective study participants were asked to complete the ethically approved study questionnaire (available at www.gknowmix.com) developed in collaboration with a registered dietician and approved by the HREC at Stellenbosch University. This questionnaire was used to document socio-demographic information, clinical data such as body mass index (BMI) and lifestyle factors (dietary fat intake, alcohol consumption, smoking). In patients who reported a positive family history of AD, the relation to the affected individual was also documented in addition to age of onset, as previously described by Lückhoff et al. (2015). Alcohol intake was differentiated into abstain, occasionally (1- 2 units/week), moderate (1-13 units/week) and high (14 or more units/week). This corresponded to the ordinal categories 0-3 considered for statistical analyses. Study participants were also asked how frequently they ate certain foods rich in saturated or trans-fats over the course of the preceding three months. This was used to derive a dietary saturated and trans-fat score, considered to be moderate when ranging between 22 and 26, low between 16-21, very low if <16, and high if ≥27. The practical relevance of this dietary fat score as a useful clinical tool in the assessment of lifestyle habits in relation to cardio-metabolic risk was previously described in the South African setting by Davis et al. (2014).

APOE Genotyping

Genomic DNA was extracted from whole blood (QIAamp® DNA Midi-kit) or saliva samples (Oragene® reagents). Single nucleotide polymorphisms (SNPs) in the *APOE* gene (ε-2 rs7412 and ε-4 rs429358) were genotyped as previously reported (Luckhoff et al. 2015), using high throughput real-time polymerase chain reaction (PCR) at the Pathology Research Facility, Stellenbosch University. The wild type, heterozygous and homozygous SNP genotypes generated for Taqman® SNP Genotyping Assays (Applied Biosystems, Werterstadt, Germany) on the Corbett Rotor-Gene™ 6000 series Multiplexing System were validated against direct sequencing as the gold standard.

Statistical Analysis

All statistical analyses were performed using the Statistica software package (StatSoft,Inc) and R Studio package (freely available at www.r-studio.org). The clinical characteristics of participants included in the validation phase of this study (Lückhoff et al. 2015) were described and compared between individuals with and without a family history of AD. Qualitative characteristics were described using cross-tabulation and frequency tables and compared between study groups using the Pearson's Chi-squared or Fisher's exact test as appropriate. Quantitative phenotypes were described as the means along with standard deviations (SD) and compared between study subgroups using a Student's t-test assuming normality of distribution. In cases where quantitative outcomes showed a non-symmetrical distribution, such variables were log-transformed for analyses. If the best-fitting log-transformed model still showed a non-normal distribution, these were described as the median with interquartile range (IQR) and compared between study groups using the Wilcoxon signed rank test. The linear relationship between the fat score and metabolic risk phenotypes was assessed using the Spearman rank correlation coefficient test, for the total study groups as well as subgroups defined based on the presence or absence of an AD family history. Analysis of covariance (ANCOVA) was used to determine whether *APOE* genotype modified the association between dietary habits and metabolic risk phenotypes. A p-value of <0.05 was seen as statistically significant with trend-associations noted where applicable.

RESULTS

Clinical characteristics

The clinical characteristics of the study group are summarized in Table 1 and compared between participants with (75) and without (505) a family history of AD. No significant differences in age, sex, BMI, serum lipid profiles, alcohol consumption, smoking status or dietary fat intake were detected between the two study subgroups ($p>0.05$). In a separate analysis, no significant difference in these parameters were noted for female study participants stratified based on the presence or absence of AD family history. There was also no difference in the clinical characteristics of patients with and without a family history of AD considering the subgroup for whom the full serum lipid profile was assessed (data not shown).

Table 1. Clinical characteristics of study participants summarized and compared based on the presence (75) or absence (505) of AD family history. Numerical data is presented as the means along with standard deviations (SD) unless otherwise indicated.

Characteristics	Positive AD family history (n=75)	Negative AD family history (n=505)	Unadjusted P-value
Age (years)	44.4 (13.0)	46.4 (12.1)	0.19
Sex (male/female)	18/57	158/347	0.19
Body mass index (kg/m²)	27.3 (7.1)	27.3 (6.0)	0.97
Alcohol intake	1.1 (0.8)	1.2 (0.9)	0.30
Fat score (units)	16.4 (7.8)	16.7 (8.4)	0.79
Current smoker (yes/no)	5/69	56/437	0.45
Total cholesterol (mmol/L)	5.1 (1.1)	5.4 (1.1)	0.53
HDL cholesterol (mmol/L)	1.5 (0.5)	1.4 (0.4)	0.17
LDL cholesterol (mmol/L)	3.5 (0.8)	3.4 (0.9)	0.72
Triglycerides (mmol/L)	1.2 (0.8)	1.3 (0.9)	0.50

Association between alcohol as well as dietary fat intake and cardio-metabolic risk phenotypes in relation to AD family history

Relationship between smoking and cardio-metabolic risk phenotypes

In the total Caucasian study group, smoking was associated with higher triglyceride levels ($p<0.001$) and lower HDL cholesterol ($p=0.004$). No significant association between smoking status and BMI ($p=0.110$), total ($p=0.167$) or LDL cholesterol levels ($p=0.457$) was otherwise noted. In study participants with a negative family history of AD, the association between smoking and triglycerides ($p=0.002$) as well as HDL cholesterol levels ($p=0.022$) retained significance. No significant relationship between smoking and the selected metabolic risk phenotypes of interest were noted for study participants with a positive family history of AD ($p>0.05$).

Relationship between alcohol consumption and cardio-metabolic risk phenotypes

In the total Caucasian study group, a trend was observed for alcohol intake to be positively associated with total ($p=0.070$) as well as LDL cholesterol levels ($p=0.071$). A statistically significant inverse association was identified between alcohol intake and HDL cholesterol ($p=0.009$). Alcohol consumption was not otherwise associated with BMI ($p=0.603$) or triglyceride levels ($p=0.449$) in the total study group. A trend towards a positive association between alcohol intake and total cholesterol was also noted for study participants with a negative family history of AD ($p=0.082$). In this subgroup, alcohol intake was not associated with BMI ($p=0.587$), LDL ($p=0.121$), HDL cholesterol ($p=0.201$) or triglyceride levels ($p=0.182$). In study participants with a positive family history of AD, a positive association was noted between alcohol intake and BMI ($p=0.008$) as well as triglyceride levels ($p=0.021$). In this subgroup, alcohol intake was not otherwise associated with total ($p=0.560$), LDL ($p=0.219$) or HDL cholesterol levels ($p=0.138$). In summary, the association between alcohol intake and metabolic risk phenotypes differed based on the presence or absence of AD family history.

APOE genotype as a modifier of the association between alcohol intake and cardio-metabolic risk traits

No significant modifying influence for *APOE* genotype on the association between lifestyle habits and cardio-metabolic risk traits was noted when the total study group was considered. A number of differential effects were however observed when participants were stratified based on the presence or absence of a self-reported family history of AD. Firstly, *APOE* genotype modified the association between alcohol intake and total cholesterol in study participants with a positive family history of AD (p=0.026), with a significant positive association between these parameters being limited to ε-4 allele carriers (Figure 1). In this subgroup, alcohol intake was also positively associated with total cholesterol levels in 25 *APOE* ε-4 allele carriers (Rho=0.35, p=0.085). There was however no significant relationship between alcohol intake and total cholesterol levels in 26 *APOE* ε-4 non-carriers (Rho=-0.29, p=0.155). In study participants with a negative history of AD, *APOE* genotype also modified the association between alcohol intake and BMI (p=0.026) with a significant positive correlation between these variables again being limited to ε-4 allele carriers (Figure 2). There was a significant positive association between alcohol intake and BMI in 163 *APOE* ε-4 allele carriers (Rho=0.16, p=0.050). There was however no significant relationship between alcohol intake and BMI in 346 *APOE* ε-4 non-carriers (Rho=-0.05, p=0.413). A trend was also noted for *APOE* genotype to modulate the relationship between alcohol intake and HDL cholesterol (p=0.056) with an inverse association being restricted to ε-4 allele non-carriers. In summary, the interaction between *APOE* ε-4 and alcohol intake on BMI as well as serum lipid profiles was dependent on the presence or absence of a family history of AD.

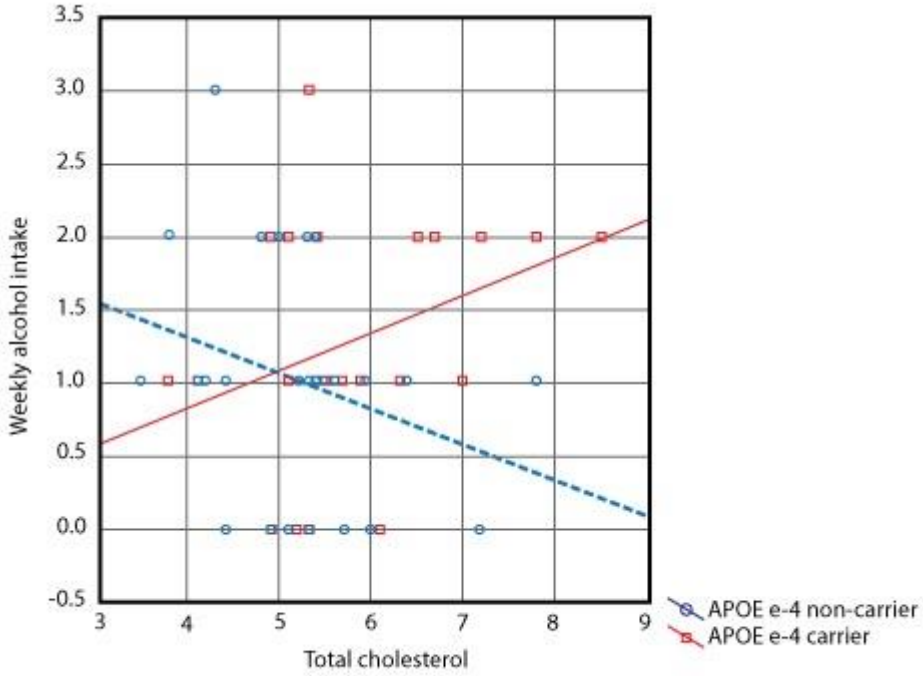

Figure 1. Scatterplot diagram illustrating the significant modifying influence of *APOE* genotype on the association between alcohol intake and total serum cholesterol in South African patients with a positive family history of AD.

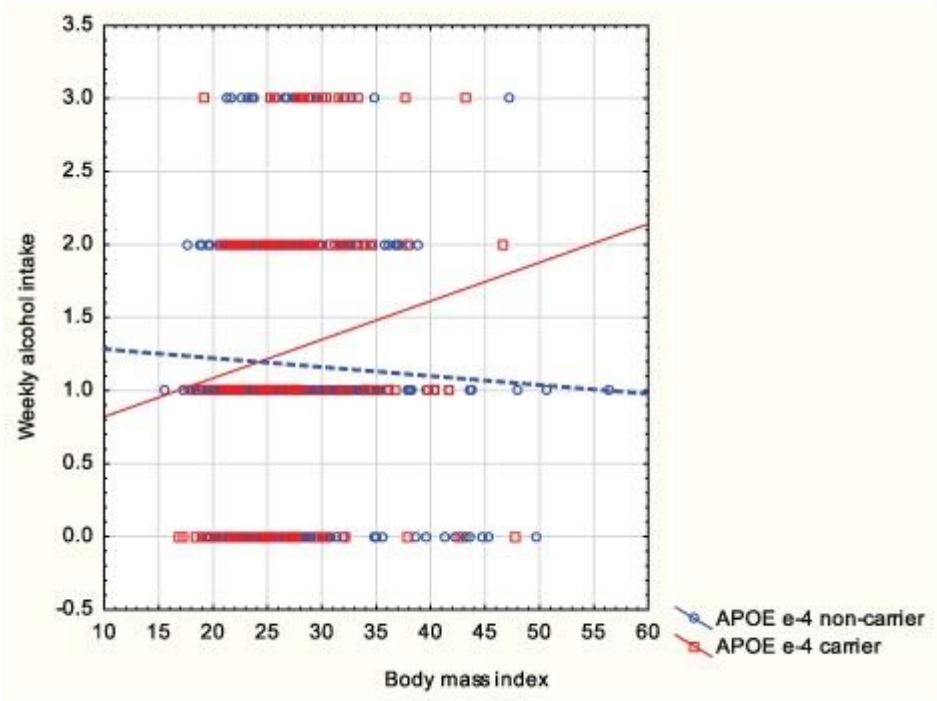

Figure 2. Scatterplot diagram illustrating the significant modifying influence of *APOE* genotype on the association between alcohol intake and body mass index (BMI) in patients without a family history of Alzheimer's disease (p=0.026).

DISCUSSION

In this study, we provide additional evidence substantiating our previous finding that the modifying influence of *APOE* genotype on the association between lifestyle habits and selected cardio-metabolic risk phenotypes is influenced by an AD family history. In a recent South African cross-over intervention study, a beneficial increase in HDL cholesterol associated with moderate alcohol consumption was shown to be less pronounced in *APOE* ε-4 allele carriers (Kotze et al. 2014). In the present questionnaire-based cross-sectional study, *APOE* genotype was identified as a genetic determinant of the relationship between alcohol intake and BMI as well as total cholesterol levels, with a deleterious increase in these parameters associated with excessive consumption being limited to ε-4 allele carriers. The role of AD family history as a determinant of genotypic effects evident from this study

was deemed in keeping with our previous observation that its interaction with *APOE* genotype influences the clinical expression of a hypercholesterolaemic phenotype in ε-4 allele carriers (Lückhoff et al. 2015). These findings therefore collectively support the relevance of lifestyle-based assessment positioned alongside clinical inquiry concerning AD family history as part of a multidisciplinary approach to chronic disease risk screening incorporating *APOE* genotyping. The added value of genetic testing in this context lies not only in prioritizing the need for lifestyle-based intervention aimed at decreasing cumulative cardio-metabolic risk in a genetic subgroup of dyslipidaemic patients, but also improving the identification as well as guiding the management of South African patients with FH (Kotze and van Rensburg 2012).

Realization that the clinical expression of *APOE* ε-4 is context-dependent has created interest in the value of lifestyle-based interventions to decrease cumulative cardio-metabolic risk to prevent the onset and progression of AD in high-risk genetic patient subgroups with advancing age. Emerging evidence suggests that simple dietary modifications decrease cumulative risk for both CVD and AD while posing minimal potential for added harm (Barnard et al. 2014). Although the detrimental effects of environmental exposures on AD risk are exacerbated in *APOE* ε-4 allele carriers (Kivipelto et al. 2008) this patient group shows a more favourable response to lifestyle-based as opposed to pharmacological interventions compared to non-carriers (Masson et al. 2003; Baptista et al. 2011). The *APOE* ε-4 allele is known to aggravate the deleterious effects of excessive alcohol consumption on deranged cholesterol parameters (Ordovas and Mooser 2002). In this study, the unfavourable cholesterol-raising effects of excess alcohol intake were restricted to ε-4 allele carriers, in keeping with the established role of *APOE* genotype as a determinant of the relationship between alcohol intake and altered serum lipid profiles (Corella et al. 2001). In patients with established AD, the synergistic interaction between *APOE* ε-4 and excessive alcohol intake has been associated with a more adverse clinical presentation (Harwood et al.

2010). Other studies have however failed to demonstrate the significance of such gene-diet interactions on risk for vascular disease including ischemic stroke (Djoussé et al. 2009).

The modulating effect of *APOE* ε-4 on the relationship between alcohol intake and HDL cholesterol is well-established (Djoussé et al. 2004). *APOE* genotype has also been shown to influence the extent of cardio-protective benefits associated with polyphenol-rich red wine consumption in South African patients (Kotze et al. 2014). A questionnaire-based approach was used in an attempt to replicate this finding in this study, in which we demonstrated that *APOE* genotype again modified the association between alcohol intake and HDL cholesterol, with significantly higher mean levels noted for ε-4 allele carriers compared to non-carriers. Multiple prospective studies have demonstrated that long-term moderate alcohol consumption is associated with a decreased risk for overall- as well as cardiac mortality (Camargo et al. 1997; Gaziano et al. 2000: Fuller 2011; Pai et al. 2012). Adherence to a Mediterranean style diet, which places emphasis on the health-promoting effects of moderate consumption of certain alcoholic beverages including red wine, has been associated with decreased incidence risk for AD as well as cardiovascular mortality (Scarmeas et al. 2006; Bonaccio et al. 2015). The cardio-protective benefits of alcohol consumption in this context have been attributed to its favourable effects on serum lipid profiles (Agarwal 2002). In contrast, hypoalphalipoproteinemia as a component of the metabolic syndrome has been shown to confer independent risk for CVD (Reiner et al. 2011). Collectively, the abovementioned observations suggest that *APOE* ε-4 carriers are not only least likely to derive the same cardio-protective benefits from moderate alcohol consumption as non-carriers, but are also more susceptible to the deleterious effects of excessive alcohol intake on serum lipid profiles.

APOE genotype modified the relationship between dietary fat consumption and HDL cholesterol in our study cohort, with a detrimental decrease in mean levels with increased saturated and trans-fat intake being limited to ε-4 allele carriers with a positive family history of AD. It is well-established that the cholesterol-raising effects of *APOE* ε-4, and

homozygosity for this risk-associated allele in particular, are mediated by a high-fat, high-cholesterol diet (Tikkanen et al. 1990). The differential effects of saturated as opposed to moderate unsaturated fat intake on dementia risk is also known to be modified by the *APOE* ε-4 allele. In this context, unsaturated fatty acid intake exerts a protective effect on risk for AD, while high saturated fat intake during midlife increases disease risk with advancing age (Petot et al. 2003; Laitinen et al. 2006). Excessive dietary intake of saturated and trans-fats also increases risk for ischemic vascular disease, which is in turn associated with cognitive decline and dementia (Puglielli et al. 2003). Despite the well-evidenced role of *APOE* genotype as a determinant of inter-individual variation in serum lipid profiles, conflicting evidence suggests that ε-4 allele carriage does not modify the association between diet and body mass on total and LDL cholesterol levels (Petkeviciene et al. 2012). It is appreciated that, to date, the majority of studies concerning this topic have failed to consider the role of AD family history as a potential confounder in relation to its role as a determinant of genotypic effects on disease risk. To what extent the benefits of dietary omega-3 and omega-6 fatty acids on dementia risk are attenuated and exacerbated respectively in *APOE* ε-4 carriers however remains incompletely understood (Barberger-Gateau et al. 2007; Morris 2009). Findings from a recent study by Liang et al. (2013) suggest that, in addition to its direct association with dyslipidaemia, *APOE* genotype is also considered a partial determinant of the favourable effects of icosapentaenoic acid (EPA) supplementation on the normalization of serum lipid profiles (Liang et al. 2013).

Findings from our study collectively support the importance of a questionnaire-based approach to the assessment of lifestyle risk factors to facilitate the appropriate interpretation of information gathered from *APOE* genotyping performed as an integral component of cardiovascular risk screening and management. In this context, genetic testing could prove useful in identifying a high-risk subgroup of non-FH dyslipidaemics set to derive the greatest benefit from the timely implementation of lifestyle-based intervention strategies aimed at decreasing cumulative cardio-metabolic risk to prevent the onset and progression of AD in

later life. In particular, emphasis is placed on the role of physical activity in normalizing serum lipid profiles and related metabolic abnormalities in *APOE* ε-4 allele carriers, as well as mitigating the deleterious effects of advancing age on multiple biochemical disturbances associated with dementia risk (Okonkwo et al. 2014). In this context, the favourable effects of exercise in maintaining normal cognition and decreasing the rate of cognitive decline are known to be more pronounced in *APOE* ε-4 carriers (Bernstein et al. 2002; Podelwis et al. 2005; Obisesan et al. 2012; Farina et al. 2014).

Several case-control studies (Zamrini et al. 2004; Wong et al. 2013) as well as at least one meta-analysis (Rockwood 2006) have reported that statins may prevent the onset and clinical progression of dementia. Findings from large-scale prospective follow-up studies however fail to support the notion that lipid-lowering pharmacotherapy alters the clinical course of AD in patients with an established diagnosis (Feldman et al. 2006). *APOE* ε-4 allele carriers are not only least sensitive to the health-promoting benefits of lipid-lowering medications (Masson et al. 2003), but statins may in fact exacerbate risk for cerebrovascular insults in this genetic patient subgroup (Woo et al. 2013; Romero et al. 2014). In contrast, patients with certain monogenic dyslipidaemias including FH (Kotze et al. 1993) require long-term treatment with lipid-lowering medications to decrease risk for ischemic vascular disease (Najam and Ray 2015). *APOE* genotyping could therefore prove useful in increasing case finding for FH in the local healthcare setting while simultaneously preventing statin overtreatment in a genetic patient subgroup for whom such treatment is either not indicated or could pose additional harm.

Despite the fact that only 75 participants enrolled in the present study noted a positive family history of AD, our findings are in accordance with existing evidence implicating *APOE* polymorphisms in the differential effects of lifestyle habits on metabolic risk phenotypes. An important limitation identified for the current study was the fact that a full lipid profile was only available for a subset of patients included in the present investigation. However, data concerning the parameters for which *APOE* ε-4 showed a modulating effect were available

for the majority of patients enrolled in this study. In addition, restriction of the study group to only include Caucasian participants of European Ancestry limited the ability to investigate the role of ancestral background as a modifier of functional gene effects in risk allele carriers. In this context, future studies are required to further elucidate the putative modulating influence of *APOE* genotype on the association between lifestyle habits and cardiovascular risk in underrepresented African population groups (Tishkoff et al. 2009). It is further acknowledged that the majority of patients included in this study were female (70%), which raises the question to what extent gender modifies the gene effect of *APOE* polymorphisms on AD risk. The selection of a largely female patient group in this study reflects the demographics of the total cohort enrolled in a chronic disease screening program incorporating a genomics component as discussed above. There was however no difference in age, BMI, serum lipid profiles, dietary habits or alcohol consumption noted between females with and without a family history of AD. Sex was therefore not considered as an important modifier of the gene effect in this study.

The strengths of this study however lie in the provision of novel local evidence which supports the assessment of relevant non-genetic data to facilitate the appropriate clinical interpretation of results obtained from *APOE* genotyping used to optimize the management of vascular risk in a high-risk subgroup of dyslipidaemic patients. This notion is based on evidence gathered from a practical healthcare setting instead of a controlled research environment, which also minimized the potential for pre-selection of the FH phenotype (Lückhoff et al. 2015). Biochemical determination of serum lipid profiles in relation to BMI relevant to *APOE* genotyping is positioned as a means of assessing the phenotypic expression of the ε-2/ε-3/ε-4 alleles in order to monitor the response to individualized treatment aimed at decreasing cumulative risk for CVD and AD with advancing age. The lifestyle and dietary questionnaire utilized in this study therefore provides a useful tool for the assessment of cumulative environmental risk in relation to genetic disease susceptibility conferred by the *APOE* polymorphism. This approach allowed for the validation of previously

described genotype-phenotype associations as well as evaluation of the relationship between metabolic risk traits and lifestyle risk factors relevant to vascular disease and dementia.

In summary, we showed that *APOE* ε-4 genotype modifies the association between alcohol and BMI as well as total cholesterol levels, with a tendency for the adverse effects of these lifestyle habits to be exacerbated in risk-allele carriers. In addition, our findings suggest that the differential expression of these effects is itself influenced by the presence or absence of a family history of AD. This notion is in accordance with findings reported in a recent local study (Lückhoff et al. 2015) in which we demonstrated that the interaction between *APOE* genotype and AD family history determines the expression of a hypercholesterolaemic phenotype in ε-4 allele carriers. Findings from this particular study (Lückhoff et al. 2015) support the relevance of clinical inquiry concerning AD family history alongside the assessment of serum lipid profiles as part of a novel pre-screen algorithm used to determine eligibility for *APOE* genotyping performed as an integral component of chronic disease risk screening to optimize vascular risk management in a high-risk subgroup of dyslipidaemic patients. In the present study, we demonstrated the benefits of a questionnaire-based approach used for the assessment of lifestyle risk factors to allow for the appropriate clinical interpretation of *APOE* genotyping results within a multidisciplinary framework (Figure 3). This approach is positioned as a means to assist clinicians in the development and timely implementation of lifestyle-based intervention strategies to decrease cumulative environmental risk in a genetic subgroup of dyslipidaemic patients. In this context, *APOE* genotyping could also prove useful in prioritizing the need for statin treatment in addition to facilitating the differential diagnosis of FH, which remains underdiagnosed and undertreated in the South African healthcare setting (Marais 2004).

We conclude that, in order to advance the routine clinical implementation of a genomics-based preventive strategy aimed at combating the health burden imposed by the growing global prevalence of AD and dementia, an effective framework is required to support a

continuum of research translation aimed at realizing the application of emerging genomic technologies to improve quality of life and promote wellness throughout life.

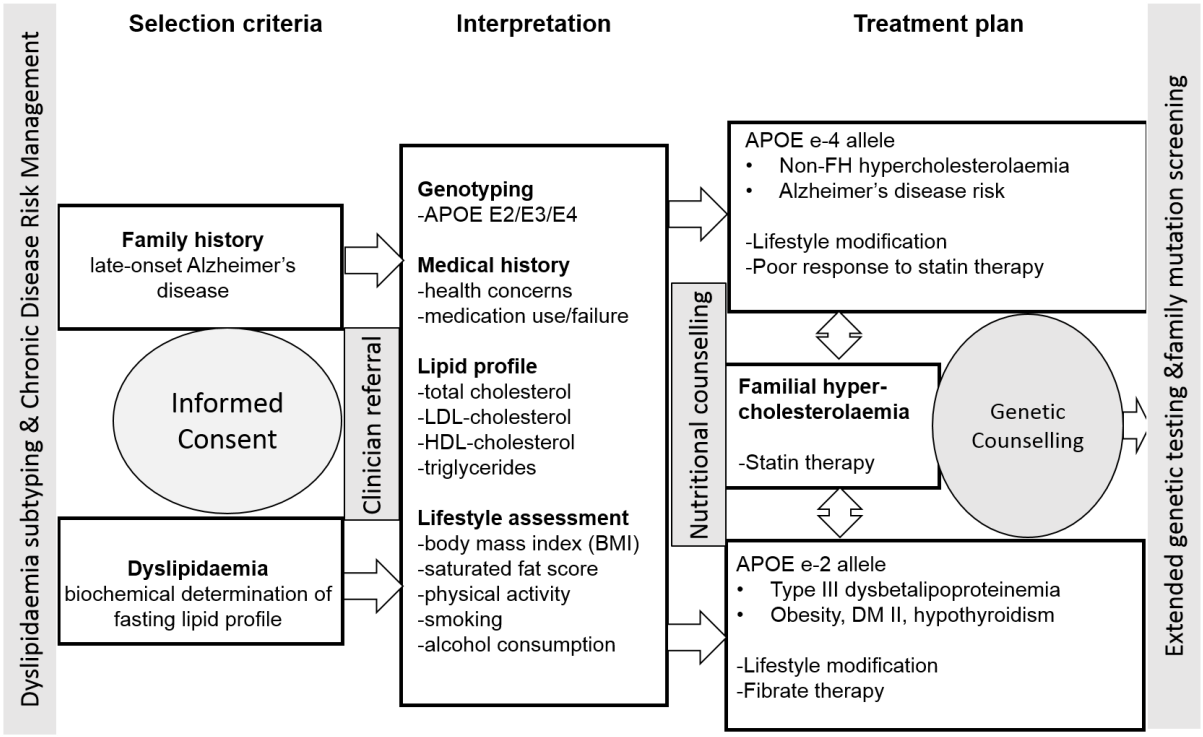

Figure 3. A framework for application of *APOE* genotyping as an integral part of chronic disease risk management focused on prevention of cumulative cardio-metabolic risk associated with Alzheimer's disease in later life.

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Disclosure

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CHAPTER 4

DISCUSSION AND CONCLUSIONS

There is ongoing interest in validating whether genetic testing could add value to risk management of patients with cardiovascular disease (CVD). The clinical application of genetic testing has long been restricted to a limited diagnostic scope of focusing on high-penetrance mutation screening for accurate diagnosis of Mendelian disorders such as FH (Kotze et al. 1993). In South Africa, the use of a CVD multi-gene assay performed in conjunction with biochemistry testing and a questionnaire-based clinical and lifestyle assessment (Kotze and Thiart 2003) provided one of the first examples of how genetic testing can be applied to identify patient subgroups requiring different treatment strategies (Kotze et al. 2015). The development and implementation of a combined research and service delivery platform allowed for the characterization of treatable dyslipidaemia subtypes using a multidisciplinary approach. The clinical application of this pathology-supported genetic testing (PSGT) approach to chronic disease screening provides the opportunity for development and refinement of testing algorithms that may help inform clinical and therapeutic decision making at an individual level (Kotze et al. 2015).

Recent evidence supports the added value of genetic testing in the diagnosis of mixed hyperlipidaemias associated with the *APOE* ε-2/ε-3/ε-4 polymorphism, which may result in impaired LDL receptor cholesterol binding (Marais et al. 2014). *APOE* genotyping could therefore play an important role in the differential diagnosis of FH and less severe forms of dyslipidaemia with the goal of prioritizing the need for tailored pharmacological interventions, including statin therapy. The assessment of *APOE* genotype has however been limited in general medical practice due to questions surrounding the clinical utility of *APOE* genotyping

as well as ethical issues inherent to genetic testing of multi-functional variants with reduced penetrance. In particular, public anxiety that the use of genetic information for actuarial underwriting may negatively impact upon their insurability may contribute to a general unease surrounding the relationship between genetics and the insurance industry. To ensure the appropriate use of genetic testing in clinical practice, a code of conduct has been compiled for the insurance industry in South Africa, which aims to promote the fair, equitable, confidential and evidence-based handling of genetic information. Kotze et al. (2004) previously reviewed this topic and concluded that genetic testing should not negatively impact upon insurability in cases where 1) patients with treatable genetic conditions where proof substantiating the efficacy of interventional strategies is sufficient, 2) when a family history or clinical symptoms of disease are already present, and 3) when the disease-causing mutation is excluded in at-risk relatives of the index patient. The authors contrasted different genetic services, emphasizing the distinction between high-penetrance mutations used in diagnostic tests for certain monogenic disorders and multi-gene assays for complex diseases with a genetic component. The latter is focused on the identification of genetic subtypes of treatable multifactorial conditions to guide the development and timely implementation of tailored risk reduction and therapeutic strategies aimed at decreasing cumulative risk for disease development and progression as well as limiting exposure to treatment side-effects and therapeutic failure.

4.1. Ethical considerations

The issue of cardiovascular genetic testing involving *APOE* genotyping and actuarial underwriting for insurance claims is complicated by the interaction between established legal principles and scientific evidence of clinical relevance. The development of increasingly complex genomic tools has further complicated the debate on genetic discrimination in the context of life insurance. Public fear that genetic data gathered as part of clinical and diagnostic work-up can result in discrimination, exclusion, loss of privacy, higher premiums

and ultimately negatively impact on their insurability could stifle scientific research and limit genomic innovations. This is opposed by the fear that actuarial fairness will not be possible if policy holders withhold genetic data from insurance companies, as well as that of adverse selection. Currently no consensus has been reached on the criteria and evidence required for genetic results to be used in actuarial underwriting (Zick et al. 2005). However, comprehensive case analysis is anticipated to further the development of reimbursement policies and strategies that facilitate data collection, inform payers and developers, and guard against potential discrimination or exclusion in the genomics era.

From an ethical viewpoint, a number of aspects should be considered in relation to *APOE* genotyping as well as pre- and post-test handling of patient information. *APOE* genotyping is not recommended as a component of pre-natal testing and is not advised in paediatric patients. In symptomatic patients, genetic counselling is recommended in consultation with a family member and/or legal guardian/caregiver. It is of critical importance to ascertain the clinical profile and age of onset in symptomatic patients, with emphasis on informing family members of the risk and mode of inheritance as well as potential indications for further testing and evaluation with advancing age. The ethico-legal and moral implications of *APOE* genotyping in asymptomatic high-risk patients remains unclear and the subject of ongoing debate regarding the consequences of testing on mental wellbeing and patient care. The REVEAL study showed that knowledge of *APOE* genotype does not result in significant short-term psychological distress (Chao et al. 2008). In general, patients are willing to pay for this test due to its perceived personal utility of *APOE* genotyping even in lieu of sufficient long-term evidence concerning its clinical utility at the time. Knowledge concerning individual genetic risk is however known to be integrated into pre-conceived notions regarding personal and familial susceptibility towards late-onset AD. However, the absence of *APOE* polymorphism carriage does not necessarily dissuade patients from the personal believe that they are at increased risk for developing AD (Chilibeck et al. 2011). These observations are in accordance with the notion that knowledge concerning *APOE* genotype does not add

significant diagnostic or predictive value in the assessment of dementia risk. This assumption however fails to take into account the role of *APOE* not as a diagnostic confirmation of a specific disease state, but rather as a tool for the development of tailored therapeutic strategies to combat cumulative risk for CVD/AD.

4.2. Clinical validation

APOE is the primary ligand of the LDLR underlying disease pathogenesis in patients with FH. In dyslipidaemic patients with a strong family history of cardiovascular disease which cannot be accounted for by the detection of variation in the *APOE* gene, the possibility of FH cannot be ruled out. This diagnosis should be considered in the context of clinical risk factors such as obesity and diabetes mellitus as a possible explanation for the presence of a deranged serum lipid profile. Kotze et al. (1993) previously described the role of receptor-defective (FH1 mutation, D206E) versus receptor-negative (FH2, V408M) mutations as causative for this severe monogenic dyslipidaemia known to be 5-10 times more prevalent in certain local population groups including Afrikaner patients. In this particular study (Kotze et al. 1993), the frequency of the *APOE* ε-4 allele was 0.17 in non-FH Afrikaner controls, 0.13 in patients with FH2 and 0.10 in patients with FH1. The authors demonstrated that disease severity was related to the type of FH mutation associated with the expression of different a phenotypes. Inter-individual variation in phenotypic presentation could not be accounted for by the *APOE* ε-4 allele. Presence or absence of this allele failed to contribute towards an additive or synergistic derangement in total serum cholesterol in patients already affected by FH as the cause of their medical condition. The aforementioned consideration that high-penetrance mutation screening for FH should be considered in a subgroup of *APOE* ε-4 non-carriers with elevated cholesterol levels is further supported by underrepresentation of this allele in South African patients with FH. This effect may be ascribed to selection against the *APOE* ε-4 genotype in FH patients who carry high-penetrance LDLR mutations responsible

for their disease. A similar phenotype selection could also partly account for the apparent protective effect of the ε-2 allele in the context of ischemic stroke and dementia.

Sandholzer et al. (1995) have shown that the frequency of the *APOE* ε-4 allele is two-fold higher, and homozyogisty 3-5 fold more common, in the Khoi San population compared to Caucasians in South Africa. A number of subsequent studies have confirmed that the *APOE* ε-4 allele is overrepresented in African population groups, which infers an associated increase in dose-dependent risk for vascular disease and dementia in the context of a Western lifestyle. For example, Zekraoui et al. (1997) demonstrated the frequency of the *APOE* ε-4 allele is 0.41 in African Pygmies and 0.18 in Sub-Saharan study participants. Corbo and Scacchi (1999) similarly noted the frequency of the *APOE* ε-4 allele as 0.41 in African Pygmies and 0.37 in the Khoi San population. Overrepresentation of the APOE ε-4 allele in African patients is also clear from a study conducted by Atadzhanov et al. (2014), who demonstrated a frequency of 0.27 in Zambians. The function of the *APOE* gene product was found to be homogeneous across ethnic groups despite differences in population-based genetic makeup (Hallman et al. 1991).

The *APOE* ε-2 allele has been associated with decreased LDL cholesterol levels in female patients with FH (Ferrières et al. 1994). Despite the dissociation between *APOE* ε-2 and hypercholesterolemia, a positive correlation between this allele and increased triglyceride levels has been reported in South African patients of African Ancestry (Masemola et al. 2007). In patients with FH, *APOE* ε-2 carriage is also associated with hypertriglyceridemia uncharacteristic of this severe dyslipidaemia subtype, in addition to being causative of an autosomal dominant form of type III dysbetalipoproteinemia (Blom et al. 2002). In South African patients, the *APOE* ε-2 allele was found to be associated with the metabolic syndrome (van Velden et al. 2011). These findings collectively argue against the notion that *APOE* ε-2 exerts a protective effect against the development of cardiovascular disease and associated complications. The cholesterol-raising effects of the *APOE* ε-4 allele, as well as the association between *APOE* ε-2 and increased triglyceride levels, were replicated in this

translational study despite the fact that the investigation was not performed in a controlled research environment. Our findings therefore provide important evidence substantiating the clinical validity of *APOE* genotyping in the local healthcare setting. Analytical validation of APOE genotyping has been performed previously as part of the postgraduate study of LR Fisher (personal communication).

4.3. Clinical application of APOE genotyping

The focus of *APOE* genotyping is mostly on disease management but it also has great potential for health management when combined with routine check-ups of serum lipid profiles and body weight in addition to related metabolic derangements. The results of these assessments are used together with information on medication use, lifestyle and family history to determine whether the genetic variations identified have current or future clinical relevance. The purpose of the combined pathology and genetic testing approach is to 1) identify treatable disease subtypes caused by a combination of genetic and environmental factors, 2) facilitate the prevention or lowering of cumulative risk by structured elimination of environmental drivers of the disease process and 3) to formulate a risk reduction plan for each patient based on the underlying disease mechanisms identified by using well-established health guidelines adjusted according to the clinical and genetic profile of the patient. This can only be accomplished if environmental exposures known to affect the expression of the genes included in the test are taken into account and correlated with the clinical picture and biochemical blood parameters. This can in turn be used to monitor the response to the intervention plan provided in the report.

For successful incorporation of *APOE* genotyping as a routine component of cardiovascular risk management, it is essential that both the indications for referral as well as the implications for treatment are well-established, as supported by the results of this study. Understanding that the *APOE* ε-2/ε-4 alleles investigated in this study are not causal, but

exert their detrimental effects under specific circumstances, holds great potential for lowering of cumulative disease risk. A lack of referral guidelines for *APOE* genotyping used to optimize cardiovascular risk management therefore limits the potential for clinical translation of genomic research in the context of chronic disease risk screening. The selection of a well-defined target population set to derive optimal benefit from *APOE* genotyping was identified as an important step towards clinical application in South Africa and elsewhere. Assessment of relevant clinical and lifestyle data could assist clinicians in determining eligibility for genetic testing in the context of dyslipidaemia to allow for the appropriate interpretation of *APOE* genotyping results.

In accordance with our goal to develop a novel pre-screen algorithm for cardiovascular genetic testing, we demonstrated the clinical relevance of inquiry concerning AD family history in determining eligibility for *APOE* genotyping. This was based on clinical expression of a hypercholesterolaemic phenotype in ε-4 allele carriers being dependent on the interaction between AD family history and *APOE* genotype. The observation that AD family history constituted a determinant of gene expression supports the notion that patients with a family history should be considered at high risk and need to be subjected to more regular routine follow-up to monitor lipid levels over time. The finding that the cholesterol-lowering benefits of physical activity were limited to patients with a positive family history of AD further supports the relevance of this variable in the development and implementation of tailored lifestyle-based interventions. In addition, we demonstrated that the modifying influence of *APOE* genotype on the association between alcohol, dietary fat intake, BMI as well as total cholesterol levels, were also related to cumulative risk associated with a family history of AD. These findings collectively support the role of a questionnaire-based clinical and lifestyle assessment to facilitate the appropriate clinical interpretation of *APOE* genotyping results performed as part of a comprehensive multidisciplinary approach to cardiovascular risk screening and management.

From converging lines of evidence, the value of a questionnaire-based approach was demonstrated as a useful tool to validate the relationship between metabolic phenotypes and lifestyle risk factors in the South African population. In addition, we demonstrated that the relationship between these parameters was in certain circumstances also influenced by the presence or absence of a family history of AD. In our study, BMI was positively associated with LDL cholesterol and inversely associated with HDL cholesterol levels irrespectively of AD family history. The dietary fat score was positively correlated with LDL cholesterol and inversely associated with HDL cholesterol levels only in study participants with a positive family history of AD. Smoking was further associated with higher triglycerides and lower HDL cholesterol levels in the total study group. Alcohol intake was positively associated with BMI as well as triglyceride levels only in patients with a positive family history of AD. These differential effects on the relationship between cardio-metabolic risk traits and modifiable lifestyle habits highlighted the importance of clinical inquiry concerning AD family history not only as a pre-screen selection step for *APOE* genotyping, but also as an integral component of a multidisciplinary approach to cardiovascular risk management guided partly from a genetic background.

Findings from this research project collectively support the routine clinical application of *APOE* genotyping performed in the context of cardiovascular risk assessment. Importantly, *APOE* genotyping could assist in increasing case finding and diagnosis of monogenic dyslipidaemias such as FH, while simultaneously preventing overtreatment with lipid-lowering pharmacotherapy in cases where it is not indicated or could cause adverse drug side effects. In this context, detection of very high LDL-cholesterol levels and/or a strong family history of early-onset coronary heart disease that cannot be accounted for by variation in the *APOE* gene should alert clinicians to a possible diagnosis of FH. This condition require early initiation of lipid-lowering statins to effectively combat risk for cardiovascular complications. Confirmation of *APOE* ε-4 carrier status in non-FH dyslipidaemic patients would allow for the timely implementation of tailored lifestyle-based interventions aimed at

decreasing cumulative cardiovascular risk. *APOE* genotyping is also proposed as a means of 1) facilitating clinical follow-up and monitoring for ischemic vascular complications and dementia over time, 2) guiding the selection of appropriate existing and emerging therapeutic modalities in patients with a confirmed diagnosis of CVD/AD, and 3) informing clinical and therapeutic decision making to improve health and well-being.

The aforementioned clinical applications for *APOE* genotyping are considered as ancillary to its existing role in improving diagnostic reliability for late-onset AD in patients who present atypically. In this context, *APOE* genotyping performed as part of a CVD multi-gene risk assay (Kotze and Thiart 2003) could prove useful not only in optimizing cardio-metabolic risk screening and intervention, but accurately identifying patients with FH which remains both underdiagnosed and undertreated in South Africa (Marais 2004). It however remains imperative to perform ongoing validation and comparative effectiveness studies to provide further evidence supporting the clinical utility of *APOE* genotyping. In our study, analytical validation of the CVD multi-gene assay as well as replication of the well-established disease-associated and risk-modulating effects of the *APOE* polymorphism plays an important role in this regard. It is important to establish whether *APOE* genotyping alters clinical and therapeutic decision making to improve patient outcomes and increase quality of life to promote overall wellbeing in the South African population.

Based on the abovementioned observations, a framework is presented which supports the implementation of a comprehensive pathology-supported genetic testing strategy incorporating *APOE* genotyping as an integral component of a chronic disease risk screening program (Figure 1).

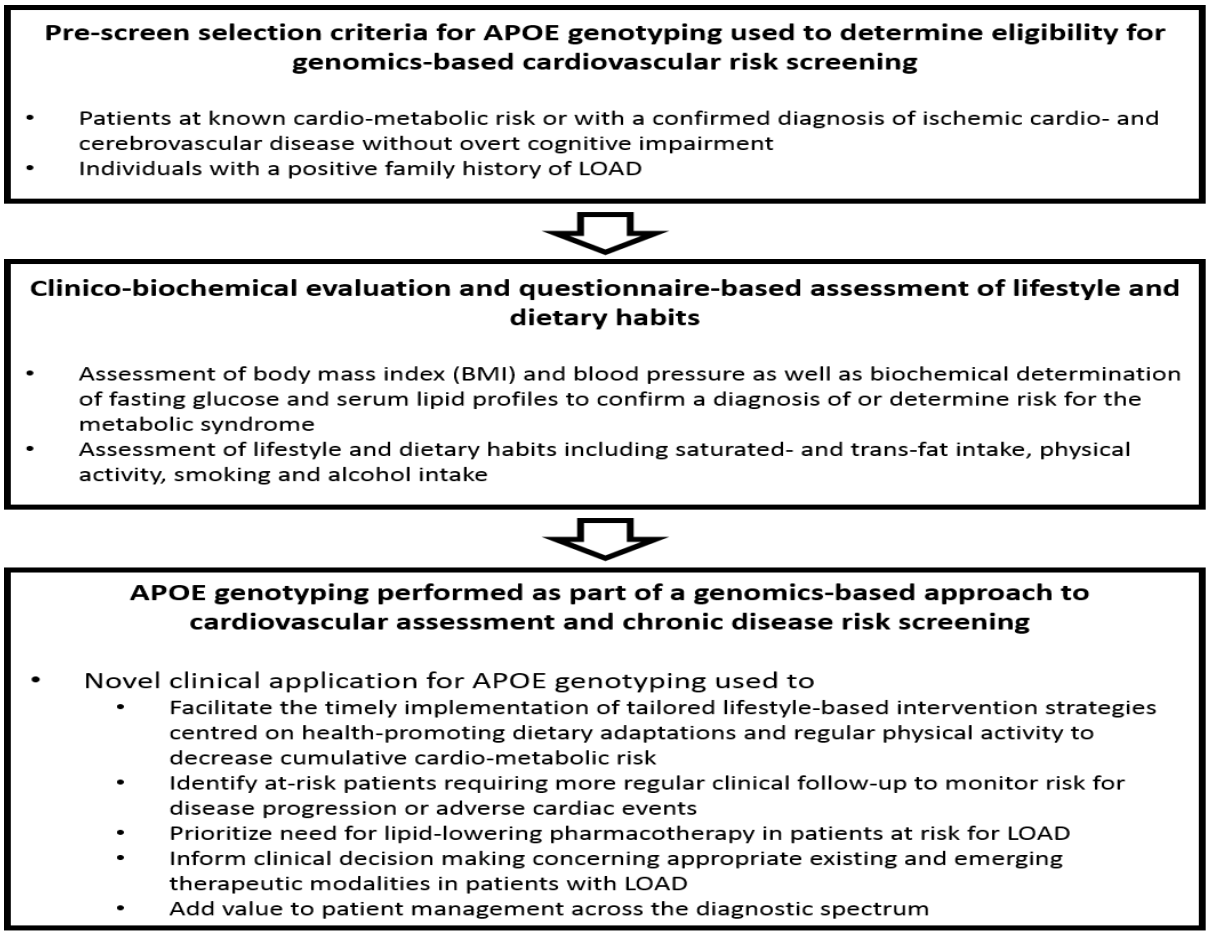

Figure 1. Pipeline for the development of a multidisciplinary framework supporting the routine implementation of *APOE* genotyping as a routine component of patient care in the context of chronic disease risk screening.

4.4. Conclusions

In this study, a combined research and service delivery approach was used to develop a pre-screen algorithm incorporating AD family history as part of the selection criteria used to determine the appropriateness of *APOE* genotyping. Previously, only clinical indicators including dyslipidaemia and other relevant non-genetic lifestyle factors were considered suitable selection criteria for application of the CVD multi-gene test. In this context, genetic testing is applied to improve cardiovascular risk assessment and optimize cumulative risk reduction in a susceptible subgroup of dyslipidemic patients. Evaluation of the wellness

genomic profiling performed as an integral part of a chronic disease screening program enables selection of the most appropriate therapy for the patient. Treatment failure or drug side-effects may however occur in a subset of patients who would then be eligible for extended clinical / exome sequencing to identify the causative mutation(s). Application of pathology-supported genetic testing may therefore also serve as a pre-screening step to determine the appropriateness of extended genetic testing in genetically uncharacterized patients. This will be the case when a medical condition exists or the pathology test is abnormal in the absence of relevant tested genetic risk factors, meaning that other genetic and/or environmental risk factors not covered in the initial test / assessment are responsible for the condition. In such situations an extended mutation analysis or pathology testing may be recommended as appropriate. A genetic test is usually performed once in a lifetime and patients will benefit most when relevant pathology test results are integrated with the genetic test results. By looking at the personal and family medical history in the context of the test results, it is possible to identify whether any biochemical abnormalities detected could be caused by genetics or whether it is most likely environmentally induced and may interact with specific genes.

Regular updating (approximately once a year) of the clinical information (e.g. personal medical conditions, cholesterol, weight and diet assessment) online will enable the generation of follow-up reports to determine the effectiveness of the proposed intervention plan as provided in the report. If no improvements are seen according to pre-set goals, adjustment of the treatment plan may be necessary. This is important as an intervention plan that may be provided as a patient handout is considered experimental due to the fact that the clinical utility has not previously been tested in the context of the unique clinical and genetic profile of each individual patient. The incorporation of testing results obtained from *APOE* genotyping into a universally accepted body of knowledge is done according to well-established principles to optimize cardiovascular risk management with the added goal of preventing the onset and progression of AD with advancing age. In accordance with the

results obtained in this study, *APOE* genotyping could assist in identifying a genetic subgroup of dyslipidaemic patients set to derive optimal benefit from weight management and lifestyle modification. This includes limiting alcohol consumption and decreasing the dietary intake of saturated and trans-fats. Regular clinical follow-up and monitoring of serum lipid profiles is advised in order to establish the efficacy of lifestyle-based interventions, with non-responders considered as potentially eligible to receive statins as part of their treatment plan.

Our findings provided the basis for implementation of a novel pre-screen algorithm for *APOE* genotyping used to assist cardiovascular risk assessment and management. The implementation of a research pipeline created an incentive to develop a reimbursement policy for the conditional approval of this genetic test by certain local medical aid schemes, using an integrated research and service delivery approach. Given the fact that variation in the *APOE* gene affects response to cholesterol-lowering treatment, prevention of statin overtreatment is an important consideration in this approach, as illustrated in Figure 2.

In addition to a confirmed diagnosis of dyslipidaemia, it is proposed that patients with a positive family history of late-onset AD should be considered eligible for *APOE* genotyping following pre-test counselling and obtaining informed consent even if serum lipid profiles are normal. In accordance with our finding that AD family history constitutes a determinant of the expression of a hypercholesterolaemic phenotype in a genetic subgroup of dyslipidaemic patients, *APOE* polymorphism carriers should be considered at increased cardiovascular risk and should be subjected to more regular follow-up to monitor lipid levels. If the patient's clinico-biochemical profile is consistent with a diagnosis of FH, early initiation of statin therapy is indicated. Lifestyle-based interventions including weight loss and dietary adjustments should be considered first-line therapy in *APOE* ε-4/ε-2 carriers as well as patients at increased cardio-metabolic risk (e.g. obesity) in non-carriers. In accordance with our findings, statins should best be avoided in asymptomatic *APOE* ε-4 carriers, with a primary focus on lifestyle-based interventions, including more regular physical activity as well

as limiting alcohol consumption and decreasing the intake of dietary saturated and trans-fats. In *APOE* ε-2 allele homozygotes, the assessment of "second-hit" pathologies including obesity and hypothyroidism which increase the risk of developing type III dysbetalipoproteinaemia is warranted. In the presence of hypertriglyceridaemia, these patients may also require treatment with lipid-lowering fibrates if a favourable response to diet intervention is not achieved. Patients at increased cardiovascular risk for whom severe dyslipidaemia could not be accounted for by *APOE* ε-2/ε-4 carriage, next-generation sequencing may be indicated if their clinical profile as well as personal and family medical history is not in keeping with FH.

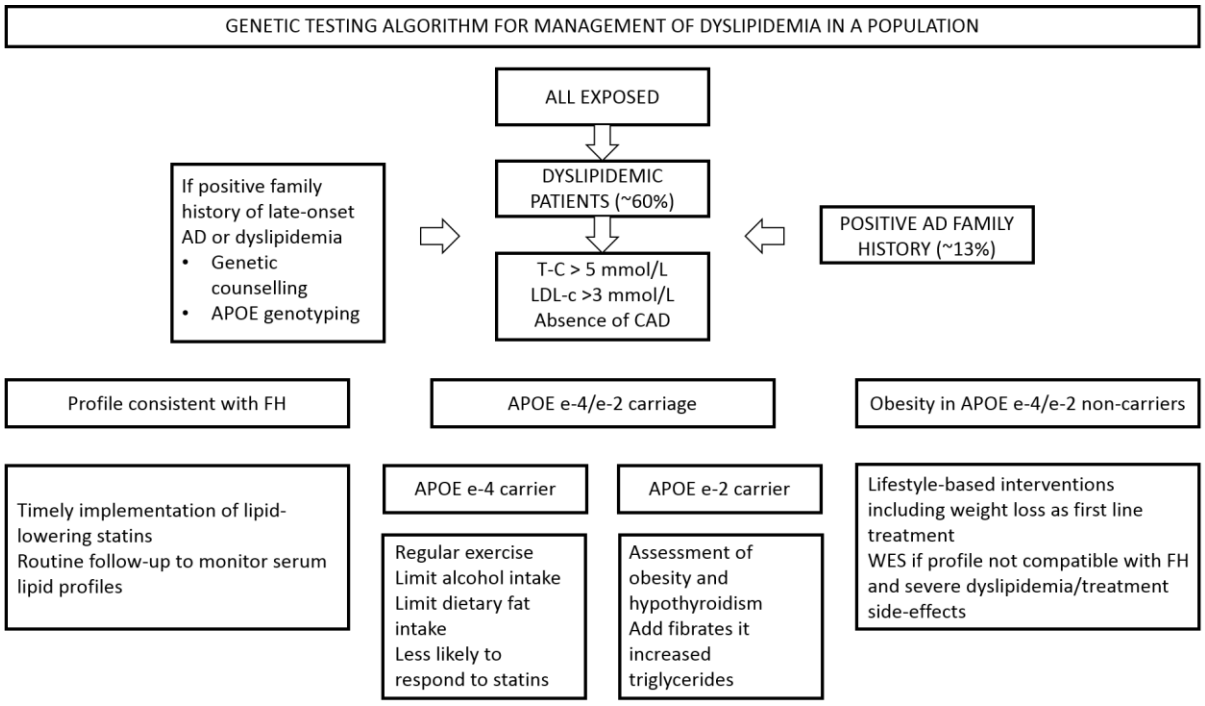

Figure 2. Pre-screen algorithm used to determine eligibility for *APOE* genotyping performed as an integral component of vascular risk management to optimize treatment in dyslipidaemic patients. The novel component of this algorithm is the inclusion of assessment of AD family history to identify a subgroup of patients set to derive benefit from *APOE* genotyping even in the absence of a deranged lipid profile at baseline/referral. *APOE* genotyping performed in conjunction with a questionnaire-based assessment of clinical and

lifestyle risk factors aims to prioritize the need for lifestyle-based as opposed to pharmacological interventions to normalize serum lipid profiles.

In summary, collective evidence gathered from a local healthcare setting supports the clinical validity as well as utility of *APOE* genotyping performed as an integral component of cardiovascular risk screening used to optimize cumulative risk reduction across the disease spectrum in a high-risk genetic subgroup of dyslipidemic patients. Firstly, the role of clinical inquiry concerning AD family as part of a novel pre-screen algorithm is supported to determine eligibility for *APOE* genotyping beyond the assessment of serum lipid profiles. Our findings further support the use of a questionnaire-based approach to the assessment of lifestyle risk factors to facilitate the accurate clinical interpretation of *APOE* genotyping results within a multidisciplinary framework. The consideration of genetic testing results within the context of diverse patient data gleamed from a variety of sources is positioned as a response to the limitations inherent to genomics-only risk assessment.

In conclusion, we demonstrated the appropriateness including the assessment of AD family history as a novel component to the pre-screen algorithm used to determine eligibility for *APOE* genotyping. In accordance with international studies, we provided local evidence of the interaction between *APOE* genotype and lifestyle factors known to influence the course and progression of both CVD and AD. In particular, the finding that cardiovascular risk may be ameliorated by regular physical activity emphasizes the potential of *APOE* genotyping to direct clinical management in high-risk patients to decrease cumulative risk for CVD as well as AD. The routine clinical application of *APOE* genotyping in the local healthcare setting has the potential to inform clinical and therapeutic decision making to ultimately improve wellness and promote quality of life in dyslipidaemic patients at risk for AD.

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APPENDIX

Ethics Letter

22-Sep-2015

Ethics Reference #: N09/08/224
Title: Application of personalized medicine using an integrated service and research approach.

Dear Prof Martha Kotze,

The HREC approved the following progress report:

Progress Report dated 01/06/2014 - 31/08/2015

The approval of this project is extended for the further year.

Approval date: 17 September 2015

Expiry date: 17 September 2016

If you have any queries or need further help, please contact the REC Office 219389819.

Sincerely,

REC Coordinator
Ashleen Fortuin
Health Research Ethics Committee 2

Ethics Letter

05-May-2015

Ethics Reference #: N09/08/224
Title: Application of personalized medicine using an integrated service and research approach.

Dear Professor Martha Kotze,

Your letter dated 10 April 2015 refers.

The Health Research Ethics Committee approved the amended documentation.

The following amendments were approved:

Inclusion of children for genetic testing under the circumstances described in the revised protocol. Use of available DNA samples and frozen blood of six children from project N09/03/092 with diabetes and permission for recontacting of the parents to obtain a new sample if the available samples are insufficient. Approval for inclusion of the attached article written by Dr Hilmar Luckhoff as part of this study for his masters study entitled: Development of a novel pre-screen algorithm for cardio-metabolic risk management using a genomics database resource.

If you have any queries or need further help, please contact the REC Office 219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

SUMMARY ON RELEVANT STUDY METHODOLOGY

The information below describes the methodology used in more detail and the specific contributions of the candidate are summarised in relation to each article on pages 54 and 63.

The study in question involved the retrospective assessment of patient information included in a research database linked to a pathology-supported genetic testing (PSGT) service conducted at the interface between the laboratory and clinic. This approach has been described in the review by Kotze MJ, Luckhoff HK, et al. (2015) as referred to in the thesis.

This study was not a clinical controlled trial, but performed in an uncontrolled translational research/clinical setting. Different private practicing clinicians referred patients for inclusion in a chronic disease / wellness screen including a genomics component. We therefore did not have control over the process of obtaining a peripheral blood sample for biochemical testing performed as part of routine clinical practice. It was however confirmed in personal communication with Professors RT Erasmus and SJ van Rensburg that all biochemical analyses were performed at accredited laboratories using standardized tests. The pathology test results were entered into the database at enrolment of study participants.

Description of the research model used in this study

The *apolipoprotein E* (*APOE*) ε-2/ε-3/ε-4 polymorphism is the most extensively investigated genetic risk factor for late-onset Alzheimer's disease (AD). The risk-associated *APOE* ε-4 allele provides a genetic link between cardiovascular disease (CVD) and AD owing to its pathogenic associations with numerous metabolic abnormalities including impaired lipid homeostasis which formed the basis of this study. A number of factors however limits the clinical application of *APOE* genotyping as a biomarker with probable clinical utility in general medical practice. These include the lack of well-defined eligibility criteria used to identify a target population of dyslipidaemic patients set to derive optimal benefit from *APOE* genotyping used to inform clinical and therapeutic decision making. Another clinical dilemma is represented by concerns regarding the ethical implications of *APOE* genotyping. Towards the goal of overcoming these limitations, a local study was designed utilizing a combined research and service delivery approach facilitated by the retrospective assessment of comprehensive patient information included in a secure research database. The implementation of a learning healthcare system (Figure 1) in this context provided an invaluable tool to allow research findings to inform and influence practice guidelines and clinical decision making. Results obtained outside a controlled research setting was evaluated against existing international studies for implementation during a pilot study. This preliminary data served as the basis for the development of a pre-screen selection algorithm for *APOE* genotyping incorporating clinical inquiry concerning AD family history as a novel

component used to direct eligibility for *APOE* genotyping as part of a multi-gene CVD assay. This approach served in itself as the basis for development of a reimbursement policy for *APOE* genotyping as discussed by Prof Manie de Klerk (presented at the 10th Applied Genetics Workshop, 30 October 2015). The assessment of large-scale prospective patient data based on this prescribed algorithm would allow for its continual improvement, based on evidence that *APOE* genotyping does influence patient care, with specific regard to the necessity for lipid-lowering statin therapy in ε-4 carriers. Risk-benefit and cost-effectiveness studies would prove useful in determining the feasibility and clinical utility of *APOE* genotyping in the local clinical domain. The dissemination of these findings through a continuum of research translation would allow for large-scale improvements in patient care as well as assist in identifying potential issues which could hinder the routine implementation of *APOE* genotyping as a component of chronic disease risk screening and management.

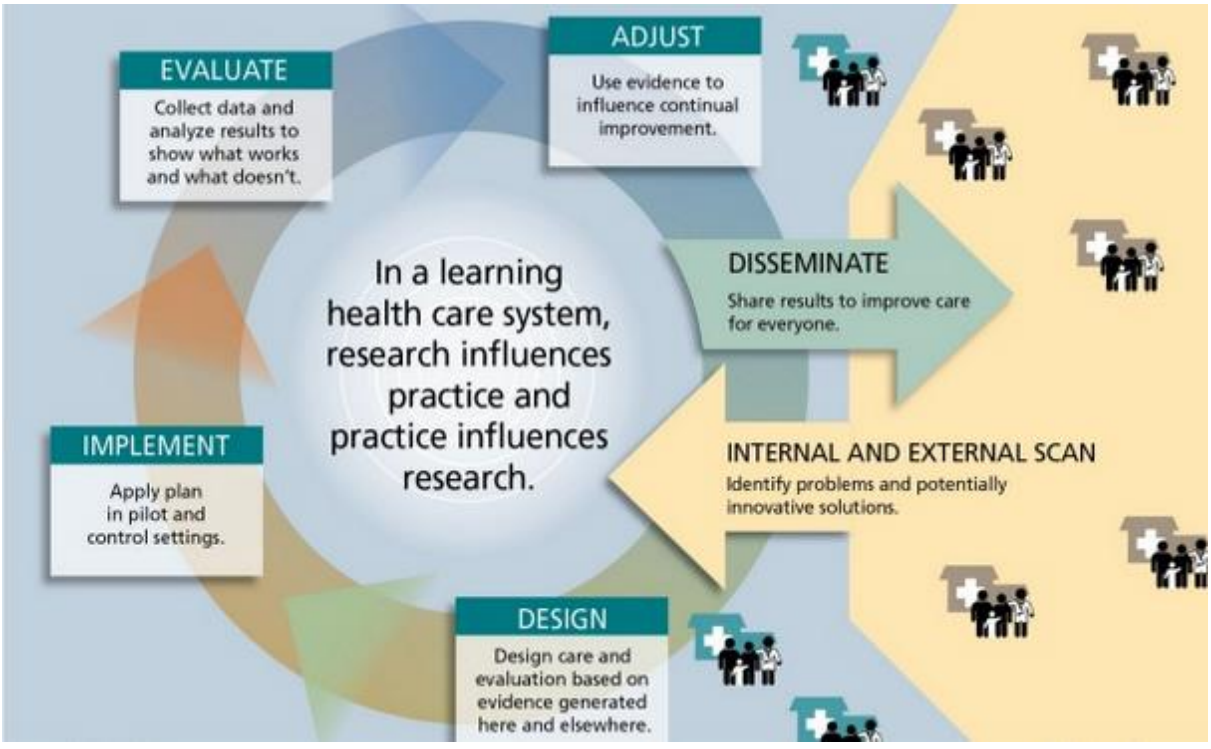

Figure 1. Pipeline for the implementation of a learning healthcare system to allow for the conditional approval of APOE genotyping used to direct patient care and inform clinical decision making in the South African setting (source: http://www.slideshare.net/grouphealth/learning-health-care-systems).

Data analysis and statistical methods examples

The pathology-supported genetic testing (PSGT) approach emphasizes the importance of considering genetic testing within the context of diverse phenotypic data gleamed from a variety of sources and linked by the common thread of pathological assessment. Prior to genetic testing using an analytically validated multi-gene assay, all prospective patients are asked to complete an ethically approved Medical History and Lifestyle Questionnaire (available at www.gknowmix.com) used to document information on personal and family medical history, relevant clinical data and medication use. This information is used to determine eligibility for genetic screening with the goal of preventing unnecessary testing or initial referral in cases where the health concerns are unlikely to be addressed. Results from genetic testing are considered within the context of clinical data and provided as an integrated interpretive report to the referring clinician, including suggestions as to 1) how lifestyle changes and adjustment to dietary habits could possibly be used to mitigate relevant risk factors as guided partly from a genetic background, as well as 2) to what extent existing treatment could be altered or dosages changed to decrease risk for treatment-related side-effects in addition to therapeutic intolerance and/or failure. This approach is aimed at increasing treatment compliance and improving quality of life.

Information obtained from patients who provided written informed consent for research participation were filtered through to the back-end of the patient database (accessed at www.gknowmix.org), which contains the data suitable for research use. In an attempt to maintain anonymity, each patient was assigned an automatically generated reference number with patient names and other identifiers removed as appropriate for uploading to the research database. After the necessary data fields are selected for a specific study, the patient information is extracted and exported to an Excel document for statistical analysis.

In order to facilitate communication between the researcher and statistician, it is essential that a standardized report containing a summary of the relevant patient data as well as research aims and objectives is made available. Towards this goal, a semi-automated online template was developed for presenting data to a bioinformatician or statistician. This template includes the necessary preliminary statistical tests which was performed by the candidate in order to describe patient data and communicate this effectively in line with the project goals.

Input of dataset into statistical program and testing whether numerical data is normally distributed	1) Input dataset into R Studio >read.table(file="dataset.csv",header=T,sep=";",dec=".",colClasses= c("numeric","factor"))->dat1 2) Determine whether continuous numerical data is normally distributed using the Shapiro-Wilk test (example for age) >m1=mean(dat1$AGE,na.rm=T) >m1 >s1=sd(dat1AGE,na.rm=T) >s1 >normvec1=rnorm(150,mean=m1,sd=s1) >shapiro.test(normvec1) [p<0.05 – normally distributed] 3) Describe normally distributed numerical data as the means along with standard deviation (SD) and non-normal data as the median along with interquartile range (IQR)
Sex-based comparison of clinical and biochemical characteristics as well as lifestyle and dietary habits of study participants	Create subset for males and females >subset(dat1,SEX==0)->df1 >subset(dat1,SEX==1)->df2 Calculate the means and standard deviation for each numerical variable for both males and females Compare numerical data between males and females using a Student's t-test (example for age) >df1$AGE->vect1 >df2$AGE->vect2 >t.test(vect1,vect2) = provides p-value Compare categorical data by creating a contingency table and using the Chi-squared test or Fisher's test if cell counts are equal to or less than five >contingency1=matrix(c(10,20,30,30,20,10),nrow=3)

	>chisq.test(contingency1) >contingency2=matrix(c(5,10,20,20,10,5),nrow=3) >fisher.test(contingency2)
Comparison of genotype distributions and minor allele frequency for genetic variant(s) of interest between patient groups	Determine genotype distribution by allele count and test for Hardy-Heinberg equilibrium (HWE) for both groups using an exact test [link: HWE calculator] and describe findings in a separate table. Compare genotype distribution(s) and minor allele frequencies using either the Chi-squared test or logistic regression analysis to comment on to what extent the variant of interest confers risk for the condition of interest Chi-squared test: >contingency3=matrix(c(40,50,10,30,50,20),nrow=3) >chisq.test(contingency) Logistic regression analysis (considering one variant of interest) Create a column (PC) with patients and controls coded as controls=0 and patients=1, and considering the variant of interest e.g. MTHFR 677C>T coded as M677 with CC=0, CT=1 and TT=2: >library(MASS) >step_with_mult=stepAIC(glm(PC~M677,data=dat1,family=binomial(link=”logit”))) >summary(step_with_mult) Describe with odds ratios (OR) and 95% confidence intervals (CI) >exp(cbind(OR=coef(step_with_mult),confint(step_with_mult)))

For the two sub-studies, the qualitative characteristics of interest in this study were: sex, alcohol intake, physical activity, smoking status and statin use. The quantitative phenotypes of relevance to this study were: age, body mass index, the fat score, as well as the components of the lipid profile (total, HDL, LDL cholesterol and triglyceride levels).