Thyroxine in Acute Myocardial Infarction (ThyrAMI)

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Importance: Thyroid hormones play a key role in modulating myocardial contractility and vascular function. Subclinical hypothyroidism is associated with worse cardiovascular outcomes, in those without cardiovascular disease, and a poor prognosis in patients with acute myocardial infarction.

Objective: To evaluate the effect of levothyroxine treatment on left ventricular function, markers of vascular function and patient reported outcomes in patients with acute myocardial infarction and subclinical hypothyroidism.

Hypotheses: Levothyroxine treatment will improve left ventricular function as a primary outcome measure. Furthermore, levothyroxine treatment will decrease thrombus burden, improve clot kinetics, platelet reactivity and endothelial function as well as patient reported outcomes.

Design, Setting, and Participants: A double blind, randomized clinical trial conducted in six hospitals in the United Kingdom. Patients with acute myocardial infarction including ST-segment elevation and non-ST-segment elevation were recruited between February 2015 and December 2016 with the last participant being followed up in December 2017.

Interventions: Levothyroxine treatment (n=46) commencing at 25 mcg titrated to aim for serum thyrotropin levels between 0.4 and 2.5 mU/L or identical placebo (n=49), both provided in capsule form, once daily for 52 weeks.

Main outcomes and measures: The primary outcome measure was left ventricular ejection fraction at 52 weeks, assessed by magnetic resonance imaging, adjusted for age, sex, type of acute myocardial infarction, affected coronary artery territory and baseline left ventricular ejection fraction. Secondary outcomes were surrogate markers of vascular function and patient reported outcome measures of health status, health-related quality of life, and depression.

Results: Among the 95 participants randomized, the primary outcome measurements at 52 weeks were available in 85 (89.5%) patients. The mean left ventricular ejection fraction at baseline and at 52 weeks was 51.3% and 53.8% in the levothyroxine group compared to 54.0% and 56.1% in the placebo group; adjusted difference in groups (95% confidence interval) of 0.76% (-0.93% to 2.46%), p=0.37. Levothyroxine treatment did not significantly decrease thrombus burden, improve clot kinetics, decrease platelet reactivity or improve endothelial function. Furthermore, patient reported outcomes were not significantly different between both groups at the study end. There were 15 (33.3%) and 18 (36.7%) cardiovascular adverse events in the levothyroxine and placebo groups, respectively.

Conclusions and relevance: In this preliminary study involving patients with subclinical hypothyroidism and acute myocardial infarction, treatment with low dose levothyroxine, compared to placebo, did not significantly improve left ventricular ejection fraction, markers of vascular function and patient reported outcomes after 52 weeks. These findings do not support treatment of subclinical hypothyroidism in patients with acute myocardial infarction.

Dedications

I dedicate this work to my two children, Zark and Zarlush, my mentors and all those patients who volunteered to help.

Acknowledgements

I would like to thank everyone who helped with this project and despite the help of so many; I can only acknowledge a handful of names in this section. It would not have been possible to complete this work without the guidance, patience and sincere support of all those involved with this project.

Firstly, I would like to thank my supervisors Dr Salman Razvi and Professor Azfar Zaman without whom this work would never have been a certainty. My biggest gratitude goes to Dr Salman Razvi for giving me this wonderful opportunity to come to Newcastle to undertake this PhD project and for having the trust in me to deliver on this project. I am grateful for his tireless enthusiasm, valuable guidance and persistent encouragement throughout the project and for helping me to develop a good foundation in research. His passion for research, strive for excellence and sharp judgment has provided me with the perfect role model to follow in my future scientific endeavours and clinical practice. I am grateful to Professor Zaman for all his guidance and supervision, whilst I was at the Freeman Hospital, and for supporting me in all the studies undertaken at the Clinical Research Facility. I would like to thank my PhD panel members, Professor Helen Arthur and Dr Tim Cheetham for their continued support, encouragement and insightful comments. I would also like to thank Lorna Ingoe for all her hard work and support throughout the three years of my research. She worked tirelessly in helping to recruit patients, in recording data and performing patient follow-ups.

I would like to thank my wife Spogmay for playing a pivotal role throughout my three years as a research fellow including her persistent patience during the write-up phase. Her continued support and encouragement throughout the research period made every step that much easier to accomplish. Finally, yet importantly, I thank every patient who volunteered to help with this study; without whom this work would never have been a reality.

Declaration

I declare that this thesis submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy to Newcastle University is a product of my original work and not submitted elsewhere for a degree or diploma. The studies were conducted by me at the premises of Newcastle University and Newcastle Upon Tyne NHS foundation Trust. I was responsible for recruitment of participants and their care, conduct of all the studies, performing follow up visits, collection and analysis of the samples, data management, data analysis and research governance. I have correctly acknowledged the specific contributions by others in the relevant sections of the thesis.

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1.1 Basic principles of thyroid hormone action on the cardiovascular system

1.1.1 Background

Acute myocardial infarction (AMI) and its related complications represent a major public health problem. Despite improvements in reperfusion therapy the 2020 World Health Organisation projections view the high incidence of post-ischaemic heart failure as the most important cause of morbidity and mortality (Kitakaze, 2010). Over the years improvements in coronary reperfusion, first by thrombolysis and later by angioplasty, have significantly improved outcomes in patients with AMI (Keeley *et al.*, 2003). Limitation of infarct size extent and recovery of dysfunctional contractile segments are the key elements for preventing post-ischaemic left ventricular remodelling. Despite improvements in coronary perfusion, many patients go on to develop heart failure due to pathological ventricular remodelling. In addition to research into new reperfusion strategies, a new perspective involves studying mechanisms by which myocytes can self-protect at times of stress which can prevent irreversible cell death. Such mechanisms include preventing myocyte apoptosis, preserving mitochondria, targeting survival pathways such protein kinase B (PKB) and protein kinase C (PKC) as well as promoting angiogenesis (Kitakaze, 2010; Nicolini *et al.*, 2013).

In this regard, thyroid hormones (TH) are increasingly being recognised as significant players both in the pathogenesis of and the recovery and repair after an AMI. The role of TH in aggravating cardiovascular disease is recognised, given that TH receptors are present in both the myocardium and vascular endothelial tissues thereby allowing changes in circulating TH concentrations to modulate end organ activity. This involves regulating genes which encode key proteins involved in myocardial contractility such as the myosin heavy chain, and the sarcoplasmic reticulum calcium pump as well as activating key survival pathways which will be explored in more detail. It is likely that interventional trials investigating the role of thyroid hormones in ameliorating post AMI complications will be performed in the coming few years (Pantos *et al.*, 2009b; Pantos *et al.*, 2010). Low TH in different cardiac states has traditionally been thought as an adaptive process, which lowers energy demands. However it is also likely that low TH levels in heart disease states is due to a permissive state, which favours cardiac failure. This is shown by the presence of low TH in states such as AMI, heart failure and post coronary artery bypass grafting (CABG) being associated with a worse prognosis (Hamilton *et*

al., 1990; Novitzky *et al.*, 1996; Friberg *et al.*, 2001; Lymvaios *et al.*, 2011). Furthermore, experimental studies have demonstrated that abnormal cardiac remodelling, angiogenesis, gene expression and systolic functional changes in low TH states are reversible with TH supplementation (Pantos *et al.*, 2008a; Forini *et al.*, 2011).

Even subclinical hypothyroidism (SCH) is associated with worse cardiovascular outcomes (Parle *et al.*, 2001; Walsh *et al.*, 2005). From a clinical perspective 'subclinical' denotes the presence of disease without manifest symptoms and suggests the presence of either mild or early disease. SCH has been associated with increased cardiovascular morbidity and mortality, especially in younger individuals (Imaizumi *et al.*, 2004; Kvetny *et al.*, 2004). However, the clinical significance of SCH and mild thyroid overactivity (subclinical hyperthyroidism) is uncertain. The absence of high-quality clinical trials has adversely impacted on optimal protocols for diagnostic testing and created uncertainty regarding the appropriateness of treating the mildly abnormal serum thyroid test. Furthermore, thyroid function tests are commonly checked by health professionals: one in four people in the United Kingdom have their thyroid function assessed annually (Allahabadia *et al.*, 2009). Frequent testing therefore can lead to detection of incidentally abnormal thyroid function parameters and, in the absence of high quality evidence either for or against treatment, lead to confusion as to the best evidence-based management of subclinical thyroid diseases.

Another challenge in the diagnosis of SCH includes the variation of the median TSH and reference ranges with age and time of day with the latter related to the circadian variation of TSH, which current TSH reference ranges do not account for. Normal TSH reference ranges are based according to the 2.5^{th} and 97.5^{th} percentiles of the distribution in the tested population. The National Health and Nutrition Examination Survey (NHANES) study showed the 97.5^{th} percentile to be 3.5 mU/L in 20-30 year olds and 7.5 mU/L in those above the age of 80 whereas Vadiveloo et al in the TEARS Study assessed thyroid function tests retrospectively in over 150000 subjects without thyroid disease and found the 97.5^{th} median TSH centile to increase from 3.98, in subjects 30-40 years of age, to 5.94 in those above 90 (Hollowell *et al.*, 2002; Vadiveloo *et al.*, 2013). Another retrospective laboratory database interrogation of TSH data from more than 19,000 outpatient visits concluded that the prevalence of SCH reduced from 14.1% to 7.9% and that of SHyper increased from 6.8% to 7.9% between 7-8am and 2-3pm, respectively (Andersen *et al*., 2015). This shows there are clear confounders in relation to the

normal TSH reference range and therefore any decision on treating an individual should not be according to one TSH value above the normal range.

1.1.2 Historical perspective

The association between thyroid dysfunction and cardiovascular disease (CVD) is not new. In 1878 William Greenfield, decades before thyroid function tests became available, described the autopsy findings of a middle-aged woman with myxoedema as having *"much serous effusion in the pericardium…….the heart was large……the arteries were everywhere thickened, the larger ones atheromatous"* (Ord, 1878). Soon after, clinical and autopsy accounts describing atherosclerosis in myxoedema patients were beginning to be reported (T, 1883; TM, 1888). It soon became apparent that the clinical condition of myxoedema was linked to a poorly functioning thyroid gland. However, the diagnosis of myxoedema (a severe form of hypothyroidism) was based purely on clinical grounds and, in the absence of modern thyroid function assays, milder and less clinically apparent forms of the disease would have been missed. In 1891, George Murray, injected sheep thyroid hormone extract subcutaneously to a patient with clinical features of myxoedema with dramatic effect. Over the subsequent 30 years Murray switched to oral thyroid extract collected from a pool of sheep - the glands being obtained from an abattoir. It took many years (1949) before commercial production of thyroxine was available but, even then, for many years, tablets of desiccated thyroid extract were the mainstay of replacement (Slater, 2011). This new therapeutic agent was shown in clinical trials of myxoedema patients to have positive effects in reducing high cholesterol levels (Barnes, 1959) and improving cardiovascular morbidity (Barnes, 1973) and mortality (Kountz, 1950). From 1965 onwards, the first generation of radio-immunoassays to estimate serum Thyroid Stimulating Hormone (TSH) with limited functional sensitivity were developed (Carole Spencer Thyroid Disease Manager). In 1967, Belgian researchers, in a case-control study of 25 inadequately-treated hypothyroid autopsies matched to 50 age and gender-matched controls, reported that the presence and severity of coronary artery disease was more common in the hypothyroid patients (96%) than in controls (60%) and that left ventricular hypertrophy and dilatation was more frequent in the hypothyroid group. In 1977, Tunbridge *et al* performed the first cross-sectional cohort study in Whickham, North-East England, and concluded there was no association between SCH and presence of CVD however, a follow up of the population cohort over 20 years found SCH to be associated with increased cardiovascular events and

mortality with subsequent treatment with levothyroxine improving such outcomes (Tunbridge *et al.*, 1977b; Razvi *et al.*, 2010).

These initial data led investigators to consider using TH analogues in euthyroid individuals at high CV risk with the aim to reduce the deleterious effects of thyroxine excess while preserving its recognised beneficial effects on CV risk factors ('The coronary drug project. Findings leading to further modifications of its protocol with respect to dextrothyroxine. The coronary drug project research group,' 1972; Brenta *et al.*, 2007). The results of a trial of Dextro-thyroxine, a low-activity dextro-isomer of thyroxine in male AMI survivors, conversely, curbed the enthusiasm for using TH therapy in patients with CVD. This trial showed increased arrhythmias and higher mortality in the treated group probably due to contamination with levo-thyroxine (Stamler, 1977) and the supra-physiological doses of Dextro-thyroxine (more than double the normal endogenous thyroxine production) (Pingitore *et al.*, 2012). Another trial using a TH analogue diiodothyropropionic acid (DITPA) in heart failure patients showed higher heart rate and symptoms suggestive of hyperthyroidism (Brenta *et al.*, 2007; Goldman *et al.*, 2009). It is worth noting that both studies used TH analogues rather than native THs. The results of these early trials prejudiced clinicians and curbed enthusiasm of researchers as well as pharmaceutical companies in developing and researching the use of TH and its analogues at therapeutic doses in patients with CVD. More recently, studies using a liver-selective TH receptor agonist eprotirome in patients with resistant and familial hypercholesterolemia, respectively, showed a beneficial impact on lipids but haven't progressed to large –scale phase III studies due to concerns over liver and cartilage injury (Sjouke *et al.*, 2014).

1.1.3 Cellular activity of thyroid hormones

Thyroid function is regulated by the hypothalamic-pituitary-thyroid axis via a classic endocrine negative feedback mechanism (Figure 1-1). Thyrotropin releasing hormone (TRH) is secreted by the hypothalamus, and stimulates the anterior pituitary to produce TSH - which in turn drives the thyroid gland to release TH. TH have a negative feedback effect on the hypothalamus and pituitary. TSH has a log-linear relationship with T4 levels meaning that even mild changes in TH concentrations lead to large changes in TSH. Thus, serum TSH is a robust marker of systemic TH status (Larsen, 1982).

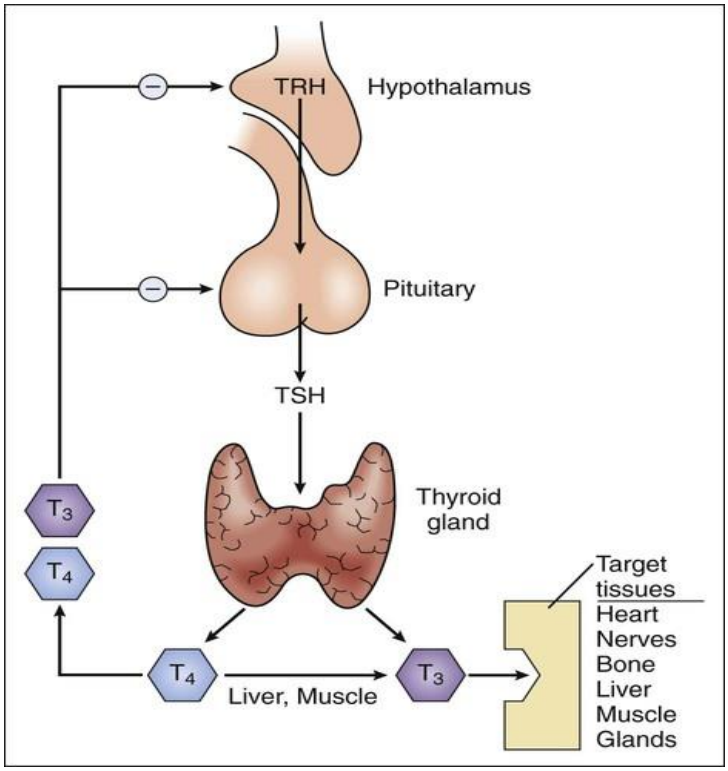


Figure 1-1 Hypothalamic-pituitary-thyroid axis

TRH released by the hypothalamus causes TSH release from the pituitary gland leading to TH release from the thyroid gland in the form of T4 and T3. These have a negative feedback effect on both the hypothalamus and pituitary gland.

The thyroid gland secretes two main iodinated hormones, thyroxine (T4) and triiodothyronine (T3). Both molecules can generate biological activity in responsive tissues, however, T3 is considered the biologically active hormone that elicits its effect by binding to the thyroid hormone receptor (TR) (Klein and Danzi, 2007). The affinity of TR is approximately 10 times higher for T3 than for T4 and thus makes T3 very potent (Sandler *et al.*, 2004). Less than 20% of circulating T3 is secreted by the thyroid gland whereas the bulk is produced in extra-thyroidal tissues from T4, by a process of deiodination in which T4 is converted to T3 by a process of deiodinising a single iodine atom, leading to the potent thyroid hormone receptor-mediated effects of T3. The metabolism of TH is regulated by three selenocysteine enzymes called deiodinases (Gereben *et al.*, 2008). Two deiodinase pathways [deiodinase 1 (D1) and deiodinase 2 (D2)] lead to extra-thyroidal T3 production. D1 is active mostly in the liver and the kidney and is thought to contribute 15 – 20% of total circulating T3. D2 activity is located in brown adipose tissue, pituitary gland, brain and the heart and is responsible for the majority (two thirds) of T3 production (Croteau *et al.*, 1996; Salvatore *et al.*, 1996). The third deiodinase

(D3) catabolises both T4 and T3 to inactive products, leading to termination of thyroid hormone action. Increased D3 activity has been shown to decrease T3 and cause a local hypothyroid state in cardiomyocytes (Pol *et al.*, 2011). Therefore the effect of intracellular T3 on TR defines the thyroid status of an organism and is dependent on circulating (serum) thyroid hormone levels, as well as intracellular tissue levels regulated by deiodinasing enzymes.

TH have a broad range of effects, particularly on the heart, but also on the cardiovascular system in general (Figure 1-2). TH influence cardiac status in 3 ways: 1) the direct effect of T3 on cardiac myocytes by binding to nuclear receptors which influences gene expression 2) effects of T3 on the sympathetic nervous system 3) effects of T3 on the peripheral circulation which determines cardiovascular haemodynamics, cardiac filling and systolic contractility. T3 activity in the cardiomyocyte regulates genes that encode proteins related to myocardial contractility such as the myosin heavy chain, sarcoplasmic reticulum calcium pump and phospholamban (He *et al.*, 1997; Kaasik *et al.*, 1997; Holt *et al.*, 1999). TH receptors are not membrane bound, but located in the intracellular compartment. Within the cardiac myocyte, T3 binds to thyroid nuclear receptors (TRs), which in turn bind to thyroid hormone response elements (TREs) of the regulated genes to induce transcription. The two main thyroid receptors are TRα, which is highly expressed in cardiac myocytes, and TRβ. Thyroid receptors are unique in that they can bind to TREs in the absence of TH and therefore repress transcription and regulate specific genes. Therefore different key proteins within the myocyte are dependent on the availability of TH (Brent, 1994). Proteins which are TH dependent include alpha-myosin heavy chain (αMHC), sarcoplasmic reticulum proteins such as calcium-activated ATPase (SERCA), and Na+/K+-ATPase whereas proteins which are negatively regulated by TH include β-Myosin heavy chain (βMHC) and phospholamban. The two myosin heavy chains form an important component of the cardiac myocyte contractile apparatus whereas both SERCA and its inhibitor phospholamban regulate the release and reuptake of calcium from the sarcoplasmic reticulum**.** SERCA pumps calcium out of and back into the sarcoplasmic reticulum during contractility and relaxation of the cardiac myofilaments respectively. This efficient calcium release and reuptake from the sarcoplasmic reticulum is essential for efficient cardiomyocyte function. (Ojamaa *et al.*, 1996; Fazio *et al.*, 2004; Kranias and Hajjar, 2012). Therefore cardiac contractility is not only dependent on myosin heavy chains but also calcium handling by SERCA and its inhibitor phospholamban.

TH influence cardiac chronotropy by both genomic and non-genomic effects on components of the adrenergic-receptor complex as well as sodium, potassium and calcium channels. This

manifests as tachycardia and increased risk of atrial fibrillation (AF) in hyperthyroid states, whereas bradycardia and reduced cardiac contractility are the main clinical features of hypothyroidism. The genomic effects include encoding for the αMHC and SERCA which are positively regulated by T3 whereas βMHC and phospholamban are negatively regulated. Other important cardiac genes regulated by TH include TR protein themselves and the sodium/calcium ion (Na+/Ca2+) exchanger.

The non-genomic effects on the cardiac myocyte and systemic vasculature are usually receptor independent, largely occur on the plasma membrane and are identified by their rapid rate of action compared to the genomic effects which have longer to take effect. These include activation of sodium, potassium and calcium membrane ion channels, effects on the mitochondrial membrane and involvement in signalling pathways of the cardiac myocyte and vascular smooth muscle. One signalling pathway is the P13K/AKT pathway, which causes endothelial nitric oxide (NO) production and a subsequent reduction in the systemic vascular resistance (SVR) (Kuzman *et al.*, 2005). In addition, TH also significantly affect cardiac mitochondrial function (Marin-Garcia, 2010) and changes in their circulating levels may lead to impaired myocardial bio-energetic status and function.

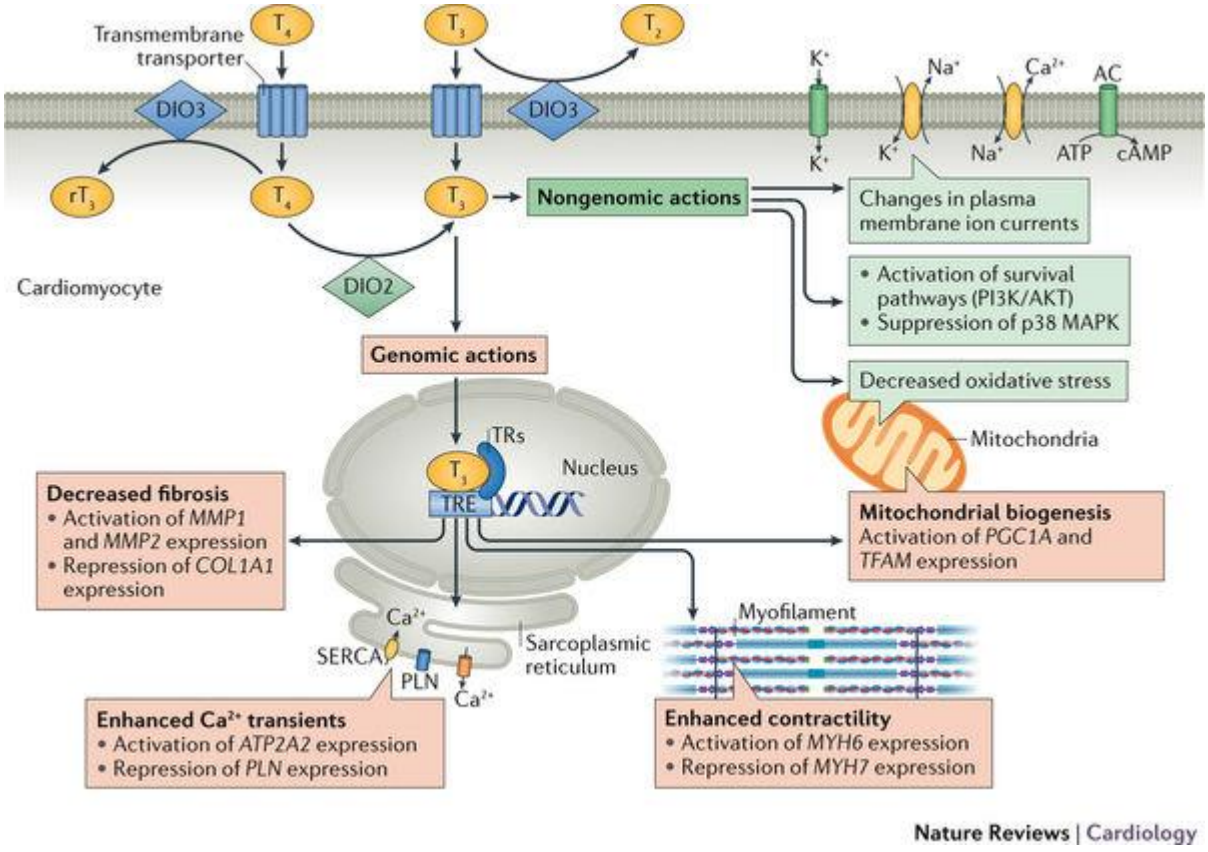


Figure 1-2 The effect of TH on the cardiomyocyte.

Schematic representation of the effect of thyroid hormones T4 and T3 on the cardiomyocyte via genomic and non-genomic actions. T3 enters the cardiomyocyte directly as well as being produced by the conversion of T4 by D2. T3 binds to thyroid nuclear receptors (TRs) which in turn bind to thyroid hormone response elements (TREs) of the regulated genes to induce transcription. Proteins which are TH dependent include alpha-myosin heavy chain (αMHC), sarcoplasmic reticulum proteins such as calcium-activated ATPase (SERCA) whereas proteins which are negatively regulated include β-Myosin heavy chain (βMHC) and phospholamban. D3 breaks down both T4 and T3. From: Jabbar et al, Nat Rev Cardiol. 2017 Jan;14(1):39-55 (Jabbar *et al.*, 2017).

1.1.4 Thyroid hormone effects on cardiovascular haemodynamics

TH has an inotropic effect on the heart by direct action on beta adrenoreceptors and increasing αMHC, which forms a key component of the myocyte contractile function (Pantos *et al.*, 2007a; Pantos *et al.*, 2010). TH also effects systolic function by regulating the amount of calcium available within the sarcoplasmic reticulum for systolic contraction (Dillmann, 1990). These effects are genomic and take time to take effect. TH effects diastolic function by increasing the expression of SERCA within the sarcoplasmic reticulum which subsequently leads to reuptake of calcium during diastole leading to improved ventricular relaxation (Klein and Danzi, 2007). TH also decreases systemic vascular resistance (SVR) by causing smooth muscle relaxation within the arterioles by increasing calcium reuptake via SERCA, inhibiting phospholamban and increasing tissue metabolism and thermogenesis (Kiss *et al.*, 1994; Carr and Kranias, 2002). This decrease in SVR with the direct inotropic effects leads to an increase in cardiac output (Figure 1-3). The renin-angiotensin system plays a key role in the haemodynamic effects by TH. An initial decrease in the SVR by TH leads to decreased perfusion within the kidneys which leads to increased renin and aldosterone levels. This leads to an increase in the blood volume and hence cardiac preload which is another explanation for an increase in cardiac output by TH (Klein and Ojamaa, 2001; Klein and Danzi, 2007).

TH also have a regulatory effect on blood pressure. Hyperthyroidism causes a hyperdynamic circulation characterised by an increase in cardiac contractility and heart rate, increased preload and a decrease in SVR with the overall result increasing the cardiac output by 5-300%. Although hyperthyroidism can increase systolic blood pressure, the net effect is variable depending on the increased cardiac output versus the decreased systemic vascular resistance (Ching *et al.*, 1996; Danzi and Klein, 2003). The relationship between subclinical hyperthyroidism and blood pressure is less clear with most observational studies including a meta-analysis showing no link (Volzke *et al.*, 2006; Volzke *et al.*, 2009; Cai *et al.*, 2011). Hypothyroidism is associated with bradycardia, a decreased cardiac output, diastolic hypertension and a narrowed pulse pressure. This is due to impaired vascular smooth muscle relaxation which causes an increase in SVR and therefore an isolated rise in the diastolic blood pressure which can reverse after TH replacement (Streeten *et al.*, 1988; Dernellis and Panaretou, 2002; Taddei *et al.*, 2003). With overt hypothyroidism and SCH, the increase in SVR combined with impaired diastolic relaxation of the smooth muscles and ventricles increases the afterload. It is this diastolic impairment combined with a low heart rate that impairs ventricular filling and therefore results in a low cardiac output (Brenta *et al.*, 2003;

Ripoli *et al.*, 2005). Hypothyroidism can also lead to myocardial fibrosis, by stimulating fibroblasts as well as leading to progressive systolic dysfunction and a reduction in myocardial blood flow due to a loss of arterioles as has previously been demonstrated in animal studies (Tang *et al.*, 2005; Chen *et al.*, 2013).

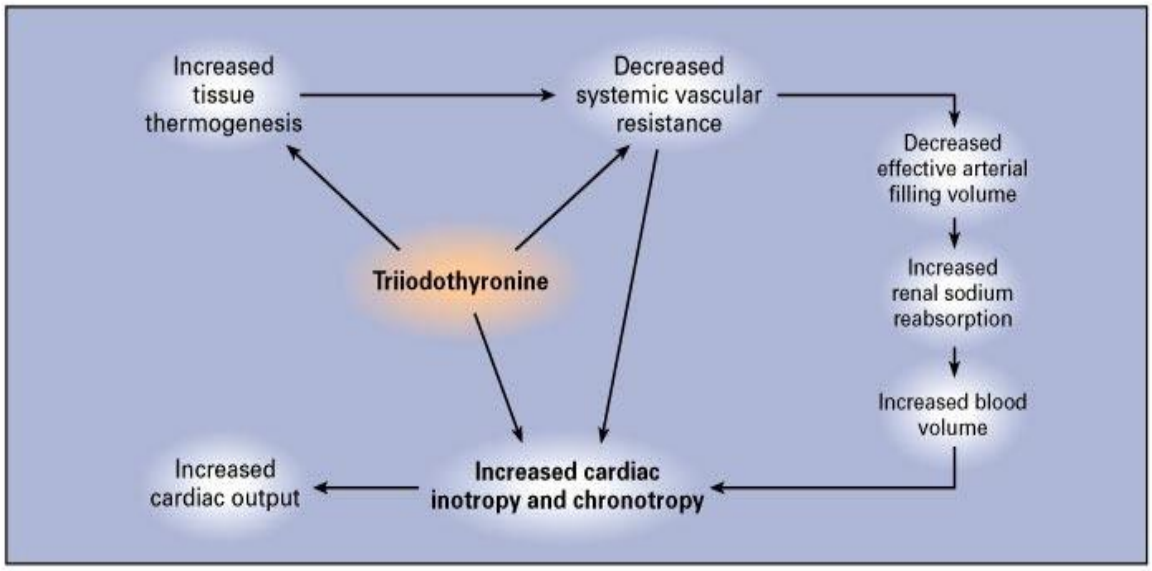


Figure 1-3 Effect of TH on cardiovascular haemodynamics.

TH increases cardiac output by affecting thermogenesis, blood volume, vascular resistance, cardiac contractility and heart rate. From: Klein et al, N Engl J Med. 2001; 344(7):501-9 (Klein and Ojamaa, 2001).

1.1.5 Overt and subclinical hypothyroidism

Overt hypothyroidism is prevalent in 0.2 to 2% of non-pregnant adults (Tunbridge *et al.*, 1977a; Canaris *et al.*, 2000) and, by definition, is diagnosed when serum TSH is elevated (usually more than 10 mU/L) and circulating TH (FT4) is low. Overt hypothyroidism has several cardiac manifestations including a reduction in cardiac output and cardiac contractility, a decrease in heart rate, and an increase in peripheral vascular resistance (Klein and Danzi, 2007). There are also significant changes in modifiable atherosclerotic risk factors, including hypercholesterolemia, diastolic hypertension, increased carotid intimal media thickness, and reduced endothelial derived relaxation factor (nitric oxide), which accompany overt hypothyroidism. All these clinical features are reversible with TH replacement.

SCH is diagnosed in the presence of serum TH within their reference range with raised serum TSH concentrations and can be mild (TSH > 4.0 or 4.5 but <10mU/L) or severe (>10mU/L). However, there is a lack of consensus on the "normal" upper range of TSH leading to controversy on both definition and clinical significance of SCH (Hamilton *et al.*, 2008). The common causes of hypothyroidism and SCH are outlined in Table *1-1*. The prevalence of SCH ranges between 4–20% of the adult population (Biondi and Cooper, 2008). The wide range is a result of differences in age, sex, body-mass index, race, dietary iodine intake, and the cut-off concentrations of serum TSH that are used to define the condition. The prevalence of raised serum TSH concentrations is higher in white than in black populations, supporting a genetic effect on TSH secretion (Hollowell *et al.*, 2002). It is estimated that at least 10% of older women have SCH; this has potential significance as a vascular risk factor in the wider population.

Cause	Hypothyroidism and SCH
Autoimmune disease	Hashimoto's autoimmune thyroiditis, TSH-receptor blocking antibodies
Structural disease	Thyroid damage due to thyroidectomy or radiation damage (radioactive iodine therapy or external radiotherapy of head and neck)
Release of preformed thyroid hormones	Post thyroiditis state in those with underlying thyroid disease
Pituitary disease	Secondary hypothyroidism due to TSH deficiency
Drugs	Lithium, amiodarone, tyrosine kinase inhibitors and interferon therapy.

Table 1-1 Common causes of hypothyroidism and SCH.

SCH can impair relaxation of vascular smooth muscle cells, inducing increases in systemic vascular resistance and arterial stiffness, as well as changes in endothelial function by reduction of nitric oxide availability, without apparent clinical significance (Owen *et al.*, 2007). Population studies support these findings with the Whickham Survey cohort revealing higher systolic and diastolic blood pressures and total cholesterol concentrations in subclinical hypothyroidism than in euthyroid controls (Razvi *et al.*, 2010) and the EPIC-Norfolk study reporting that in spite of the worse cardiovascular risk profile seen in SCH subjects, coronary heart disease and all-cause mortality did not increase across 10·6 years of follow-up (Boekholdt *et al.*, 2010). There is conflicting evidence from population studies about the association of SCH, with increased risk of cardiovascular disease and mortality in some prospective population-based cohort studies (Walsh *et al.*, 2005; Razvi *et al.*, 2010; McQuade *et al.*, 2011) which hasn't been confirmed in others (Rodondi *et al.*, 2005; Boekholdt *et al.*, 2010). Patient-level meta-analysis of several prospective cohort studies providing 542,494 person-years of follow-up showed that SCH is associated with a higher risk of CV events and mortality in people with higher serum TSH levels particularly in those with TSH levels greater than 10 mU/L (Rodondi *et al.*, 2010).

There is evidence from observational studies to suggest a link between SCH and adverse cardiovascular outcomes. There are however no randomised clinical trials of TH treatment of SCH to determine whether improvements in cardiovascular events accrue. Small intervention trials of levothyroxine in SCH using surrogate markers have shown improvement in left

ventricular function, vascular endothelial function and atherogenic lipid particles (Biondi and Cooper, 2008; Cooper and Biondi, 2012). Several other studies have shown similar results (Biondi *et al.*, 1999; Monzani *et al.*, 2001; Ripoli *et al.*, 2005). In an observational study of 3093 patients with a raised serum TSH treated in primary care, those under 70 years of age who were treated with levothyroxine had fewer cardiovascular events than those who weren't treated (Razvi *et al.*, 2012). However, in individuals over the age of 70 years (n=1642), there was no benefit to levothyroxine detected (Razvi *et al.*, 2012). Recently, a small before-after trial in SCH patients showed improvement in cardiac mitochondrial function with levothyroxine treatment (Madathil *et al.*, 2015). This study provides a novel sub-cellular level insight into the action of TH in cardiac tissue (Figure *1-4*).

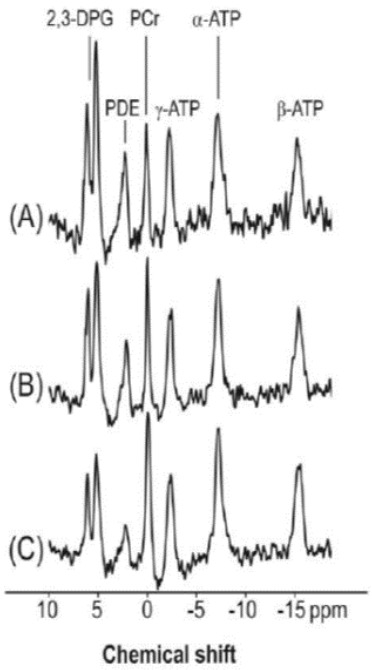


Figure 1-4 Cardiac 31P spectra from SCH and euthyroid controls

Sample cardiac 31P spectra from a SCH patient (A) before and (B) after LT4 treatment demonstrating PCr/ATP ratios that improve with treatment and are comparable to (C) euthyroid control. PCr/ATP is a marker of mitochondrial function. (Adapted with permission from the M.D. thesis of A Madathil, Newcastle University)

SCH is associated with CV risk factors which will be explored in more detail (Table *1-2*). As there is lack of evidence from prospective randomised controlled trials in SCH, international guidelines have suggested that treatment should only be considered in those with more severe disease (serum TSH > 10 mU/L), symptoms of hypothyroidism or individuals younger than 70 years old particularly if they also have other CV risk factors (Pearce *et al.*, 2013).

	Hypothyroidism and SCH
Lipids	Higher total and LDL cholesterol
Hypertension	Diastolic hypertension
Thrombogenicity	Unclear
Endothelial dysfunction	Impaired endothelium-dependent vasodilatation and arterial stiffness
Cardiac structure and function	LV systolic and diastolic dysfunction

Table 1-2 Different mechanisms by which underactive thyroid states are associated with cardiovascular risk.

1.2 Potential mechanisms linking thyroid dysfunction with CV disease

1.2.1 Thyroid hormones and hyperlipidaemia

Thyroid hormones are involved in lipid metabolism. The association between hypothyroidism and hypercholesterolemia and elevated low-density lipids (LDLs) has been known for many years with some estimates showing a link present in up to 90% of cases (Duntas, 2002; Klein and Danzi, 2007). Hypothyroidism is also associated with increased oxidation of LDL which leads to an increase in atherogenesis which can reverse with thyroxine treatment (Costantini *et al.*, 1998; Diekman *et al.*, 1998). Lipoprotein (a) is known to be more atherogenic that other lipoproteins and has been shown to increase in overt hypothyroidism and subsequently decreases with TH treatment (Martinez-Triguero *et al.*, 1998; Tzotzas *et al.*, 2000).

Elevated levels of lipids are also evident in patients with SCH suggesting an increased risk for atherosclerosis; however evidence remains controversial based on different population based studies. In some studies such as the Whickham Survey and National Health and Nutritional Examination Survey (NHANES) III, there was no link between SCH and hyperlipidaemia whereas other studies showed a link as well as a relative increase in serum lipids with an increase in serum TSH in SCH (Tunbridge *et al.*, 1977a; Hueston and Pearson, 2004; Asvold *et al.*, 2007). In other studies, such as those by Bauer et al, there was a relative increase in LDL and cholesterol levels with an increase in serum TSH in SCH in the older age group (Bauer *et al.*, 1998; Kanaya *et al.*, 2002). Furthermore, other population-based studies have shown that SCH not only increases lipid levels but is strongly associated with atherosclerosis (Hak *et al.*, 2000; Kvetny *et al.*, 2004).

The causes of hyperlipidaemia in an underactive thyroid state is due to a decrease in LDL receptors, which reduces the clearance of cholesterol from the liver, and decreased activity of the enzyme cholesterol 7α-hydroxylase which is usually activated by thyroid hormones in breaking down cholesterol (Pandak *et al.*, 1997; Duntas, 2002). Randomised placebo-controlled trials have shown differences in whether thyroxine treatment has a beneficial effect on LDL and cholesterol levels. Some of these trials have shown a beneficial effect (Caraccio *et al.*, 2002; Monzani *et al.*, 2004; Razvi *et al.*, 2007), whereas others have not (Jaeschke *et al.*, 1996; Kong *et al.*, 2002). Such differences in all these studies are probably due to different population groups, varying severity of hypothyroidism, the presence of thyroid antibodies, the dose of thyroxine and the age group of the participants. A Cochrane review of the literature including 6 RCTs concluded that thyroxine treatment of SCH had a trend towards improving

LDL levels greater than 155mg/dL in a subgroup analysis although there were no overall effects in reducing total cholesterol, high density lipids (HDLs) and low density lipids (LDLs) in the interventional groups (Villar *et al.*, 2007).

1.2.2 The role of vascular function in cardiovascular disease

The vascular endothelium is an organ with secretory and synthesising functions. It is semi-permeable with different receptors which regulates the flow of molecular substances through the body. The endothelium has a multifactorial role in vascular haemostasis by maintaining the balance between vasodilation and vasoconstriction, inhibiting smooth muscle cell proliferation and regulating thrombogenesis and fibrinolysis (Davignon and Ganz, 2004). A disturbance is this function leads to endothelial dysfunction, marked by modifications in the secretory function of the endothelium and transformation to a thrombogenic medium, which causes arterial wall disease. Endothelial dysfunction is a marker of atherosclerosis preceding plaque formation and is associated with traditional risk factors such as, hypertension, hypercholesterolemia, diabetes, stress and diseases marked by systemic inflammation (Gokce *et al.*, 2002).

Vascular tone is determined by the production of vasodilators and vasoconstrictors. Vasodilators include nitric oxide (NO), prostacyclin, bradykinin and endothelium-derived relaxing factor (EDRF). All these vasodilators inhibit platelet aggregation whereas bradykinin also stimulates the production of tissue plasminogen activator (t-PA) which breaks down thrombus, indicating a role in fibrinolysis. Vasoconstrictors produced by the endothelium include endothelin and angiotensin II which promote smooth muscle proliferation therefore contributing to plaque formation. Endothelial dysfunction disrupts the balance between vasodilators and vasoconstrictors leading to atherosclerosis by increasing platelet aggregation, leukocyte adhesion and smooth muscle proliferation (Levine *et al.*, 1995; Davignon and Ganz, 2004).

The progression of fatty deposits to atheromatous plaques within the endothelium is dependent on continuous insults which can either be endogenous such as hypercholesterolemia and hypertension or exogenous such as smoking (Chien, 2003). In response to such insults, the endothelium expresses vascular cell adhesion molecule-1 (VCAM-1) and P-selectin which are adhesion molecules for leucocytes aiding in self repair (Linton and Fazio, 2003). Continuous insults cause these adhesion molecules to be overexpressed causing increased leucocyte

attachment (Figure *1-5*). Overtime the leucocytes undergo transformation to tissue macrophages within the tunica media and lead to the release of various mediators and cytokines which act on the endothelial receptors to impair the secretory and regeneration mechanisms within the endothelium. The macrophages cause transformation of lipoproteins into foam cells which leads to enlargement of the atheromatous plaque (Libby, 1995).

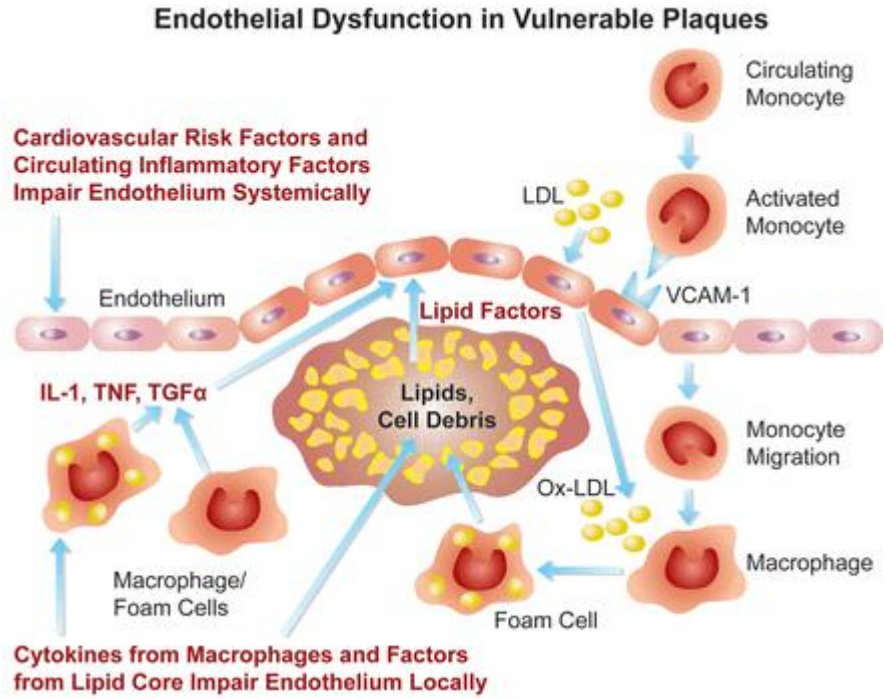


Figure 1-5 Endothelial dysfunction leading to plaque formation.

Endothelial function is impaired by cardiovascular risk factors and systemic cytokines. Cardiovascular insults over a prolonged period lead to adhesion molecule expression which attracts leukocytes. These leukocytes transform to tissue marcophages in the tunica media and release cytokines leading to lipid transformation. From: Ganz et al, Eur Heart J. 2013;34(27):2025-7 (Ganz and Hsue, 2013).

Given the link between cardiac risk factors and endothelial dysfunction, the use of endothelial function has been used to predict clinical outcomes related to atherosclerosis. In one study of patients with mild CAD followed up for 28 months, significant coronary artery endothelial dysfunction resulted in increased cardiac events whereas no events occurred in patients with mild endothelial dysfunction (Suwaidi *et al.*, 2000). One problem with this study was the majority of cardiac events were driven by the need for revascularisation rather than MI therefore representing a softer end point however; the study still did show the importance of

measuring endothelial function with regard to cardiac status. In another study of 281 patients followed up over 4.5 years, endothelium dependent and independent vasodilation was assessed using venous occlusion plethysmography, which measured blood flow responses, with impaired vasodilator responses predicting cardiovascular events during follow up (Heitzer *et al.*, 2001).

1.2.3 Thyroid hormones and vascular function

Overt and SCH are associated with diastolic hypertension and impaired vascular function. The causes of hypertension in these patients are numerous. Firstly, there is an increase in systemic vascular resistance due to a lack of the vasodilatory effects of TH on the smooth muscle cells of blood vessels. Other causes include endothelial dysfunction, as well as increased arterial stiffness (Danzi and Klein, 2003; Biondi and Cooper, 2008). Some studies have shown an isolated elevation of systolic blood pressure in hypothyroidism (Saito *et al.*, 1983; Fommei and Iervasi, 2002), of which one study used ambulatory blood pressure measurements and showed a significantly elevated systolic blood pressure readings in hypothyroid patients (Fommei and Iervasi, 2002). The Tromso study showed a significant positive relationship between TSH and both systolic and diastolic blood pressure (Iqbal *et al.*, 2006). Studies have also shown a relationship between SCH and hypertension. Two community-based studies concluded that subjects with SCH had a significantly higher systolic blood pressure (SBP) than euthyroid subjects (adjusted for age, sex and body mass index)(Duan *et al.*, 2009; Liu *et al.*, 2010). In a further smaller study which compared 57 women with SCH to 34 healthy controls, mean diastolic blood pressure was increased in SCH patients (Luboshitzky *et al.*, 2002). These studies are further supported by a meta-analysis which showed SCH to be associated with increased systolic and diastolic pressures (Cai *et al.*, 2011).

Central arterial stiffening increases cardiac afterload and is an important determinant of all-cause mortality as well as being a precursor for atherosclerosis. Pulse wave velocity, a measure of arterial stiffness, is an accurate means by which a relationship between cardiac risk factors and arterial stiffness can be sought (Amar *et al.*, 2001). Two studies have shown central arterial stiffness, as measured by pulse wave velocity, to be higher in hypothyroid patients compared to controls with levothyroxine therapy fully reversing stiffness and improving vascular function (Dernellis and Panaretou, 2002; Obuobie *et al.*, 2002). Three other studies have shown arterial stiffness to increase in SCH patients with and without autoimmune thyroiditis and this subsequently decreases post thyroxine therapy (Nagasaki *et al.*, 2006; Nagasaki *et al.*,

2007; Nagasaki *et al.*, 2009). These studies demonstrate that treating underactive thyroid disease improves cardiovascular risk profile as measured non-invasively.

Flow-mediated dilatation (FMD) is a non-invasive test to measure endothelium function and refers to the dilatation of the brachial artery in response to an increase in blood flow after a period of reactive hyperamia, in which the brachial artery is occluded. It is dependent on the effects of NO on the vascular endothelium. The principle of reactive hyperaemia involves occluding the brachial artery and measuring a change in blood vessel diameter by ultrasound post occlusion. Healthy vasculature responds to NO resulting in an increased vessel diameter post occlusion. Flow mediated endothelium dependent vasodilation has been compared in hypothyroid and SCH patients with FMD decreasing with a subsequent increase in TSH and reduced thyroid function (Lekakis *et al.*, 1997). This finding is further supported by two randomised placebo controlled trials (RCTs) which showed thyroxine treatment to improve endothelial function in SCH patients (Taddei *et al.*, 2003; Razvi *et al.*, 2007). The RCT by Razvi et al showed thyroxine to reduce LDL levels, improve the quality of life and improve endothelial function as measured by FMD in SCH patients (Razvi *et al.*, 2007).

Intima-media-thickness (IMT) as measured by ultrasonography of different arteries such as the carotid artery has been associated with atherosclerosis and CAD (Bots *et al.*, 1997; Mannami *et al.*, 1997). The thickening of the intima-media of an artery not only indicates regional changes but also generalised atherosclerosis and IMT is a non-invasive method for assessing early atherosclerosis and providing an end point to assess the effects of intervention (Allan *et al.*, 1997; O'Leary and Polak, 2002). Increased IMT is considered to be an early marker for systemic atherosclerosis and CAD in both overt and SCH subjects and thyroxine therapy has shown to improve IMT. In SCH, a positive correlation between TSH and intima-media thickness has been found on regression analysis independent of other variables (Monzani *et al.*, 2004).

Numerous factors are likely to cause vascular dysfunction and elevated blood pressures in patients with an underactive thyroid state. The role of hyperlipidaemia in low thyroid states is one cause due to its role in atherosclerosis and arterial stiffening as well causing inflammation (Dart *et al.*, 1991; Taddei *et al.*, 2003). The deposition of fatty deposits with the endothelium results in the expression of vascular adhesion molecules which starts the process of atherosclerosis. Such adhesion molecules such as E-selectin and P-selectin are also increasingly expressed in patients with Hashimoto's thyroiditis, suggesting there is a close

interplay between autoimmune thyroid antibodies and inflammation (Marazuela *et al.*, 1995). Further evidence shows that in patients with SCH associated with autoimmunity, the improvement in endothelial dysfunction is far less than would be expected in such patients who had SCH alone (Taddei *et al.*, 2003). Other studies have also shown the role autoantibodies in inflammation when investigating endothelial and vascular function in patients with SCH with autoantibodies (Cikim *et al.*, 2004; Kvetny *et al.*, 2004; Taddei *et al.*, 2006; Turemen *et al.*, 2011). In one such study by Taddei et al, patients with SCH had higher CRP and interleukin levels compared to those who were euthyroid and administering an anti-inflammatory agent in the form of indomethacin significantly improved the vasodilatory response to acetylcholine (Taddei *et al.*, 2006). In the Rotterdam study, the prevalence of arterial disease, aortic calcification and MI was not only increased in patients with SCH but the strongest association was observed in those patients with SCH and positive thyroid auto-antibodies (Hak *et al.*, 2000).

Finally, TH have beneficial effects on the endothelial cell through the TRα1 and TRβ. Activation of TRα1 increases coronary blood flow as well as decreasing coronary resistance and improving cardiac contractility in ischaemia–reperfusion injury (Suarez *et al.*, 2014). Activating TRα1 also causes vascular myocyte relaxation by increasing the production of nitric oxide in endothelial and vascular smooth muscle cells through the activation of nitric oxide synthase and the important signalling pathway PI3K/AKT (Carrillo-Sepulveda *et al.*, 2010). Activation of TRβ by TH activates the mitogen-activated protein kinase (MAPK) pathway inducing angiogenesis (Suarez, 2010). This leads to the transcription of several proangiogenic genes, such as vascular endothelial growth factor (*VEGF*) (Cheng *et al.*, 2010), angiopoietin (Cheng *et al.*, 2010) and basic fibroblast growth factor (*bFGF*) (Tomanek *et al.*, 1998a).

1.2.4 Assessing endothelial dysfunction

A key process in endothelial dysfunction is impaired NO bioavailability leading to decreased vasodilation. Endothelial dysfunction is assessed using both invasive and non-invasive methods. Invasive methods include the infusion of acetylcholine into the coronary artery which promotes vasodilation if the endothelium is intact whereas on the contrary, vasoconstriction occurs in the presence of atherosclerosis due to the direct constricting effects of acetylcholine. Infusing agents such as nitroglycerine on the other hand test vasodilation independent of the endothelium; however these tests are limited by their invasive nature.

Because endothelial dysfunction is a systemic process affecting different vascular beds, non-invasive tests for endothelial function have been developed which are thought to be a better representation of coronary vascular function by assessing the peripheral vasculature. These tests are dependent on the physiological stimulation of NO produced post-ischaemic hyperaemia and include FMD, finger plethysmography and EndoPAT. FMD is the most common method for assessing endothelial function and involves ultrasonography of the brachial artery and assessing changes in luminal diameter following transient ischaemia for 5 minutes using a pneumatic cuff around the forearm. On cuff deflation, there is an increase in the volume of blood flow which increases shear stress and activates NO leading to vasodilation. Therefore FMD measures the percentage change in diameter of the brachial artery (Charakida *et al.*, 2010). Despite its frequent use, there are certain problems with the use of FMD. This includes careful operator training due to significant operator variability which includes probe positioning. Importantly environmental factors need to be controlled as FMD is influenced by room temperature, medications, use of caffeine as well as recent infection. Furthermore, the literature shows variations in mean FMD when measurements are taken in similar populations for different studies (Bots *et al.*, 2005).

EndoPAT is a non-invasive test which uses a peripheral arterial tone (PAT) signal for non-invasively measuring arterial tone in the peripheral arterial beds. The PAT signal is measured from the fingertip by recording peripheral arterial volume changes using a pneumatic probe with a plethysmographic cuff (Figure *1-6*). It quantifies changes in endothelium mediated arterial tone by occluding the brachial artery for 5 minutes and then measuring reactive hyperaemia manifested by an increase in the PAT signal amplitude. By using one arm for the experiment and the other arm as a control, EndoPAT can account for general systemic changes that occur during the test and corrects for this when giving a final reactive hyperaemia index (RHI) score. A significant advantage of using EndoPAT is that it is both operator and interpreter independent as the procedure involves very few variables that are interoperator dependent. Selamet et al demonstrated excellent reproducibility in 30 adolescents who had their test repeated within seven days. A further study confirmed EndoPAT to be more reproducible when compared to FMD even though FMD was measured by an experienced sonographer (Selamet Tierney *et al.*, 2009).

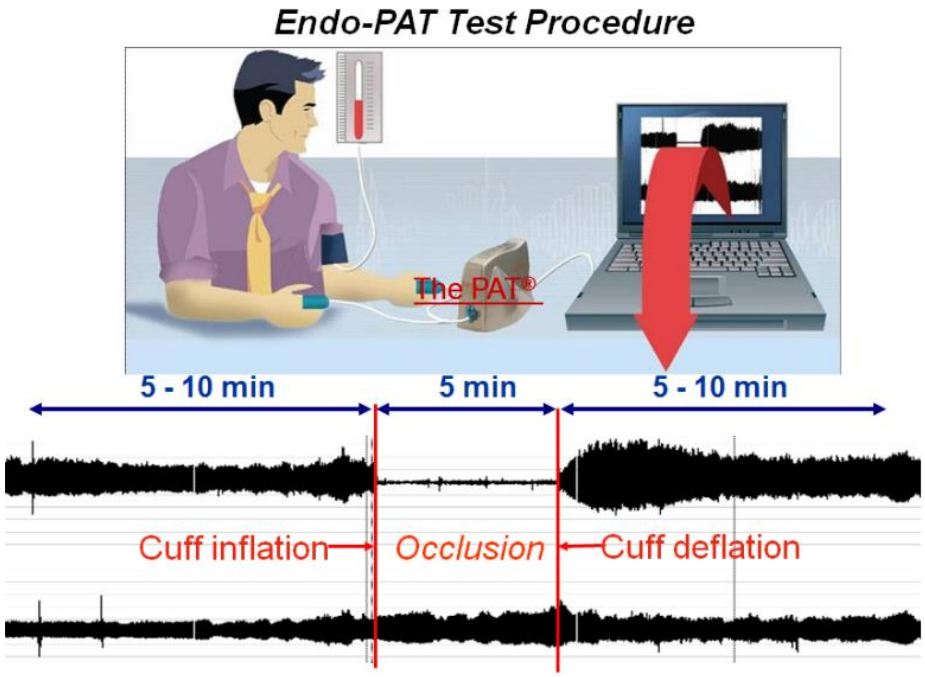


Figure 1-6 EndoPAT test procedure.

One arm is used as the experiment arm and the other arm as a control. An increase in the PAT signal post occlusion is a measure of increased arterial tone and hence normal endothelial function.

1.2.5 Thyroid hormones and thrombosis

Alterations in coagulation parameters in SCH might play a role in the potential development of atherosclerosis. In one study comparing women with SCH with euthyroid controls, factor VII activity and the factor VII activity:factor VII antigen ratio were significantly increased in women with SCH, whereas no differences in vWF or other haemostatic factors were observed (Muller *et al.*, 2001). In another similar study, decreased antithrombin III activity and increased levels of fibrinogen, factor VII and plasminogen activator inhibitor antigen were found in SCH patients which may explain a hypercoagulable state (Canturk *et al.*, 2003). This is further supported in a study by Guldiken et al who found the global fibrinolytic activity, such as tissue plasminogen activator, to be lower in SCH subjects than in controls (Guldiken *et al.*, 2005). These studies demonstrate how TH may act on both the coagulation and fibrinolytic pathways leading to decreased thrombus formation. A recent study using the Badimon chamber, a model which simulates ex vivo coronary artery blood flow through a diseased artery, has shown the thrombus area in patients with SCH 7-10 days post non-ST elevation myocardial infarction to be larger than in euthyroid patients despite the use of dual antiplatelets

in the form of aspirin and clopidogrel (Figure 1-7) (Viswanathan *et al.*, 2014a). This may also help explain the increased adverse outcomes in SCH patients post AMI as such a state is likely to be more thrombogenic.

The mean platelet volume (MPV) which reflects platelet size has shown to be associated with cardiovascular risk (Endler *et al.*, 2002; Chu *et al.*, 2010). In one retrospective analysis, patients with SCH had a higher MPV than euthyroid subjects and there was a positive correlation between TSH and MPV which could explain thrombotic risk (Kim *et al.*, 2013). This could suggest another reason for increased thrombogenesis in SCH however more research is needed with regard to this. The Tromso Study on the other hand did not show a significant difference in coagulation markers between SCH and euthyroid subjects (Jorde *et al.*, 2006). Other studies have also not shown a difference in homocysteine levels between SCH and euthyroid subjects (Deicher and Vierhapper, 2002; Christ-Crain *et al.*, 2003). Minimal research has been undertaken to assess the effect of TH replacement therapy on haemostasis and thrombus burden. In one study, SCH subjects exhibited a prothrombotic state as shown by increased levels of factor VII, plasminogen activator inhibitor antigen and lower d-dimer levels compared to healthy controls which fully reversed with 6 months of thyroxine therapy (Lupoli *et al.*, 2015). These findings are supported by a further study which showed the same findings in SCH patients treated with thyroxine (Canturk *et al.*, 2003). In another smaller study, although SCH subjects exhibited a prothrombotic state as shown by increased levels of antithrombin III, fibrinogen and homocysteine, 6 months of thyroxine therapy had no effect on coagulation although it was unclear how many patients attained euthyroidism post-treatment (Anagnostis *et al.*, 2014).

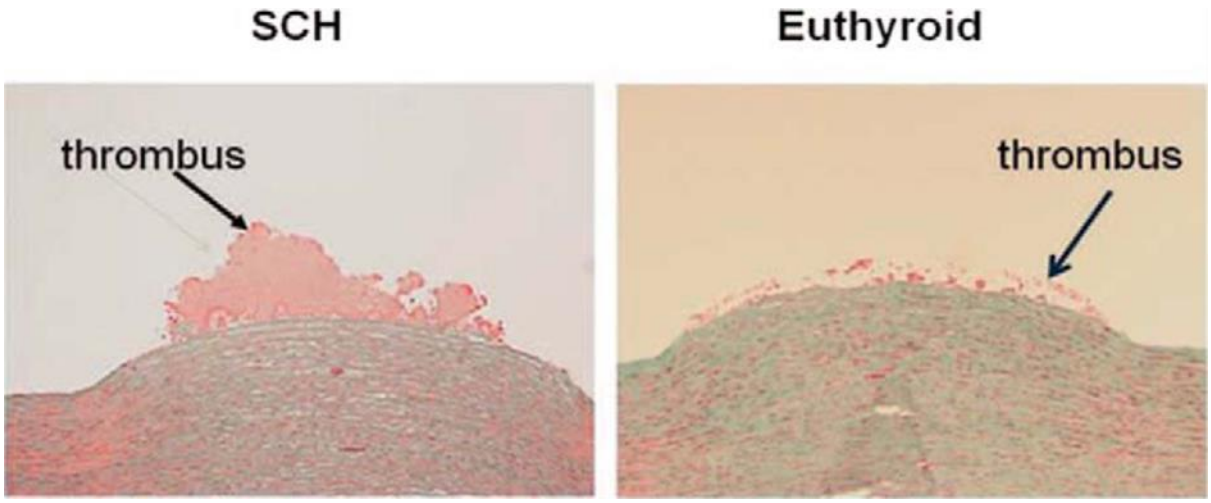


Figure 1-7 Difference in thrombus burden between SCH and euthyroid patients.

The Badimon chamber assesses ex-vivo thrombus burden. SCH patients post MI, on dual antiplatelets, have a higher thrombus burden in comparison to euthyroid patients. This may explain the higher cardiovascular risk in SCH. From: Viawanathan et al, J Clin Endocrinol Metab. 2014 Jun;99(6) (Viswanathan *et al.*, 2014a).

Studies assessing coagulability in hypothyroidism have produced conflicting results with some studies showing hypercoagulability whereas other studies demonstrated increased fibrinolysis. Erem et al found a hypofibrinolytic state in patients with hypothyroidism as shown by increased levels of fibrinogen and plasminogen activator inhibitor (Erem *et al.*, 2003). In other studies serum thyroxine levels were inversely related to fibrinogen and d-dimer levels in patients with hypothyroidism leading to a hypercoagulable state (Chadarevian *et al.*, 1998; Chadarevian *et al.*, 1999). Studies have also found elevated homocysteine levels in hypothyroidism with Christ-Crain et al showing elevated levels of CRP and homocysteine in patients with hypothyroidism which did not reverse with thyroxine therapy whereas Catargi et al found homocysteine levels to increase with a subsequent increase in TSH levels, and total homocysteine levels in hypothyroid patients were higher compared to controls (Catargi *et al.*, 1999; Morris *et al.*, 2001; Christ-Crain *et al.*, 2003).

Hypothyroidism has also been associated with increased fibrinolysis with studies showing reduced von Willebrand factor antigen which effects primary haemostasis and increases the bleeding time (Nitu-Whalley and Lee, 1999; Michiels *et al.*, 2001; Gullu *et al.*, 2005). In one study comparing moderate and severely hypothyroid patients with euthyroid controls, patients with moderate hypothyroidism had decreased fibrinolytic activity and were more susceptible to clot formation whereas patients with severe hypothyroidism had increased fibrinolysis and

lower tissue plasminogen activator antigen (Chadarevian *et al.*, 2001). A systematic review taking into account the main studies looking at coagulation and fibrinolysis showed patients with hypothyroidism to have an increased risk of bleeding (Squizzato *et al.*, 2007). However, the authors state that no high quality study with regard to the effects of hypothyroidism on coagulation was identified. Furthermore, subjects in the studies did not have the same degree of thyroid dysfunction with many studies including subjects with subclinical thyroid disease. Finally, in some studies thyroid function tests were not repeated at the time of coagulation tests (Squizzato *et al.*, 2007).

In summary, there is considerable heterogeneity in all these studies making it difficult to ascertain the impact of an underactive thyroid state on the coagulation pathway. Studies have demonstrated that TH deficiency can alter the coagulation pathway but more research is needed by having higher quality studies with robust methodologies which include a larger number of participants, the type of tested TH, TH measurements at similar intervals as coagulation tests and including subjects with the same degree of thyroid dysfunction. At present, one can conclude the likely increased risk of AMI in SCH subjects may be due to a prothrombotic effect, however more robust studies are needed relating to SCH and thrombogenesis.

1.2.6 Thyroid hormones and cardiac function

Overt hypothyroidism can cause left ventricular diastolic dysfunction by reducing the activity of SERCA and this subsequently leads to reduced reuptake of calcium during diastole leading to impaired ventricular relaxation (Tielens *et al.*, 2000; Virtanen *et al.*, 2001). This impairs left ventricular filling during diastole. Similar findings also occur in SCH patients as shown from imaging studies using doppler echocardiography and radionuclide ventriculography to compare SCH patients with euthyroid controls. These studies have demonstrated an increase in the peak A wave velocity, a decrease in early diastolic mitral flow velocity/late diastolic mitral flow velocity ratio (E: A) and mitral acceleration and deceleration times (Figure *1-8*, Table *1-3*) which are important measures of diastolic function (Biondi *et al.*, 1999; Monzani *et al.*, 2001; Brenta *et al.*, 2003; Aghini-Lombardi *et al.*, 2006). Other impaired parameters in these studies include a more prolonged isovolumetric relaxation time (IRT) and impaired peak filling rate during diastole. Biondi et al, Yazici et al and Aghini-Lombardi et al have all shown a significant reduction in early diastolic mitral flow velocity/late diastolic mitral flow velocity ratio (E: A) in SCH subjects in comparison to controls with the use of Doppler echocardiography (Biondi *et al.*, 1999; Yazici *et al.*, 2004; Aghini-Lombardi *et al.*, 2006). In

other studies diastolic dysfunction has been demonstrated at both rest and exercise as well as systolic dysfunction on exertion with thyroxine therapy normalising such parameters (Biondi *et al.*, 1999; Biondi *et al.*, 2002; Brenta *et al.*, 2003). Diastolic heart failure has implications in hypothyroid and SCH patients due to the high prevalence in the elderly and that it likely reduces exercise tolerance in younger patients (Biondi *et al.*, 1999).

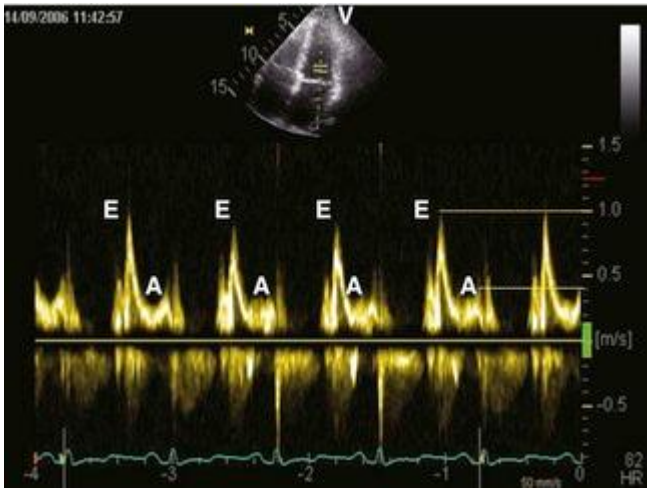


Figure 1-8 Diastolic function is derived by doppler echocardiography which measures the inflow velocity across the mitral valve into the left ventricle during relaxation.

The E wave represents peak velocity in early diastole whereas the A represents peak velocity in late diastole due to atrial contraction. The E velocity should be greater than A velocity with a ratio >1. In diastolic dysfunction, the ventricle can become stiff which impairs ventricular filling therefore increasing the back pressure as it fills leading to a decrease in the E velocity and a decrease and even reversal of the E:A ratio.

Study	No. of patients	Age (yr)	TSH (mIU/liter)	Cardiac findings	Cardiac methods
Biondi, 1999	26	36 ± 12	8.6 ± 4.8	↑ A, ↓ E/A, ↑ IRT	Doppler echo
Di Bello, 2000	16	32 ± 12	5.3 ± 1.9	↑ A, ↔ E/A, ↑ IRT	Doppler echo
Monzani, 2001	20	33 ± 12	5.4 ± 2.4	↔ E/A, ↑ A, ↑ IRT	Doppler echo
Vitale, 2002	20	38 ± 12	10.6 ± 4.05	↔ E/A, ↑ IRT	Doppler echo
Brenta, 2003	10	50 ± 8.7	11.0 ± 4.2	↑ TPFR	Radionuclide ventriculography
Yazici, 2004	45	40 ± 7.9	8.41 ± 2.1	↑ A, ↑ IRT, ↓ E/A	Doppler echo
Aghini-Lombardi, 2006	24	35 ± 6.2	5.3 ± 1.1	↑ A, ↑ IRT, ↓ E/A	Doppler echo

Table 1-3 Table to demonstrate studies which have compared SCH and euthyroid subjects with regard to LV function.

The studies demonstrate that SCH causes a decrease in E:A, a prolonged isovolumetric relaxation time and an increase in the total peak filling rate which are important measures of diastolic dysfunction. From: Biondi et al, Endocr Rev. 2008 Feb;29(1):76-131 (Biondi and Cooper, 2008).

Even in euthyroidism, subjects with lower TH levels have impaired cardiac function whereas an increase in thyroid hormone is associated with enhanced ventricular contraction and relaxation indicating that variations in TH within the reference range can effect cardiac function (Roef *et al.*, 2013). Cardiac MRI (CMR) is considered the gold standard test for accurately assessing LV volumes and function. In one robust study, CMR was used in otherwise healthy SCH patients to assess cardiac volumes and function with the results demonstrating a reduced end diastolic volume (EDV), a reduced ejection fraction of 5% and an increase in SVR in SCH subjects compared to controls. Repeat CMR post thyroxine showed normalisation of these parameters which were similar to controls (Ripoli *et al.*, 2005).

In summary, the current evidence demonstrates diastolic dysfunction to be the most frequent cardiac abnormality in SCH in a clinical setting due to impaired ventricular filling and relaxation although the number of participants in these studies were small. Although these studies have not yielded conclusive results with regard to systolic function, a robust study using cardiac MRI has demonstrated systolic dysfunction which reversed with thyroxine therapy. Overall an underactive thyroid state is a risk factor for heart failure by causing diastolic dysfunction and increasing the SVR.

1.2.7 Assessing cardiovascular function and scar size

Cardiovascular magnetic resonance imaging (CMR) is the gold standard method for assessing ejection fraction since this modality achieves superior image quality. It is non-invasive, avoids exposure to radiation and is the most reproducible method for quantifying left ventricle (LV) volumes and hence ejection fraction. (Hudsmith *et al.*, 2005). Volumetric measurements of the LV cavity is dependent on a time-volume relation of the LV cavity and this is obtained by a series of breath held short-axis planes whilst in the scanner.

Late gadolinium enhancement (LGE) CMR is the most accurate modality for scar detection post AMI due to its high spatial resolution which can detect even subendocardial infarctions (Wagner *et al.*, 2003). Gadolinium contrast given to patients during CMR accumulates in the extracellular spaces of both healthy and diseased myocardium to exhibit enhancement. The rate of gadolinium uptake, accumulation and washout is influenced by diseased myocardium post MI leading to increased enhancement, due to accumulation and delayed washout, in comparison to healthy myocardium in which the gadolinium is washed out after a specific time period. The relative contrast in healthy and diseased myocardium is dependent on when

imaging occurs post gadolinium administration. Up to 3 minutes post administration, gadolinium resides in the blood pool and healthy myocardium. After approximately 10 minutes, the gadolinium is washed out from the normal tissues but is retained in diseased tissue post MI leading to late enhancement on T1-weighted imaging (Figure 1-9). This is due to cellular necrosis, oedema and lysis leading to an increased extracellular space in diseased tissue.

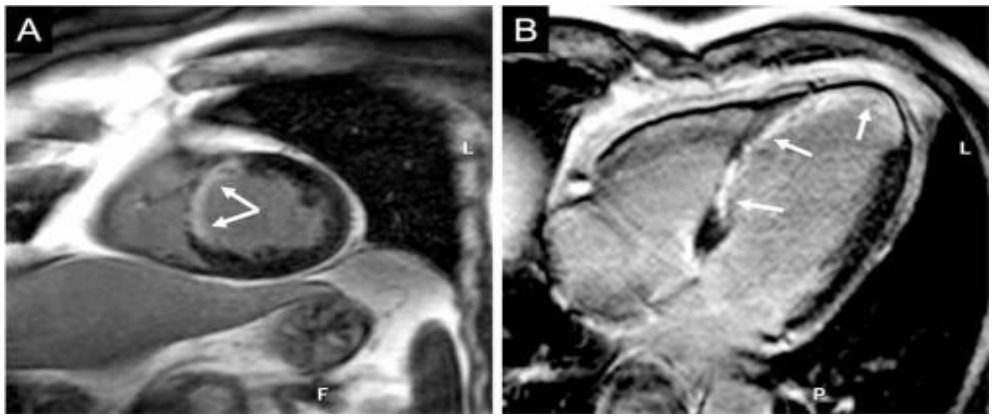


Figure 1-9 The detection of scar by the use of LGE on CMR. Myocardial cell necrosis post AMI increases the extracellular space leading to a delay in the gadolinium washout at 10 minutes and hence increased enhancement compared to normal tissue.

1.3 Experimental studies involving TH

1.3.1 Thyroid hormones and cardioprotection

Post myocardial infarction cardioprotection is a new therapeutic target for pharmacological intervention in both the acute and chronic phase of AMI when post-ischaemic heart failure develops. The main goal of cardioprotection is to reduce or limit myocardial damage in order to avoid impairment of left ventricular function. Furthermore, the additional goal is to limit the progression toward irreversible HF syndrome. Myocardial damage in AMI is caused by two main processes: ischaemic damage due to the abrupt occlusion of the coronary vascular system and damage caused by revascularisation leading to reperfusion injury. Cardioprotection is a complex phenomenon involving the stimulation of cell growth, neo-angiogenesis, metabolic adaptation, and maintenance of mitochondrial integrity is a new emerging aspect. In this post myocardial infarction cardioprotection scenario, the emerging role of TH in influencing different molecular, tissue, and cellular mechanisms requires further exploration, particularly as data accumulates with regards to its regenerative properties (Columbano *et al.*, 2006; Naqvi *et al.*, 2014). The targets of TH cardioprotective effects are the limitation of infarct extent, mainly involving the border zone, and the limitation of the LV post-ischemic remodelling process, involving both the border and the remote zone through the antifibrotic and pro-angiogenetic effects. LV remodelling involves changes in cardiomyocytes, extracellular matrix, and microcirculation affecting the infarct region, AMI border zone, and remote regions (Gerdes *et al.*, 1992; Dixon and Spinale, 2010). This causes thinning of the infarct area, infarct expansion at the site of the necrotic border zone, and hypertrophy and fibrosis of the remote zone (Pfeffer, 1995). The net result of this process is progressive LV dilatation, its functional impairment, and the progression to heart failure.

1.3.2 Thyroid hormones and the foetal genotype

Cardiac remodelling is the stress response of the heart to myocardial ischaemia, metabolic changes and changes in mechanical loading to restore the function of the heart. Haemodynamic overload leads to cardiac dilatation which in the short term helps to maintain cardiac output and blood flow (Pantos *et al.*, 2010). Therefore remodelling involves changes in cardiomyocytes, the vasculature and the extracellular matrix. A key feature of this process is activation of the foetal gene program in which the myocardium has characteristics similar to the foetal heart in regard to genetic expression, isoform switches of proteins, and energy consumption in which glucose is metabolised over fatty acids (Taegtmeyer *et al.*, 2010).

The experimental setting has shown how α-myosin heavy chain (αMHC) and SERCA decrease post AMI whereas β-myosin heavy chain (βMHC) increases as in the foetal genotype (Pantos *et al.*, 2007a; Henderson *et al.*, 2009). In experimental studies, TH supplementation reverses these changes and improves myocardial function by increasing αMHC which is the main contractile protein (Chang *et al.*, 1997; Ojamaa *et al.*, 2000; Pantos *et al.*, 2007a; Henderson *et al.*, 2009). The reasons for why there is a change in myocyte expression to the foetal mode post AMI is not clear although we do know TH deficiency has a large part to play. Ojamaa et al have shown T3 to be low up to 4 weeks post AMI whereas another study showed that T3 remained low for up to 8 weeks post AMI (Ojamaa *et al.*, 2000; Olivares *et al.*, 2007). Furthermore, TH is low during the foetal stage of development and subsequently increases in the post-natal period leading to maturation of the myocardium with transcriptional gene changes which cause alterations in metabolism, myocyte shape and cellular function (Pantos *et al.*, 2008b). Rajabi et al have shown that a low T3 in the injured myocardium could be a benefit in conserving energy after an AMI however this is also considered to be a maladaptive state in the long term, if not reversed, which has poor prognostic benefits (Rajabi *et al.*, 2007). The different mechanisms by which a low TH state effects the heart will be discussed in detail below.

1.3.3 Thyroid hormones and LV function

Suppression of TH post AMI causes calcium overload within cells which contributes to reperfusion injury and hence LV impairment (Krause *et al.*, 1989). Although TH levels decrease in rats post AMI, supplementation of TH post AMI has shown beneficial effects on LV function. TH administered for 2 weeks after an AMI was shown to attenuate remodelling and improve LV function in the viable myocardium (Pantos *et al.*, 2007a), whereas TH administered for 13 weeks post AMI was found to increase LV function, improve LV systolic and diastolic diameters and improve myocyte geometry (Pantos *et al.*, 2008a). Even administration of a high dose of TH 13 weeks after AMI showed TH to partially reverse cardiac dysfunction in rats with an old MI by changing cardiac geometry and myosin expression, indicating how myocyte remodelling can take place over a long period with the potential of reversal of pathological remodelling to physiological (Pantos *et al.*, 2009b). Chen et al, Henderson et al and Forini et al have also shown similar beneficial effects of TH on LV function in experimental studies post MI (Henderson *et al.*, 2009; Forini *et al.*, 2011; Chen *et al.*, 2013). Although the doses of TH in some of these studies were higher than optimal,

Henderson et al used a dose that was physiological and found an improvement in both systolic and diastolic function (Henderson *et al.*, 2009). TH has also been shown to limit the extent of infarct size and apoptosis in the border area of the infarcted tissue leading to an improvement in LV function (Chen *et al.*, 2008; Kalofoutis *et al.*, 2010). This limitation of infarct extension is through the activation of the cellular pro-survival pathways PI3K/Akt and PKC and suppression of the p38 MAPK pathway, which reduces myocyte apoptosis (programmed cell death), as shown experimentally in rodent models of AMI treated with T3 (Rybin and Steinberg, 1996; Kuzman *et al.*, 2005; Pantos *et al.*, 2009a).

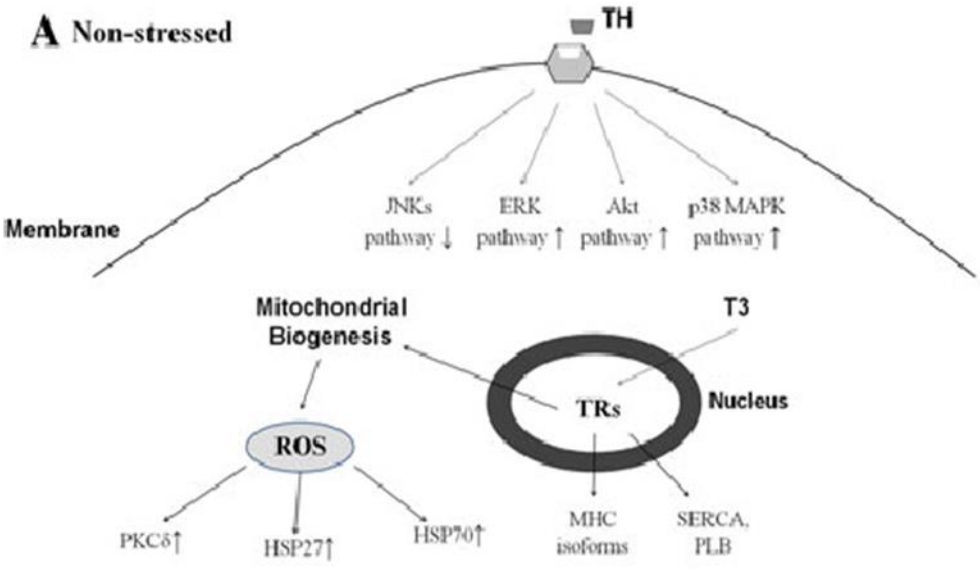


Figure 1-10 Mechanisms of TH cardioprotection

TH activates pro-survival signalling pathways (non-genomically) which has a benefit post MI. TH also regulate myocyte contractile proteins (direct genomic action) and upregulate cardioprotective molecules via mitochondrial biogenesis (non-genomic action).

These studies in the experimental setting show how LV function not only improves due to the inotropic effects and anti-apoptotic of TH non-genomically but also genomically due to an increase expression of αMHC, SERCA and inhibition of phospholamban. This is supported by a study by Pantos et al in which T3 treated cells expressed 51% αMHC and 49% βMHC compared to non-treated cells which expressed 100% βMHC (Pantos *et al.*, 2007b). In another study by Forini et al, human atrial cardiomyocytes cultured with and without T3 demonstrated how T3 deprived cardiomyocytes had larger dimensions and altered morphology resembling

impairments observed in heart failure. One of the key findings was a reduction in SERCA which resulted in poor calcium handling and this is well known to cause myocardial impairment as shown by studies in which reuptake of calcium during diastole leads to impaired ventricular relaxation (Forini *et al.*, 2001).

1.3.4 Thyroid hormones and myocyte shape and geometry

Compensatory mechanisms are activated post AMI to try to restore cardiac function but sustained activation in the long term can also lead to cardiac failure. Cardiac function deteriorates due to myocyte changes in the viable, non-ischaemic myocardium. A key aspect of maladaptive cardiac remodelling is myocytes becoming large and spherical which causes an increase in wall stress and a decline in LV function (Sehgal and Drazner, 2007). Wall stress is increased with an increase in myocyte diameter and subsequently decreases with an increase in wall thickness. Therefore progression to dilated failure has been show to occur with increased myocyte diameter without an increase in myocyte width and thickness, which therefore reduces the cross sectional area (Gerdes *et al.*, 1992; Gerdes and Iervasi, 2010).

TH has been shown to alter myocyte size and diameter. In hypertensive rats with heart failure, TH reduced LV stress by decreasing the ratio of LV diameter to wall thickness indicating that physiological remodelling entails reducing the myocyte major diameter and increasing the minor diameter (Thomas *et al.*, 2005). In rats with an old MI, TH changed myocyte geometry from a spherical shape to a more elliptical shape leading to an increase in ejection fraction (EF) (Pantos *et al.*, 2008a; Pantos *et al.*, 2009b). The beneficial effects of TH on cardiac geometry immediately after an AMI have also been investigated. Myocytes from the non-infarcted myocardium showed increased lengthening and TH treatment increased the cross sectional area by increasing myocyte thickness which lead to an increase in LV function (Chen *et al.*, 2013). This provides more evidence on how TH therapy can prevent pathological remodelling post AMI. TH has been shown to effect myocyte shape and geometry by activating survival pathways such as the ERK signalling pathway (Pantos *et al.*, 2007b; Mourouzis *et al.*, 2012). This is shown by the administration of PD98059 (an inhibitor of ERK signalling) which prevents a change in cardiac geometry with TH (Pantos *et al.*, 2007b).

1.3.5 Thyroid hormone effects on angiogenesis and mitochondrial function

Small arterioles within the myocardium are important for oxygen delivery and therefore myocardial function. A reduction in myocardial arterioles and blood flow has been demonstrated in dilated cardiomyopathy, hypothyroidism and neonatal hypothyroidism (Heron and Rakusan, 1996; Khalife *et al.*, 2005; Tang *et al.*, 2005; Liu *et al.*, 2009). In cardiomyopathic hamsters with SCH, coronary blood flow was shown to be impaired which contributed to a reduction in LV function and myocyte loss. Such changes were fully reversed with TH treatment (Khalife *et al.*, 2005). TH has been shown to induce arteriolar growth in hypertrophy secondary to hyperthyroidism (Tomanek *et al.*, 1995; Tomanek *et al.*, 1998b), and to prevent the loss of arterioles during induction of hyperthyroidism (Liu *et al.*, 2008). In a MI setting, TH increase arteriolar length and density which helps oxygen delivery to the injured myocardium (Forini *et al.*, 2011; Chen *et al.*, 2013).

TH can induce angiogenesis via the integrin receptor αVβ3 which leads to activation of the ERK 1 and 2 pathway. This leads to the transcription of different angiogenic genes such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin (Mousa *et al.*, 2005; Mousa *et al.*, 2006). Another proangiogenetic effect of TH is via the interaction of TH with TRβ receptor leading to hypoxia-inducible factor 1 alpha (HIF-1α) expression which is an important factor in cell proliferation and promoting vascular growth (Eckle *et al.*, 2008). Furthermore, TH also has non genomic angiogenic effects by causing coronary smooth muscle cell relaxation via TRα receptors and this represents another likely mechanism for increasing coronary artery blood flow by decreasing coronary vascular tone (Taddei *et al.*, 2003).

Mitochondrial dysfunction plays a key role in the development of heart failure post MI (Ide *et al.*, 1999; Ikeuchi *et al.*, 2005). T3 administration has been shown to rescue mitochondrial function and therefore prevent cardiac remodelling as well as reduce myocyte apoptosis in a post ischemic rat model (Forini *et al.*, 2011; Nicolini *et al.*, 2013). The cardioprotective mechanisms of T3 on mitochondria is through generating an antioxidant response and controlling the calcium flux into the myocardium which prevents the development of heart failure. The beneficial effect of thyroid hormones through SERCA activation has to be weighed against actions on mitochondrial oxygen demand, because SERCA is one of the most sensitive enzymes to ATP depletion. Therefore, mitochondrial dysfunction during ischaemia–reperfusion could undermine calcium removal by SERCA and affect myocyte function (Willis

et al., 2015). T3 also activates important pathways such as the mitochondrial ATP-sensitive K channels (mitoKATP) pathway, peroxisome proliferator-activated receptor gamma coactivator1-alpha, and the mitochondrial transcription factor A. This results in an increased expression of factors involved in mitochondrial DNA transcription such as HIF-1α and mitochondrial transcription factor A (mtTFA). An overexpression of mtTFA has shown to increase mitochondrial DNA and inhibit LV remodelling post AMI whereas a reduction in mtTFA causes mitochondrial dysfunction and attenuates cardiac failure (Wang *et al.*, 1999; Ikeuchi *et al.*, 2005). By targeting the expression of mtTFA, mitochondrial DNA and mitochondrial electron transport system can be restored which reduces myocardial oxidative stress. The mitoKATP pathway is also an important pathway for myocardial protection with T3 being shown to protect myocytes against oxidative stress (Ardehali *et al.*, 2005; Forini *et al.*, 2011) (Figure *1-11*). One study has shown T3 induced cardiprotection via the mitoKATP pathway in cardiomycoytes exposed to hydrogen peroxide (Forini *et al.*, 2011). Therefore the mitoKATP pathway could be a key target for preventing cardiomyocyte cell death secondary to oxidative stress.

The TH system has multiple roles in maintaining mitochondrial integrity. Thyroid hormones are critical regulators of tumour suppressor protein p53 a protein that accumulates within the myocardium in the acute phase of MI leading to apoptosis (Figure *1-11*). Experimental studies have shown that the intracellular increase in p53 in cardiomyocytes is more pronounced in rats developing low T3 syndrome early after AMI than in those without low T3 syndrome (Forini *et al.*, 2014). Early T3 administration blunts the increase of p53 levels in the AMI border zone after ischaemia–reperfusion, and this effect is associated with preserved mitochondrial function, decreased apoptosis, and reduced extent of necrosis (premature cell death) in the infarct border zone (Forini *et al.*, 2014; de Castro *et al.*, 2016).

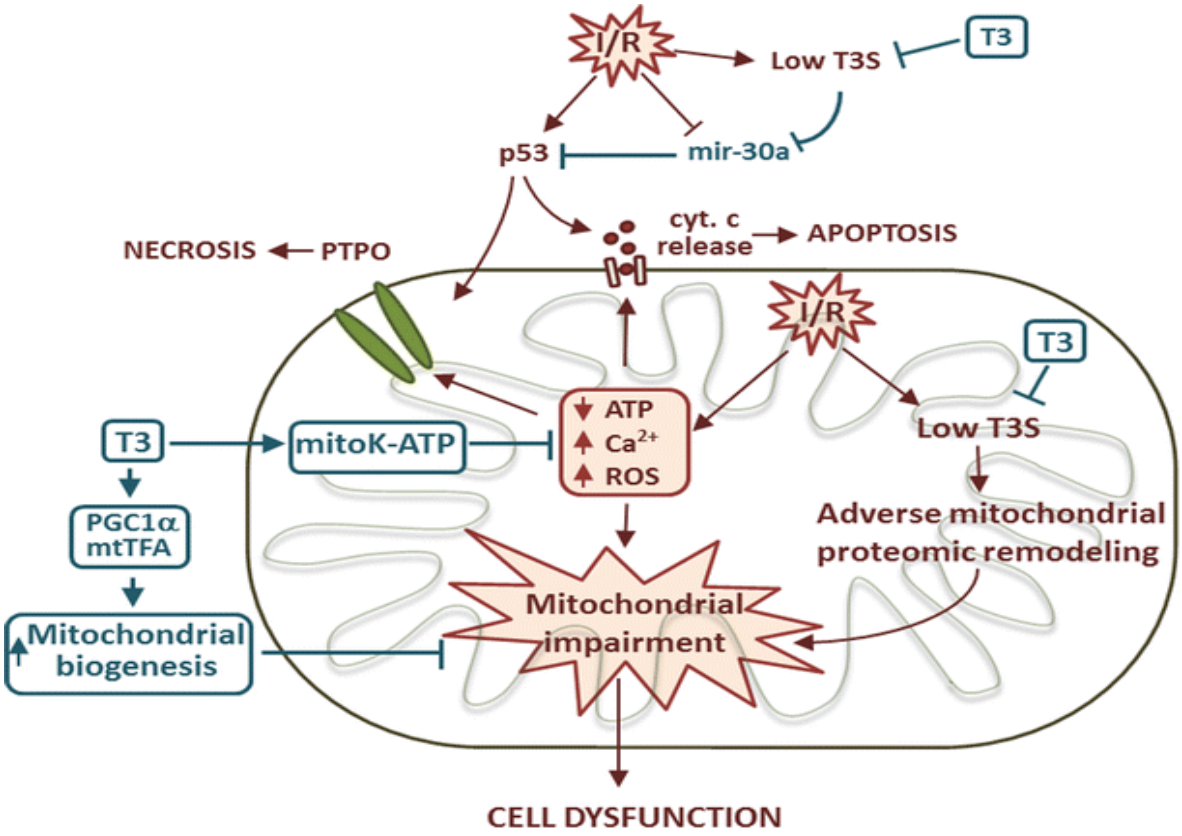


Figure 1-11 Main mechanisms of mitochondrial preservation and cardiomyocyte protection.

Ischaemic reperfusion (IR) causes myocyte injury by decreasing ATP, increasing intracellular calcium and reactive oxygen species. This insult is further enhanced by p53 and low T3 which leads to mitochondrial dependent apoptosis. Increasing TH levels leads to 1) opening of the protective mitoKATP channel 2) increasing mitochondrial biogenesis via mtTFA which leads to mitochondrial DNA transcription 3) reducing p53 levels post ischaemic reperfusion injury by inhibiting miRNA involved in production. From: Pingitore et al, Cardioprotection and thyroid hormones. Heart Fail Rev. 2016;21(4):391-9 (Pingitore *et al.*, 2016).

1.3.6 Thyroid hormones and apoptosis

Controlling survival signalling pathways which prevent myocyte apoptosis and preserve myocytes can reduce the severity of heart failure (Figure 1-12). The AKT/PKB pathway is an important survival signalling pathway which reduces apoptosis in hypoxia and ischaemia reperfusion to improve cardiomyocyte function (Matsui *et al.*, 1999; Fujio *et al.*, 2000; Matsui *et al.*, 2001). Furthermore T3 administration activates the PKB pathway in serum starved myocytes to protect against cell death as well as activating the pathway in the area affected by myocardial infarction to reduce apoptosis (Kuzman *et al.*, 2005; Chen *et al.*, 2008). The AKT pathway is also important for physiological hypertrophy. Hypertrophy of surviving cardiomyocytes is an integral part in cardiac remodelling. Cardiac hypertrophy develops as a

result of different signalling pathways which should be targeted in order to prevent pathological hypertrophy which is associated with changes in extracellular matrix composition, myocardial fibrosis and impaired capillary growth (Walsh, 2006; Nicolini *et al.*, 2013). TH activates PI3K/AKT (phosphatidylinositol 3′ kinase/protein kinase B) and 2 GSK3β (glycogen synthase kinase 3 beta) which have a role in normal physiological hypertrophy by reducing fibrosis, increasing vascular growth and activating genes which code for key regulatory proteins (DeBosch *et al.*, 2006; Maillet *et al.*, 2013).

TH also has an important role in ischaemic reperfusion injury with the administration of T3 during the reperfusion period reducing myocyte apoptosis via the PKB pathway resulting in cardioprotection (Pantos *et al.*, 2009a). TH can also protect against ischaemia-reperfusion via heat shock proteins. Heat shock protein 27 (HSP27) is an important cytoskeletal protein which becomes increasingly expressed, secondary to TH, in the cytoskeleton during ischaemic stress and therefore increasing tolerance to ischaemia (Pantos *et al.*, 2003a) whereas heat shock protein 70 (HSP70) expression is associated with decreased p38 MAPK activation in an ischaemic model pre-treated with TH (Pantos *et al.*, 2001; Pantos *et al.*, 2003b).

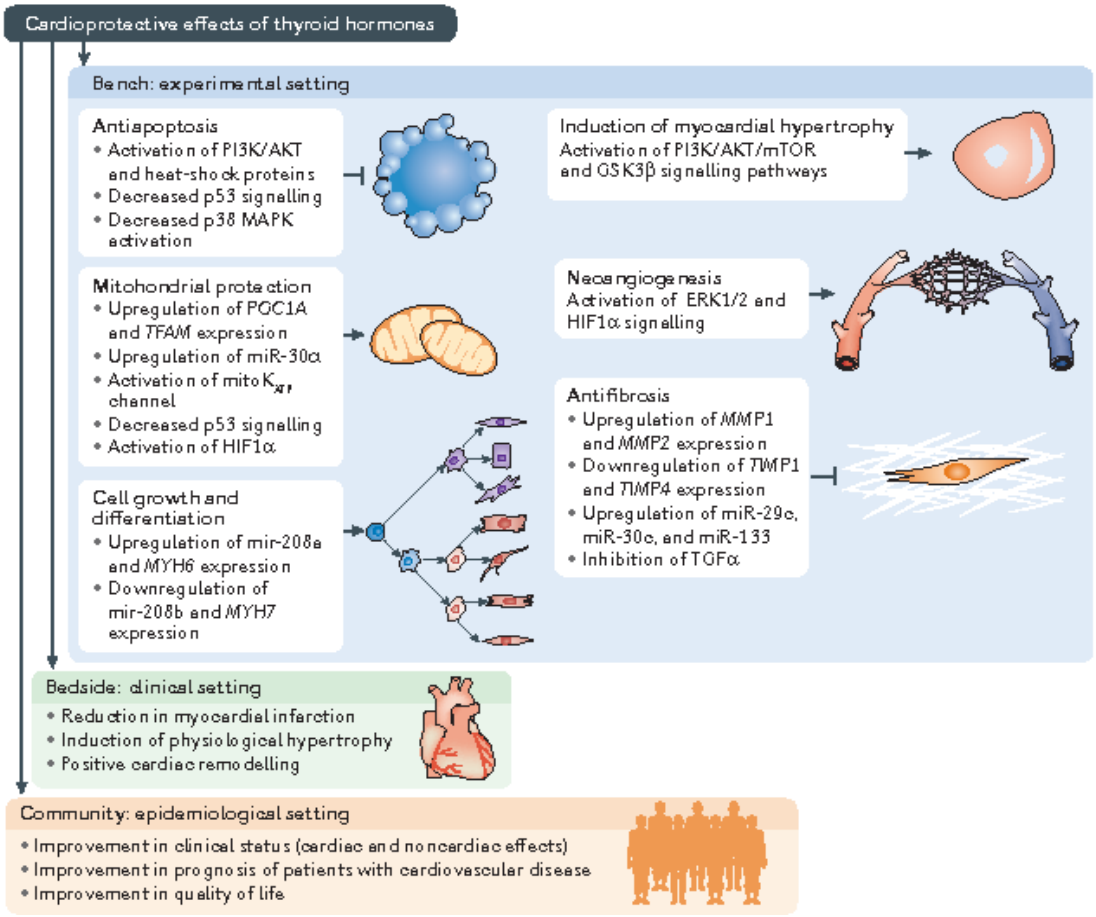


Figure 1-12 The translational effect of thyroid hormones on cardioprotection: from experimental setting to clinics and community.

1.4 Clinical studies involving thyroid hormones and cardiovascular disease

1.4.1 Clinical outcomes of SCH in cardiovascular disease

A number of observational studies have shown individuals with subclinical hypothyroidism to have an increased risk for cardiovascular disease (Hak *et al.*, 2000; Imaizumi *et al.*, 2004; Walsh *et al.*, 2005; Iervasi *et al.*, 2007). The Whickham Survey evaluated cardiovascular events in a population cohort over 20 years and found SCH to be associated with increased cardiovascular events and mortality and subsequent treatment of SCH with levothyroxine improved such outcomes (Razvi *et al.*, 2010). Walsh et al showed SCH to be a strong independent risk factor for coronary heart disease whereas Hak et al in the Rotterdam Study demonstrated those with SCH were more likely to have a myocardial infarction and calcification of the aorta (Hak *et al.*, 2000; Walsh *et al.*, 2005). These findings are supported by a meta-analysis which shows the association of SCH with an increased risk of coronary heart disease (Rodondi *et al.*, 2006).

Not all prospective cohort studies have shown a link between SCH and cardiovascular disease (Rodondi *et al.*, 2005; Cappola *et al.*, 2006). This is likely due to such studies including a population age group whom were significantly older and above the age of 65 whereas other studies which did show a significant difference included a younger age group. Age variation and cardiovascular risk in SCH is supported by a study in Denmark which showed the risk to be only significant in those people less than 50 (Kvetny *et al.*, 2004). Furthermore, a retrospective study of patients from the United Kingdom General Practitioner Research Database showed that treatment of SCH with levothyroxine in healthy individuals was associated with fewer IHD events in younger individuals but this was not evident in older people (Razvi *et al.*, 2012). This further supports the view of SCH being a risk factor in the younger age group. A patient-level meta-analysis by Rodondi et al of 55,287 participants from 11 prospective cohort studies showed SCH to be associated with coronary heart disease and mortality in those with higher TSH levels>10 whereas a mild increase in TSH was not associated with CHD (Rodondi *et al.*, 2010).

Studies relating to heart failure and SCH have shown such patients to be more likely hospitalised and have a worse prognosis in comparison to euthyroid patients (Iacoviello *et al.*, 2008; Rodondi *et al.*, 2008; Silva-Tinoco *et al.*, 2011). The Health, Aging, Body Composition population-based study showed elderly patients with a TSH>7 to have an increased risk of developing CCF in comparison to euthyroid individuals (Rodondi *et al.*, 2005). In an analysis

of more than 3000 participants aged 65 years or more without heart failure at baseline, who were followed up for 12 years in the Cardiovascular Health Study, showed that SCH subjects treated with levothyroxine had a 72% reduction in heart failure events and furthermore, a TSH above 10mu/L was associated with a higher risk for heart failure (Rodondi *et al.*, 2008). This is supported by the Prosper Study which was a prospective cohort study assessing the link between SCH and heart failure. Prosper showed subjects with SCH were more likely to be hospitalised for heart failure and once again heart failure was higher in subjects with TSH greater than 10 (Nanchen *et al.*, 2012). Previous observational studies have also shown SCH to be associated with worse cardiovascular outcomes including mortality in patients with acute cardiovascular disease (Iervasi *et al.*, 2007; Rodondi *et al.*, 2010; McQuade *et al.*, 2011; Molinaro *et al.*, 2012; Rhee *et al.*, 2017; Seo *et al.*, 2018).

In summary, despite known cardiovascular risks of SCH, there is not enough evidence for treating all patients at present due to current data being from observational studies or from small interventional trials with cardiac risk factor change as outcomes. For this reason randomised controlled trials are needed to evaluate the benefits of treating SCH in reducing cardiovascular risk. In the meantime, it may be beneficial to treat SCH patients who are at high risk of cardiovascular disease or those that may have evidence of cardiac impairment on imaging. Current data suggests treatment of SCH is likely to be more beneficial in younger patients than the elderly however more studies are needed to ascertain the risks or benefits of thyroxine treatment in the elderly with SCH. Further studies are needed to assess the outcomes of patients with SCH post myocardial infarction.

1.4.2 Low thyroid hormones in myocardial infarction and the role of inflammation

Within hours of an acute illness, the changes in thyroid hormone levels are referred to as non-thyroidal illness (NTI). The most common abnormality is a reduction in T3 whereas in unwell patients T4 also decreases. Both a low T4 and T3, which are sustained, are associated with a worse prognosis in NTI (Fliers *et al.*, 2015). A reduction in TH levels post MI may be considered beneficial by lowering oxygen consumption and energy requirements in the injured myocardium however; the net effect on the heart may still be detrimental due to the role of TH in LV remodelling post MI. A decrease in TH levels post myocardial infarction is related to increased cytokine expression, decreased expression of deiodinase 1 enzyme (D1) and increased expression of deiodinase 3 enzyme (D3). Three studies in patients post AMI showed both interleukin-6 (IL-6) and interleukin-10 (IL-10) to increase post AMI which correlated

significantly with low TH levels (Kimura *et al.*, 2000; Nishino *et al.*, 2000; Kimur *et al.*, 2001). Kimur et al showed how subjects post AMI had lower TH levels in comparison to healthy controls whereas the levels of IL-6 and IL-10 were significantly higher and correlated with low FT3 levels (Kimur *et al.*, 2001). In a study by Kimura et al, all patients post AMI had an elevated IL-6 and low concentrations of TH, compared to controls, and the mechanism of action was via glycoprotein (gp) 120 receptors (Kimura *et al.*, 2000). Cytokines such as IL-1 and IL-6 decrease TH by inhibiting D1 activity and competing for receptor coactivators leading to decrease conversion of T4 to T3 (Yu and Koenig, 2000). TNFα is also upregulated in AMI and has been shown to increase IL-6 in ischaemia-reperfusion and in the viable border area of the myocardium further supporting the role of cytokines in decreasing TH (Hirschl *et al.*, 1996). This decrease in TH is considered to be protective by decreasing energy consumption as has previously been discussed but can be maladaptive in the long term if not reversed.

TH signalling can also be impaired post AMI due to an increase in D3 activity. A study by Wassen et al, in a rat model of right ventricular (RV) failure and hypertrophy, demonstrated how D3 activity was increased in the overloaded RV with no change in the normal functioning LV. Furthermore D3 activity was increased in the RV of rats with heart failure compared to those with hypertrophy alone (Wassen *et al.*, 2002). The largest clinical study assessing the relationship between increased D3 activity and low TH levels in pathological states was conducted in intensive care patients by Peeters et al who showed such changes to occur locally within the heart and at the systemic level (Peeters *et al.*, 2003). These studies show how markers of inflammation and deiodinase enzymes make the heart hypothyroid post myocardial infarction by reducing the availability of T3.

1.4.3 Clinical studies investigating thyroid hormones in AMI

Experimental studies have shown enough evidence of how a low TH which does not reverse in cardiac disease is maladaptive rather than adaptive and can lead to a reduction in LV function post AMI. In comparison to other organs, the heart is unique and more susceptible to a reduction in TH in the circulation as cardiomyocytes have reduced ability in converting T4 to T3 (Klein and Danzi, 2007; Gerdes and Iervasi, 2010). Therefore even mild changes in circulating TH can make the heart relatively hypothyroid at times when TH will be required to repair the myocardium. This may explain the high mortality amongst patients who have SCH and cardiovascular disease (Rodondi *et al.*, 2010; Molinaro *et al.*, 2012), low T3 and heart

failure (Hamilton *et al.*, 1990; Pingitore *et al.*, 2005) and in patients with low T3 who are undergoing CABG (Holland *et al.*, 1991; Cerillo *et al.*, 2014).

Previous observational studies have shown SCH to be associated with worse outcomes including mortality in patients admitted with cardiovascular disease including AMI (Iervasi *et al.*, 2007; Rodondi *et al.*, 2010; Molinaro *et al.*, 2012; Seo *et al.*, 2018). Iervasi and colleagues found cardiac patients with SCH (6.7% of the total cohort) and low circulating T3 levels (29.2%) to have a higher risk of cardiovascular and all-cause mortality than euthyroid individuals within a large cohort of patients admitted with cardiac events (Iervasi *et al.*, 2007). SCH after admission for an acute cardiac problem has been associated with an up to 3.6 fold increase in cardiac mortality and a 2.3 fold increase in overall death (Molinaro *et al.*, 2012). In another study of AMI patients followed for 3.5 years post coronary intervention, patients with SCH had a worse outcome including mortality; with TSH being a very strong predictor for all-cause mortality (Seo *et al.*, 2018). A prospective study by Friberg et al showed T3 levels to rapidly decline within a week after an AMI whereas in another study by Friberg, in-hospital and post discharge mortality was higher in patients with the most suppressed TH levels post AMI (Friberg *et al.*, 2001; Friberg *et al.*, 2002). In a study by Zhang et al, 501 patients had TFTs measured post AMI with 171 patients having a low T3. During a 1 year follow-up, patients with low FT3 had a higher mortality in comparison to euthyroid patients indicating how low TH levels post AMI are predictor of short-term and long-term poor prognoses (Zhang *et al.*, 2012). A further study showed a low T3 in up to 20% of patients below the age of 75 with an AMI and such patients were shown to have a lower survival rate despite percutaneous coronary intervention, further supporting the role of TH in myocardial recovery (Lazzeri *et al.*, 2012). Two further studies have shown a higher mortality in patients with a low T3 post AMI in comparison to euthyroid patients (Pavlou *et al.*, 2002; Iervasi *et al.*, 2003). In Iervasi's study, 173 of the 573 consecutive cardiac patients had a low T3 compared to 400 patients with normal thyroid function. At 1 year, 25 deaths occurred in the low T3 group compared to 12 in the euthyroid group with low TH levels being the biggest predictor of mortality on multivariate analysis. Furthermore TH were lower in patients developing heart failure and progressing to death post AMI (Iervasi *et al.*, 2003). Imaging studies have shown how a low T3 post AMI is associated with a worse cardiac function (Ceremuzynski *et al.*, 2004; Lymvaios *et al.*, 2011). In one study, the 48 hour post percutaneous coronary intervention LV function strongly correlated with serum TH whereas at 6 months an improvement in LV function correlated with an improvement in TH levels (Lymvaios *et al.*, 2011). Low TH has also been shown to

correlate with the severity of coronary artery disease on angiography as well as cardiac mortality indicating that a reduction in TH is not only associated with a worse outcome post MI but may also be a risk factor for ischaemic heart disease as has previously been discussed (Auer *et al.*, 2003; Coceani *et al.*, 2009). In the study by Coceani et al, patients with suspected coronary artery disease (CAD) who were having angiography had their TH measured to see if there was a relationship with disease burden on angiography (Coceani *et al.*, 2009). The study showed individuals with CAD on angiography to have lower T3 levels in comparison to those who did not have CAD, with free T3 being a significant predictor of CAD using multivariate logistic regression. The level of T3 was not statistically significant between patients who had single and multi-vessel disease (Coceani *et al.*, 2009). From all these studies it is difficult to illicit whether low TH levels contribute to disease progression or are a consequence of disease severity. Factors favouring the former view include T3 being the active form of thyroid hormone and therefore a low T3 state could be a risk factor for CAD as are both hypothyroidism and subclinical hypothyroidism which are known to be associated endothelial dysfunction, hyperlipidaemia, cardiac dysfunction and thrombosis. Unfortunately, the relationship between a low T3 state and such parameters has not been assessed previously as in both hypothyroidism and subclinical hypothyroidism. This is further supported by an improvement in cardiovascular risk factors with thyroid therapy in patients with SCH. These studies demonstrate how TH may also be used as a risk stratifier in patients with an AMI due to its correlation with disease burden on angiography, LV impairment on imaging as well as long-term mortality.

There are limitations with all these studies. Firstly, the timing of T3, T4 and TSH is not stated in most of these studies with such measurements taken only once. Therefore these studies cannot account for variations in thyroid function over a period of time from an AMI. For this reason we do not know whether a change in TH was due to sick euthyroid syndrome, in which TH levels normalise after a short time period, or due to a maladaptive response? It is very likely that TH levels would have normalised days after an MI in some patients recruited to these studies. Furthermore, AMI and CAD are not clearly specified in these studies making it difficult to ascertain if subjects were stable CAD patients or patients with chest pain and unstable symptoms characterised by troponin and ECG changes. Therefore, there is always potential for selection bias and heterogeneity in all these studies. In summary, despite acknowledging worse outcomes in patients with AMI who have supressed TH, current studies at present are either observational, retrospective or prospective studies. For this reason

randomised controlled studies are necessary to investigate whether TH replacement can improve myocardial function and improve mortality outcomes in a clinical setting.

1.4.4 Thyroid hormones and heart failure

SCH and low T3 syndrome are the more frequent metabolic TH alterations in HF (Silva-Tinoco *et al.*, 2011). In particular low T3 syndrome occurs in almost 20-30 % of HF patients (Passino *et al.*, 2009). Low T3 syndrome and SCH have been associated with a worse prognosis in HF (Iervasi *et al.*, 2003; Iervasi *et al.*, 2007; Iacoviello *et al.*, 2008).

Studies have been conducted to assess the relationship between low TH and heart failure. In one study, 21% patients with dilated cardiomyopathy had a low T3 which was associated with worse cardiac function on echocardiography. All patients with a Ft3/ft4 ratio less than 1.7 had worse cardiac function, increased mortality or subsequently needed a shock from cardiac arrest whereas this was not the case with a ratio greater than 1.7 (Kozdag *et al.*, 2005). In a prospective study by Opasich et al the prevalence of low T3 in patients with severe heart failure being considered for heart transplantation was 18%. Low T3 correlated with NYHA class as shown by 31% of patients in NYHA class 3 and 4 having low T3 compared to 7% in class 1 and 2. Furthermore, mortality was higher in patients with low T3 in comparison to euthyroid subjects, 48% v 21% (Opasich *et al.*, 1996). This is supported by another study assessing the relationship between T3 and mortality in patients with both ischaemic and non-ischaemic dilated cardiomyopathy admitted to hospital. In such patients ejection fraction (EF) and T3 were the only dependent factors associated with mortality (Pingitore *et al.*, 2005). In another study looking at ambulatory patients with heart failure with an ejection fraction (EF) of less than 35, the prevalence of patients with a low T3 was 34% which correlated with a worse NYHA class and disease severity (Ascheim and Hryniewicz, 2002).

Similar results have been sought for studies evaluating the prognostic role of SCH in heart failure. In stable heart failure patients followed up for 1-2 years, serum TSH was considered a stronger variable than T3 in heart failure progression as measured by echocardiography and B natriuretic peptide (BNP). Even slight changes in TSH resulted in worse outcomes as shown in subjects with heart failure progression who had an average TSH of 4.8 compared to 2.2 in those who remained stable (Iacoviello *et al.*, 2008). The Health, Aging, Body Composition population-based study also showed that elderly patients with a TSH>7 had an increased risk of developing heart failure in comparison to euthyroid individuals (Rodondi *et al.*, 2005).

Iervasi et al in a retrospective study assessed the effects of subclinical thyroid disease on cardiac outcomes in patients who had known cardiac disease. The study showed that survival rates and overall death was higher in subclinical thyroid disease and low T3 syndrome compared to euthryroidism (Iervasi *et al.*, 2007).

Although these studies have shown the association between a low thyroid state and a worse prognosis in patients with heart disease, there are limitations to take into account. Firstly, in many of these studies thyroid function tests were performed only once making it difficult to ascertain how many of these patients had sick euthyroid syndrome and how many patients with mild hypothyroidism may have progressed to overt hypothyroidism which needed treating. Other limitations include exclusion of patients with acute cardiac states such as myocardial infarction which itself increases mortality risk. With some of these studies being retrospective, there is the potential for selection bias and misclassification of the causes of death based on death certificates which may not have been cardiac. Finally, there is no differentiation to the different cardiac states in the studies meaning more serious cardiac disorders were likely associated with a low thyroid state and that disease severity rather than thyroid state resulted in worse outcomes in some patients.

1.4.5 Thyroid hormone therapy in heart failure

Studies have also examined the effects of TH therapy in heart failure. Moruzzi and colleagues investigated both the short and medium term effects of levothyroxine therapy in a small sample of patients with dilated cardiomyopathy. Giving short-term levothyroxine improved LVEF, improved cardiac exercise ability, decreased diastolic dimensions and SVR without causing adverse side effects. Both the cardiac and exercise ability of patients were increased without changes in adrenergic support (Moruzzi *et al.*, 1994). In another study, similar changes were observed with medium-term levothyroxine therapy over a period of 3 months with an increase in EF% and cardiac output, a decrease in ventricular diastolic dimensions and SVR, and an enhance response of cardiac output and heart rate to a dolbutamine infusion. Surprisingly despite the levels of thyroxine increasing, patients did not show symptoms or features of hyperthyroidism as shown by the findings (Moruzzi *et al.*, 1996). Pingitore et al showed a low T3 to be associated with a higher mortality in patients with dilated cardiomyopathy and therefore the short term effects of TH replacement in patients with low T3 and dilated cardiomyopathy were investigated in this randomised, placebo-controlled study. TH replacement improved ventricular performance as shown by an increase in stroke volume and

there were no side effects such as arrhythmias or haemodynamic compromise. Furthermore, the neuroendocrine profile significantly improved with a reduction in aldosterone, BNP and IL-6 levels (Pingitore *et al.*, 2005; Pingitore *et al.*, 2008). In a prospective study by Hamilton et al, low T3 was an increased predictor of mortality in patients with CCF. The 1 year survival in subjects with a normal T3/reverse T3 ratio of 100% compared to 37% in those with low ratio (Hamilton *et al.*, 1990). In a second study, the safety and haemodynamic effects of intravenous triiodothyronine was assessed in patients with congestive cardiac failure. Triiodothyronine was well tolerated with no adverse effects such as arrhythmias or ischaemia. Cardiac output increased with a significant decrease in SVR (Hamilton *et al.*, 1998).

2 Chapter: Hypothesis and aims

2.1 Hypothesis

Treating SCH with levothyroxine following an AMI improves left ventricular systolic function, thrombus area, endothelial function, health status, quality of life and is safe.

2.2 Aims

There is enough evidence to state that even minor changes in TH concentration may impact adversely on the cardiovascular system. SCH affects both endothelial function and cardiac function in healthy individuals and is likely to be prothrombotic. Furthermore, several observational studies have demonstrated that SCH in high-risk patients is associated with worse cardiovascular outcomes. This apparent increased risk could be due to the fact that both the pathogenesis of atherosclerosis, and recovery and myocardial-repair after AMI are influenced by TH levels. Much research on SCH and cardiovascular disease has focussed on large population studies and there is no RCT to date investigating the effect of thyroid hormone therapy in patients post AMI, when repair and recovery of myocytes is essential to prevent pathological remodelling.

This study was therefore designed with the following aims in mind:

1. To perform a prospective longitudinal observational study to investigate the prevalence of thyroid dysfunction in patients presenting with AMI and its association with troponin levels.
2. To assess the association between thyroid dysfunction post AMI and long term mortality.
3. To perform a double-blind placebo-controlled trial of levothyroxine treatment in patients with SCH post AMI, in an adequately powered sample to detect a significant difference on left ventricular systolic function with LT4 versus placebo.
4. To determine the effect of LT4 therapy on endothelial function, platelet dependent thrombus, viscoelastic properties of thrombus and platelet reactivity in SCH post AMI.
5. To assess the effect of LT4 therapy on health status, quality of life, depression and safety.

3 Chapter: Methods

3.1 General methods

3.1.1 Study design

The study was divided into two parts ThyrAMI 1 and ThyrAMI 2

ThyrAMI 1 - This was a prospective longitudinal observational study of patients with AMI (both ST-elevation AMI and non-ST-elevation AMI) that were followed up to study the association between thyroid status at the time of AMI (within 24 hours of diagnosis) with long-term mortality.

ThyrAMI 2 - This study was a double blinded, randomised placebo-controlled trial of levothyroxine of 12 months duration in patients with SCH (serum TSH persistently 4.01 to 10.0 mU/l, normal FT4) starting within 21 days following AMI. The day of AMI was the date of diagnosis or the date of admission to hospital, whichever was later.

3.1.2 Primary outcome measure

ThyrAMI 1 - The primary outcomes were the prevalence of thyroid dysfunction in patients presenting with AMI and the association between thyroid status at the time of AMI (within 24 hours of diagnosis) with markers of severity of AMI and subsequent all-cause mortality.

ThyrAMI 2 - The primary outcome measure was a change in LV ejection fraction as assessed by magnetic resonance imaging.

3.1.3 Secondary outcome measure ThyrAMI 2

I. Left ventricular systolic and end diastolic volumes and myocardial viability assessed by cardiac MR imaging.
II. Thrombus burden measured by the Badimon chamber, a highly reproducible clinical ex vivo model of thrombosis that mimics flow conditions within the coronary circulation of man.
III. Efficiency and quality of *ex vivo* thrombus formation utilising Thromboelastography (TEG™), a noninvasive measure. Quantitative measures include clotting time parameters, clot strength and clot lysis were also measured. These parameters have been validated as highly correlated with longer-term cardiac outcomes in several conditions of risk.

IV. Endothelial function assessed by measuring peripheral arterial tone using a validated tool, EndoPAT™. Endothelial function assessment using the EndoPAT™ has been shown to have a high degree of correlation with coronary artery endothelial function, the severity and extent of coronary artery disease, and traditional cardiovascular risk factors and is useful in predicting future cardiovascular events.

V. Platelet reactivity: The reactivity (inhibition) of platelets to anti-platelet agents such as aspirin, clopidogrel, prasugrel and ticagrelor were quantified by the point-of-care monitor VerifyNow™ (Accumetrics, CA, USA).

VI. Safety assessments: The safety of levothyroxine therapy in post-AMI patients was assessed at each study visit by enquiry of symptoms by New York Heart Assessment (NYHA) category classification, ECG recording for rhythm disturbance and adverse and serious adverse events during the course of the study.

3.1.4 Participant population

ThyrAMI 1

I. Inclusion criteria

1. Adult males and females (aged 18 years or older).
2. Acute myocardial infarction diagnosed in the preceding 24 hours (defined as chest pain with dynamic ECG changes or increased troponin enzymes (at least a fourfold increase above the local laboratory reference range).
3. Participants in other research studies were eligible for inclusion as this is an observational study.

II. Exclusion criteria

1. Patients who were unable to provide informed consent.
2. Those with advanced malignancy (who were unlikely to survive for more than 6 months in the opinion of local investigator).
3. Those on medications that can affect thyroid function such as amiodarone, lithium, carbimazole and propylthiouracil. Patients on levothyroxine will be included, but their results were analysed separately.

ThyrAMI 2

I. Inclusion criteria:

1. Males and females aged between above the age of 18.
2. Serum TSH ≥4.01 mU/L with normal free thyroxine levels (9 to 25 pmol/L) on two occasions with at least one TSH result between 4.01 to 10.00 mU/L (on day of admission for AMI and 7 to 10 days after AMI).
3. Acute myocardial infarction diagnosed on admission to hospital (chest pain with dynamic ECG changes or increase troponin enzymes (at least a fourfold increase above the laboratory reference range).

II. Exclusion criteria:

1. Patients on medications that affect thyroid function (levothyroxine, carbimazole, propylthiouracil, amiodarone, lithium).
2. Patients who were unable to provide written informed consent.
3. Patients with advanced malignancy (who, in the opinion of the investigator, are unlikely to survive for more than 6 months).
4. Patients with sustained ventricular tachycardia requiring treatment that occurs >24 hrs after myocardial reperfusion/revascularisation.
5. Patients who had contra-indications to MR scanning (cardiac pacemaker, metallic heart valves, cochlear implants, coronary artery stents incompatible with MR scanning, etcetera).
6. Patients who were unlikely or unwilling, in the opinion of the investigator, to attend for study-specific visits.
7. Participants whose serum TSH is >10.0 on both occasions or <4.0 on either occasion.
8. Patients who are already participating in another interventional study.

3.1.5 Screening and recruitment, consent, and biochemical analysis

i. Screening and recruitment

Eligible participants were identified from the cardiology units of acute hospitals in the North of England (Gateshead, Newcastle, Sunderland, North Tyneside, Leeds and Middlesbrough). The Freeman Hospital in Newcastle was the biggest site for recruitment to the study. All individuals who met the study inclusion and exclusion criteria for ThyrAMI 1 had their thyroid function checked, after informed consent was obtained, within 24 hours of diagnosis of AMI.

A 24 hour timeframe post AMI was used to prevent the influence of sick euthyroidism on thyroid function, which usually takes place with maximal changes between 24 and 36 hours (Friberg *et al*., 2002). Other blood tests, routinely performed post AMI such as total cholesterol, troponin T/troponin I and serum creatinine were also evaluated on admission. A troponin was repeated in all patients within 12 hours (median 6, range 3 – 12 hours) as per local clinical practice. Other variables measured on admission including demographic details, clinical features, and coronary angiographic findings and ischaemia time for STEMI patients.

Of those patients that had TSH and FT4 levels within inclusion range were provided with a participant information sheet for ThyrAMI 2. Furthermore, these individuals were invited to have a repeat thyroid blood test 7 to 10 days after the day of AMI (Figure *3-1*). The day of AMI was the date of diagnosis or the date of admission to the hospital, whichever was later. Those individuals who had a TSH and FT4 levels within the inclusion range on the second occasion were asked to participate in ThyrAMI 2 and sign the consent form. Those who did not attend for their second visit blood test were contacted to enquire whether they are still interested in participating. Ethical approval was granted by the UK National Research Ethics Service (Ref: 14/NE/0151). The study was conducted as per the guidelines outlined in the Helsinki Declaration, and patients had the right to refuse to take part in the study and furthermore could withdraw anytime without giving a reason.

Once a participant had thyroid blood results that were within the desired range for inclusion in the study on two occasions and had consented to participate in the RCT, the study team arranged a convenient date and time for the baseline tests and randomisation to the treatment groups. The dates for the baseline tests were within 21 days of the patients' AMI dates.

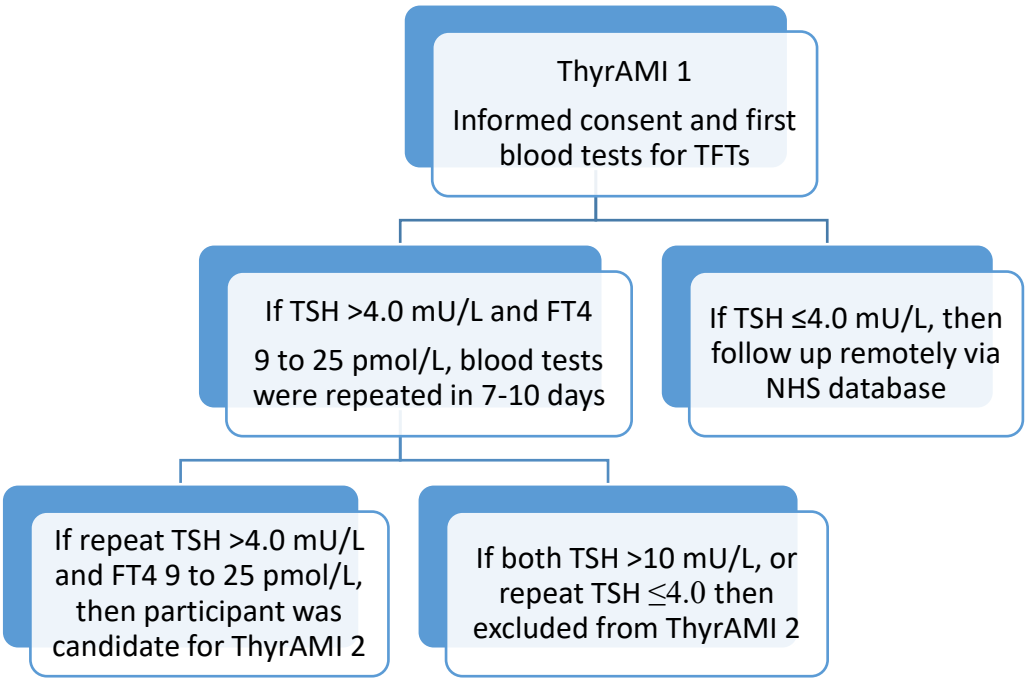


Figure 3-1 Flow chart representing ThyrAMI 1 observational study and identification of potential cohort for the interventional trial ThyrAMI 2.

ii. Consent procedures

All informed consent discussions were undertaken with an opportunity for participants to ask any questions. Following receipt of information about the study (that is, provision of the patient information sheets), participants were given reasonable time to decide whether or not they wanted to participate. Those wishing to take part provided written informed consent by signing and dating the study consent form and initialling the relevant sections, which was witnessed and dated by myself.

The original signed consent form was retained in the Investigator Site File, with a copy in the relevant clinical notes and a copy provided to the participant. The participant consented to their GP being informed of their participation in the study. The right to refuse to participate without giving reasons was respected. The information sheet and consent form for the study were available only in English. Participants who lacked capacity to consent for themselves were excluded from the study.

iii. Biochemical analysis

Biochemistry: Serum TSH, FT4 FT3, high sensitive (hs) troponin T, troponin I, total cholesterol, and urea and electrolytes were analysed using the Roche assay (Roche cobas, Roche Diagnostics UK). At two of the sites, the same analytes were measured by the Advia Centaur immunoassay (Siemens Healthineers, Surrey, UK). Reference ranges were as follows: TSH (0.4 - 4.0 mU/L), FT4 (9.0 - 25.0 pmol/L), FT3 (3.0 - 7.0 pmol/L), hs troponin T (0 - 14 ng/L), hs troponin I (0-45 ng/L), creatinine (70 - 110 □mol/L), total cholesterol (0 - 4.5 mmol/L). Anti-thyroid peroxidase antibodies (TPOAb) were measured by the Roche immunoassay and levels below 35 mU/L were classed as negative.

3.1.6 Study specific procedures undertaken by participants

For patients meeting the study criteria for the RCT, a visit to the Clinical Research Facility (CRF) was arranged, with the tests including: the Badimon chamber, EndoPAT, VerifyNow and TEG. Either on the same day or on a separate day, patients also had a visit to the Cardiac MRI Centre, Newcastle University, where the cardiac MRI was performed. All these tests were performed within 21 days of the MI. Each patient was randomised the previous day to enable the investigational medicinal product (IMP) to be collected and assigned to the participant.

For the CRF tests, patients attended having fasted from midnight which included not taking their morning medications till the initial tests had been completed. The patients' understanding of the study was checked and any final queries or questions were answered. The baseline demographic data including blood pressure, height, and weight were recorded. The first test to be performed was EndoPAT and this was followed by the Badimon chamber. Prior to starting the chamber, patients had bloods taken for the VerifyNow, TEG and storage of the serum and plasma by the biochemistry laboratory for later date analysis. Participants were then given breakfast. Patients were then transferred to the MRI Centre for their cardiac MRI. Alternatively, some patients opted to have the scan on a separate day. After completion of all baseline tests, the study medication was given to the patient with strict instructions to take one capsule daily on an empty stomach 30mins before breakfast. A wallet sized card was also provided containing emergency contact details for both the principal investigator and myself.

Patients were seen for visits 2, 3, and 4 on a monthly basis during which their blood pressure was taken, ECG performed and changes in other medications or medication dose verified. Compliance to the IMP was also assessed with the number of capsules left being counted. Importantly, patients were asked about any adverse events with serious adverse events being reported to the trial manager within 24 hours (Figure 3-2). A blood test was taken to recheck the thyroid level as this determined whether patients needed a slight change in dose of levothyroxine if they were in the active group. The blood was taken to biochemistry department for analysis with the result being sent to the independent physician who confirmed the next dose on the Randomisation system, at the Newcastle Clinical Trials Unit. I was notified by email regarding confirmation that the next bottle was ready to be issued. A pharmacy script was generated from the Randomisation system to give to pharmacy who usually issued the bottle on the same day. This was then delivered to the patient. Visit 5 took place three months after visit 4 (6 months into the study). All patients were seen 52 weeks after taking the IMP (visit 6) with the baseline tests repeated (Figure *3-2*).

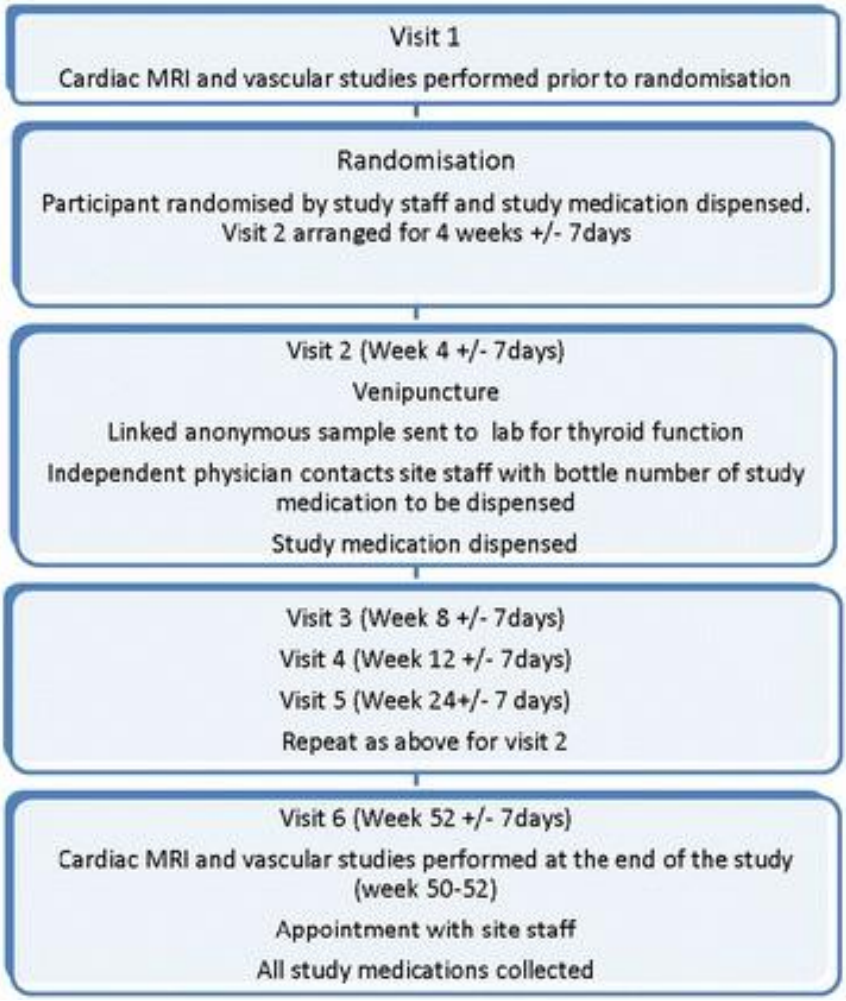


Figure 3-2 Flow chart representing the ThyrAMI 2 RCT study

3.1.7 Data collection and outcome assessments

The schedule of events for ThyrAMI-2 were as follow:

Visit 1A (7 – 10 days post AMI)

The following procedures and assessments and carried out at the screening visit:

- Written informed consent
- Physical examination: Height in cm (or participant reported if apparatus not available), Weight (kg), Blood pressure (mm/Hg), Pulse rate (beats/minute).
- Venipuncture: 20mls blood (TSH, Free T4, Free T3, TPO antibodies, Total cholesterol, HDL, Triglycerides).
- Clinical history: (Relevant medical history, Medication list)

- Questionnaires (in the order stipulated below). If patients refused to answer certain questions, their wishes were respected.
- SF-12: Participant completed
- Minnesota Living With Heart Failure Questionnaire
- CES-D questionnaire
- ECG (12 lead) and NYHA classification

Visit 1B: (day 0)

All recruited participants underwent the following at Newcastle Magnetic Resonance Centre, Newcastle University.

- Cardiac MR scanning.

Visit 1C: (day 0)

The following procedures and assessments were carried out at the baseline visit:

- Badimon chamber, TEG®, VerifyNow® and EndoPat® analyses at Clinical Research Facility, Royal Victoria Infirmary.

Visits 2 - 5: Follow-up (weeks 4 - 24 +/- 7 days)

The following procedures and assessments were carried out at visits 2 – 5 at either the local hospital or participant's home:

- Venepuncture: 5mls blood (TSH, Free T4, FT3).
- Clinical History (Adverse event, Serious adverse events, changes to concomitant medication)
- Safety assessments: ECG (rhythm only), NYHA classification assessment
- Study Medication:
    - o Assessed LT4 dose according to dose titration guidelines
    - o Prescribed study medication (prescribed by independent blinded physician)
    - o Dispensed study medication
    - o Assessed compliance.

Visit 6A: Follow-up (week 52 +/- 7 days)

All participants underwent the following at Newcastle Magnetic Resonance Centre, Newcastle University.

- Cardiac MR scanning

Visit 6B:

- Badimon chamber, TEG®, VerifyNow® and EndoPat® analyses at Clinical Research Facility, Royal Victoria Infirmary.

Visit 6C:

The following procedures and assessments were be carried out at the local hospital at this visit:

- Physical examination: Weight (kg), Blood pressure (mm/Hg), Pulse rate (beats/minute).
- Venepuncture: 20mls blood (TSH, Free T4, Free T3, Total cholesterol, HDL, Triglycerides)
- Questionnaires (in the order stipulated below).
  - o SF-12: Participant completed
  - o Minnesota Living With Heart Failure Questionnaire
  - o CES-D questionnaire
- ECG (12 lead) and NYHA classification
- Clinical History (Adverse event, Serious adverse events, changes to concomitant medication).
- Study Medication collected (see Section 17 for further details):
  - o Assessed compliance
  - o Assessed integrity of the blind
- At this visit the study participants were asked to contact their General Practitioner to discuss LT4 treatment or monitoring of thyroid status at regular intervals.

Visit 7: Telephone follow-up (week 53 +/- 7 days)

A telephone call was arranged by a member of the local study team to the participant to enquire about any serious adverse effects or to answer any queries that the participant had related to the study.

3.1.8 Investigational medicinal product

I. Intervention and control

This was a double-blinded RCT study in which levothyroxine and placebo were used. Levothyroxine is the active investigational medicinal product (IMP) and was used to achieve a target TSH of 0.4 to 2.5 mU/L. The placebo served as the control. Both the levothyroxine and placebo were prepared and appeared the same to ensure blinding in accordance with the specifications of the Medicine Health Regulatory Authority (MHRA).

The study drug was sourced, assembled and packaged by Newcastle Specials (Pharmacy Production Unit) at The Newcastle upon Tyne Hospitals NHS Foundation Trust, MIA (IMP) 17136. The LT4 used in the study was the generic Levothyroxine 25 mcg and 50 mcg tablets (Amdipharm Mercury Ltd). This was provided in blister packs.

The double-blind was achieved by de-blistering and over-encapsulation, using a capsule filler of Lactose BP. For doses that were multiples of 50 mcg, we over-encapsulated Levothyroxine 50 mcg tablets; for the remaining 25 mcg, 75 mcg and 125 mcg dose increments, we over-encapsulated Levothyroxine 25 mcg tablets. This ensured the capsule size was kept as small as possible for swallowing purposes. Capsules were re-packaged into an appropriate bottle container (polypropylene) and labelled.

The side effects of LT4 (25 mcg and 50 mcg) are well known and documented ('EMC Mercury Pharmaceuticals. http://www.medicines.org.uk/emc/medicine/27213. Accessed 08 Nov 2014.,' ; 'EMC Mercury Pharmaceuticals. http://www.medicines.org.uk/emc/medicine/22557. Accessed 08 Nov 2014,'). The total daily dose provided to each participant was between 25 mcg and 150 mcg.

II. Randomisation and Blinding

Participants were randomised using a computerised randomisation algorithm, stratified by type of MI (NSTEMI versus STEMI), in a 1:1 ratio to levothyroxine therapy or placebo (as container or bottle numbers), starting within 21 (+/− 7) days of the AMI (date of diagnosis or the date of admission to hospital, whichever was later). In addition, investigators were unaware of allocation and were blinded to treatment groupings. Randomisation was administered centrally via Newcastle Clinical Trials Unit using a secure password-protected web-based system.

Assignment to either the LT4 or placebo arm was blinded to the participant as well as study team (double-blind). Code break envelopes were available at the pharmacy at each site. Study medication was prescribed by the study team at each site using a trial-specific prescription (documenting the required IMP pack number), and dispensed according to local pharmacy practice. Independent study physicians, who were unblinded, adjusted study drug dose as per protocol. The relevant pharmacy held a corresponding list allowing pharmacy staff to correlate IMP pack number with relevant packaged IMP dose for any particular IMP bottle, thus maintaining the double-blind.

III. Administration of the study drug

Participants were randomised to either LT4 or placebo to be taken orally once daily. This was discussed with the patient at the time of consent and was made clear in the patient information sheet. Initial starting dose of LT4 was 25 mcg daily. To achieve the desired target TSH levels in the LT4-treated group (target TSH levels 0.4 to 2.5 mU/L), participants had their TSH levels checked every 4 weeks and concomitant dose of their LT4 altered by 25 mcg daily, if required. The IMP was provided as 5 week supplies of LT4 or matching placebo (dispensed separately at each visit), packaged into appropriate individual polypropylene bottles. The label on the bottle did not indicate details of the arm of the study to which the participant had been randomised.

Study medication was be prescribed by the PI or co-investigator at each site using a trial-specific prescription (documenting the required IMP pack number), and dispensed according to local pharmacy practice. Independent study physicians who were unblinded adjusted the study drug dose as per protocol. The relevant pharmacy held a corresponding list allowing the pharmacy staff to correlate the IMP pack number with

relevant packaged IMP dose for any particular IMP bottle, thus maintaining the double-blind.

Participants were informed of potential adverse reactions and advised to contact the relevant study team if required. A study-specific participant contact card was provided to each participant.

Once randomised, the participant began their study medication on Day 0.

At 4, 8, 12 and 24 weeks (+/− 7 days), serum TSH levels were checked and LT4 doses adjusted as follows (the next bottle of IMP was started at week 4, 8, 12, 24 +/− 7 days, respectively):

LT4 group:

TSH 0.4 to 2.5 mU/L: Continue LT4 at current dose once daily

TSH > 2.5 mU/L: LT4 increased by 25 mcg daily

TSH < 0.4 mU/L: LT4 reduced by 25 mcg, or if already on 25 mcg daily (the lowest possible dose), then recheck TFTs in 4 weeks. If TSH is ≥0.4 mU/L then continue on current dose. Otherwise, if TSH continues to be <0.4 mU/L on the repeat blood test, then the participant will be withdrawn from the study.

Placebo group:

Individuals in this group received a fresh bottle of IMP starting at 4, 8, 12, 24 +/− 7 days after blood tests to maintain the double blind.

LT4 treatment based on the above regimen continued for a total of approximately 52 weeks at which point the final set of study specific assessments were be made (with a further follow-up phone call at approximately 53 weeks). If a participant in the placebo arm had a TSH >10 mU/L then their TFTs were rechecked in 4 weeks. If, the TSH was ≤10 mU/L on the repeat test then the participant continued with the study and, if, the TSH continued to be >10 mU/L then the participant was withdrawn from the study. At the final visit, participants on each arm of the study stopped their IMP and were referred back to their GP to have their thyroid function checked in 6 to 12 weeks. The GP and the patient were encouraged to discuss whether there was a need to commence LT4 therapy in these individuals based on the clinical situation. The GP letter (sent once the participant was randomised) and the GP follow-up letter (sent after each participant

completed their IMP) outlined the suggestion to recheck TSH levels at 6 to 12 weeks for each participant once they have completed their IMP.

At the end of each visit, participants were asked to return any surplus study drug in the original packaging to the study team, who verified and documented compliance. All unused study medication and packaging was sent to the local pharmacy for documentation and destruction as per local policy (following appropriate reconciliation by the Trial Manager). Documentation of prescribing, dispensing and return of study medication was maintained for study records.

IV. The role of the independent clinical team

In double-blinded randomised controlled trials, participants and investigators are unaware of intervention assignments throughout the trial. In the ThyrAMI 2 RCT where dose adjustment of the active study drug took place, it was important that an independent study physician and at least two third party representatives, not directly involved within the trial, kept accurate documentation of each participant's randomisation arm, the dose of active study medication prescribed, and thyroid function test results during the trial.

For each randomised participant the independent study physician or third party representative created and stored a prescribing record which consisted of an excel spreadsheet for each participant detailing the centre number, the randomisation number, the trial arm to which the participant has been randomised, the thyroid function test result for each relevant study visit and the dose of medication prescribed according to TSH results for those on the interventional arm. The prescribing records were stored on a separate computer drive at Gateshead which was password protected with access restricted to only the independent study physician and third party representatives

Thyroid function tests (TFTs) were performed at 6 visits (visit 1a, 2, 3, 4, 5, 6c). The participant's blood sample for each study visit was sent to the Pathology Department in the local laboratory. After processing the sample, the Pathology Departments sent the results electronically to a generic e-mail address: thyrami@ghnt.nhs.uk. Access to this e-mail account was restricted to the independent study Physician and the Third Party representatives. The independent study physician or third party representative logged into the e-mail address at least once daily to assess participants' blood results.

At Visit 1a the randomisation system allocated a bottle number corresponding to the stocks of study drug held at the recruiting site. The dose of LT4 at this visit was 25mcgs once daily. The study staff at each site logged into the computer system and generated the site specific prescription for the study drug. The system automatically uploaded the required IMP pack number. The study staff printed out the prescription and the PI or delegated individual at site signed the prescription and took to the hospital Pharmacy for dispensation. At Visits 2,3,4,5 the independent study physician or third party representative adjusted the study drug dose if necessary. If the participant had been allocated to the levothyroxine arm, participants with a TSH 0.4 – 2.5 mU/L continued LT4 at their current dose once daily. With a TSH > 2.5mU/L, the LT4 dose was increased by 25mcgs once daily and if the TSH < 0.4 mU/L, the LT4 dose was reduced by 25mcgs once daily. The maximum dose of LT4 to be prescribed was 150 mcgs once daily.

At Visits 4 and 5 if a participant had a TSH level < 0.4 mU/L, and the LT4 dose prescribed was currently 25mcgs once daily then the TFTs were checked in 4 weeks and the independent study physician emailed the study staff at each individual site with a standard e-mail. If the TSH level subsequently raised to ≥0.4mU/L then LT4 25mcgs once daily was to be continued. If the repeat TSH level remained <0.4mU/L the independent study physician or third party representative informed the chief investigator and the principal investigator at the relevant study site with a standard e-mail. If a participant in the placebo arm has a TSH >10 mU/L then their TFTs were rechecked in 4 weeks and the independent study physician emailed the study staff at each individual site with a standard e-mail. If the TSH is ≤10 mU/L on the repeat test then the participant continued with the study and, if, the TSH continued to be >10 mU/L then the participant was withdrawn from the study. The independent study physician or third party representative informed the chief investigator and the principal investigator at the relevant study site with a standard e-mail.

The third party representative checked all blood results and allocation of study drug bottles and doses to the participant. In the absence of the independent physician, the third party representative performed the above functions. For the LT4 arm, if the third party representative had any queries in regard to the dose of LT4, advice from the CI, PI or research fellow were sought as long as blinding was maintained. Advice gathered was recorded in the file stored by the independent study physician.

3.1.9 Statistical methods

Left ventricular ejection fraction (LVEF) is considered to be the strongest predictor for adverse outcomes after an acute myocardial infarction (AMI). Previous randomised trials in patients with AMI and a significant improvement in ventricular ejection fraction demonstrated concurrent absolute differences in LVEF of 3 percentage points or more (Sharpe *et al.,* 1991; Doughty *et al.,* 2004). The ThyrAMI 2 study was designed with 90% power to detect an overall difference of 3% in LVEF between the two groups (3% improvement in the placebo group and 6% in the LT4 group) 12 months after AMI, at a two-sided significance level of 5%. Using ANCOVA (differences in change in EF from baseline between placebo and LT4 taking into account variability in the baseline with correlation between baseline and follow-up of 0.75), we calculated that 47 patients would need to be enrolled in each group, allowing for a 10% drop-out.

Statistical analyses: For baseline characteristics, categorical data are expressed as numbers and percentages and compared using the Chi squared test. Continuous variables are expressed as mean±SD and were compared using unpaired Student's t test or ANOVA where necessary. Non-parametric continuous variables are expressed as median with their interquartile ranges (IQR) and were logarithmically transformed prior to analysis. Multiple regression was used in each results chapter to assess variables that predicted each dependent variable. Linear relationship between the dependent variable and other continuous predictor variables was assessed using scatter plots using sequential analysis of variance. Firstly, for each analysis, the relationship of all the main independent variables was checked with the dependent variable to see if there was a linear relationship prior to performing multiple regression. Multivariate regression analysis was then performed. Residuals were evaluated by Q-Q plots to ensure that they were normally distributed. Multicollinearity was assessed by variance inflation factor and values above 2.5 were deemed indicative of multicollinearity.

Predictors of thyroid dysfunction were assessed using multivariable binary logistic regression analysis. Missing data was dealt with by using multiple imputation method. Ten imputed datasets were created and pooled results were summarised. Both troponin T and I levels are reflective of the severity of myocardial damage and are used to diagnose AMI, but their absolute values differ. Therefore, we first standardised, centred and then combined the two values to form a single hs-standardised Troponin (st Troponin) variable. This combined single variable was utilised in all analyses.

The relationship between thyroid parameters and all-cause mortality was evaluated using Cox proportional hazards multivariable analysis. Survival times were calculated from the date of the AMI till the date of death or date of being known to be alive. A p value of <0.05 was deemed as being indicative of statistical significance in all analyses. Analyses were performed using the statistical software package SPSS v22 (Ill, Chic, USA).

3.1.10 Data handling and record keeping

Medical information obtained at each visit was recorded in the subject's medical notes or other source documentation in real time. Data was collected and entered into a secure, validated clinical data management system (MACRO), by an authorised member of the research study team. Data for each visit was entered by relevant local staff at each site. The clinical data management system was web based; allowing access to authorised staff via password protection. Data was handled, computerised and stored in accordance with the Data Protection Act 1998. No participant identifiable data will leave the study site (CRFs identified participants by initials, date of birth and unique patient number only). Strict confidentiality was ensured while dealing with patient-sensitive data in accordance with the Caldicott Guardian's recommendations (applications will be made to the relevant Caldicott Guardian for use of NHS patient data).

All study data was held in strict confidence by the investigators/research team. Data and documents were be stored in locked cupboards. A confidential list of trial identifiers and corresponding patient identifying details were held at site in a locked cupboard by the Principal Investigator. The quality and retention of study data was the responsibility of the CI. All study data retained in accordance with the latest Directive on GCP (2005/28/EC) and local policy.

3.1.11 Compliance and withdrawal

i. **Assessment of compliance**

Compliance with study medication (IMP) was assessed and documented by the research team by checking and recording the number of returned capsules at each visit. This allowed any issues to be addressed immediately with the participant. Compliance was classed to be good if between 80 to 100%. The local pharmacy documented all unused study medication/packaging prior to appropriate reconciliation by the Trial Manager.

ii. **Withdrawal of participants**

Participants had the right to withdraw from the study at any time for any reason without giving a reason. The investigator also had the right to withdraw patients from the study drug in the event of inter-current illness, adverse events, and serious adverse events, suspected unexpected serious adverse reactions, protocol violations, administrative reasons or other reasons.

A participant who decided to withdraw from the study, their wishes were respected. These participants were asked to complete a "confirmation of withdrawal" form to document their decision. However, for participants that withdrew due to reasons other than related to tolerance of MR scanning, they were offered the chance to have the primary outcome assessment (cardiac MR imaging) performed at the time of withdrawal. Participants who had serum TSH <0.4 or >10.0 mU/L on two separate occasions at least 4 weeks apart whilst on study drug were withdrawn from the study due to safety reasons.

3.2 Measuring left ventricular volumes and ejection fraction by cardiac magnetic resonance imaging

3.2.1 Methods

All CMR examinations were performed on a 3 Tesla MR scanner (Philips) at the Magnetic Resonance Centre at Newcastle University. Participants were scanned supine using a 6-channel cardiac coil and ECG gating (Philips Achieva). Balanced steady-state free precession images were obtained covering the entire left ventricle (field of view (FOV) 350 x 314mm^2, repetition time/echo time (TR/TE) = 3.9/1.9ms, turbo factor 17, flip angle (FA) 40^o, slice thickness 7mm, 25 phases, resolution 1.37mm). After piloting using localisation, cine images were acquired in the long-axis and short-axis views during breath holding in end-expiration. The images were acquired during breath holding to avoid image artefact whereas ECG gating was important to ensure image acquisition at the start of the QRS complex which marked ventricular contraction. The end result is a series of short axis views across the left ventricle during each cardiac cycle from diastole (relaxation) to systole (contraction).

3.2.2 Image analysis

CMR image analysis was performed using the IntelliSpace software version 6.0 (Philips Achieva). The Cine images open with the short axis images on the left and the long axis images on the right as seen in the image below (Figure *3-3*). The range of LV segmentation for analysis is defined by indicating the valve and apical slice on the short axis using the long axis as a guide.

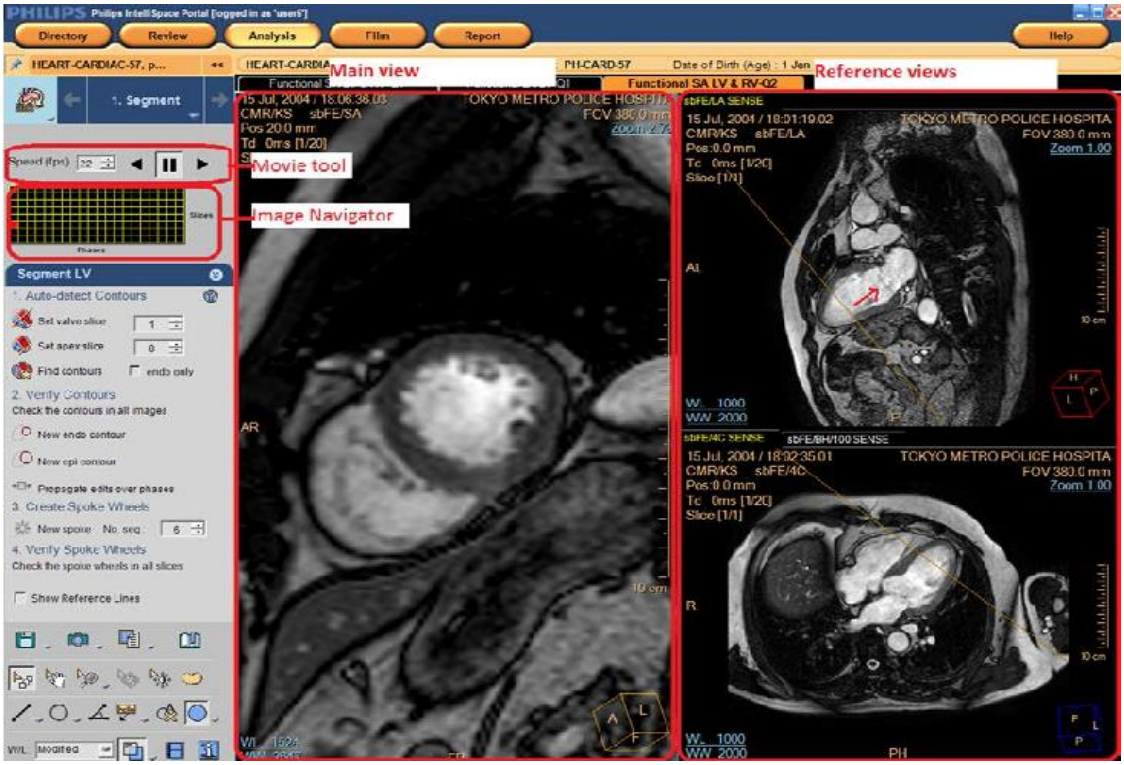


Figure 3-3 Long-axis and short-axis views of the left ventricle

Long-axis views are shown on the right whereas short-axis views on the left. Moving up and down the slices of the ventricle in the long-axis gives a corresponding short axis view during the cardiac cycle from diastole to systole. The apical slice is the last slice furthest from the valves whereas the basal slice is slice of the ventricle closest to the valves.

Manual tracing of the endocardial and epicardial contours of the short-axis slices at end-diastole and end-systole was performed across each slice which covered the left ventricle (Figure *3-4*). The basal slice was selected for end-diastole and end-systole when at least 50% of the blood pool was surrounded by myocardium whereas the apical slice was defined as the final slice in the apex showing blood volume. This method has been approved by the Society for Cardiovascular Magnetic Resonance Board of Trustees Task Force (Schulz-Menger *et al.*, 2013).

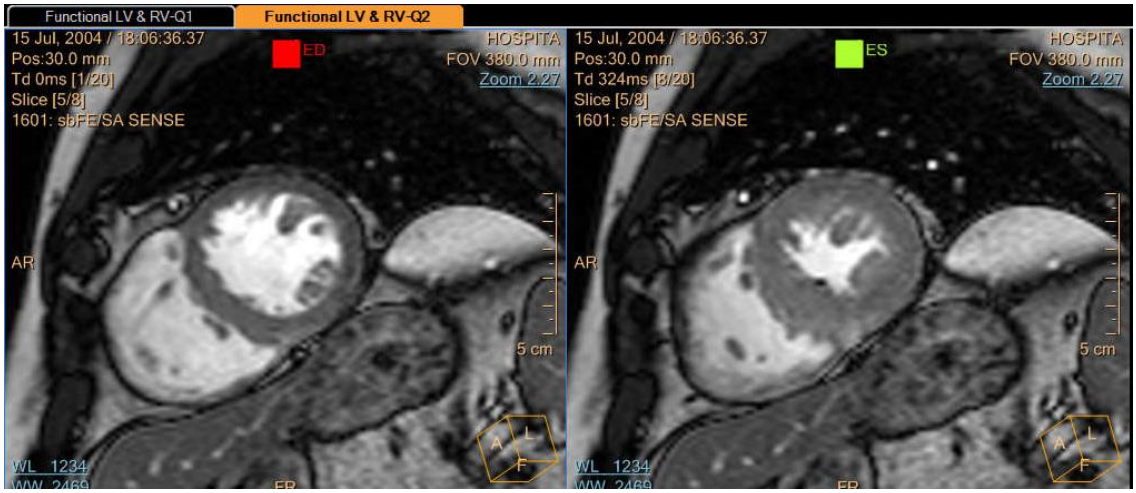


Figure 3-4 Contours in diastole and systole of a single slice of the left ventricle

The image on the left shows a short-axis view in end-diastole representing the maximum volume during that slice of the cardiac cycle whilst the heart relaxes whereas the right image shows a short-axis view in end-systole representing the minimum volume whilst the heart contracts.

Tracing of the endocardial and epicardial contours of the short-axis views at end-diastole and end-systole across each slice covers the left ventricle and enables the relevant results to be obtained:

- **End diastolic phase** (ED): The phase at the beginning of a heartbeat where the heart is 'at rest', i.e. where the blood volume is at a maximum.
- **End systolic phase** (ES): The phase where the heart is fully contracted, i.e. where the blood volume is at a minimum.
- **End diastolic volume (VED)**: The amount of blood in the heart at the end diastolic phase; expressed in milliliters (ml).
- **End systolic volume (VES)**: The amount of blood in the heart at the end systolic phase; in ml.
- **Stroke volume (SV)**: The amount of blood pumped out per heartbeat, i.e. the difference between the blood volume at the end diastolic phase and the end systolic phase; in ml.

SV = VED - VES

- **Stroke index (SI)**: The stroke volume relative to the body surface area; in ml/beat/m2.

SI = SV/BSA

- **Body Surface Area (BSA)**: The estimated (not measured) area of the patient's body surface; in m2. For adults, the approximate value of BSA can be calculated using Mosteller's formula: BSA = √ (Height [cm] x Weight [kg]) / 3600

- **Ejection fraction (EF)**: The amount of blood pumped out per heartbeat relative to the blood volume at the end diastolic phase in percentages.

EF = (SV / VED) × 100

- **Cardiac output (CO)**: The amount of blood that is pumped out per minute; in liter (l). The heart rate is in beats per minute (bpm). CO = (SV × HeartRate) / 1000

3.2.3 Volume over time curve and diastolic function

The final aspect of the LV function analysis involves looking at the volume over time curve of the left ventricle. This shows the changes in the volume within the left ventricle over a period of time during which the heart contracts and relaxes during one cycle. This is not only important for showing systolic function, which is the ability of the ventricle to contract, but also demonstrates diastolic function which the ability of the ventricle to relax and fill during relaxation.

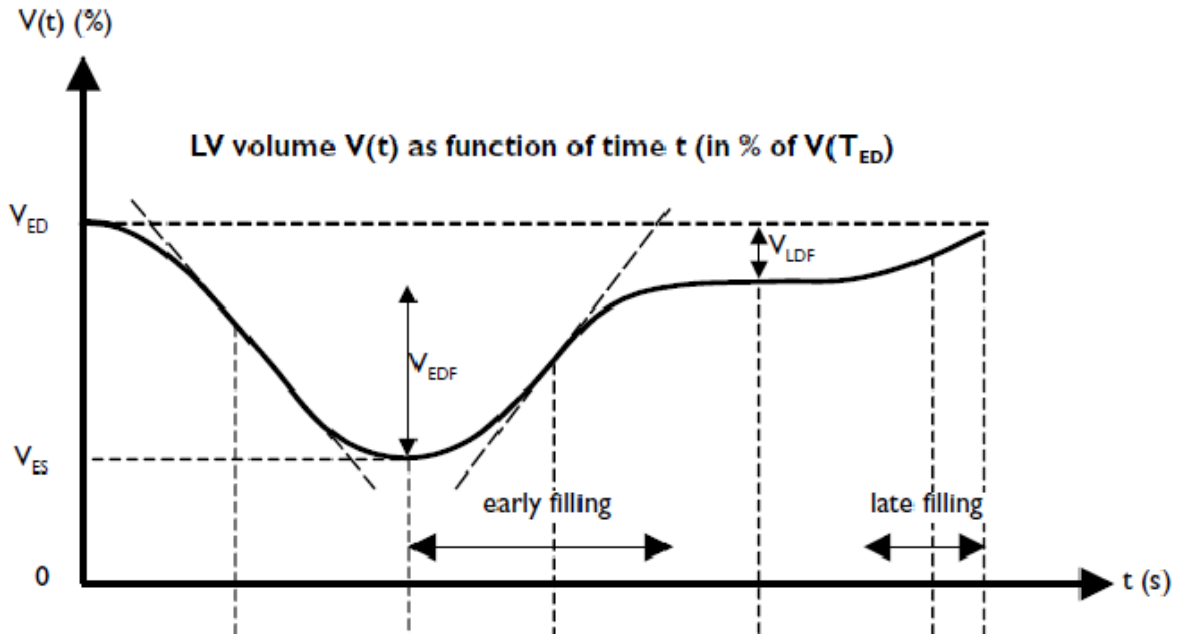


Figure 3-5 Volume over time graph within the left ventricle

The graph represents one cardiac cycle during systole and diastole. The downward slope represents systole whereas the upward curve represents diastole when the heart relaxes and fills. The volume over time curve enables early filling and late filling to be calculated which represents diastolic function.

3.2.4 Quality control for cardiac MRI

Prior to performing cardiac MRI analysis, inter- and intra-observer variability was assessed for LVEF which was the primary end point for the study. The LVEF is derived from the end-diastolic volumes (EDV) and end-systolic volumes (ESV). For inter-observer variability, two researchers analysed 19 patient scans on separate days over a one-month period. As shown in Table *3-1*, the coefficient of variation for inter-observer variability was 2.62% for ejection fraction. For intra-observer variability (Table *3-2*), one researcher repeated the analysis for 18 patient-scans giving a coefficient of variation of 2.20% for ejection fraction, which is within the accepted limits (Grothues *et al.*, 2002).

	Researcher 1			Researcher 2			
Patient	EDV (mls)	ESV (mls)	EF (%)	EDV (mls)	ESV (mls)	EF (%)	Mean difference in EF
1	256.9	183.7	29	256.7	188	27	-2
2	226.7	154.9	32	225.5	155.5	31	-1
3	111.7	62.5	44	110.3	61.7	44	0
4	136.6	60.8	56	133.4	57.9	57	1
5	137.4	53.8	61	136.2	52.6	61	0
6	144.1	71.5	50	146.2	73	50	0
7	185.7	97.9	47	183.2	96.9	47	0
8	162.7	69.3	57	158.3	69.2	56	-1
9	114.8	38.7	67	118.8	36.3	69	2
10	93.8	27.2	71	95.1	26.5	72	1
11	163.2	76.1	53	164.2	72.9	56	3
12	151.5	59.3	61	138.9	40.4	71	10
13	104.1	36.8	65	104.8	32.8	69	4
14	155.2	69.5	58	148.8	62.9	58	0
15	128.6	66	51	130.2	65.6	50	-1
16	119.2	60.8	49	116.7	58.2	50	1
17	138	56.7	59	133	54.4	59	0
18	118.8	56.2	53	122.3	54.4	55	2
19	139.7	62.1	56	149	65.9	56	0

Average mean difference	1.0
Standard deviation	2.62
Coefficient of variation	2.62

Table 3-1 Table showing inter-observer variability for LV function analysis with a coefficient of variation of 2.62 which is well within the recommended guidelines for reproducibility.

	Researcher 1-1			Researcher 1-2			
Patient	EDV (mls)	ESV (mls)	EF (%)	EDV (mls)	ESV (mls)	EF (%)	Mean difference in EF
1	185.7	97.9	47	186.2	98	47	0
2	162.7	69.3	57	163.5	69	58	0
3	114.8	38.7	67	115.5	40.2	65	-2
4	93.8	27.2	71	95.4	29.9	69	-3
5	163.2	76.1	53	164	77.2	53	-1
6	151.5	59.3	61	152.1	60.4	60	-1
7	104.1	36.8	65	100.9	36.2	64	1
8	155.2	69.5	58	154.2	65.2	55	4
9	128.6	66	51	126.8	61.6	51	4
10	111	38.2	66	110.9	36.9	67	1
11	156.3	62.6	60	153.3	63.2	59	-1
12	185.9	108.2	42	183.5	105.7	42	3
13	115.7	47.5	59	116	46.8	60	1
14	152	69	54	154.4	70.8	54	-1
15	119.2	60.8	49	115.9	59.7	49	1
16	138	56.7	60	137.1	55	59	2
17	118.8	56	53	117.1	54	53	2
18	139.7	62	57	143.7	62.7	56	-1

Average mean difference	0.5
Standard deviation	1.10
Coefficient of variation	2.20

Table 3-2 Table showing intra-observer variability with a coefficient of variation of 2.20 which is well within the recommended guidelines for reproducibility.

3.3 Late gadolinium enhancement (LGE) using cardiac MRI

3.3.1 Methods

Following completion of acquiring the Cine images, 0.2 mmol/kg of gadolinium was administered intravenously for LGE assessment. LGE images were obtained ten minutes after injecting to allow gadolinium to enter and leave the extracellular space within the myocardium. The images were obtained during breath holding with a cardiac triggered 3 dimensional phase sensitive inversion recovery sequence (multi shot gradient echo TR/TE = 5/2.4, FA = 15°/5°, acceleration factor 31, parallel imaging factor 2, 1.8mm resolution zero filled to 1.3mm). LGE was only administered in patients with normal or mildly impaired renal function with an estimated glomerular filtration rate above 60.

3.3.2 Myocardial segmentation and analysis

The American Heart Association 17 segment model is the standardised way of the dividing the myocardium into 17 segments in the short axis to establish the number of segments possessing LGE (Cerqueira *et al.*, 2002). As shown in Figure *3-6*, the basal, mid-cavity and apical short axis segments are derived from the location on the long axis of the left ventricle. Figure *1-9* demonstrated the detection of myocardial scar on CMR. For the analysis, both Tim Hodgson (senior cardiac radiographer) and myself analysed all the scans together and agreed on what segments had LGE. The Phillips Intellispace software was then used to quantify LGE using the 'n'-SD technique in which the endocardial and epicardial borders for the myocardial region of intetest (ROI) were outlined and then a normal 'remote' (dark) segment ROI within the myocardium was selected and used to define the reference signal intensity (mean and standard deviation, SD) (Figure *1-9*). Semi-automated thresholding to n+5SD was used as a threshold between normal myocardium and LGE to quantify infarct size. This method has been approved by the Society for Cardiovascular Magnetic Resonance Board of Trustees Task Force (Schulz-Menger *et al.*, 2013).

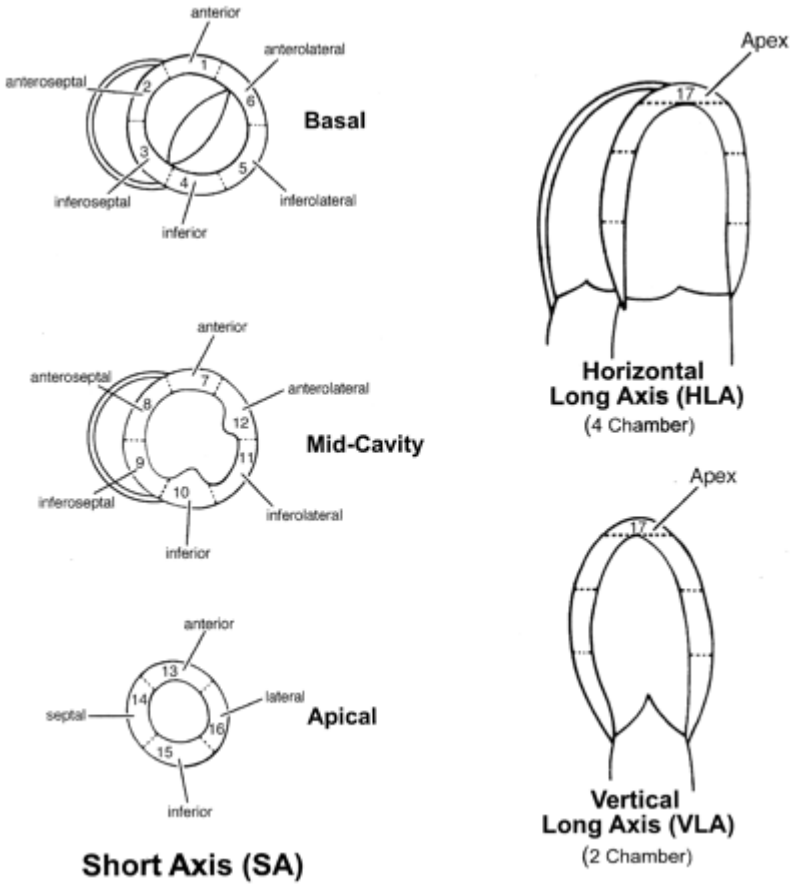


Figure 3-6 17 segment representation of the LV cavity

The diagram shows the basal, mid-cavity and apical segments in the short axis corresponding to the respective long axis view. The number of segments showing LGE were verified between 2 analysts who respectively analysed the short axis views in detail.

3.4 Badimon Chamber to assess thrombus burden

3.4.1 Principles of the Badimon chamber

The Badimon chamber is an *ex vivo* model of thrombosis which assesses thrombus formation on a substrate which has undergone deep arterial injury. The chamber has previously been used in studies to assess the effect of anti-thrombotic and antiplatelet agents on thrombus formation (Balasubramaniam *et al.*, 2014; Viswanathan *et al.*, 2014b). The main advantage of using the chamber is that it mimics physiological blood flow conditions of the coronary artery and has been shown to be highly reproducible when assessed within and between days reducing inter- and intra-observer variability (Lucking *et al.*, 2010). The chamber has previously been used in our laboratory and has demonstrated higher thrombus burden in NSTEMI patients with SCH compared to those with euthyroidism (Viswanathan *et al.*, 2014a).

The generation of thrombus in the chamber depends on two principles:

1) A substrate which is highly thrombogenic, similar to a diseased coronary artery to which platelets are likely to bind.
2) Shear stress which induces platelet activation

Freshly slaughtered pig aortas were the source of substrate in the Badimon chamber. The endothelium was surgically removed to expose the tunica medium, which is rich in collagen, and this permitted platelet aggregation in a manner similar to a diseased coronary vessel which undergoes changes prior to a MI. Platelet activation occurs under high shear stress at sites where there are narrowing within the blood vessel secondary to atheromatous plaques. This is due to the normal laminar blood flow being disrupted at narrowings which encourages blood flow under high shear stress. Therefore, reducing the luminal diameter within the chamber increases the shear stress causing a subsequent increase in the velocity of blood flow and subsequent thrombus formation.

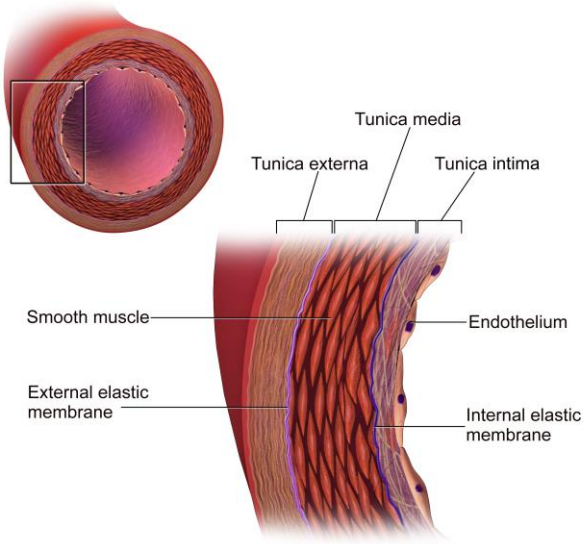


Figure 3-7 A cross sectional image of an aorta segment.

Removing the endothelium and tunica intima exposes the underlying media which is highly thrombogenic and rich in collagen

3.4.2 Set up the Badimon chamber

The Badimon chamber consists of:

1) one low shear stress plexi glass chamber
2) two high shear stress plexi glass chambers
3) three plexi glass over chambers with a screw in which each plexi glass chamber is placed and secured
4) four plastic connectors
5) a plexi glass container into which all the plexi glass over chambers are placed in line with each other

Each of three plexi glass chambers consist of an upper lid unit and a lower core unit which are secured together with the substrate in between (Figure 3-8). The substrate is held in place by the pressure of the upper lid onto the lower core unit. The lower core unit has cylindrical channels which have a dimension of 1mm*25mm in the two high shear chambers and 2mm*25mm in the low shear stress chamber. The purpose of these channels is to allow direct contact of the substrate with the flowing blood. The over chamber with a screw holds together the upper lid and lower core unit securely with substrate in between. All three chambers are connected together by plastic connectors which connect to the channels in the lower core unit.

In the study the 3 chambers were arranged in the following order: low shear chamber → first high shear chamber →second high shear chamber. The rheological conditions in the first chamber (low shear) simulate those of patent coronary arteries whereas the second and third chambers (high shear) simulate a stenosed coronary artery. The whole assembly was kept aligned in a plexiglass container which was in a water bath at a temperature of 37 degree Celsius.

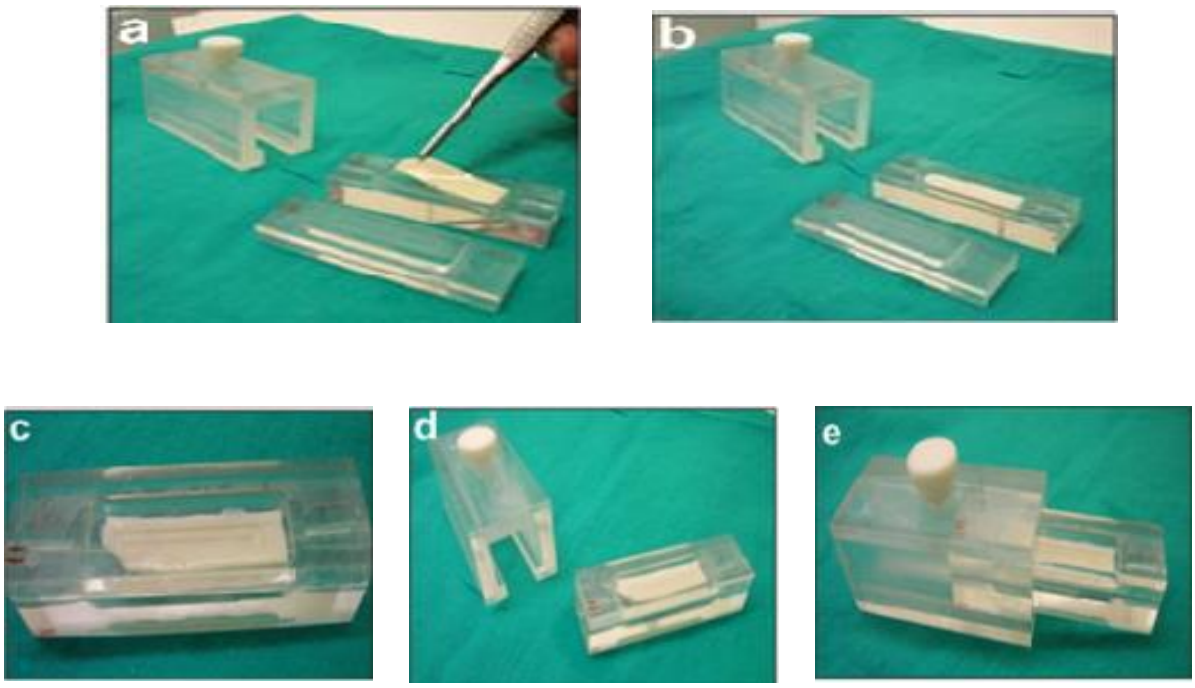


Figure 3-8 Set up of the plexi glass chambers.

Each chamber consists of a lower core unit into which the aortic substrate is placed (A). The upper lid is placed on top of the lower core unit with the substrate in between (B and C). The unit is then placed in an over chamber with a screw which ensures that the units are held securely. There are three chambers in total (2 high shear stress and 1 low shear stress).

Aortas from freshly slaughtered pigs were obtained from a local abattoir. Each aorta was surgically dissected into numerous segments measuring 15mm*35mm. The thin endothelial layer was stripped off to expose the underlying tunica media, which was rich in collagen, and excess tunica adventitia was also removed to ensure fitting into the chamber. The aortas were thoroughly inspected with a magnification glass to ensure there was no damage to the tunica media as well as to remove any dissection flaps which could predispose to excess to thrombus formation. They were then stored in a 0.01M phosphate buffer solution at minus 80 degree Celsius. One day before the chamber study, the aorta segments were placed into a fridge at 5

degress Celcius to enable thawing. The segments were once again examined for any damage or flaps and only aortas without dissection flaps were used for each chamber study.

The chamber was kept at a temperature of 37 degrees Celcius to mimic body temperature. A peristaltic pump was used to draw blood from a large vein in the antecubital fossa via tygon tubing through the three perfusion chambers (Figure 3-9). 14.0 tygon tubing (Cole Palmer, IL, USA) was used to connect the distal end of the chamber to a peristaltic pump (Masterflex, model 7013) which ensured the flow was 10ml/min. Tygon tubing was used to connect the proximal end of the chamber to one port of a 3 way tap (Alaris, MFX2280E, Cardinal Health Inc, Rolle, Switzerland) which was subsequently connected to a cannula in the cubital fossa of the subject's right arm via further tubing. The final port of the 3-way tap was used to flush and prime the system 0.01M buffer solution prior to starting the experiment to ensure there was a continuous flow within the system without air bubbles. The venous blood was then allowed to flow into the system for 5 minutes after which further buffer solution was again passed through the chamber for 30 seconds to remove blood which had not attached to the substrate.

It was important that the connectors of the Badimon chamber were non-thrombogenic and sustained the expected shear rates. Tygon tubing which satisfied these conditions was used.
0.01M phosphate buffer solution (PBS) was prepared from PBS in concentrate powder form (Cole-Palmer inc, MA, USA). Deiodinased water was used to reconstitute the powder to produce fresh 0.01M solution.

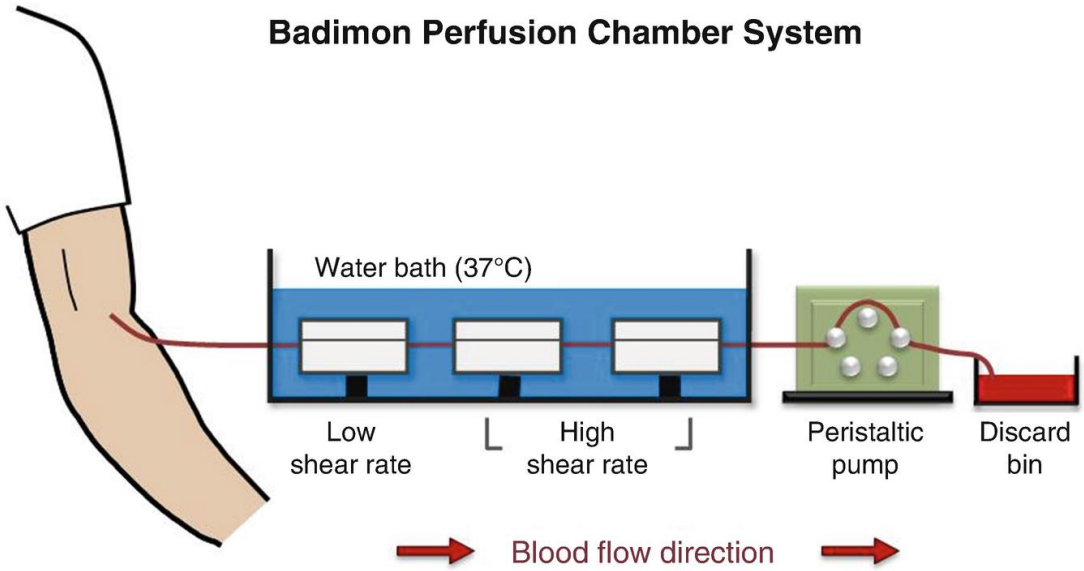


Figure 3-9 Setup and principles of the Badimon chamber.

Blood from the antecubital fossa flows through the chamber at 10mls/min due to the peristaltic pump drawing blood. The chamber was kept at temperature of 37 degrees Celsius. The blood flowed through the chamber via the tygon tubing. Upon entering the low shear unit the blood came into direct contact with the overlying aortic substrate and then continuously flowed into the subsequent 2 high shear units before being discarded.

3.4.3 Histopathology methods

Aorta tissue from each chamber was carefully removed from the chamber unit and then placed in 10% formalin solution for preservation. After 72 hours of fixation in formalin, the three aorta segments were removed (one low shear and two high shears) and each segment was sectioned into eight equal pieces with a width of 1mm with the central cross-sectional area of each aorta piece representing the area over which the thrombus had formed. All the pieces were placed in a total of six histocassettes and labelled as follows:

1) Low shear unit: A1,A2,A3,A4 B1,B2,B3,B4
2) High shear unit: C1,C2,C3,C4 D1,D2,D3,D4
3) High shear unit: E1,E2,E3,E4 F1,F2,F3,F4

The samples were then embedded in liquefied wax and each aorta piece was then sectioned to 0.4 µm width sections using micotome. Post sectioning each sample was stained using modified Masson trichrome stain and then placed in a glass slide. These procedures were carried out in the Cellular Pathology department at the Royal Victoria Infirmary.

The Masson trichrome staining of the aorta segments enables differentiation of the thrombus from the tunica media over which it has formed. Post staining the thrombus with fibrin appears red, the elastin of the aorta segment appears green the smooth muscle within the media appears pink. This modification has been validated by the Thrombosis Research Unit, Mount Sinai Hospital, New York (Osende *et al.*, 2001).

3.4.4 Image acquisition

The slides were viewed using a high precision light microscope (Leica DM 2000, Leica Microsystems, Wetzler, Germany). The slides were placed on the microscope lens and the thrombus images were magnified X10 with the red visible thrombus on top of the tunica media which was pink (Figure 3-10). A digital camera with a high resolution (KY-F1030, JVC, Japan) was used to capture the images electronically. The microscope was calibrated on a regular basis and was cleaned, inspected and serviced by a technician once a year.

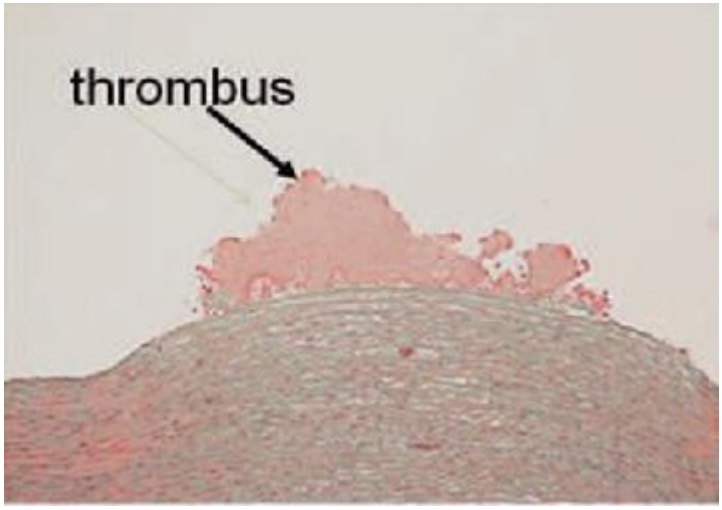


Figure 3-10 Visualisation of thrombus when placing each slide under a microscope.

Magnification X10 shows the thrombus in red having formed over the tunica media outlined in pink. For each chamber study there were 24 such images to analyse which represented the 24 segments of aorta (8*low shear and 16*high shear).

3.4.5 Measurement of thrombus area

Each thrombus image was opened in jpeg format and analysed using the software Image-Pro Plus Version 4 (Media Cybernetics Inc, MD, USA). The software enabled the brightness and contrast of each image to be standardised enabling clearer visualisation of the thrombus. A cursor was used to trace carefully around the thrombus and at the border of the thrombus and tunica media where extra care was taken to ensure that no portion of the tunica media was included as this would lead to overestimation in thrombus area. The thrombus area was calculated by the software's differentiation of dark objects within the cursor border which was recognised as developed thrombus. The thrombus area was attained for each of the 24 segments of aorta (8*low shear and 16*high shear) with the measurement expressed as μ^2 per millimetre aorta surface area (μ^2/mm). This enabled a mean thrombus area to be attained for both low shear and high shear aorta segments

There were sections which were unsuitable for analysis. Such reasons included:

a) Excess dye used whilst preparing the slides
b) Sections which were damaged whilst preparing the samples as this would led to underestimation
c) Sections with flaps as this would lead to sluggish flow and excess thrombus formation
d) Sections with minimal thrombus at less than 500 μ^2/mm as this was indicative of wash artefact.

The images were checked by two observers which included an experienced chamber researcher, who had significant experience in using the Badimon chamber, and myself. Sections of aorta unsuitable for analysis were excluded.

3.4.6 Quality control

For the Badimon chamber, the performance of the circuit was checked with 0.01M phosphate buffer solution prior to starting each patient test and involved priming with the solution at 10ml/min (standard laminar flow) and at 150ml/min. Secondly, the chamber was performed on 2 individuals on 3 separate occasions one month apart in exactly the same conditions to the study and the mean thrombus area μ2/mm for subject 1 was 7543, 7928 and 7399 whereas for subject 2 the thrombus area was 6099, 6781 and 6542. These were not statistically different. For analysis of thrombus area, intra-observer variability was performed on fifteen slides, two

weeks apart, giving a coefficient of variation of 4.1%. The same slides were analysed by an experienced chamber researcher giving an inter-observer coefficient of variation of 4.5%.

3.5 Thromboelastography (TEG) and platelet mapping in assessing visco-elastography

3.5.1 Background and technique

TEG is a viscoelastic haemostatic assay which measures the viscoelastic properties of clot formation within the blood. Current laboratory tests used in hospitals have limitations by measuring individual components of the haemostasis process which does not represent how such components interact in clot formation. Therefore, such tests do not necessarily indicate how well haemostasis is functioning. TEG measures the rate and strength of clot formation induced by thrombin which activates platelets and the coagulation cascade. By assessing thrombus formation continuously over a period of time, TEG reflects whole blood thrombosis by showing the interaction between platelets leading to platelet aggregation; and the interaction of platelets with fibrin and the coagulation cascade. This involves measuring each cycle of clot formation and the susceptibility of the clot to autolyse. Therefore, the rate of clot formation is determined by platelet function, fibrinogen levels, the coagulation system, fibrinolysis and medications which inhibit these systems. Measuring the process of clot formation and auto lysis enables the researcher to understand both the thrombotic and bleeding tendency and this can guide adequate blood product replacement if needed such as during and after cardiopulmonary bypass surgery. This has been shown to decrease transfusion rates during surgery (Shore-Lesserson *et al.*, 1999). TEG can also be used acutely to measure the inhibitory effect of aspirin and P2Y12 inhibitors on platelet aggregation to help decide timing for surgery (Mahla *et al.*, 2012).

TEG is a point of care test in which a result can be attained within 30 minutes after taking blood from a patient. The measurement of the viscoelastic properties is via a pin suspended in a cup (heated to 37 degrees Celsius) from a torsion wire which is connected to an electrical transducer. The blood is placed within the cup and the motion of the cup does not affect the pin initially as the blood is unclotted allowing free movement (Figure 3-11). The cup rotates in an oscillating manner every 10 seconds at 4.45 degrees. As clot starts forming, the rate of cup movement decreases around the pin with the rate of movement being inversely proportional to clot formation. The interlinking of clotting factors, fibrin and platelets form a strong

connection between the cup and pin and this energy is measured by the torsion wire as kinetic energy which is a measure of viscoelastic clot strength over time. This energy is converted to an electric signal which is displayed as a graphical and numerical output (Figure 3-11). Therefore, TEG measures the rate of thrombus formation and the maximum strength of the clot by giving a measurement of maximum amplitude (MA) on the graph. TEG also evaluates clot lysis by assessing the decrease in amplitude from the MA over a period of time. In addition to TEG, another viscoelastic analyser is rotational thromboelastometry (ROTEM). The main difference with ROTEM is that the cup is immobile whereas the pin slowly oscillates. No randomised controlled trial has compared both TEG and ROTEM. The use of both analysers is dependent on location with ROTEM being used more in Europe and TEG in North America.

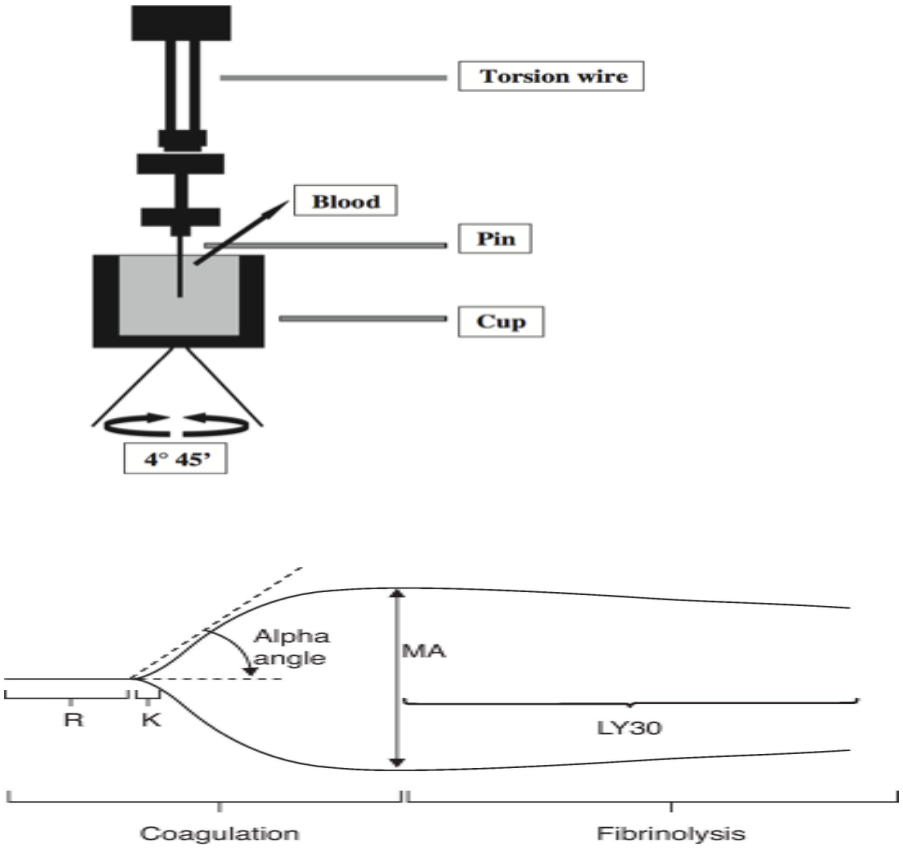


Figure 3-11 Thromboelastography.

The top diagram shows the basic principle of TEG with the pin extending from a torsion wire which is connected to a transducer. As the cup oscillates, changes in the rate of cup movement is dependent on blood viscosity and this is transmitted as kinetic energy giving a corresponding trace as demonstrated in the lower image. Therefore, the TEG tracing represents clot kinetics.

3.5.2 Parameters derived from TEG graph

R time, in min - reaction time; time taken from placing blood in the cup till initial fibrin formation. This is the time from beginning of the TEG trace when the test starts till initial fibrin formation with an amplitude of 2 mm. It is dependent on clotting factors of the initial enzymatic cascade which start the process of thrombus formation. This value will be shortened if the blood is hypercoagulable due to excess clotting factors and prolonged in a coagulopathy. Normal time is 2-8 mins.

K time, in min - kinetics; time taken to reach a fixed level of clot strength i.e 20 mm. This is the time for the thrombus to become firm and is dependent on the conversion of fibrin to fibrinogen. Normal time is 1-3 mins.

α-angle - the angle of the slope between R and K when a clot strength of 20 mm is reached. It measures the rate of fibrin build up and the cross linking with platelets and therefore represents the speed of thrombus formation. Normal angle is 55-78.

MA, in mm - maximum amplitude; measures the maximum distance between the two arms of the graph. This is the maximum viscoelastic strength of the thrombus and is dependent on fibrin and platelet interaction. This is the most consistent measurement among all the TEG measurements and can be used to study the effect of different anti platelets on thrombus formation. Normal time is 51-69 mins.

Clot index - dimensionless parameter which takes into account each of the 4 stages of thrombus formation in TEG (R, K, α-angle and MA) into one equation CI = - (0.1227)R + (0.0092)K + (0.1655)MA - (0.041)α - 5.0220. It is an overall measure of thrombus strength and measures the combined effect of fibrinogen and platelets on thrombus. Normal value is -3 to 3.

LY30 - the percentage decrease in amplitude 30 mins after reaching MA. This gives a measure of early fibrinolysis during clot breakdown. Normal value is 0-8.

LY60 - the percentage decrease in amplitude 60 mins after reaching MA. This gives a measure of late fibrinolysis during clot breakdown. Normal value is 0-15.

L-parameter, mm/min – the thrombus lysis parameter which measures the average reduction in amplitude per unit of time. This measures the changes in the viscoelastic property of the thrombus due to fibrinolysis. Normal value 30-60.

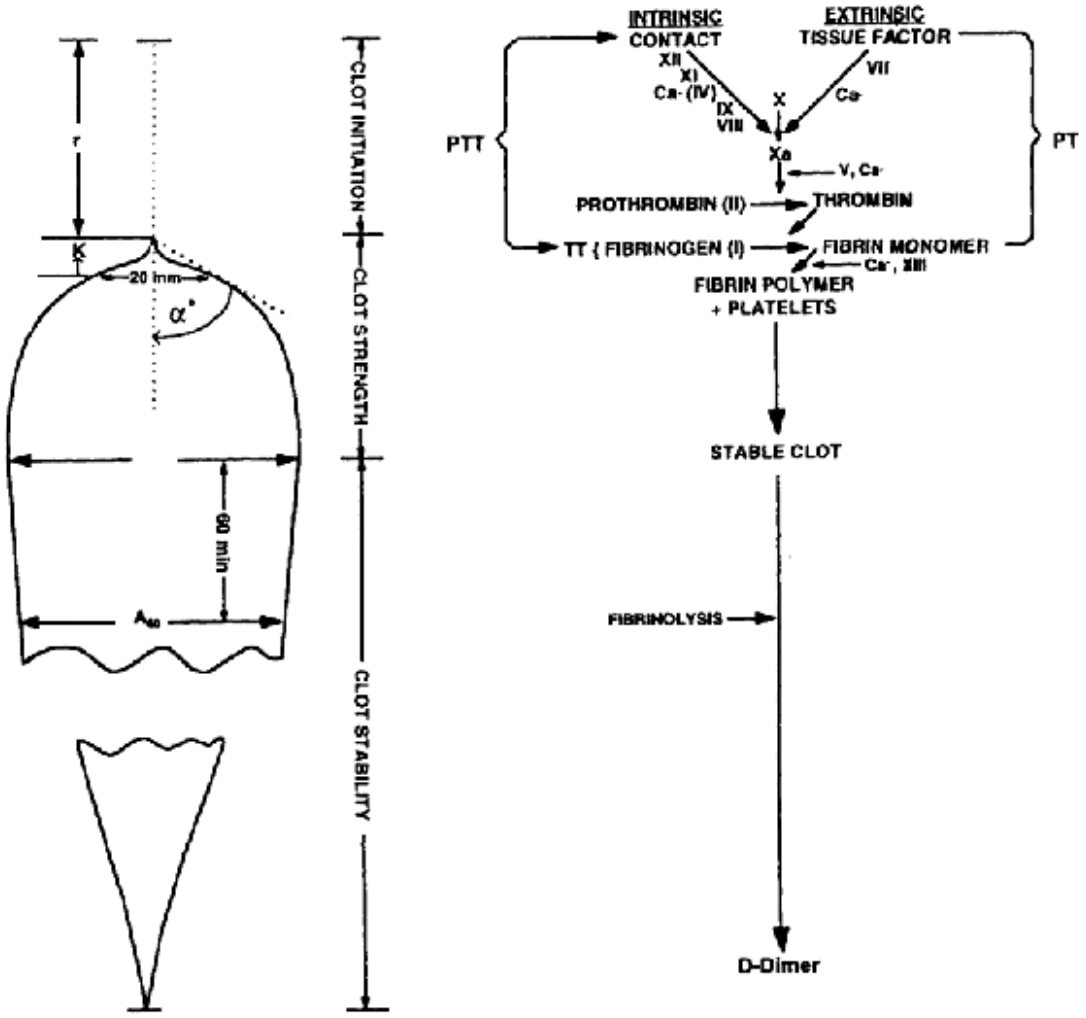


Figure 3-12 The different components of clot formation corresponding to the TEG trace.

The interpretation of the TEG trace enables the researcher to identify the contribution of the coagulation cascade, fibrin and platelets in clot formation and this guide further therapy.

3.5.3 Platelet mapping

Clot formation is determined by the interaction of the coagulation cascade with platelets. Standard TEG measures the rate of clot formation via thrombin which is important in the coagulation cascade by converting fibrinogen to fibrin as well as being the most potent platelet activator leading to platelet aggregation. Therefore, in routine TEG, kaolin is added to citrated blood which leads to thrombin activation of platelets and the coagulation cascade with the end result being the strongest clot formation possible independent of antiplatelet agents. Therefore, the MA derived from the TEG graph is the maximum viscoelastic strength of the clot in that individual.

Platelet mapping is an addition to TEG which enables the operator to know the effects of each antiplatelet agent on platelet inhibition. This is achieved by measuring the effects of the platelet activators arachidonic acid and ADP on platelet receptors which have not been inhibited by antiplatelets. Usually thrombin activation by kaolin is so significant that it will mask the effect of platelet activation by other activators such as AA and ADP. Therefore platelet mapping is carried out on heparinised blood in which the thrombin is inhibited. By inhibiting thrombin, activator F (labelled AP1) was added leading to production of the fibrin meshwork by conversion of fibrinogen to fibrin and activation of Factor XIIIa. This was followed by the addition of either AA or ADP activators in individual cups to measure platelet inhibition by each antiplatelet (Figure 3-13).

The end result of standard TEG and platelet mapping is 4 tracings (Figure 3-14). The first trace represents thrombin activation by kaolin which represents the highest MA (MA thrombin) whereas the pending three traces are for activator F (MA activator), ADP (MA ADP) and AA (MA AA). Platelet mapping isolates the contribution of fibrin and platelets to the clot's strength. The trace for MA activator represents the estimation of fibrinogen contribution to clot strength independent of platelets. The MA ADP and MA AA tracings represent the contribution of the platelets with fibrin to clot strength based on activation by the activators ADP and AA. The difference between the MA of these curves gives the individual contribution of fibrin, ADP and AA to clot strength. Additionally, the inhibitory effect of aspirin and the P2Y12 antagonist is derived from the equation:

Percentage maximum amplitude reduction or MA reduction

$= 100 - (([MA_{AA \text{ or } ADP} - MA_{activator}]/[MA_{thrombin} - Ma_{activator}])*100)$

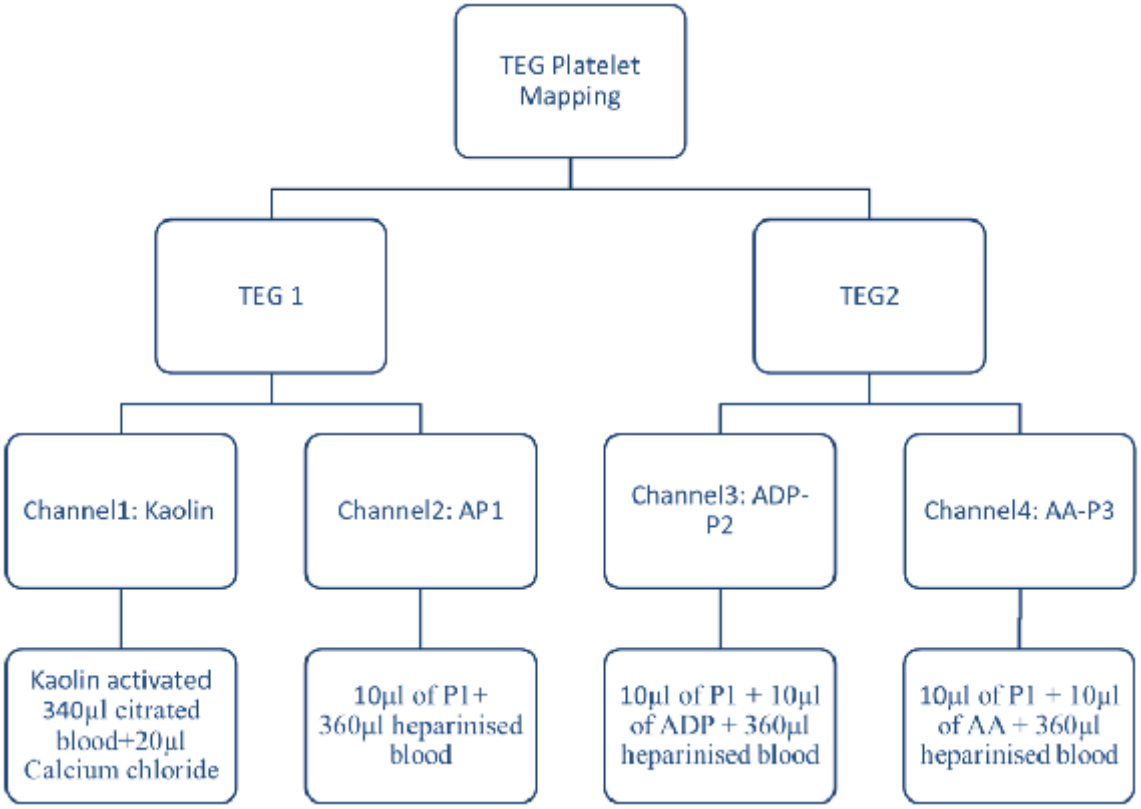


Figure 3-13 Diagram representing the 4 channels of TEG and the individual components.

- Channel 1: Kaolin activated citrated blood which leads to thrombin activation. The TEG results from thrombin activation of fibrinogen and platelets leading to the strongest clot possible
- Channel 2: Activator was added to heparinised blood without a platelet agonist. The TEG curve results from fibrin dependent clot formation.
- Channel 3: Activator added with ADP platelet agonist to heparinised blood. ADP activates platelet receptors not inhibited by the P2Y12 inhibitor. The activator converts fibrinogen to fibrin. The TEG curve results from a clot formation dependent on fibrin and non-inhibited platelet receptors activated by ADP. This shows how effective the P2Y12 inhibitor has been in inhibiting platelets.
- Channel 4: Activator added with AA platelet agonist to heparinised blood. AA activates platelet receptors not inhibited by aspirin. The TEG curve results from a clot formation dependent on fibrin and non-inhibited platelet receptors activated by AA. This shows how effective the aspirin has been in inhibiting platelets.

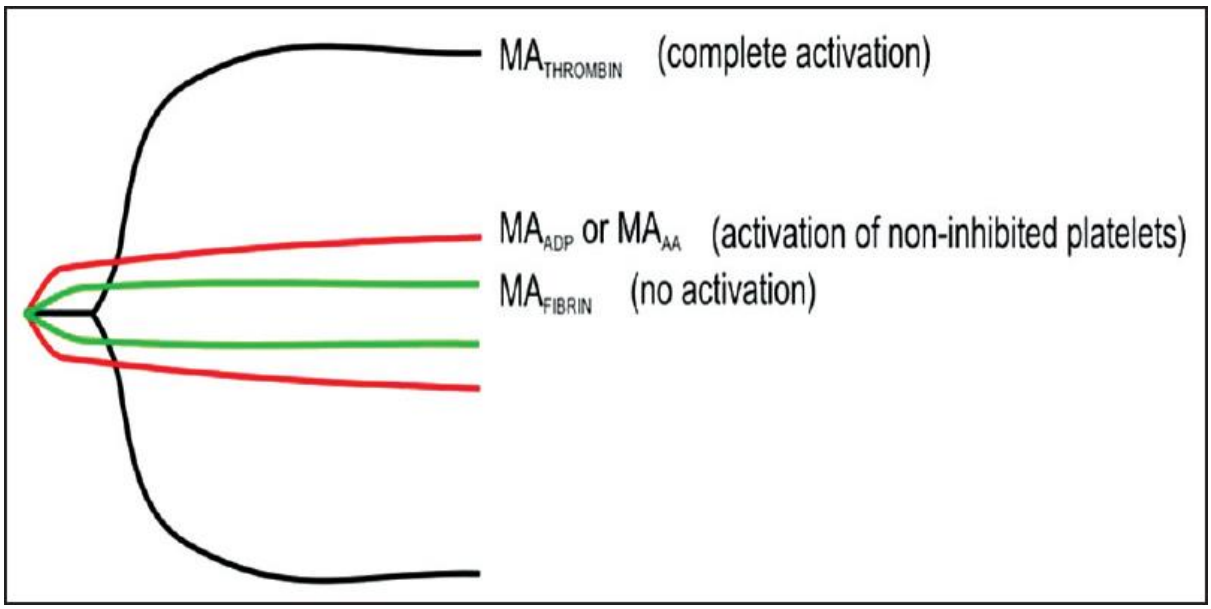


Figure 3-14 TEG and platelet mapping tracings.

The MA thrombin tracing represents maximum platelet activation due to thrombin resulting in the highest amplitude curve. The MA fibrin tracing represents clot strength solely due to fibrin activation resulting in the lowest amplitude curve. The MA ADP and MA AA curves are thrombus formation from activation of non-inhibited platelets by the respective antiplatelet agents. The amplitude of these curves is dependent on platelet inhibition. Platelet inhibition is calculated using the formula: MA reduction = $100 - (([MA_{AA\ or\ ADP} - MA_{activator}]/[MA_{thrombin} - Ma_{activator}])*100)$. This represents percentage maximum amplitude reduction. The higher the MA reduction, the better the response to the antiplatelet.

3.5.4 Study protocol

The protocol as per the manufacturer's guidelines were followed (Hemoscope, CA, USA). Citrated and heparinised bloods were collected from each patient. For the standard TEG, 1 ml of citrated blood was added to kaolin and then 340µl of this dilution was added to the cup in channel 1 into which 20µl of CaCL2 had already been pipetted. For platelet mapping, heparinised blood was added to the cups in channel 2, 3, and 4 into which the activator (AP1) and respective agonists (ADP and AA) had been pipetted. The TEG ran for 90 mins and all data was displayed graphically in time as the samples were being processed.

Kaolin activated TEG

1. Add 1ml of citrated blood into kaolin tube supplied by the manufacturer.
2. Mix by gentle inversion 5 times.
3. Pipette 20µl of $CaCL_2$ into a cup in channel 1 of the TEG machine.
4. Pipette 340µl of kaolin dilution into cup and start, slide the carrier up and move lever to the test position and click the start button on the software main toolbar.

Platelet mapping

1. Add 50µl of distilled water to aliquot vial containing A-P1 (activator) and give 5 mins to mix.
2. Add 100µl of distilled water to the aliquot vial containing ADP and a 100µl of distilled water to the vial containing AA.
3. Pipette 10µl of A-P1 into each cup of channel 2, 3 and 4.
4. Add 10µl of ADP into cup of channel 3.
5. Add 10µl of AA into cup of channel 4.
6. Pipette 360µl of heparinised blood into A-P1 cup in channel 2, mix gently by pipetting sample up and down 5 times and then start test.
7. Pipette 360µl of heparinised blood into ADP cup in channel 3, mix gently by pipetting sample up and down 5 times and then start test.
8. Pipette 360µl of heparinised blood into AA cup in channel 4, mix gently by pipetting sample up and down 5 times and then start test.

3.5.5 Quality control for TEG

For TEG, quality control prior to starting each test was performed which ensured equilibrium of both the torsion wire and central pin. These results were stored electronically. Once every fortnight, quality control on the control sample provided by the manufacturer was also performed, which was repeated for each new batch of reagents to ensure that the results were within the manufacturer's recommended range. Furthermore, the reproducibility was assessed by performing TEG on 6 individuals on 2 separate occasions and a coefficient of variation of MA thrombin of 2.9% was obtained.

3.6 Measuring platelet aggregation with VerifyNow

3.6.1 Principles of VerifyNow

VerifyNow (Accumetrics, CA, USA) is a point of care test which measures platelet aggregation and reactivity allowing the operator to determine the antiplatelet effects of aspirin and P2Y12 inhibitors. Platelet aggregation is determined by the use of different agonists such as AA, ADP and thrombin receptor activating peptide (TRAP). The system consists of a cartridge into which the blood is drawn. The blood then passes into a staging well allowing heating to 37 degrees Celsius. The blood is then passed into 4 wells which contain the specific platelet agonist and fibrinogen coated beads. The platelet agonist causes platelet activation whereas the fibrinogen interacts with the glycoprotein IIb/IIIa receptors leading to platelet aggregation. The cartridge is placed between a light source and an optical sensor which detects the light. The light absorbance of the sample is measured up to 16 times per second with the light transmittance being proportional to platelet aggregation. The effect of an antiplatelet in inhibiting platelets is measured by changes in light transmittance over a unit of time using arbitrary units. The greater the antiplatelet inhibiting effect, the less platelet aggregation takes place in response to the specific agonist leading to less light transmittance and hence lower reactive units (Figure 3-15).

Two different cartridges were used for measuring platelet inhibition: one for measuring the effects of aspirin and the second for measuring the effects of P2Y12 inhibitors (clopidogrel, ticagrelor and prasugrel). The cartridges differed based on the platelet agonist within them.

3.6.2 VerifyNow aspirin assay

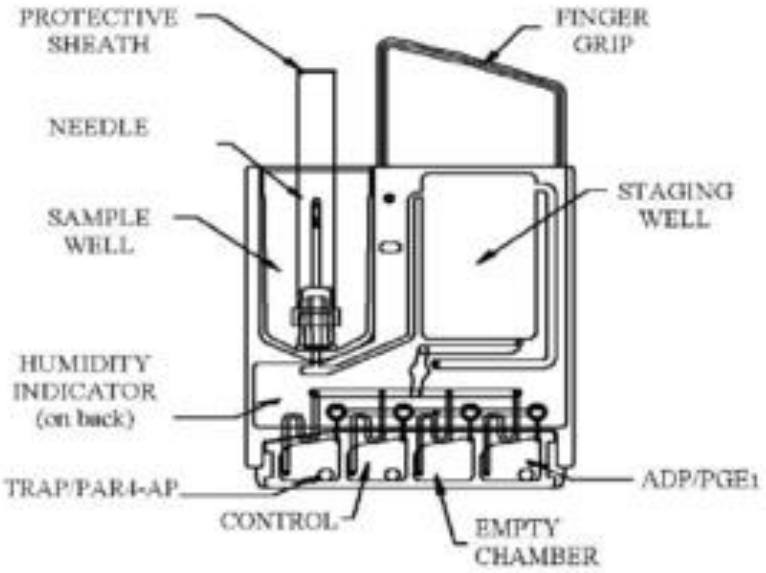
The VerifyNow aspirin assay consists of a cartridge with AA as the agonist to activate platelets. The AA is converted by cyclooxygenase (COX) to thromboxane A_2 leading to platelet activation. As platelet aggregation increases, the transmittance of light increases and the change in light transmittance is reported as aspirin reactive units (ARU). An ARU cut off of 495 was used with a value ≥495 representing platelet hyperactivity despite being on treatment (hyporespnder).

The use of VerifyNow has been validated in studies. In one study, the use of VerifyNow was compared with traditional platelet aggregometry (Malinin *et al.*, 2004). The overall agreement between the two methods for determining aspirin response was 87% and the correlation was 0.9. In another study the VerifyNow was compared to light transmittance aggregometry and

the Platelet Function Analyzer (PFA)-100 in patients treated with aspirin post ischaemic stroke (10). Seventeen percent of patients were identified as hyporesponders to aspirin by VerifyNow, 22% by PFA-100 and 5% by light transmittance aggregometry (Harrison *et al.*, 2005).

3.6.3 VerifyNow P2Y12 assay

The VerifyNow P2Y12 assay consists of a cartridge with ADP as the agonist to activate platelets. Platelet reactivity is measured as P2Y12 reactive units (PRU). The change in light transmittance due to platelet aggregation by ADP is reported as PRUz. Responsiveness to P2Y12 inhibitors is expressed as a percentage of the baseline platelet reactivity. For this reason, in one of the channels iso-TRAP is used as an agonist as it is unaffected by P2Y12 inhibitors allowing a baseline value for platelet function to be obtained in the form of PRUb. Therefore, the VerifyNow provides 3 measurements: PRUz (inhibited platelet function), PRUb (baseline platelet function) and % inhibition. Percentage inhibition due to the P2Y12 inhibitor is calculated from the formula $(1\text{-}PRU_Z/PRU_B)*100$. Consequently if a P2Y12 inhibitor produces significant platelet inhibition, the PRU_Z value will be lower and the percentage inhibition higher. A PRU_Z of 240 and a percentage inhibition of 40% was used as a cut off with a value $\geq$240 and inhibition less than 40% representing platelet hyperactivity despite being on treatment (hyporesponder). This is based on a paper by Marcucci et al who demonstrated a higher risk of cardiovascular death and non-fatal myocardial infarction in patients with a PRUz value above 240 (Migliorini *et al.*, 2009).



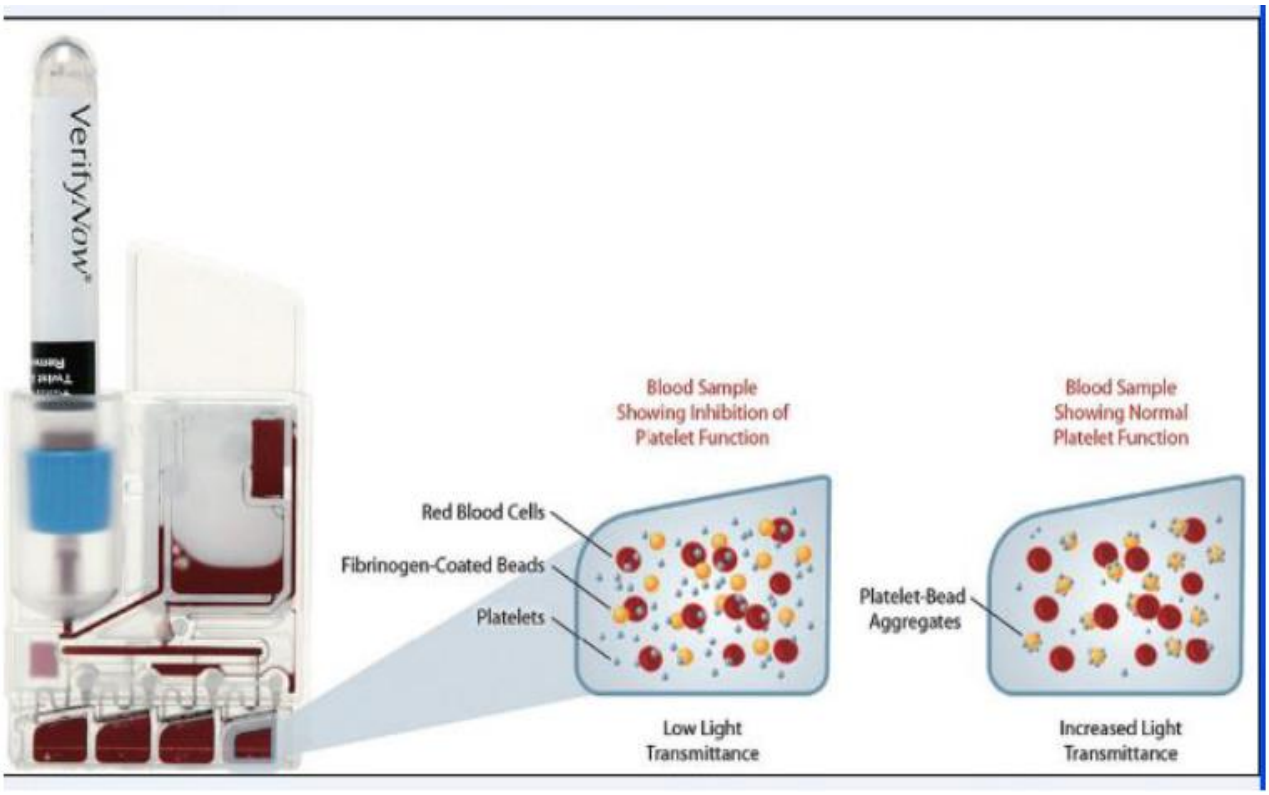


Figure 3-15 The principles of VerifyNow.

Blood is drawn into the 4 chambers which contain fibrinogen coated beads to initiate platelet aggregation. In addition, each chamber contain a specific agonist such as ADP or arachidonic acid and therefore platelet aggregation depends on residual antiplatelet activity. The greater the antiplatelet inhibiting effect, the less platelet aggregation takes place in response to the specific agonist leading to less light transmittance

3.6.4 Study protocol

Blood was collected in sodium citrate tubes and gently mixed by inverting at least 5 times. The VerifyNow cartridges were taken out of the packaging and inserted into the well of the device. The blood tubes were inverted further before being fixed to the needle within the cartridge. The cover of the assay device was closed to prevent light from entering. Platelet aggregation was measure between 3-5 minutes with results displayed as ARU or PRU respectively. Further details are provided below.

1. Perform electronic QC on cartridge provided by manufacturer.
2. With the aspirin citrated plasma tubes, mix gently by inverting 5 times.
3. Open the aspirin assay cartridge from the pack and insert the device into the machine column.
4. Remove the needle covering being careful in avoiding any needle injuries.
5. Mix the blood again 5 times in the plasma tubes and then insert the tube upside down into the well allowing contact between the needle and plasma tube.
6. Close the cover of the assay device to prevent light from entering.
7. After 300 seconds of analysing, the result will be displayed as an ARU.
8. Remove the aspirin cartridge and discard in a sharps container.
9. Repeat this procedure with the P2Y12 cartridge.
10. The results will be displayed as PRUz, PRUb and %inhibition.
11. The results were recorded electronically using the patients study ID.

3.6.5 Quality control for VerifyNow

VerifyNow is provided with its own manufacturer control which is used prior to starting each test to ensure the control results are not outside the range for both ARU and PRU. Additional quality control tests were performed on separate samples provided by the manufacturer every fortnight to ensure the results were in range. In addition, the reproducibility of the test was evaluated in 10 healthy individuals on two occasions with coefficient variation for ARU being 2.4% and for PRUZ being 3.1%.

3.7 Measuring endothelial function using EndoPAT

3.7.1 Priniciples of EndoPAT

EndoPAT (Itamer Medical LTD, Caesarea, Israel) uses a pneumatic probe with a plethysmographic cuff to measure changes in pulsatile blood volume within the fingertip vasculature with each heartbeat. By using one arm for the experiment and the other arm as a control, EndoPAT can account for general systemic changes that's occurs during the test and corrects for this when giving a final reactive hyperaemia index (RHI) score. A probe was placed on the index finger of each arm and then a blood pressure cuff was placed on the forearm of the experimental arm to induce transient ischaemia by occluding the brachial artery. Firstly, baseline blood flow measurements were taken for both arms for 5 mins which gave similar pulsatile waveform activity in both arms. Then, transient ischaemia was induced by inflating the blood pressure cuff to 60mmHg above the systolic blood pressure for 5 minutes in the experimental arm. This induced reactive hyperaemia and when the blood pressure cuff was released, PAT signal measurements were taken for a further 5 minutes with changes in PAT signal amplitude representing changes in endothelium mediated vascular tone induced by NO. EndoPAT gave a RHI measurement which represented dilatation, and this manifested as changes in the PAT signal amplitude. Since the PAT signal is influenced by the autonomic nervous system, all tests were performed in a stress-free environment which included a quiet bedroom with dimmed lights and a temperature of 21^{o}C.

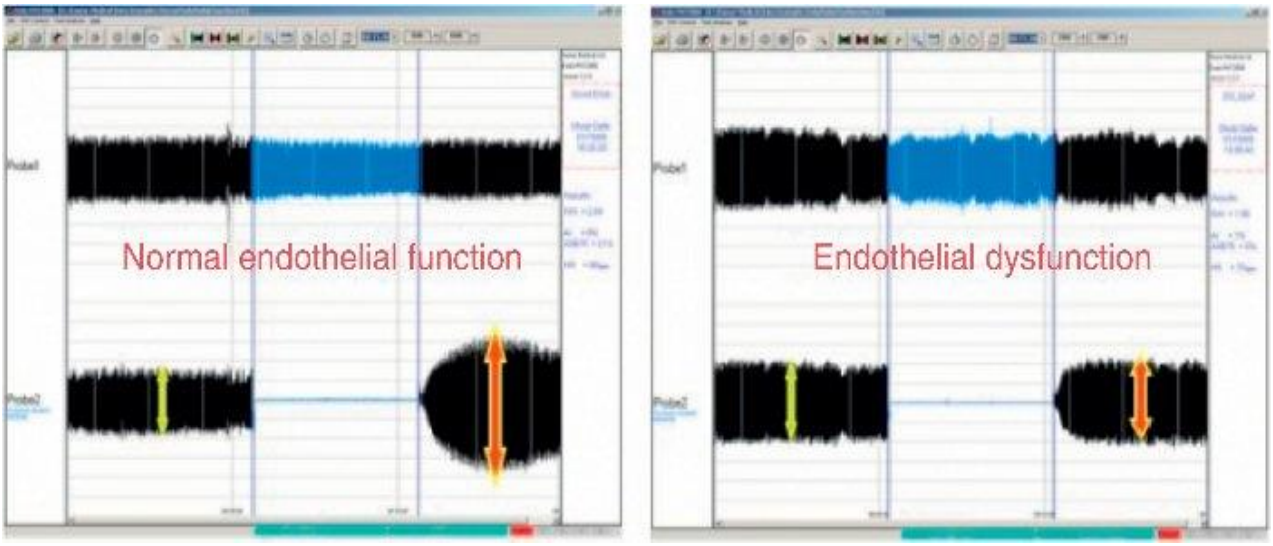


Figure 3-16 Pulsatile PAT signal waveforms showing normal endothelial function and endothelial dysfunction.

The two pairs of tracing are for two different patients. The upper PAT signal tracings represent the control arms with the same continuous waveform throughout the 15 minutes. The lower tracings represent the experimental arms. After 5 minutes, the cuff is inflated restricting blood flow resulting in no pulsatile activity as shown by the flat line. The cuff is deflated after 5 minutes of occlusion inducing reactive hyperaemia. This results in an increase in the waveform amplitude compared to baseline if the endothelial function is normal whereas in endothelial dysfunction there is no increase waveform amplitude.

The RHI score is a measurement of endothelial function and is derived from a ratio of the post to pre-occlusion PAT signal in the tested arm divided by the post to pre PAT signal in the control arm. The higher the RHI score, the healthier the endothelium giving a lower risk for future cardiovascular events. A score below 1.68 represents inadequate endothelial function indicating a significant change in lifestyle plus consideration of pharmacological therapy if there are any additional risks. A score between 1.69 and 2 represents healthy endothelial function with further improvements in life style necessary whereas a score above 2 represents optimal endothelial function and cardiovascular protection. EndoPAT has been used in the Framingham Heart Study to test endothelial function in over 5,000 subjects with Hamburg et al demonstrating an inverse relationship between RHI values and multiple cardiovascular risk factors in an analysis in 2008 (Hamburg *et al.*, 2008).

3.8 Quality of life and patient reported outcomes

Quality of life (QOL) and patient-reported outcome measures were evaluated by validated questionnaires, at baseline and at 52 weeks. The three different questionnaires used included: the Short Form 12 four-week recall (SF-12 v2), Minnesota Living with Heart Failure Questionnaire, and the Centre for Epidemiologic Studies Depression Scale (CES-D) (See appendix 1).

SF-12 v2 - has been extensively studied and used as a valid measure of health-related quality of life in variety of population groups (Gandek *et al.*, 1998). The scoring consists of a physical and a mental component and is designed to have a similar performance to the longer version SF-36, with less time to complete. The physical scores range from 20 to 56, with higher scores indicating better physical health. The mental scores range from 41 to 61, with higher scores indicating perceived better mental health.

Minnesota Living with Heart Failure Questionnaire – Has been used and approved to assess heart failure specific quality of life (Garin *et al.*, 2014). Scores range from 0 to 105, with higher scores indicating increase in symptoms from heart failure

CESD - This is a 20 item questionnaire which asked patients to rate how often they may have experienced symptoms associated with depression. The CES-D has been shown to identify an individual with clinical depression, with very good sensitivity and specificity (Lewinsohn *et al.*, 1997). Scores range from 0 to 60, with higher scores indicating greater depressive symptoms.

4 Chapter: Results

4.1 Prevalence and predictors of thyroid dysfunction and the relation of thyroid function with troponin levels in Acute Myocardial Infarction – the ThyrAMI 1 study

4.1.1 Prevalence and predictors of thyroid dysfunction

A total of 1915 patients were recruited to the ThyrAMI 1 Study of which 113 (5.9%) were on LT4 therapy for hypothyroidism. Of the remaining 1802 patients, 1440 (79.9%) patients were euthyroid, 312 (17.3%) had SCH, 22 (1.2%) had SHyper, 25 (1.3%) had LT3S, 2 (0.1%) had overt hypothyroidism and 1 (0.06%) had overt hyperthyroidism (Figure 4-1).

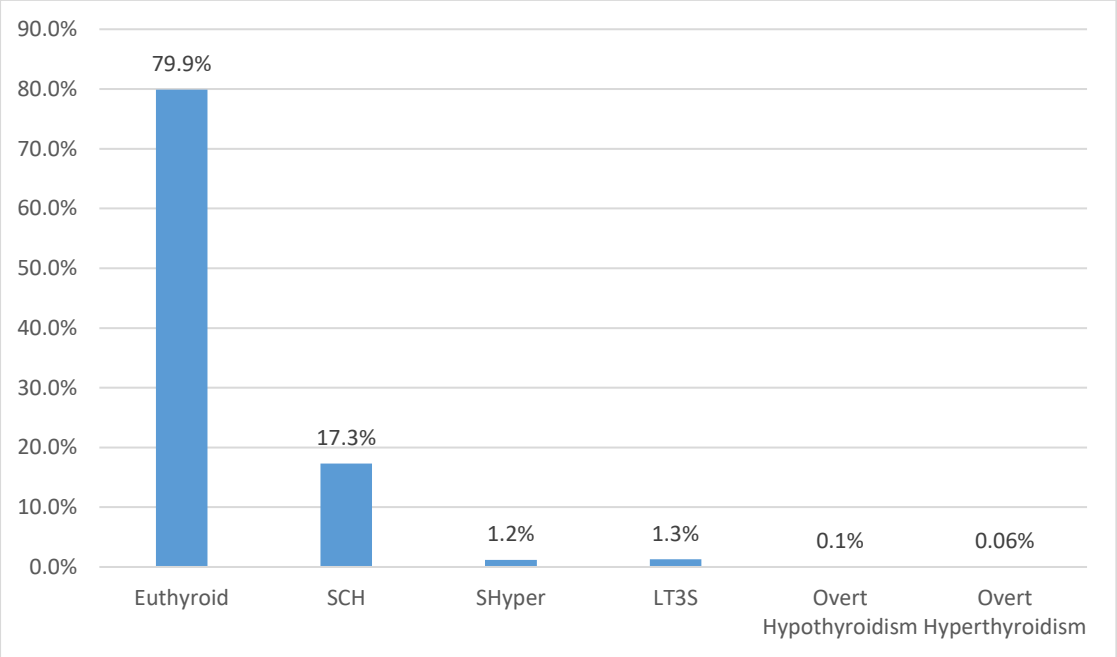


Figure 4-1 Prevalence of thyroid dysfunction in the Thyrami-1 Study.

SCH – subclinical hypothyroidism, SHyper – subclinical hyperthyroidism, LT3S – low T3 syndrome.

The baseline demographic and clinical characteristics of all participants with euthyroidism, SCH, SHyper and LT3S are outlined in Table 4-1. The SCH and LT3S patients tended to be older, included a higher percentage of females and had higher serum creatinine levels compared to the euthyroid and SHyper groups. Furthermore the SCH and LT3S groups had higher standardised troponin levels. Patients with STEMIs were observed more frequently in the SCH and SHyper groups. Interestingly, patients with LT3S had more patients with existing ischaemic heart disease than the other groups. As expected, the SCH group had higher median TSH and TPOAb levels whereas the LT3 group had lower FT3 levels.

	Euthyroid (n=1440)	SCH (n=312)	SHyper (n=23)	LT3S (n=25)	P-value
Age (years)	63.4 (±12.0)	65.8 (±11.3)	64.3 (±13.2)	70.9 (±12.5)	0.001
Males (n, %)	1074 (72.7)	213 (68.3)	18 (78.3)	16 (64.0)	0.08
Body mass index (kg/m^2)	28.5 (±5.4)	28.4 (±6.1)	26.2 (±3.9)	27.9 (±5.6)	0.25
Systolic BP (mmHg)	141.8 (±27.4)	140 (±29.3)	140.9 (±34.7)	142.9 (±35.6)	0.42
Diastolic BP (mmHg)	80.3 (±23.4)	82.1 (±16.9)	79.4 (±22.5)	82.9 (±19.7)	0.48
Pulse rate (bpm)	77.1 (±18.7)	80.3 (±18.3)	82.1 (±22.9)	79.6 (±20.4)	0.32
Current smoker (n, %)	457 (31.7)	94 (30.1)	8 (34.8)	9 (36.0)	0.87
STEMI (n, %)	695 (48.3)	181 (58.0)	13 (56.5)	11(44.0)	0.014
TSH (mIU/L)	1.8 (1.3-2.5)	5.3 (4.5-6.5)	0.3 (0.3-0.4)	1.6 (1.0-2.9)	<0.001
FT4 pmol/L	16.3 (±2.9)	15.9 (±2.9)	16.5 (±4.5)	13.9 (2.5)	0.007
FT3 pmol/L	4.7 (±0.8)	5.0 (±1.0)	4.7 (±0.6)	2.7 (±0.5)	<0.001
TPOAb U/L	39 (28-190)	128 (28-286)	35 (28-89)	30 (28-120)	<0.001
hs C-reactive protein	2 (1-9)	1 (1-7)	6 (1-46)	4 (1-52)	0.39
Total Cholesterol (mmol/L)	4.97 (±1.4)	4.91 (±1.3)	4.90 (±1.1)	4.98 (±1.5)	0.42
Creatinine (µmol/dL)	88.9 (±33.5)	95.2 (±66.2)	91.8 (±29.6)	109.0 (±59.3)	0.009
Standardised troponin	0.004 (-0.78-0.88)	0.21 (-0.59-0.90)	-0.07 (-0.83-0.90)	0.22 (-0.80-0.67)	0.04
IHD (n, %)	373 (25.9)	74 (23.7)	3 (13)	13 (52.0)	0.009
Type 2 diabetes mellitus (n, %)	237 (16.5)	55 (17.6)	5 (21.7)	4 (16.0)	0.87
Hypertension (n, %)	580 (40.3)	122 (39.1)	9 (39.1)	13 (52.0)	0.66
Hypercholesterolaemia (n, %)	369 (25.6)	71 (22.7)	4 (17.4)	5 (20.0)	0.55
Cerebrovascular disease (n, %)	65 (4.5)	18 (5.8)	2 (8.7)	2 (8.0)	0.53
Atrial fibrillation (n, %)	57 (4)	10 (3.2)	1(4.3)	1(4.0)	0.83

Table 4-1 Characteristics of patients presenting with acute myocardial infarction by thyroid status.

SCH - subclinical hypothyroidism, SHyper – subclinical hyperthyroidism, NSTEMI – non-ST-elevation myocardial infarction, STEMI – ST-elevation myocardial infarction, TSH – thyrotropin, FT4 – free thyroxine, FT3 – free triiodothyronine, TPOAb – thyroid peroxidase antibody, IHD – ischaemic heart disease.

Standardised troponin – The troponin T and troponin I were standardised, centred and then combined to form a standardised single troponin variable.

Data are presented as mean (± SD), numbers (%) or median (IQR).
Means compared using ANOVA.

Medians compared using Kruskal-Wallis test.

Proportions compared using Chi square test.

Predictors of thyroid dysfunction (SCH, SHyper and LT3S) were assessed using multivariable logistic regression analysis. Overt hypo- and hyperthyroidism were not analysed due to few patients being classed in this category. The predictor variables included age, sex, body mass index, smoking status, type of AMI, st Troponin, serum creatinine, hs CRP levels, TPOAb levels, time-period of sampling, and presence of ischaemic heart disease, hypertension, type 2 diabetes, hypercholesterolaemia, cerebrovascular disease and atrial fibrillation. Missing data was dealt with by using multiple imputation method. Ten imputed datasets were created and pooled results were summarised. A sensitivity analysis was performed for predictors of SCH, SHyper and LT3S by analysing the original non-imputed dataset.

Predictors for SCH were increasing age, female sex, higher TPOAb levels, higher serum creatinine levels, and the time of blood sampling (Table 4-2). With regard to the time of sampling, patients who had their thyroid function tested between 00:01 and 06:00 hours were more likely to have SCH than those sampled at other time points (p for trend <0.001). Significant predictors for SHyper were lower BMI and time of blood sampling (Table 4-3). With regards to the latter, those sampled between 00:01 and 06:00 had the least likelihood of being diagnosed with SHyper (p for trend 0.02). The only significant predictors for LT3S were increasing age, higher creatinine levels and presence of ischaemic heart disease (Table 4-4). Neither time of sampling, larger infarcts (as measured by peak st troponin levels) or higher hsCRP levels were significant predictors.

In the sensitivity analysis, the complete case data was analysed without the imputed values. The overall strength and direction of associations remained similar to the main analysis although the parameters of uncertainty (95 percent confidence intervals) were larger (data not shown).

	Odds ratio (95% CI)	**P-value**
Age (years)	1.03 (1.01-1.05)	<0.001
Sex		
Male	1.0 (Ref)	
Female	1.4 (1.04-1.90)	0.034
Body mass index (kg/m^2)	1.00 (0.98-1.03)	0.54
Smoking		0.41
Never smoked	1.0 (Ref)	
Current smokers	1.01 (0.70-1.44)	
Ex-smokers	0.81 (0.59-1.11)	
Type of AMI		
NSTEMI	1.0 (Ref)	
STEMI	1.37 (0.98-1.92)	0.06
Standardised Troponin	1.16 (0.99-1.36)	0.071
Creatinine (µmol/dL)	1.00 (1.00 – 1.00)	0.040
hs CRP (mg/L)	0.99 (0.99-1.00)	0.40
TPOAb (mU/L)	1.01 (1.01-1.03)	<0.001
Time of sampling (24-hour clock)		<0.001
00:01-06:00	1.0 (Ref)	
06:01-12:00	0.42 (0.29-0.61)	
12.01-18:00	0.32 (0.22-0.47)	
18:01-00:00	0.69 (0.48-0.99)	
Ischaemic heart disease		
Absent	1.0 (Ref)	
Present	0.88 (0.63 – 1.24)	0.39
Hypertension		
Absent	1.0 (Ref)	
Present	1.14 (0.85 – 1.53)	0.75

Type 2 diabetes mellitus		
Absent	1.0 (Ref)	
Present	1.08 (0.75 – 1.57)	0.62
Hypercholesterolaemia		
Absent	1.0 (Ref)	
Present	0.95 (0.68-1.33)	0.64
Cerebrovascular disease		
Absent	1.0 (Ref)	
Present	1.38 (0.77-2.46)	0.21
Atrial fibrillation		
Absent	1.0 (Ref)	
Present	0.63 (0.31-1.30)	0.21

Table 4-2 Predictors of SCH in patients with acute myocardial infarction.

BMI - Bodymass index, AMI – Acute myocardial infarction, CRP – C reactive protein STEMI – ST elevation myocardial infarction, NSTEMI – Non ST elevation myocardial infarction, IHD – ischaemic heart disease, TPOAb – thyroid peroxidase antibodies.

	Odds ratio (95% CI)	**P-value**
Age (years)	0.99 (0.95-1.04)	0.68
Gender		
Male	1.0 (Ref)	
Female	1.11 (0.37-3.33)	0.85
Body mass index (kg/m^2)	0.88 (0.78-0.98)	0.02
Smoking		0.34
Never smoked	1.0 (Ref)	
Current smokers	1.37 (0.34-5.51)	
Ex-smokers	2.29 (0.69-7.60)	
Type of AMI		
NSTEMI	1.0 (Ref)	
STEMI	2.34 (0.80-6.83)	0.12
Standardised Troponin	0.71 (0.43-1.15)	0.17
Creatinine (µmol/dL)	1.00 (0.98-1.02)	0.87
hs CRP (mg/L)	1.00 (0.98-1.02)	0.84
TPOAb (mU/L)	0.99 (0.99-1.00)	0.28
Time of sampling (24-hour clock)		0.02
00:01-06:00	1.0 (Ref)	
06:01-12:00	1.60 (0.16-15.7)	
12.01-18:00	6.99 (0.89-55.2)	
18:01-00:00	2.45 (0.25-24.4)	
Ischaemic heart disease		
Absent	1.0 (Ref)	
Present	0.25 (0.05-1.19)	0.07
Hypertension		
Absent	1.0 (Ref)	
Present	1.12 (0.39-3.15)	0.84

Type 2 diabetes mellitus		
Absent	1.0 (Ref)	
Present	2.49 (0.81-7.69)	0.13
Hypercholesterolaemia		
Absent	1.0 (Ref)	
Present	0.53 (0.15-1.96)	0.32
Cerebrovascular disease		
Absent	1.0 (Ref)	
Present	2.92 (0.58-14.7)	0.24
Atrial fibrillation		
Absent	1.0	
Present	- -*	0.99

Table 4-3 Predictors of Subclinical Hyperthyroidism in patients with acute myocardial infarction.

*Too few to calculate odds ratio.

	Odds ratio (95% CI)	**P-value**
Age (years)	1.06 (1.01-1.11)	0.01
Gender		
Male	1.0 (Ref)	
Female	1.60 (0.65-3.93)	0.31
Body mass index (kg/m^2)	1.03 (0.95-1.11)	0.54
Smoking		0.41
Never smoked	1.0 (Ref)	
Current smokers	3.06 (0.92-10.2)	
Ex-smokers	0.93 (0.33-2.61)	
Type of AMI		
NSTEMI	1.0 (Ref)	
STEMI	1.43 (0.49-4.1)	0.51
Standardised Troponin	1.06 (0.65-1.74)	0.81
Creatinine (µmol/dL)	1.01 (1.00-1.03)	0.03
hs CRP (mg/L)	1.00 (0.99-1.01)	0.42
TPOAb (mU/L)	0.99 (0.99-1.00)	0.75
Time of sampling (24-hour clock)		0.34
00:01-06:00	1.0 (Ref)	
06:01-12:00	0.73 (0.22-2.53)	
12.01-18:00	0.61 (0.17-2.10)	
18:01-00:00	1.36 (0.41-4.54)	
Ischaemic heart disease		
Absent	1.0 (Ref)	
Present	2.84 (1.12-7.18)	0.03
Hypertension		
Absent	1.0 (Ref)	
Present	1.43 (0.57-3.59)	0.40
Type 2 diabetes mellitus		

Absent	1.0 (Ref)	
Present	0.50 (0.15-1.66)	0.23
Hypercholesterolaemia		
Absent	1.0 (Ref)	
Present	0.51 (0.17-1.50)	0.24
Cerebrovascular disease		
Absent	1.0 (Ref)	
Present	1.61 (0.34-7.62)	0.57
Atrial fibrillation		
Absent	1.0 (Ref)	
Present	- -*	0.99

Table 4-4 Predictors of low T3 syndrome in patients with acute myocardial infarction.

* Too few to calculate odds ratio.

4.1.2 Thyroid dysfunction and time of sampling

Prevalence of Subclinical thyroid disease and baseline characteristics by time of sampling

Logistic regression analysis had shown a higher prevalence of SCH between 00:01 and 06:00 and SHyper between 12:01 and 18:00. Therefore the baseline characteristic of participants across each time period was analysed and described (Table 4-5). Clinical, biochemical and pre-existing medical conditions were similar across the various time-periods. However, there were less STEMI admissions in the 12:01–18:00 hrs time period than the other time-periods. In addition, serum TSH and FT3 were highest between 00:01–06:00 hrs and lowest in the 12:01–18:00 time-period whereas there was no significant difference between FT4 levels. Consequently, the diagnosis of SCH was highest in the 00:01–06:00 time-period (20.9%) and lowest in the 12:01–18:00 time-period (8.7%). Conversely, the prevalence of SHyper was lowest in the 00:01–06:00 time-period (0.7%) and highest in the period between 12:01–18:00 hrs (2.5%).

	00:01 – 06:00 N=360	**06:01 – 12:00 N=570**	**12:01 – 18:00 N=570**	**18:01 – 00:00 N=419**	**P value**
Age, mean (± SD), years	63.7 ± 12.3	64.5 ± 12.4	64.9 ± 11.7	62.9 ± 11.8	0.06
Females, n (%)	108 (30.0)	175 (30.7)	160 (28.1)	118 (28.2)	0.73
Current smokers, n (%)	128 (35.6)	164 (28.8)	161 (28.2)	143 (34.3)	0.07
BMI, mean (± SD), kg/m^2	28.4 ± 5.7	28.2 ± 5.1	28.6 ± 5.6	28.9 ± 5.7	0.22
STEMI, n (%)	191 (53.1)	288 (50.5)	252 (44.2)	204 (48.7)	0.04
Pulse rate, mean (±SD), beats/minute	76.8 ± 16.9	76.8 ± 17.4	75.4 ± 17.3	76.7 ± 19.2	0.75
Blood pressure, mean (±SD), mm Hg					
Systolic	136.2± 29.1	138.8± 27.2	139.6± 24.4	139.6± 27.7	0.54
Diastolic	80.2 ± 16.7	80.6 ± 17.0	80.6 ± 15.9	82.4 ± 17.2	0.55
Ischaemia time, median (IQR), minutes*	165 (118 – 252)	158 (112 – 251)	160 (114 – 248)	161 (107 – 244)	0.38
Coronary artery affected, n (%)					
Left main stem	6	11	9	9	
Left anterior descending	142	218	221	158	0.34
Right coronary	161	267	264	191	
Circumflex	49	71	76	59	
Other	2	3	0	2	
Thyrotropin, median (IQR), mU/L	2.60 (1.50 – 4.23)	1.93 (1.31 – 2.93)	1.80 (1.16 – 2.74)	2.10 (1.36 – 3.60)	<0.001
Free thyroxine (FT4), mean (± SD), pmol/L	16.4 ± 3.4	16.3 ± 4.1	16.1 ± 3.2	16.4 ± 3.9	0.43
Free triiodothyronine (FT3), mean (± SD), pmol/L	4.9 ± 1.0	4.7 ± 0.8	4.6 ± 0.7	4.7 ± 0.9	<0.001
Thyroid status, n (%)$					
Euthyroid	241 (72.2)	449 (84.4)	464 (85.5)	312 (79.4)	
SCH	92 (27.5)	79 (14.8)	65 (12.0)	77 (19.6)	<0.001
SHyper	1 (0.3)	4 (0.8)	14 (2.6)	4 (1.0)	
TPOAb, median (IQR), (U/L)	13.3 (8.8 – 28)	12.1 (9.2 – 28)	12.7 (9.3 – 28)	11.9 (9.3 – 28)	0.84
TPOAb positive, n (%)	65 (18.0)	94 (16.4)	95 (16.7)	61 (14.5)	0.77
Creatinine, mean (± SD), μmol/L	92.3 ± 39.8	90.8 ± 51.3	90.8 ± 38.3	88.9 ± 28.2	0.74

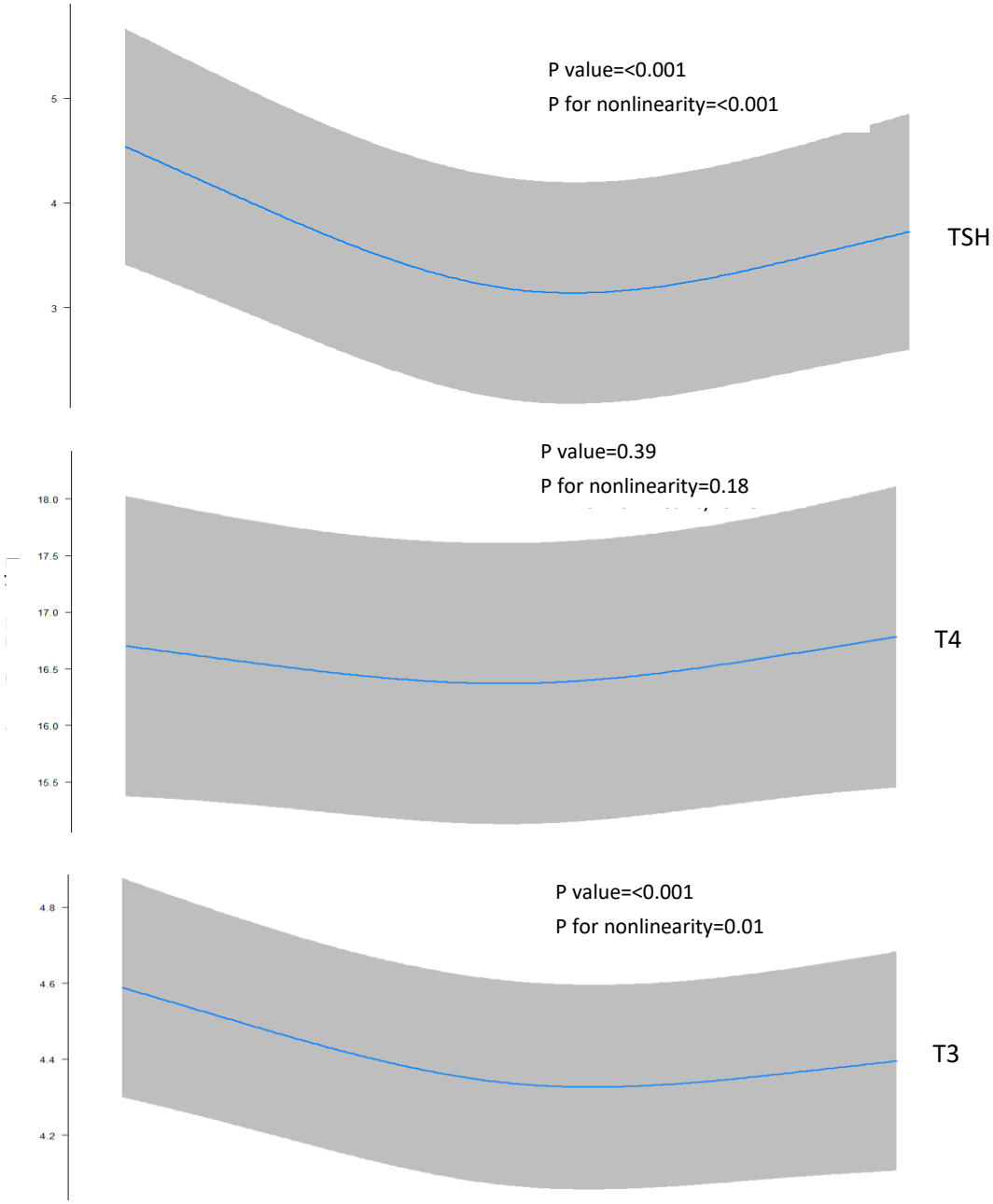
Past medical history, n (%)					
Treated hypothyroidism	25 (6.9)	36 (6.3)	27 (4.7)	25 (6.0)	0.52
Ischaemic heart disease	91 (25.3)	162 (28.4)	156 (27.4)	93 (22.2)	0.14
Hypertension	137 (38.1)	243 (42.6)	246 (43.2)	158 (37.7)	0.18
Atrial fibrillation	17 (4.7)	25 (4.4)	20 (3.5)	16 (3.8)	0.79
Type 2 diabetes mellitus	65 (18.1)	114 (20.0)	92 (16.1)	68 (16.2)	0.29
Cerebrovascular disease	15 (4.2)	27 (4.7)	38 (6.7)	15 (3.6)	0.12
PVD	16 (4.7)	10 (1.9)	20 (3.7)	15 (3.8)	0.13

Table 4-5 Baseline characteristics and prevalence of thyroid dysfunction by time of blood sampling.

* Calculated as the length of time from onset of pain till blood sample obtained.
$ After excluding individuals on levothyroxine therapy; therefore, total numbers do not add up to the sample size for the whole group for each time-period.
Means compared using ANOVA.
Medians compared using Kruskal-Wallis test.
Proportions compared using Chi square test.
BMI = body mass index, STEMI = ST-elevation myocardial infarction, SCH = subclinical hypothyroidism, SHyper = subclinical hyperthyroidism.

Association of time of sampling with thyroid function and TPOAb parameters

Time of sampling as a continuous variable over 24 hours (from 0 to 1440 minutes) was significantly associated with serum TSH and FT3 levels in an independent and nonlinear manner (Figure 4-2). No significant relationship was observed between time of sampling and FT4 or TPOAb levels.



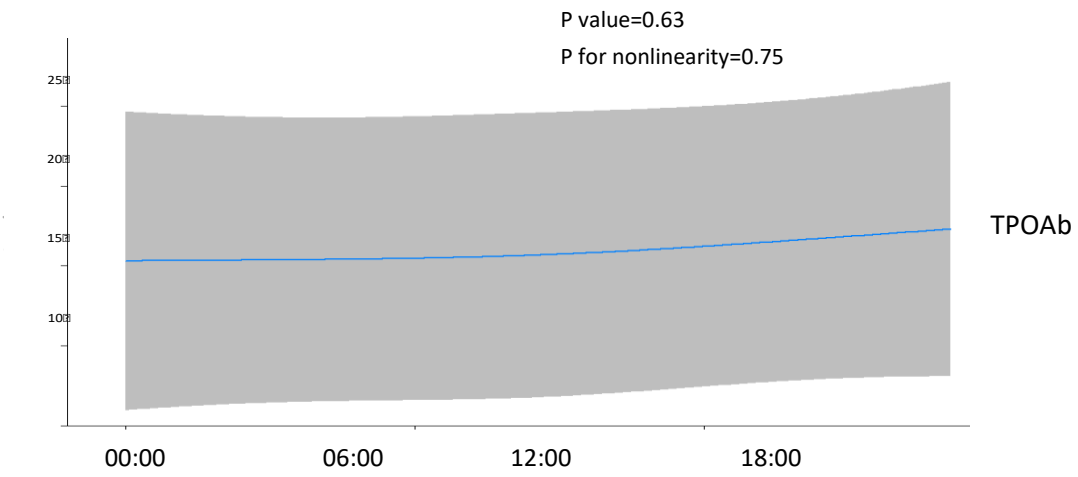


Figure 4-2 The relationship between time of sampling over 24 hours and thyroid function parameters and TPOAb levels using restricted cubic spline plots with 3 knots.

All associations adjusted for age, gender, centre, body mass index, smoking status, type of AMI, ischaemia time, serum creatinine, history of IHD, DM, CVD, BP, AF and hypothyroidism.

Normalisation of thyroid function versus those that remained in Subclinical thyroid disease state

Of the 308 patients with subclinical thyroid disease (SCTD) diagnosed on admission that had repeat thyroid function assessed, 137 (44%) of 312 of individuals with SCH and 13 (61%) of 22 of individuals with SHyper normalised serum TSH levels. There was a significant difference in normalisation rates depending on baseline time-period of sampling. In the baseline SCH group, 58% of patients from 00:01–06:00 hrs normalised serum TSH on repeat testing whereas only 28% of those from 12:01–18:00 normalised ($p<0.001$) (Figure 4-3). In the SHyper group at baseline, no analysis was performed due to the small number of participants in each time-period. The one participant with SHyper at baseline time-period of 00:01–06:00 hrs remained in SHyper state whereas 5 participants (of the 11 with repeat data available) in the 12:01–18:00 hrs normalised.

Time of sampling for the initial thyroid function testing was an independent predictor of subsequent thyroid status. Those individuals who had initial thyroid function test drawn between 12:01–18:00 hours were more likely to remain in the SCH state than those who had initial sampling between 00:01–06:00 hours [OR 2.56 (1.09 – 5.95)] or between 18:01–00:00 hours [OR 2.33 (1.01 – 5.26)]. Other variables that were significantly associated with remaining SCH were higher initial serum TSH level [OR 1.32 (1.13 – 1.55) and those that were TPO antibody positive [OR 2.56 (1.67 – 3.58)]. Participant's age, gender, interval time-period between the two thyroid function tests, type of AMI and smoking status were not significantly associated with subsequent thyroid status.

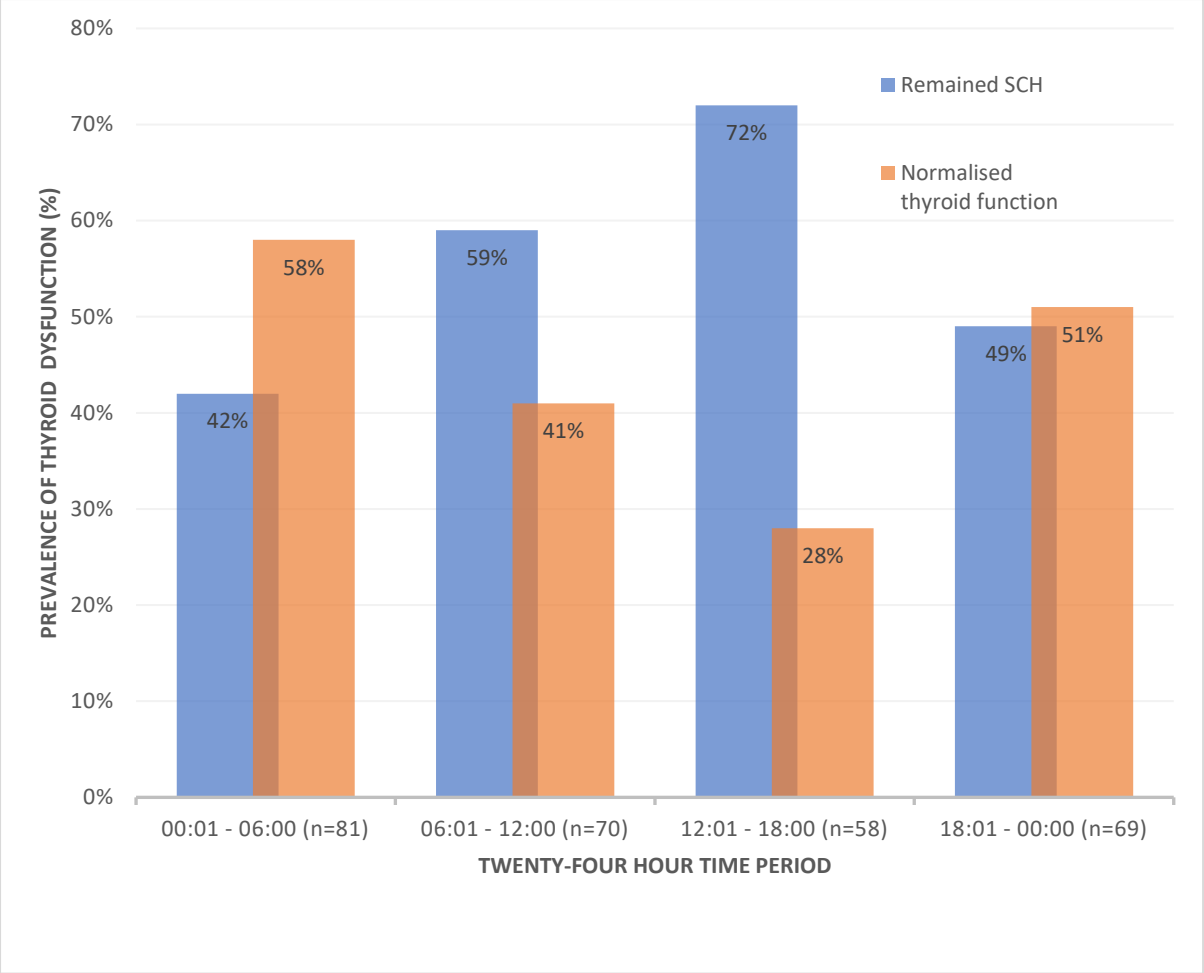


Figure 4-3 Frequency of subsequent thyroid dysfunction depending on initial time of blood sampling.

4.1.3 Relationship between thyroid dysfunction and peak troponin levels

Subclinical hypothyroidism and peak troponin

The association of SCH with troponin levels was analysed to assess whether SCH patients had higher troponin levels compared to euthyroid patients. Firstly, the baseline demographic and clinical characteristics were compared in both groups and are outlined in the Table 4-6. The SCH patients were significantly older, had a higher percentage of females, had a higher prevalence of STEMI, and as expected, had higher TSH levels. Furthermore, SCH patients had a significantly higher median TPO antibody level compared to euthyroid patients: 128 (28-286) vs. 39 (28-190), $p<0.001$. Due to the study being multi-centred, all patients had either a troponin T or troponin I measured depending on the assay in clinical use at each site. There was a significant difference in median (interquartile range) peak troponin T in SCH patients compared to those who were euthyroid: 580 (146-2378) vs 340 (101-1516) ng/L; $p=0.001$. There was no significant difference in median (interquartile range) peak troponin I in SCH patients compared to those who were euthyroid: 16984 (4157-50,000) vs 16524 ng/L (3154-50,000); $p=0.53$.

Multiple regression analysis was performed to assess whether an increase in troponin levels in SCH individuals were independent of other variables. A standardised troponin was used as the dependent variable, which took into account differences in the troponin T and troponin I assay. Standardisation of the troponin values transformed the troponin T and troponin I values, in order to combine and analyse them together. There was a significant difference in median (interquartile range) standardised troponin with SCH patients having a higher troponin than euthyroid patients: 0.21 (-0.59-0.90) vs 0.004 ng/L (-0.78-0.88), $p=0.003$ using the Student t-test. However, on multiple regression SCH was not significantly associated with elevated troponin levels after adjusting for other confounders ($p=0.077$) (Table 4-7). The significant predictors for an elevated troponin were gender ($p=0.029$), STEMI ($p<0.001$), a past history of ischaemic heart disease ($p=0.001$) and C-reactive protein (CRP) levels ($p=0.005$). Multiple regression analysis was performed using time specified reference ranges, to diagnose SCH, and showed no difference in standardized troponin levels between SCH and euthyroid patients, $p=0.078$.

Table 4-6 Characteristics of patients presenting with acute myocardial infarction by thyroid status (euthyroidism and subclinical hypothyroidism).

	Euthyroid (n=1440)	SCH (n=312)	P-value
Age (years)	63.4 (±12.0)	65.8 (±11.3)	0.001
Males (n, %)	1074 (72.7)	213 (68.3)	0.013
Body mass index (kg/m^2)	28.5 (±5.4)	28.4 (±6.1)	0.32
Systolic BP (mmHg)	141.8 (±27.4)	140 (±29.3)	0.42
Diastolic BP (mmHg)	80.3 (±23.4)	82.1 (±16.9)	0.48
Pulse rate (bpm)	77.1 (±18.7)	80.3 (±18.3)	0.52
Current smoker (n, %)	457 (31.7)	94 (30.1)	0.40
STEMI (n, %)	695 (48.3)	181 (58.0)	0.004
TSH (mIU/L)	1.8 (1.3-2.5)	5.3 (4.5-6.5)	<0.001
FT4 pmol/L	16.3 (±2.9)	15.9 (±2.9)	0.09
FT3 pmol/L	4.7 (±0.8)	5.0 (±1.0)	<0.001
TPOAb U/L	39 (28-190)	128 (28-286)	<0.001
Total Cholesterol (mmol/L)	4.97 (±1.4)	4.91 (±1.3)	0.52
Creatinine (µmol/dL)	88.9 (±33.5)	95.2 (±66.2)	0.35
Standardised troponin	0.004 (-0.78-0.88)	0.21 (-0.59-0.90)	0.003
IHD (n, %)	373 (25.9)	74 (23.7)	0.36
Type 2 diabetes mellitus (n, %)	237 (16.5)	55 (17.6)	0.71
Hypertension (n, %)	580 (40.3)	122 (39.1)	0.49
Hypercholesterolaemia (n, %)	369 (25.6)	71 (22.7)	0.22
Cerebrovascular disease (n, %)	65 (4.5)	18 (5.8)	0.44

SCH – subclinical hypothyroidism, NSTEMI – non ST-elevation myocardial infarction, STEMI – ST-elevation myocardial infarction, TSH – thyrotropin, FT4 – free thyroxine, FT3 – free triiodothyronine.

Data are presented as either mean (± SD), numbers (%) or median (IQR).

	Beta coefficient	95% confidence interval		P-value
Male gender	.104	.011	.196	.029
Age	.003	-.001	.007	.150
BMI	.005	-.003	.013	.188
Smokers	.001	-.051	.053	.959
STEMI	1.075	.990	1.159	.000
Creatinine	.001	.000	.002	.121
IHD	.199	.101	.298	.000
DM	.014	-.098	.125	.812
HTN	.024	-.068	.116	.612
Hypercholestrolaemia	.038	-.059	.135	.444
CVD	-.056	-.245	.133	.561
PVD	-.004	-.223	.214	.970
SCH	.095	-.010	.200	.077
CRP	.003	.001	.004	.005

Table 4-7 Variables associated with standardised troponin levels at the time of acute myocardial infarction whilst comparing euthyroid patients with SCH patients.

BMI - Body mass index, STEMI - ST-elevation myocardial infarction, IHD - Ischaemic heart disease, DM - Diabetes mellitus, HTN - Hypertension, Chol – Hypercholestrolaemia, CVD - Cerebrovascular disease, PVD - Peripheral vascular disease, SCH - Subclinical hypothyroidism, CRP - C reactive protein.

Relationship between FT3 levels and peak troponin levels in AMI patients

When patients with low T3 were compared with euthyroid patients, there was no significant different in median (interquartile range) standardized troponin: 0.21 (-0.82-0.65) vs -0.01 (-0.78-0.88) ng/L, p=0.85. On multiple regression LT3S was not significantly associated with troponin levels after adjusting for other confounders (p=0.75).

Multiple regression was performed to assess whether T3 levels, as a continuous variable, was associated with standardized troponin levels. Included in the analysis were euthyroid and LT3S patients whereas SCH, SHyper and patients on LT4 therapy for hypothyroidism were excluded. Positive predictors of an elevated troponin were male gender (p=0.010), STEMI (p<0.001), a past history of ischaemic heart disease (p=0.001) and CRP levels (p=0.005) (Table 4-8). FT3 levels were not associated with peak troponin levels (p=0.151) (Table 4-8).

	Beta coefficient	95% confidence interval		P-value
Male gender	.148	.036	.259	.010
Age	.002	-.002	.007	.337
BMI	.006	-.003	.015	.214
Smokers	-.002	-.062	.059	.955
STEMI	1.084	.986	1.182	.000
FT3	-.047	-.111	.017	.151
Creatinine	.001	.000	.003	.065
IHD	.199	.087	.312	.001
DM	.014	-.118	.146	.834
HTN	.010	-.097	.117	.859
Hypercholestrolaemia	.029	-.085	.142	.619
CVD	-.067	-.292	.158	.561
PVD	.145	-.099	.390	.243
CRP	.003	.001	.005	.016

Table 4-8 Multiple regression to assess the relationship between T3 and standardised troponin levels at the time of acute myocardial infarction.

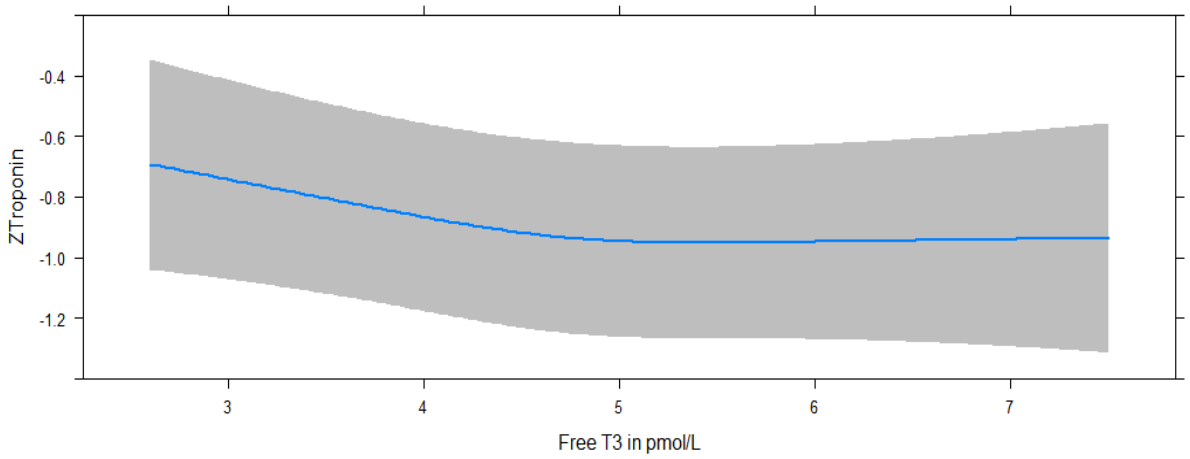


Figure 4-4 The relationship between FT3 and standardised troponin using the multivariable model, corrected for each individual variable in the multiple regression analysis. The relationship was linear and non-significant (p=0.151).

Troponin levels in patients on Levothyroxine therapy

Standardised median troponin levels in patients on LT4 therapy were not significantly lower than those not on LT4 (-0.1 v 0.01 ng/L; p=0.303). Multivariate regression analysis revealed no significant association between LT4 use and standardised troponin levels after adjusting for other confounders (p=0.90) (Table 4-9). Of the 113 patients on LT4 therapy, 26 (23%) patients had elevated and 6 (5.3%) had low serum TSH levels suggesting under and over replacement, respectively. The small number of participants with poorly controlled hypothyroidism precluded any further statistical assessments.

	Beta coefficient	95% confidence interval		P-value
Male gender	.102	.047	2.265	.024
Age	.003	.040	1.703	.089
BMI	.006	.031	1.494	.135
Smoking	-.004	-.003	-.146	.884
STEMI	1.080	.543	26.212	.000
LT4 use	.010	.002	.122	.903
Creatinine	.001	.041	2.003	.045
IHD	.189	.084	3.950	.000
DM	.043	.017	.809	.419
HTN	.011	.005	.241	.809
Hypercholestrolaemia	.032	.014	.676	.499
CVD	-.039	-.008	-.428	.669
PVD	-.023	-.004	-.216	.829
CRP	.003	.060	3.090	.002

Table 4-9 Variables predicting troponin levels post-acute myocardial infarction by euthyroid or hypothyroid (LT4 use) status.

4.1.4 Discussion

This study demonstrates a high prevalence of thyroid dysfunction in AMI patients confirming previous reports. In particular, SCH is the most frequent abnormal thyroid state noted with almost one in six individuals being diagnosed with this condition. The prevalence of LT3S and SHyper, on the contrary, are lower with approximately 1 in 100 patients being affected. Importantly, the study also provides information on predictors of these thyroid dysfunction states, which may be useful for clinicians managing patients with AMI.

Several observational studies have shown the prevalence of thyroid dysfunction in patients with AMI to be relatively high (Ertugrul *et al.*, 2011; Mukherjee *et al.*, 2018; Zahler *et al.*, 2019). The results of this analysis, for the first time as far as we are aware, add to this and identify risk factors for the common thyroid dysfunction states. Older individuals, females, those with higher TPOAb levels or higher creatinine concentrations had a higher risk of being classed as having SCH. In addition, patients who had their thyroid function samples obtained in the early hours of the morning also had a higher risk of being classed as having SCH. These findings are consistent with previous reports obtained from community-dwelling adults (Canaris *et al.*, 2000; Hollowell *et al.*, 2002; Vadiveloo *et al.*, 2011).

The NHANES III Study showed females to have higher TSH values whereas in a recent large population study, the TSH reference intervals were wider and higher in females compared to males (0.56–7.43 mIU/L vs. 0.62–6.57 mIU/L) (Hollowell *et al.*, 2002; Park *et al.*, 2018b). With regard to an increase in the prevalence of SCH with age, Vadiveloo et al in TEARS Study assessed thyroid function tests retrospectively in over 150000 subjects without thyroid disease and found the 97.5th median TSH centile to increase from 3.98, in subjects 30-40 years of age, to 5.94 in those above 90 (Vadiveloo *et al.*, 2013). The NHANES III and the cardiovascular health allstars study showed TSH levels to increase with age and be more significant after the age of 70 (Hollowell *et al.*, 2002; Waring *et al.*, 2012). However, this increase in the upper limit TSH interval with age may not represent true SCH, although international guidelines do not define SCH according to age and TSH cutoff values.

The relationship between TPO antibodies and SCH has been well studied and provides an additional explanation for the increased prevalence SCH in females. For example in the NHANES III Study, 60% of SCH cases were associated with elevated TPO antibodies and such levels were higher in females and increased with age whereas the Rotterdam Study showed individuals positive for TPO antibodies to have higher TSH levels and lower T4 levels than

those who had levels within the normal range (Hollowell *et al.*, 2002; Chaker *et al.*, 2016). In a further study by Surks et al, 67.4% of patients between 40-49 with SCH had elevated TPO antibodies whereas a 20 year follow up of the Whickham Survey demonstrated that progression of SCH to overt hypothyroidism was not only dependent on the baseline TSH but also on TPO antibody levels (Vanderpump *et al.*, 1995; Surks and Hollowell, 2007).

The results of this analysis have shown the diagnosis of subclinical thyroid disease and subsequent rates of normalisation of serum TSH levels in patients with AMI to be influenced by the time of sampling. Interestingly, the time of blood sampling from participants was a strong predictor of SCH with samples taken between 00:00 and 06:00 followed by samples between 18:00 and 24:00 being the strongest predictors of SCH. However, patients who had SCH diagnosed between 12:00 and 18:00 were more likely to remain in SCH on repeat testing. These results contest the previously held view that the time of sampling has very little impact on the width of the TSH reference range and suggest the time of sampling should be considered when evaluating whether a given TSH result is abnormal. The circadian variation of TSH, has previously been well established with the lowest TSH concentration in the afternoon and the TSH concentrations subsequently increasing after midnight (Andersen *et al.*, 2003). In an analysis of TSH values of over 400000 euthyroid individuals, there was a significant nocturnal TSH surge which resulted in a higher upper limit TSH reference interval with age indicating how the reference interval for TSH can vary by time of day (Ehrenkranz *et al.*, 2015). Similarly, another retrospective laboratory database interrogation of TSH data from more than 19,000 outpatient visits concluded that the prevalence of SCH reduced from 14.1% to 7.9% and that of SHyper increased from 6.8% to 7.9% between 7-8am and 2-3pm, respectively (Andersen *et al*., 2015). These previous studies combined with the present data suggests that the time of sampling should be considered when devising the reference range for TSH to avoid the inappropriate classification of individuals with thyroid dysfunction.

The present study demonstrated increased creatinine levels to be a significant predictor of SCH and low T3; further supporting the view that thyroid dysfunction may be related to renal function. The relationship between elevated TSH levels and kidney disease has previously been investigated with studies showing SCH and overt hypothyroidism to be strongly associated with increased creatinine levels and the progression to chronic kidney disease (Asvold *et al.*, 2011; Zhang *et al.*, 2018). Studies have also demonstrated a reduction in the decline in kidney function with levothyroxine therapy (Shin *et al.*, 2013; Lu *et al.*, 2016). A possible explanation for the observed relationship between renal impairment and underactive thyroid states include

diastolic dysfunction, reduced cardiac output and increased systemic resistance which all result in reduced renal perfusion (Klein and Ojamaa, 2001). Low T3 in kidney disease and AMI may be due to reduced deiodinase activity leading to less conversion of T4 to T3 in chronic disease states (Xu *et al.*, 2016).

Our data also assessed the relationship between thyroid dysfunction and troponin levels to see whether an elevated troponin may provide an explanation for the adverse outcomes seen in SCH and low T3 syndromes from previous studies (Friberg *et al.*, 2001; Iervasi *et al.*, 2003; Lazzeri *et al.*, 2012). For example, Iervasi and colleagues found patients with SCH (6.7% of the total cohort) and low circulating T3 levels (29.2%), after admission with cardiac disease, to have a higher risk of cardiovascular and all-cause mortality than euthyroid individuals (Iervasi *et al.*, 2007). Experimental studies have shown low circulating T3 to be associated with an increase in infarct size post AMI and restoration of T3 levels reduces progression to heart failure (Chen *et al.*, 2008; Forini *et al.*, 2011). In the present study, patients with SCH at the time of AMI had a higher serum troponin levels than euthyroid patients although this was not significant with multiple regression analysis. Furthermore FT3 levels were not associated with increased troponin levels when FT3 levels were first assessed as a categorical variable, comparing LT3 patients with patients who had normal T3 levels, and then as a continuous variable. Our results therefore show such worse outcomes in SCH and LT3 patients with AMI to be unlikely related to troponin levels or potential infarct size.

Another study of 2430 patients undergoing percutaneous coronary intervention showed patients on LT4 therapy for hypothyroidism had an increased incidence of major adverse cardiac events during follow-up in comparison to euthyroid patients. In the same study, individuals with poorly controlled hypothyroidism whilst on TH replacement similarly had higher cardiovascular events than people with serum TSH within the reference range on LT4 therapy (Zhang *et al.*, 2016). Our study demonstrated that nearly 30% of patients on levothyroxine had an abnormal TSH level indicating inadequate thyroid hormone replacement. This finding, has been previously reported, is important as both suppressed and high serum TSH have been associated with increased risk of cardiovascular disease (Flynn *et al.*, 2010). However, our analysis shows that in patients admitted with AMI, prior treatment for hypothyroidism does not impact on troponin levels. Therefore alternative mechanisms could potentially accelerate cardiovascular disease progression, in known hypothyroid patients, such as hyperlipidemia, endothelial dysfunction, systemic inflammation, thrombogenesis and cardiac dysfunction (Jabbar *et al.*, 2017).

The strengths of this study are the relatively large number of patients analysed, the prospective and uniform method of recruitment and assay analysis. For instance, samples were obtained at the first opportunity after presentation to hospital (so the impact of non-thyroidal illness was minimised) and prior to diagnostic coronary angiography (coronary angiographic dyes contain iodine in supra-physiological amounts that could affect thyroid function). Our analysis has several limitations too. Firstly, patients' thyroid status was determined based on a single thyroid function assessment within 24 hours of hospital admission (mostly immediately at the time of presentation). Although this process reduced heterogeneity and variability amongst each participating site, fluctuations of thyroid function were not taken into account by repeat testing. This may help explain, at least in part, the high proportion of patients with SCH and low number of patients with LT3 observed in our cohort. Secondly, the number of patients in certain subgroups (subclinical hyperthyroidism and low FT3) are limited and therefore no meaningful analyses could be performed. Nevertheless, to our knowledge, this is the largest cross-sectional study to date in which the complete thyroid profile including T3 has been assessed in patients presenting with an AMI. Finally, important clinical endpoints such as left ventricular function or infarct size determination via robust imaging techniques were not examined in this study. However, troponin release after AMI has been shown to be a strong predictor of outcomes and this study provides useful information regarding its association with thyroid function which was previously lacking (Matetzky *et al.*, 2000).

In conclusion, thyroid dysfunction is relatively common in patients admitted with AMI with SCH being observed in one in six individuals. Other thyroid dysfunction states such as SHyper and LT3S are relatively less frequent. Older individuals and those with higher creatinine levels are both likely to have SCH or LT3S, whereas women, and those with higher TPOAb levels and samples obtained in the early hours of the morning are more likely to be diagnosed with SCH. We have shown a high prevalence of SCH in AMI patients and this is not associated with increased myocardial damage as measured by increased troponin release. Finally, our analysis suggest that both the initial diagnosis of SCTD in patients with AMI and the proportion that subsequently normalise thyroid function is independently and significantly associated with time of sampling. This suggests the time of sampling should be considered when devising the reference range for TSH to prevent inappropriate classification of SCTD.

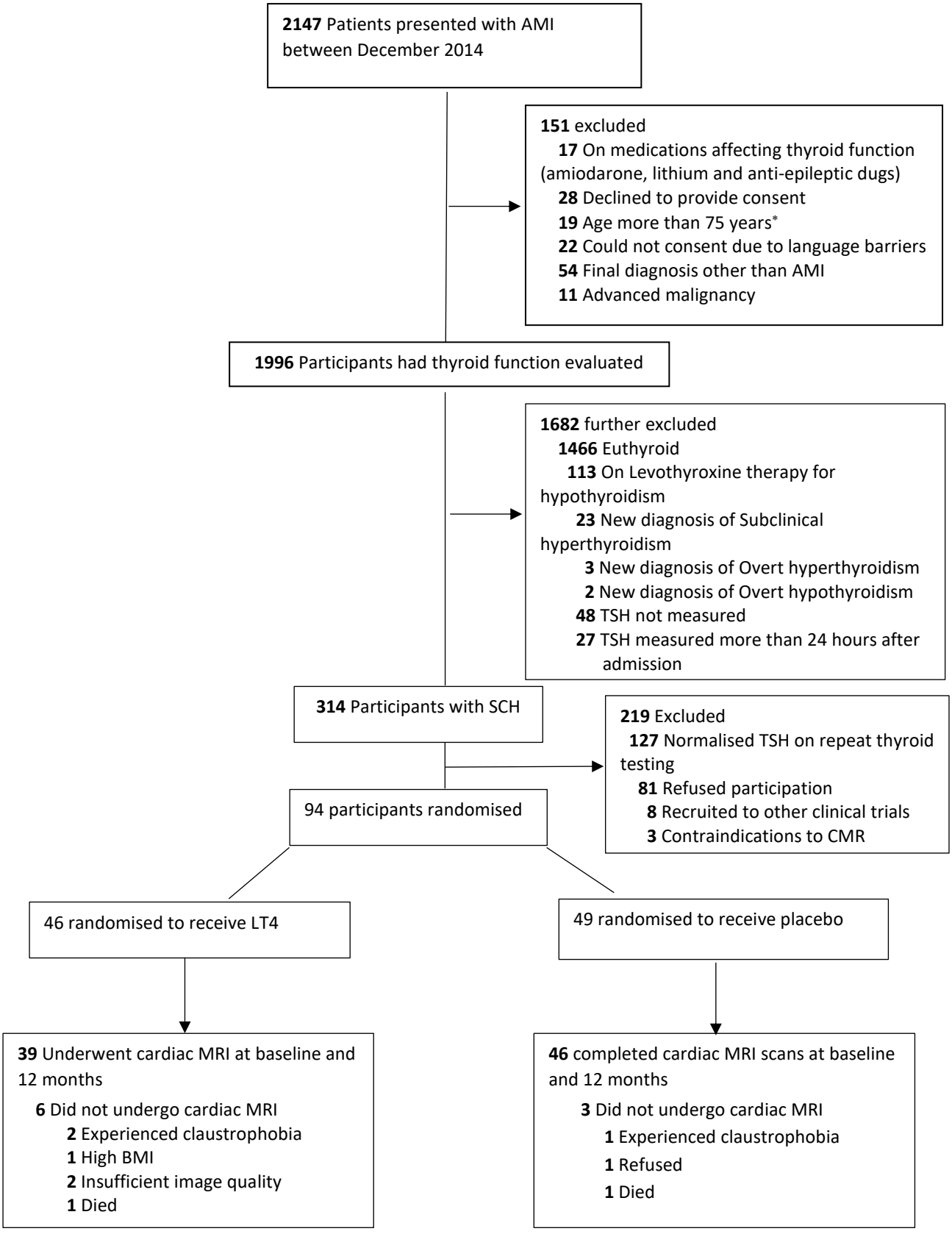
4.2 The effect of levothyroxine on clinical outcomes, cardiac function and infarct size using cardiac MRI

4.2.1 Baseline characteristics and peri-procedural data

For the RCT, patients had a baseline CMR scan after randomisation, within three weeks of their AMI, and then at 52 weeks post treatment. During the enrolment period, between January 2015 and December 2016, 2,147 patients were admitted to the participating hospitals with acute myocardial infarction and 1,996 consented to have their thyroid function screened (Figure 4-5). Of these, 314 (16%) participants were identified with subclinical hypothyroidism, and after assessments of trial inclusion and exclusion criteria, 95 participants were recruited to the trial with 46 randomised to the levothyroxine group and 49 to the placebo group. Ten randomised participants did not have either the baseline, final-visit, or both cardiac MRI scans (7 in the levothyroxine group and 3 in the placebo group), leaving 85 participants for the analysis of the primary outcome

Baseline data for both groups are shown in Table 4-10. Baseline characteristics were similar in both the LT4 and placebo groups indicating both were well matched. Both groups were similar with regard to age and past medical history which included cardiovascular risk. The median number of days of starting treatment post AMI was not significantly different in both groups, 17 (14.5-20) vs. 16 (14.0-19.5) in placebo, p=0.36. The thyroid profile was similar in both groups at the start of the study with a median TSH 5.75 (4.95-7.05) vs. 5.7 (4.7-7.3) in the placebo group, p=0.96. Furthermore the procedural data showed both groups were well matched in the treatment for their AMI which could have impacted on cardiac function (Table 4-11). For patients presenting with a STEMI, the median time from onset of symptoms to coronary intervention was 120mins (81-172) in the LT4 group and 137mins (71.5-209) in the placebo group, p=0.46. Both groups were similar with regard to the TIMI flow pre and post PCI, the use of drug eluting stents, the culprit coronary artery, the use of antiplatelet medications as well as the use of medications for secondary prevention.

Figure 4-5 Flow chart demonstrating recruitment to the ThyrAMI 2 Study.



	All (n=95)	LT4 (n=46)	Placebo (n=49)
Age (years)	63.5 (±9.5)	64.1 (±9.4)	62.9 (±9.7)
Male sex	72 (75.8%)	36 (78.3%)	36 (73.5%)
Systolic BP (mmHg)	126.6 (±16.8)	127 (±16.3)	126.3 (±17.5)
Diastolic BP (mmHg)	74.9 (±9.7)	76 (±8.56)	73.8 (±10.6)
Heart rate (bpm)	61.4 (±9.4)	60.1 (±9.5)	62.6 (±9.2)
BMI (kg/m^2)	28.7 (±4.2)	28.1 (±3.9)	29.2 (±4.4)
Current smoker	25 (26.3%)	14 (30.4%)	11 (22.9%)
Ex-smoker	37 (38.9%)	21 (45.7%)	16 (32.7%)
NSTEMI	29 (30.5%)	13 (28.3%)	16 (32.7%)
STEMI	65 (68.4%)	32 (69.6%)	33 (67.3%)
TSH (mU/L) study start*	5.7 (4.8-7.3)	5.75 (4.95-7.05)	5.7 (4.7-7.3)
FT4 pmol/L	14.7 (±2.1)	14.7 (±2.1)	14.6 (±2.5)
FT3 pmol/L	4.5 (±0.69)	4.6 (±0.76)	4.4 (±0.62)
Days since starting treatment*	17 (14.0-20)	17 (14.5-20)	16 (14.0-19.5)
NYHA 1	15 (15.8%)	7 (15.2%)	8 (16.3%)
2	74 (77.9%)	36 (78.3%)	38 (77.6%)
3	5 (5.3%)	2 (4.3%)	3 (6.1%)
Total Cholesterol (mmol/L)	4.8 (±1.39)	4.78 (±1.3)	4.83 (±1.2)
Creatinine (µmol/dl)	94.2 (±44.5)	93 (±36.5)	96.9 (±66.2)
Ischaemic heart disease	7 (7.4%)	3 (6.5%)	4 (8.2%)
Diabetes mellitus	18 (18.9%)	8 (17.4%)	10 (20.4%)
Hypertension	37 (38.9%)	18 (39.1%)	19 (38.7%)
Hypercholestrolaemia	27 (28.4%)	13 (28.3%)	14 (28.5%)
Cerebrovascular disease	5 (5.3%)	2 (4.3%)	3 (6.1%)

Table 4-10 Baseline characteristics comparing both the LT4 and placebo groups. The table shows there is no significant difference in each variable between both groups. Continuous variables are shown as mean (±SD) and median (IQR) whereas categorical variables as numbers (%).

*Non-normally distributed data, analysed after log-transformation with Student's t-testing

SCH – subclinical hypothyroidism, NSTEMI – non ST-elevation myocardial infarction, STEMI – ST-elevation myocardial infarction, TSH – thyrotropin, FT4 – free thyroxine, FT3 – free triiodothyronine.

	LT4 (n=46)	Placebo (n=49)
Symptom to PCI time(mins)*	120 (81-172)	137 (71.5-209)
Glycoprotein IIb/IIIa inhibitor	21 (45.6%)	20 (40.8%)
Timi pre-PCI grade		
Pre-PCI grade 0	10 (21.7%)	12 (24.5%)
Pre-PCI grade 1	5 (10.9%)	9 (18.4%)
Pre-PCI grade 2	18 (39.1%)	17 (34.7%)
Pre-PCI grade 3	13 (28.3%)	11 (22.4%)
Timi post-PCI grade		
Post-PCI grade 2	3 (6.5%)	2 (4.1%)
Post-PCI grade 3	43 (93.5%)	47 (95.9%)
Drug eluting stent use	40 (86.9%)	45 (91.8%)
Left main coronary artery	0	2 (4.1%)
Left anterior descending artery	11 (23.9%)	18 (36.7%)
Right coronary artery	22 (47.8%)	23 (46.9%)
Left circumflex artery	7 (15.2%)	5 (10.2%)
Other artery	6 (13%)	1 (2.0%)
Subsequent PCI	5 (10.9%)	9 (18.4%)
Aspirin	46 (100%)	49 (100%)
Second antiplatelet agent	46 (100%)	49 (100%)
Clopidogrel	13 (28.3%)	14 (28.6%)
Ticagrelor	12 (26.7%)	12 (24.5%)
Prasugrel	21 (46.7%)	23 (46.9%)
Beta blocker	43 (95.6%)	48 (97.9%)
ACE or ARB	44 (97.8%)	48 (97.9%)
Statin	46 (100%)	49 (100%)

Table 4-11 Periprocedural details comparing both the LT4 and placebo groups. Continuous variables are shown as mean (±SD) and median (IQR) whereas categorical variables as numbers (%).

Symptom to PCI time and Glycoprotein IIb/IIIa inhibitor use were for STEMI patients only.

*Non-normally distributed data, analysed after log-transformation with Student's t-testing

PCI – primary coronary intervention, ACEI-angiotensin - converting enzyme inhibitor; ARB - angiotensin receptor blocker; TIMI - Thrombolysis In Myocardial Infarction

4.2.2 TSH variability between both groups and the effect of levothyroxine on clinical outcomes and adverse events

The median TSH was significantly lower in the LT4 group after 1 year of treatment, TSH 1.8 (1.3-2.2) vs. 3.2 (2.7-4.2) in the placebo group, p=0.002 (Table 4-12). Throughout the study visits, the median TSH significantly decreased in the LT4 group with the TSH at visit V1 being 5.75 (4.9-7.1) vs. 1.8 (1.3-2.2) at V6, p<0.001. The mean FT4 was significantly higher in the LT4 group at the end of the study with a FT4 at visit 6 of 17.2±2.7 vs. 14.6±2.1 in the placebo group, p<0.001. There was no significant difference in the mean (±SD) FT3 between both groups at the end of the study, 4.7±0.6 vs. 4.8±0.6 in the placebo group, p=0.52. Furthermore, there was a significant increase in the LT4 dose prescribed at each study visit in the LT4 group starting from a median dose of 25 (25-25) mcg at V1 to 50 (50-75 mcg at V6, p<0.001.

With regard to serious adverse events (SAEs), there were 10 SAEs in 8 patients in the LT4 group and 17 SAEs in 10 patients in the placebo group (Table 4-13). The SAEs were divided into cardiovascular and non-cardiovascular events. In the LT4 group, there were 2 cardiovascular SAEs which included one patient having a NSTEMI needing a further stent and one patient developing new paroxysmal atrial fibrillation. In the placebo group there were 6 cardiovascular SAEs which included: a NSTEMI managed conservatively due to minor in stent restenosis, pulmonary oedema due to nephrotic syndrome flare, bilateral pitting oedema needing diuresis, a NSTEMI with small branch occlusion managed conservatively, dyspnoea needing admission which was secondary to ticagrelor, and a NSTEMI needing a further stent. Adverse events were compared between both groups using logistic regression. The independent predictors included age, gender, type of AMI and allocation to LT4/placebo. The cardiovascular and non-cardiovascular were not significantly different between both groups. Details of all the adverse events in both groups are outlined in Table 4-14.

		V1	V2	V3	V4	V5	V6
LT4	TSH mU/L	5.75 (4.95-7.05)	2.6 (1.8-3.5)	1.8 (1.4-2.3)	2.2 (1.6-2.9)	1.8 (1.4-2.3)	1.8 (1.3-2.2)
	FT4 pmol/L	14.7 (±2.1)					17.2±2.7
	FT3 pmol/L	4.5 (±0.69)					4.7±0.6
	Dose of levothyroxine, median (IQR), mcg/day	25	25 (25 – 25)	50 (25 – 50)	50 (25 – 68.8)	50 (25 – 75)	50 (50 – 75)
Placebo	TSH mU/L	5.7 (4.7-7.3)	3.4 (2.8-4.2)	3.8 (3.0-4.9)	3.9 (3.3-4.7)	3.8 (3-4.9)	3.2 (2.7-4.2)
	FT4 pmol/L	14.6 (±2.5)					14.6±2.1
	FT3 pmol/L	4.4 (±0.62)					4.8±0.6

Table 4-12 A comparison of the variation in TSH between both groups throughout the study visits and the mean dose of LT4 given during each visit. The median TSH decreased throughout the study with a subsequent increase in the LT4 dose in the LT4 group.

Values are shown as mean (±SD) or median(IQR).

TSH – Thyrotropin, FT4 – free thyroxine, FT3 – free triiodothyronine, IQR – interquartile range, SD – standard deviation.

	LT4 (n=46)	**Placebo (n=49)**	**Adjusted difference (95% confidence intervals)**	**P-value**
Cardiovascular events Moderate Severe	 12 (26.7) 2 (4.4)	 14 (28.6) 6 (12.2)	 OR 0.88 (0.33-2.32) OR 0.35 (0.07-1.99)	 0.79 0.19
Non-cardiovascular events Moderate Severe	 29 (64.4) 8 (17.8)	 24 (48.9) 11 (22.4)	 OR 1.33 (0.55-3.21) OR 0.73 (0.26-2.08)	 0.53 0.56
Death	1 (2.2)	1 (2.0)		0.95

Table 4-13 Adverse events and clinical outcomes reported by treatment allocation. Adverse events and clinical outcomes were compared using logistic regression.

Values are shown as numbers (%).

	LT4 group (n=46) SAEs	AEs	Placebo group (n=49) SAEs	AEs
Cardiovascular				
Atrial fibrillation	1		2	
Acute coronary syndrome	1		2	
Heart Failure			2	
Pedal edema			2	
Elective angiogram/PCI	2		1	
Bradycardia	3		2	
Left ventricular thrombus	1			
Postural drop in blood pressure	1			
Angina	3		3	
Low blood pressure	3		3	
Palpitations	2		1	
Vasovagal faint			3	
Left ventricular hypertrophy			1	
Dyspnea secondary to ticagrelor			2	
Nervous system				
Headaches	1	1		
Transient ischemic attack			1	
Essential tremor	1			
Peripheral neuropathy			1	
Sciatic back pain	1			
Respiratory				
Exacerbation of asthma	1		1	
Exacerbation of COPD			1	2
Lung malignancy	1			
Pneumonia			1	
Dyspnea other causes	1		1	1
Chest infection	3		2	
Incidental lung nodule	1			
Gastrointestinal				
Gastrointestinal bleed	1		2	
Duodenal ulcer			1	
Gastrointestinal malignancy	1			
Iron deficiency anaemia			1	1
Acute abdominal pain	1			
Incisional hernia pain	1			
Gastroenteritis	1			
Deranged liver function tests			1	
Reflux oesophagitis	3		5	
Vomiting	2			
Constipation	1			
Urogenital				
Urine infection	1			
Renal calculus	1			
Papillary bladder tumour			1	
Acute kidney injury	1			
Others				
Atypical chest pain	5		1	4
Musculoskeletal chest pain	2			1
Epistaxis			1	

Ankle tendon tear	1	
Raynaud's disease	1	
Skin rash		1
Gout	1	
Dog bite		1
Fatigue	1	
Peripheral claudication		2
Leg cramps		1
Common cold	2	1
Oral thrush	1	
Fractured clavicle		1
Low mood	1	1

Table 4-14 Details of adverse and serious adverse events by various systems in all randomized participants.

4.2.3 The effect of levothyroxine on cardiac function

At V1, the start of the study, the mean left ventricular ejection fraction (LVEF) in the LT4 and placebo groups were 51.29±9.14 vs. 54.01±7.9 in the placebo group, p=0.14 using the 2-sample t test (in %, mean±SD). Patients in the LT4 and placebo groups had no statistical difference in LVEF after 1 year of treatment, 53.8±9.67 vs. 56.09±7.91 in placebo group, p=0.237 (Figure 4-6).

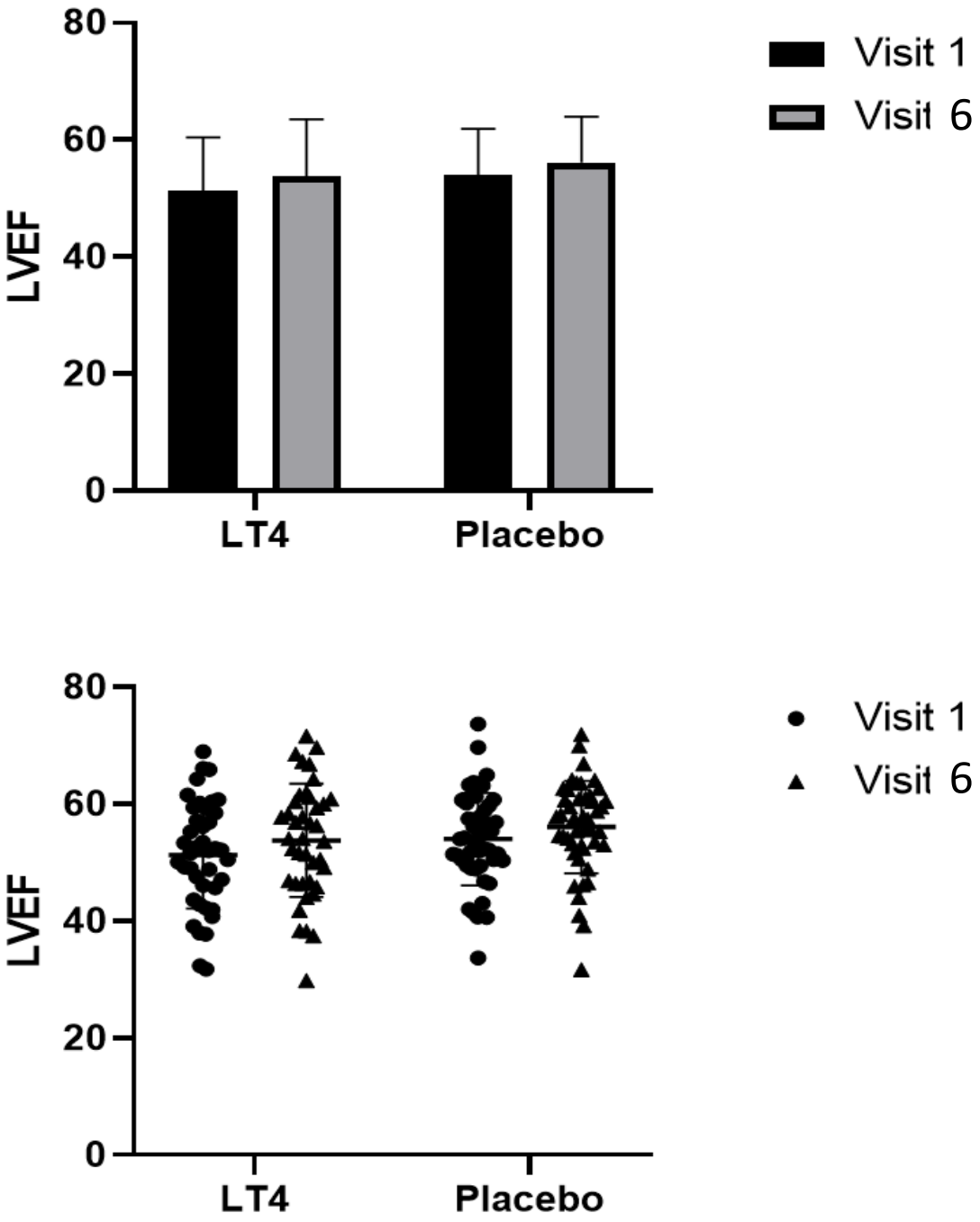


Figure 4-6 A comparison of mean LVEF between LT4 and placebo groups at baseline and after 1 year of treatment**.** The solid bars represent 1X standard deviation.

Multiple linear regression analysis was used to ascertain the relationship between LVEF at the end of study and allocation to either LT4 or placebo (Table 4-15). LVEF at the end of the study was the dependent variable whereas the various independent variables included gender, age, type of AMI, infarct territory, LVEF at visit 1 and finally allocation to either LT4 or placebo. The relationship between LVEF at the end of study and other continuous predictor variables (age and baseline LVEF) confirmed a linear relationship. Normal distribution of residuals was evaluated and confirmed by Q-Q plot (Figure 4-7). Multicollinearity was excluded as the variance inflation factor levels were below 2.5.

Allocation to LT4 was not a significant predictor (p=0.37) with the adjusted difference (95% confidence interval) in LVEF being 0.76% (-0.9 - 2.45) higher in the LT4 group compared to placebo. Gender (p=0.022), increasing age (p=0.004) and LVEF V1 (p<0.001) were the positive predictors of LVEF at end of the study. An increase in EF by 1% at V1 was associated with a 0.89% increase in EF at end of the study in the LT4 group.

	Beta coefficient	95% confidence interval		P-value
Age (years)	-0.114	-0.201	-0.026	0.004
Male gender	-2.383	-4.408	-0.357	0.022
STEMI	-0.194	-2.144	1.757	0.844
Infarct territory	-0.211	-1.167	0.745	0.662
LT4 vs placebo	0.764	-0.928	2.457	0.371
LVEF V1 (%)	0.891	0.788	0.994	0.000

Table 4-15 Variables associated with LVEF at the end of study.

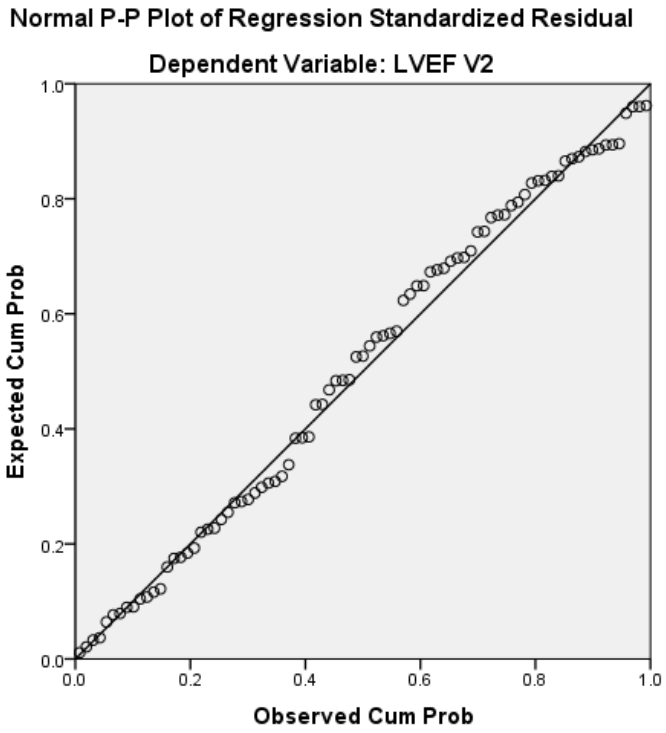


Figure 4-7 Normal distribution of residuals was evaluated and confirmed by Q-Q plot.

Sensitivity analysis assessed the interaction between allocation code and each of the variables of age group, sex, baseline LVEF, type of AMI, and baseline serum TSH, FT4 and FT3 and suggested that the result obtained was robust, and not due to differences in relevant baseline characteristics (Table 4-16). For each interaction variable, allocation to LT4 did not result in a significant improvement in LV function. For patients with an LVEF of <55%, LT4 was associated with a higher LVEF although the p for interaction was non-significant when including patients with an EF≥55%. Figure 4-8 shows the dot and whisker plot.

Variable	LT4 (n=39)	Placebo (n=46)	Δ LVEF at 52 weeks (95% CI)*, %	P for interaction
Sex				
Male	31	35	0.55 (-1.4 - 2.6)	0.68
Female	8	11	3.21 (-2.8 - 9.8)	
Age groups, years				
Below median (≤ 62.7)	18	25	0.64 (-1.9 - 3.2)	0.91
Above median (> 62.7)	21	21	0.37 (-2.5 - 3.3)	
Type of AMI				
STEMI	29	33	0.9 (-0.99 - 2.8)	0.92
NSTEMI	10	13	0.08 (-3.7-3.9)	
Baseline LVEF, %				
Normal (≥55)	13	19	-0.05 (-2.80 to 1.79)	0.44
Reduced (<55)	26	27	2.46 (0.28 to 4.60)	
Baseline serum TSH, mU/L				0.47
Below median (≤ 5.7)	17	24	0.35 (-2.96 - 3.66)	
Above median (> 5.7)	22	22	1.02 (-1.21 - 3.25)	
Baseline serum FT4, pmol/L				0.95
Below median (≤ 14.4)	22	21	0.36 (-1.91 - 2.63)	
Above median (> 14.4)	17	25	0.87 (-1.98 - 3.72)	
Baseline serum FT3, pmol/L Ω				0.39
Below median (≤ 4.5)	21	24	0.05 (-2.26 - 2.36)	
Above median (> 4.5)	15	20	1.65 (-0.89 - 4.18)	

Table 4-16 Effect of Levothyroxine compared with Placebo on LVEF according to prespecified subgroups.

* Adjusted for baseline age, sex, type of AMI, baseline LVEF and infract territory.

Ω Free T3 levels at baseline were available for 36 and 44 participants on LT4 and placebo, respectively.

LVEF – left ventricular ejection fraction; AMI – acute myocardial infarction; TSH – thyrotropin; FT4 – free thyroxine; FT3 – free triiodothyronine.

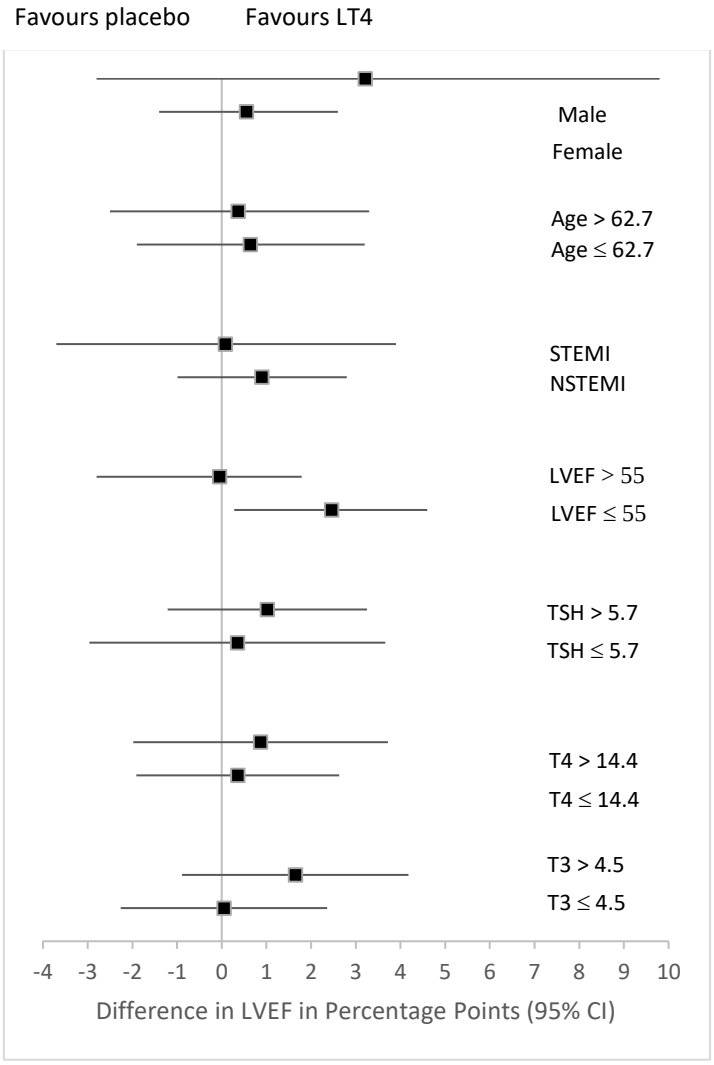


Figure 4-8 Dot and Whisker plot to show a comparison of LT4 and placebo treatment on LVEF according to baseline prespecified subgroups.

Other measurements derived from the CMR analysis are shown in (Table 4-17). Important measurements in addition to the EF were end-diastolic volume (EDV) and end-systolic volume (ESV). For each measurement, multiple regression was used to compare LT4 treatment with placebo with the independent predictors including age, gender, type of AMI, infarct territory, baseline results at visit 1, and allocation to LT4/placebo. Allocation to LT4 was not a significant predictor for each measurement with the adjusted difference (95% confidence interval) being non-significant.

For EDV/BSA at visit 2, allocation to LT4/placebo was not a significant predictor (p=0.078) (Table 4-18). The only positive predictor was EDV/BSA at visit 1 (P<0.001). For ESV/BSA at visit 2, allocation to LT4/placebo was not significant a predictor (p=0.11) (Table 4-19). The positive predictors were gender (p=0.041) and ESV/BSA at visit 1 (p<0.001). Although there was no significant difference between LT4 and placebo with regard to LV volumes, the analysis demonstrated a trend towards an improvement of volumes with LT4 therapy as shown by a decrease in both EDV and ESV.

	LT4		**Placebo**		**Adjusted difference (95% confidence intervals)**	**P value**
	Baseline	**V6**	**Baseline**	**V6**		
Secondary end points	**(n=45)**		**(n=49)**			
EDV/BSA ml/m^2	68.6 (±17.2)	66.7 (+-16.6)	70.5 (13.8)	70.4 (+- 13.1)	-4.27 (-9.03-0.49)	0.08
ESV/BSA ml/m^2	34.4 (±14.1)	31.1 (+-12.1)	32.9 (±9.9)	31.4 (+- 10.2)	-2.44 (-5.46-0.58)	0.11
Stroke index ml/ m^2	34.4 (±6.6)	34.9 (+-7.8)	37.6 (±7.9)	38.9 (+- 6.9)	-1.74 (-4.11-0.64)	0.15
Cardiac output L/min	3.9 (±0.7)	4.1 (+-0.87)	4.6 (±1.1)	4.6 (+- 0.96)	-0.14 (-0.47-0.18)	0.38
Cardiac index	2 (±0.3)	2.1 (+-0.43)	2.3 (±0.5)	2.3 (+- 0.38)	-0.07 (-0.22-0.09)	0.39

Table 4-17 Other measurements derived from CMR analysis. Outcomes were compared using multiple regression with the independent predictors used including age, gender, type of AMI, infarct territory, baseline results at visit 1, and allocation to LT4/placebo.

	Beta coefficient	95% confidence interval		P-value
Age (years)	-0.096	-0.343	0.151	0.442
Male gender	4.211	-1.253	9.675	0.129
STEMI	-1.359	-6.633	3.916	0.609
Infarct territory	1.508	-1.186	4.201	0.268
EDV/BSA ml/m2 V1	0.660	0.507	0.814	0.000
LT4 vs placebo	4.270	-0.489	9.030	0.078

Table 4-18 Variables associated with EDV/BSA at the end of the study.

EDV – End diastolic volume; BSA – body surface area.

	Beta coefficient	95% confidence interval		P-value
Age (years)	0.041	-0.115	0.196	0.606
Male gender	3.645	0.161	7.128	0.041
STEMI	-0.636	-3.997	2.724	0.707
Infarct territory	1.188	-0.509	2.885	0.167
ESV/BSA ml/m2 V1	0.731	0.605	0.856	0.000
LT4 vs placebo	2.438	-0.579	5.456	0.112

Table 4-19 Variables associated with ESV/BSA at the end of the study.

ESV – end systolic volume.

4.2.4 The effect of levothyroxine on cardiac parameters assessed by gadolinium enhancement

Overall 30 patients in the LT4 group and 30 patients in the placebo group received gadolinium. The main measurements were infarct/g, left ventricular mass/g (LVM/g) and % infarct. There was no significant difference in infarct/g at visit 2 between both LT4 and placebo groups with median values of 5.6 (1.31-15.2) vs 7.03 (1.03-13.3), p=0.82 using the Mann-Whitney test (median and IQR) (Table 4-20). The median % infarct at V2 was 5.8 (2.05-12.9) vs 8.4 (1.03-14.8), p=0.81. Multiple linear regression analysis was used to correct for each independent variable. The % infarct at V2 was used as the dependent variable whereas the independent variables were age, gender, type of MI, infarct territory, % infarct at V1 and allocation to LT4/placebo. The only significant predictor of % infarct at V2 was % infarct at V1, p<0.001 (Table 4-21). Allocation to LT4/placebo was a non-significant predictor, p=0.976 with the beta coefficient showing a difference of only 0.02% in infarct size between both groups. Table 4-22 shows the adjusted difference (95% confidence interval) for each infarct size measurement between both groups with the results being non-significant.

	LT4 (n=30)	Placebo (n=30)	P value
Infarct/g V1	7.97 (2.54-20.3)	8.96 (1.94-16.1)	0.92
LVM/g V1	106.4 (±33.4)	102.1 (±22.9)	0.56
% infarct V1	7.77 (2.65-14.7)	8.49 (1.7-16.2)	0.96
Infarct/g V6	5.6 (1.31-15.2)	7.03 (1.03-13.3)	0.82
LVM/g V6	99.2 (±29.9)	94.0 (±18.2)	0.60
% infarct V6	5.8 (2.05-12.9)	8.4 (1.03-14.8)	0.81

Table 4-20 A comparison of gadolinium enhancement in both the LT4 and placebo groups. Values are shown as mean (±SD) and median (IQR).

LVM – left ventricular mass.

	Beta coefficient	95% confidence interval		P-value
Age	0.022	-0.050	0.094	0.543
Male gender	0.921	-0.739	2.582	0.271
STEMI	-0.018	-1.753	1.718	0.984
Infarct territory	-0.465	-1.261	0.331	0.246
% infarct V1	0.808	0.742	0.874	0.000
LT4/placebo	-0.020	-1.377	1.336	0.976

Table 4-21 Variables associated with the % infarct at V2 using multiple regression. The only positive predictor was % infarct at V1, p<0.001. Allocation to LT4/placebo was non-significant with p=0.976.

Outcome	**LT4**		**Placebo**		**Adjusted difference (95% confidence intervals)**	**P value**
	Baseline	**V6**	**Baseline**	**V6**		
Infarct size	**(n= 30)**		**(n=30)**			
Infarct gms	7.9 (2.5-20.3)	5.6 (1.3-15.2)	8.9 (1.9-16.1)	7.0 (1.0-13.3)	0.23 (-1.31-1.77)	0.77
LVM gms	106.4 (±33.4)	99.2 (+- 29.9)	102.1 (±22.9)	94.0 (+- 18.2)	1.97 (-4.43-8.37)	0.54
Infarct %	7.8 (2.7-14.7)	5.8 (2.1-12.9)	8.5 (1.7-16.2)	8.4 (1.0-14.8)	0.02 (-1.34-1.38)	0.98

Table 4-22 Infarct size data compared between both groups using multiple regression.

LVM – left ventricular mass.

4.2.5 Discussion

The current RCT demonstrates no improvement in LV function in SCH patients treated with LT4 post AMI. The findings from the ThyrAMI study are important as:

I. This is the first RCT investigating the use of thyroxine in patients with SCH post AMI to assess an improvement in LV function.

II. Participants were on all recommended secondary prevention therapy and were chosen based on a strict eligibility criteria to minimise confounding variables.

III. We need to investigate further therapies to improve cardiovascular outcomes in patients with low TH levels post AMI, and this study should give investigators the confidence in the safety profile of using thyroid replacement therapy.

In this double-blind, randomised, placebo-controlled trial, treatment with LT4, albeit a low dose, for 52 weeks appeared safe but did not improve LV function in SCH patients presenting with AMI. The findings show there is no benefit of treating SCH patients with thyroxine post AMI and suggest further studies are needed to investigate alternative TH treatments in patients with a low TH state.

SCH, particularly when serum TSH is greater than 7 mU/L, is associated with a higher risk of developing cardiovascular disease and heart failure (Rodondi *et al.*, 2010; Kannan *et al.*, 2018). The presence of SCH in patients admitted with an acute cardiac event is associated with an up to 3.6-fold increase in cardiac mortality and a 2.3-fold increase in mortality (Iervasi *et al.*, 2007; Molinaro *et al.*, 2012). There is some evidence that thyroid hormone administration may be beneficial in ameliorating cardiac disease. In animal models of AMI, administration of thyroid hormone augments myocardial remodelling and improves LV function (Pingitore *et al.*, 2012). Individuals with SCH aged 65 years or older treated with LT4 have a 72% reduction in heart failure events (Rodondi *et al.*, 2008). Several other smaller trials have shown that treatment of SCH with LT4 improves LV function (Biondi *et al.*, 1999; Monzani *et al.*, 2001; Ripoli *et al.*, 2005). Thus, SCH following AMI is an important marker for poor outcome, and one that is suitable for a cost-effective intervention, if evidence of efficacy can be shown. However, it remains unknown if the relationship between SCH with poor prognosis in cardiac patients is causal or whether a raised serum TSH is a biomarker of the disease severity. To our knowledge, this is the first trial to have prospectively and systematically studied the effects of treatment of SCH with LT4 on LVEF compared with placebo, in addition to optimal medical treatment in

AMI patients. Our results demonstrate that treatment of SCH in AMI patients does not improve LV function and suggest that raised serum TSH may simply be a biomarker of worse prognosis.

Although LT4 treatment did not result in a significant improvement in LV function, there was a trend towards an improvement in LV volumes with a reduction in both EDV and ESV with treatment. Studies have demonstrated that a decrease in LVEF with subsequent increases in cardiac volumes represent adverse cardiac remodelling which is associated with worse cardiac outcomes (Konstam *et al.*, 2011). The present study therefore demonstrates a potential benefit of LT4 treatment in ameliorating pathological cardiac remodelling despite no improvement in LVEF.

One of the strengths of our study is we utilised robust imaging techniques to evaluate cardiac function. MRI is considered as the reference-standard technique to assess cardiac mass, volumes and function. Only one previous trial utilised MRI in SCH individuals to assess cardiac function, and, in this non-randomised trial, LT4 treatment in women with no previous cardiac history improved LVEF (Ripoli *et al.*, 2005). The study also addresses an important uncertainty and previous concern regarding the potential safety profile of using TH replacement therapy in a post MI setting. In the study, TH replacement therapy was safe with a low risk of potential adverse affects in such a setting. This is shown by no SAE within the study group being related to levothyroxine as well as no patients developing a sinus tachycardia or any tacharrhythmias such as atrial fibrillation. The findings from this study support three previous studies which have used intravenous triiodothyronine in patients undergoing cardiovascular surgery to show no major adverse side effects such as arrhythmias (Klemperer *et al.*, 1995; Klemperer *et al.*, 1996; Mullis-Jansson *et al.*, 1999). Therefore the findings from the present study sets more clarity on the safety profile of TH for future studies involving patients with cardiovascular disease, in particular AMI.

Despite the current negative findings, an increase in mortality in patients with a low TH state after AMI is a testament to the need for ongoing studies to reduce vascular event rates in this specific group, to levels seen in euthyroid patients. Some of the reasons which may explain the current findings may be due to the potential limitations in the study. Firstly, it can be argued that patients recruited to the study did not have a low enough thyroid function to warrant treatment in the first place. Patients with a milder form of SCH with at least one TSH value below 10.0 mU/L were recruited. This group constitutes to the majority of SCH patients and where the greatest uncertainty with regards to efficacy of treatment prevails. The results of the

present study support those guidelines which recommend treatment only if the serum TSH level is more than 10 mU/L (Pearce *et al.*, 2013). Second, the time window between coronary occlusion and initiation of LT4 therapy may have been prolonged (median of 17 days). This was considered necessary as we only wanted to recruit patients with sustained SCH in this trial. Third, due to the unknown safety profile of LT4 in this group of patients, LT4 was initiated at a small dose (25 mcg daily) and titrated over several weeks. The median dose of LT4 at the end of the study (50 mcg daily) is lower than the starting doses used in other trials that have demonstrated benefit of treatment on endothelial function and lipid profiles (Monzani *et al.*, 2004; Razvi *et al.*, 2007). This may have reduced the therapeutic efficacy of LT4 on cardiac remodelling and ventricular function, but, serum TSH levels in the LT4 group were within the target range (0.4 – 2.5 mU/L) and FT4 levels were higher at the end of the trial compared to baseline as well as the placebo group. Fourth, nearly 40% of patients recruited in this trial had evidence of preserved LV function and mean LVEF in both groups suggested only mild impairment. It is interesting that the pre-specified subgroup analysis in patients with LVEF <55% suggests that levothyroxine may be associated with some benefit. Future research will be required to evaluate efficacy in this group of patients.

Finally, the type of thyroid hormone used may have been ineffective. Experimental data has shown that in a post MI setting, a decrease in D1 and D2 activity and an increase in the D3 activity leads to a decreased conversion of T4 to T3 and T3 downregulation (Olivares *et al.*, 2007). In a large clinical study by Peeters eta al, increased D3 activity and low TH levels in pathological states was shown to occur locally within the heart and at the systemic level (Peeters *et al.*, 2003). Therefore the bioavailability of cardiac T3 could have been low in the study leading to the reduced cardiac effects and subsequent benefit of TH replacement. Owing to the impaired conversion of T4 to T3 in pathological states, T4 might not be the most suitable first line therapy in such conditions. These findings suggest further studies are needed to investigate alternative thyroid hormone treatments in patients with a low thyroid hormone state. In one recent open label by Iervasi et al, 37 patients post AMI with low T3 were investigated in which 19 patients received a T3 analogue called sodium liothyronine, which was given orally in liquid form. The 19 patients were compared with 18 untreated patients. The results showed no difference in LVEF, volumes or LGE enhancement between both groups at 6 months using CMR. This was only a pilot study with the initial study being powered to recruit more than 100 patients to detect a significant difference (Pingitore *et al.*, 2019).

Although it can be argued the study may have been underpowered, patients treated with LT4 only had a 0.76% higher EF compared to the placebo group. Even with a higher recruitment number, such a difference may have been statistically significant but would not have had any clinical significance as such a percentage increase is not associated with any clinical benefit. It is highly likely that recruiting more patients would not have changed the effect size of LT4 from the one observed (0.76%) although it may have reduced the uncertainty around this value.

In summary, among AMI patients with SCH, LT4 treatment was shown to be safe but did not improve LVEF. A role for LT4 or other thyroid hormone treatments in more severe forms of subclinical hypothyroidism cannot be ruled out.

4.3 The effect of levothyroxine on thrombus area, viscoelastography and clot kinetics, platelet reactivity and endothelial function

4.3.1 The effect of levothyroxine on thrombus area

In total, 32 patients in the LT4 group and 32 patients in the placebo group completed the Badimon chamber experiments before and after treatment. All patients were on aspirin and a second antiplatelet in the form of a P2Y12 inhibitor which included either clopidogrel, ticagrelor and prasugrel.

At V1, there was no significant difference in the median (IQR) thrombus area, in μ^2 per mm, between both the LT4 and placebo groups with values of 9906 (7206-13628) vs. placebo 9388 (7595-16631) p=0.75, 95% CI of the difference between the two groups was -2286 - 4186. At V2, there was no significant difference in the median thrombus area between both groups with values of 9668 (6712-13364) vs. placebo 9413 (7813-13621) p=0.82, 95% CI of the difference between the two groups being -2007 - 2542 (Figure 4-9). In view of the skewed nature of the data, values were log transformed for statistical analysis. In summary, although the thrombus burden decreased between visits 1 and 2 in the LT4 group, there was no difference in the thrombus area between both the LT4 and placebo groups.

Both groups were compared to see whether patients in the LT4 group may have had a lower thrombus burden depending on the specific P2Y12 inhibitor. The results showed that irrespective of the antiplatelet, there was still no significant difference in median thrombus area between both groups (Figure 4-10).

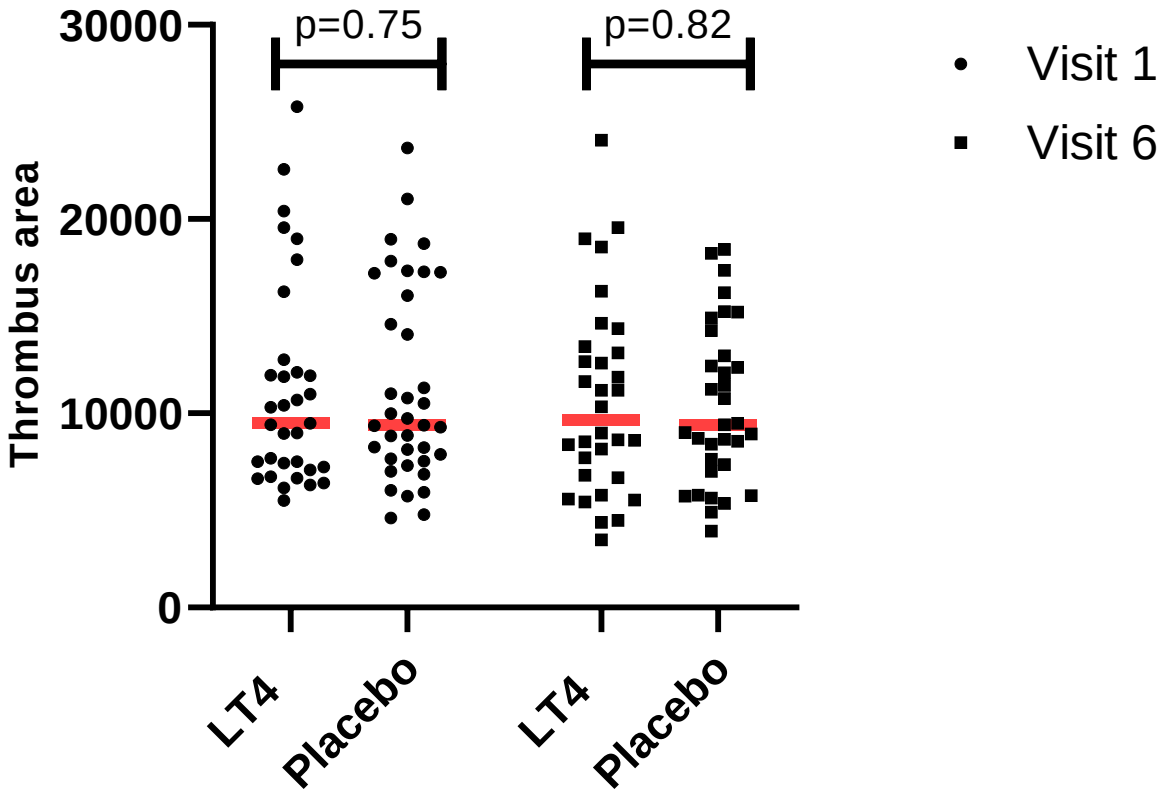


Figure 4-9 Individual plots for thrombus area in the LT4 and placebo groups before and after treatment. The solid lines represent the median.

	LT4 (n=32)	Placebo (n=32)	P-value
Clopidogrel	n=6 11758 (6750-16526)	n=6 11278 (5831-12781)	0.88
Ticagrelor	n=9 7603 (4986-16663)	n=7 8879 (4140-15010)	0.65
Prasugrel	n=17 9133 (6759-13256)	n-19 9658 (7737-14893)	0.88

Table 4-23 A comparison of median thrombus area between both the LT4 and placebo groups determined by the type of P2Y12 antiplatelet, at the end of the study visit (V6). For each antiplatelet, there was no significant difference in thrombus area between the two groups.

Values are shown as median (IQR).

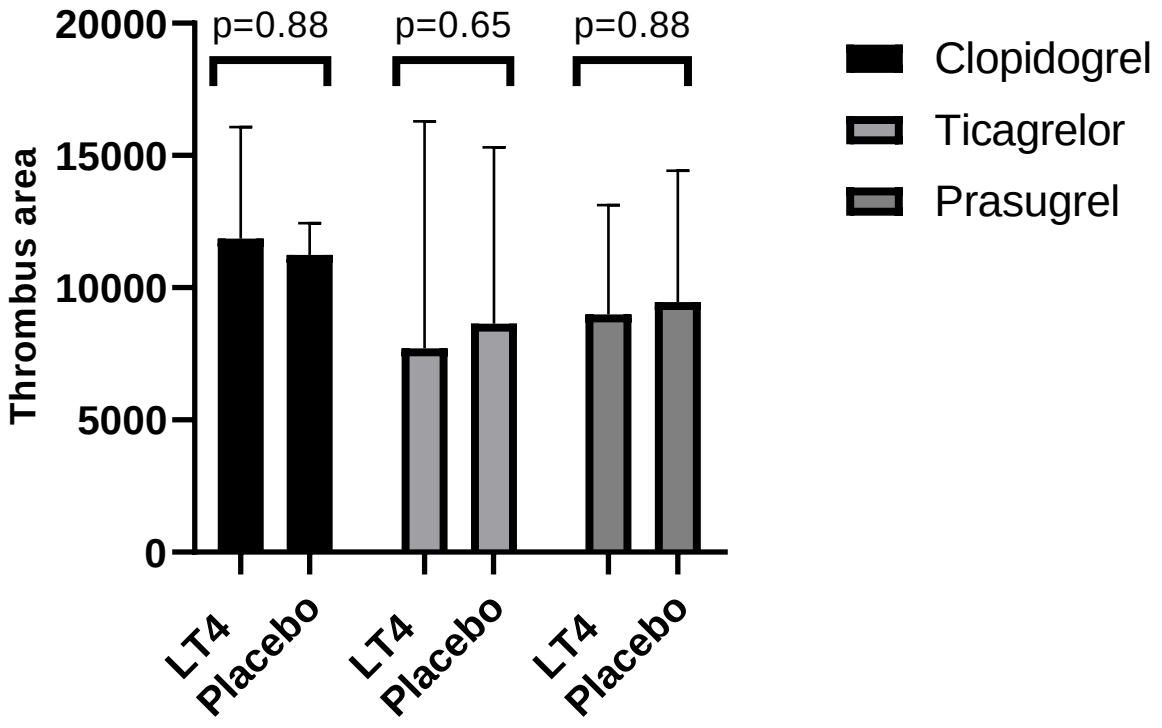


Figure 4-10 A difference in median total thrombus area between patients on LT4 and placebo after treatment (V6) determined by the specific P2Y12 antiplatelet. Overall, for each P2Y12 antiplatelet, there was no significant difference in thrombus burden when comparing both groups at the end of the study. The solid bars represent the IQR.

Multiple linear regression analysis was used to ascertain the relationship between thrombus area at visit 2 and allocation to either LT4 or placebo. Thrombus area at visit 2 was the dependent variable whereas the various independent variables included gender, age, type of AMI, smoking status, type of P2Y12 as the second antiplatelet, total mean thrombus area at visit 1 and finally allocation to either LT4 or placebo. Allocation to LT4 was not a significant predictor (p=0.56) with the adjusted difference (95% confidence interval) thrombus area being 710 μ^2 per mm (-1748 - 3170) higher in the LT4 group compared to placebo as shown from the beta coefficient. The only positive predictors of an increased mean thrombus area at V2 was smoking status with smokers having a higher thrombus burden (Table 4-24).

	Beta coefficient	95% confidence interval		P-value
Age	-40.771	-189.670	108.127	0.585
Male ender	885.368	-2130.906	3901.642	0.558
STEMI	-101.281	-3371.220	3168.658	0.951
Smokers	2057.114	-542.120	3572.108	0.009
Mean V1 µ2/mm	-0.039	-0.217	0.140	0.665
P2Y12 inhibitor	-601.867	-2467.478	1263.744	0.520
LT4 vs placebo	710.984	-1748.330	3170.299	0.564

Table 4-24 Variables associated with mean thrombus area after treatment. Type of P2Y12 antiplatelet and allocation to either LT4/placebo were non-significant predictors.

Sensitivity analysis assessed the interaction between allocation code and each of the variables of sex, age, type of AMI, baseline thrombus area, and baseline serum TSH, and suggested that the result obtained was robust, and not due to differences in relevant baseline characteristics. For each interaction variable, allocation to LT4 did not result in a significant improvement in thrombus area (Table *4-25*).

Variable	**LT4 (n=32)**	**Placebo (n=32)**	**Δ Thrombus area at 52 weeks (95% CI)*, %**	**P for interaction**
Sex				
Male	27	24	86.2 (-567 - 739)	0.61
Female	5	8	-1237 (-4924 - 2450)	
Age groups, years				
Below median (≤ 62.7)	16	20	-10.2 (-1274 - 1254)	0.84
Above median (> 62.7)	16	12	140 (-817 - 1098)	
Type of AMI				
STEMI	21	20	10.8 (-810 - 831)	0.78
NSTEMI	11	12	-8.9 (-2230-2212)	
Baseline thrombus area, µ2 per mm				
Below median (≤ 9491)	18	15	-49.4 (-988 - 1086)	0.82
Above median (> 9491)	14	17	-1523 (-5512 - 2465)	
Baseline serum TSH, mU/L				
Below median (≤ 5.7)	14	17	-2058 (-5421 - 1305)	0.058
Above median (> 5.7)	18	15	1781 (-2000 - 5564)	

Table 4-25 Effect of LT4 treatment compared with Placebo on thrombus area according to prespecified subgroups.

* Adjusted for baseline age, sex, type of AMI, smoking status and baseline thrombus area.

4.3.2 The effect of levothyroxine on platelet reactivity

Platelet reactivity was assessed using VerifyNow in 32 patients taking LT4 and 32 patients taking placebo to measure the effects of aspirin and P2Y12 inhibitors on platelet inhibition. On treatment platelet hyperactivity to aspirin, ARU, was not statistically different between both the groups at the study start (ARU, mean±SD: LT4 436±85.4 vs. placebo 412±72.3, p=0.19) and end of the study (ARU, mean±SD: LT4 452±85.1 vs. placebo 441±79.4, p=0.57) (Figure 4-11). 9 patients in the LT4 group and 8 patients in the placebo group were hyporesponders to aspirin using a cutoff value of 495 (p=0.77). On treatment platelet hyperactivity index to P2Y12 inhibition, PRUz, showed no statistical significance between LT4 and placebo patients at the study start with a median (IQR) of 50 (13-88.5) vs. placebo 39 (12-60), p=0.54 and the study end 74 (25-114) vs. placebo 54 (26-106), p=0.63 (Figure 4-12). In view of the data being non-normally distributed for both groups, the data was log transformed prior to analysis. Percentage platelet inhibition as calculated by using the manufacturer's formula for P2Y12 was 66% in LT4 group and 65% in placebo group (p=0.92). Using a PRUz cut off ≥ 240 units, no patients in either group were "hyporesponders" to P2Y12 inhibition. PRUz values did not correlate with thrombus area from the Badimon chamber (rho -0.02, p=0.91) (Figure 4-13). There was also no correlation between thrombus area and ARU values (rho 0.18, p=0.14) (Figure 4-14).

Both groups were compared to see whether patients in the LT4 group may have had a higher platelet inhibition dependent on the specific P2Y12 inhibitor. The results showed that irrespective of the antiplatelet, there was still no significant difference in on treatment platelet reactivity to P2Y12 inhibition, PRUz, between both the LT4 and placebo groups at the end of the study visit (Figure 4-15).

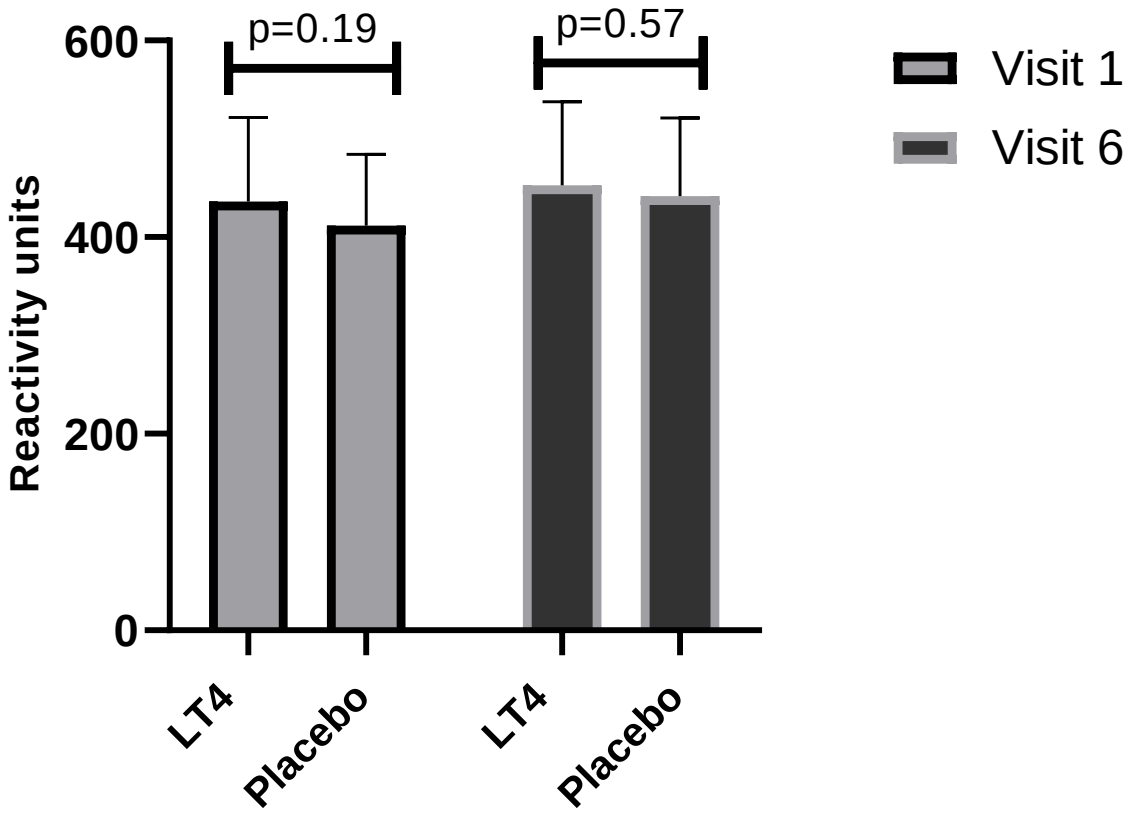


Figure 4-11 Mean ARU in the LT4 and placebo groups at both study visits. ARU represents the platelet reactivity to aspirin. The solid bars represent 1X standard deviation.

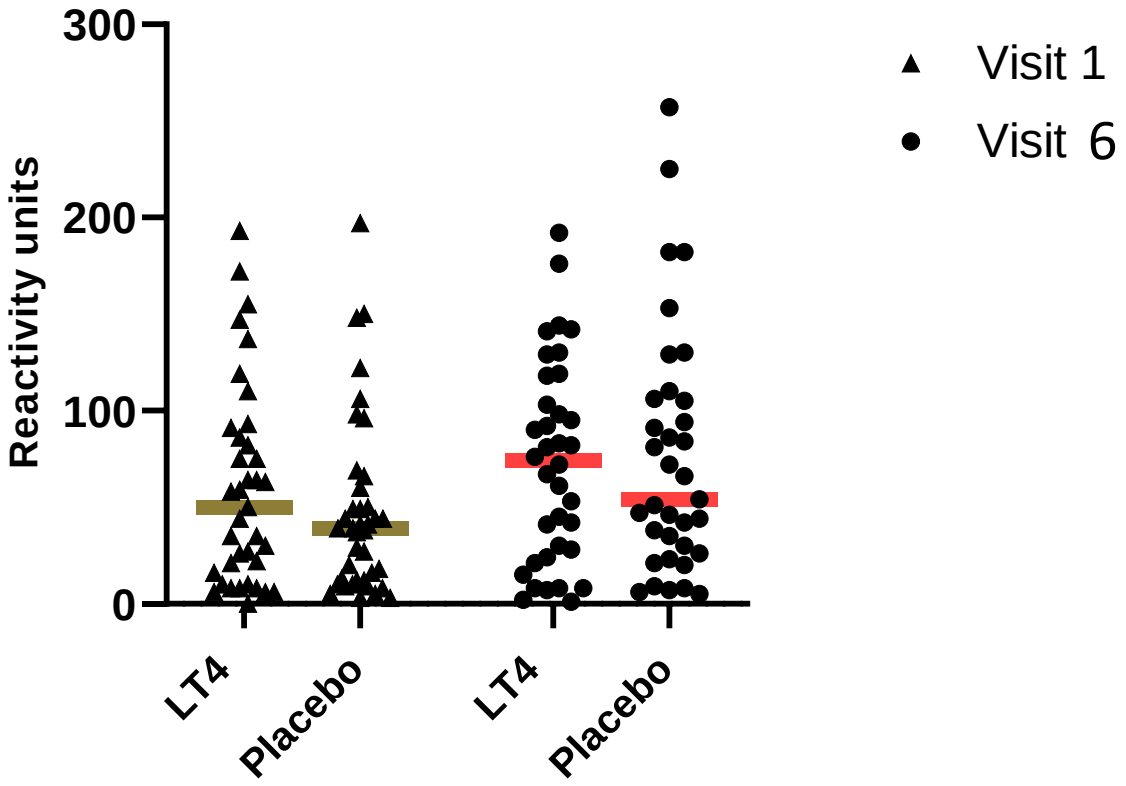


Figure 4-12 PRUz values for patients in the LT4 and placebo groups before and after treatment. Baseline and post treatment values in both groups were not statistically different. Solid lines represent the median.

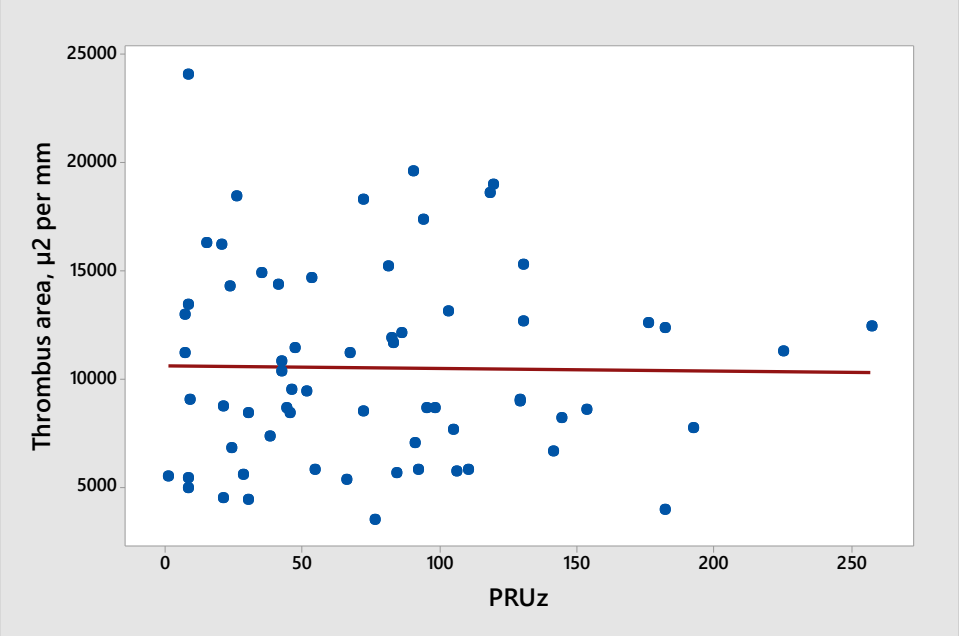


Figure 4-13 Correlation between thrombus area and VerifyNow® PRUz values in both groups combined at the end of the study. The correlation was not significant (rho -0.02, p=0.91).

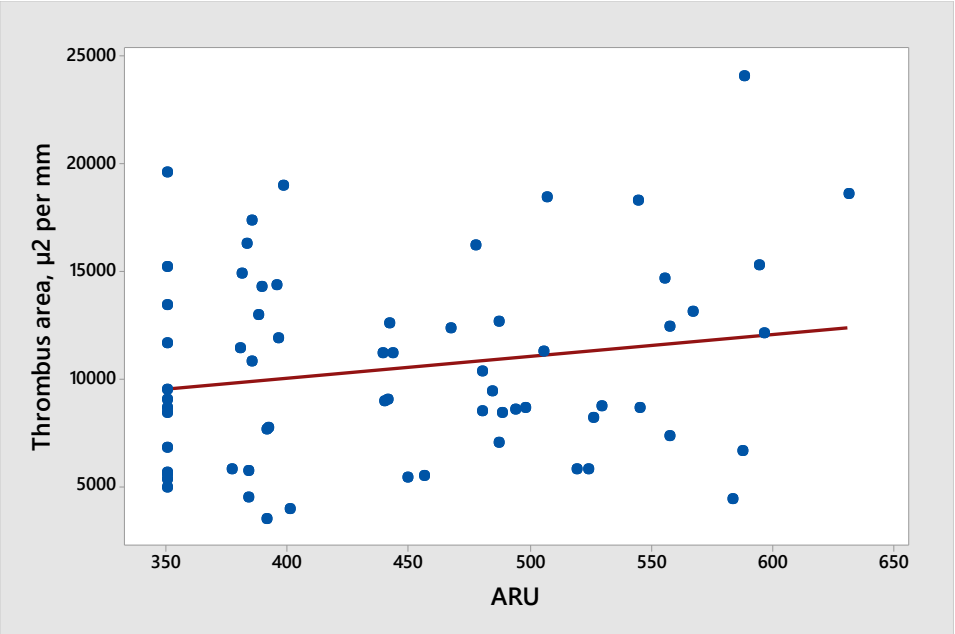


Figure 4-14 Correlation between thrombus area and VerifyNow® ARU values in both groups combined at the end of the study. The correlation was not significant (rho 0.18, p=0.14).

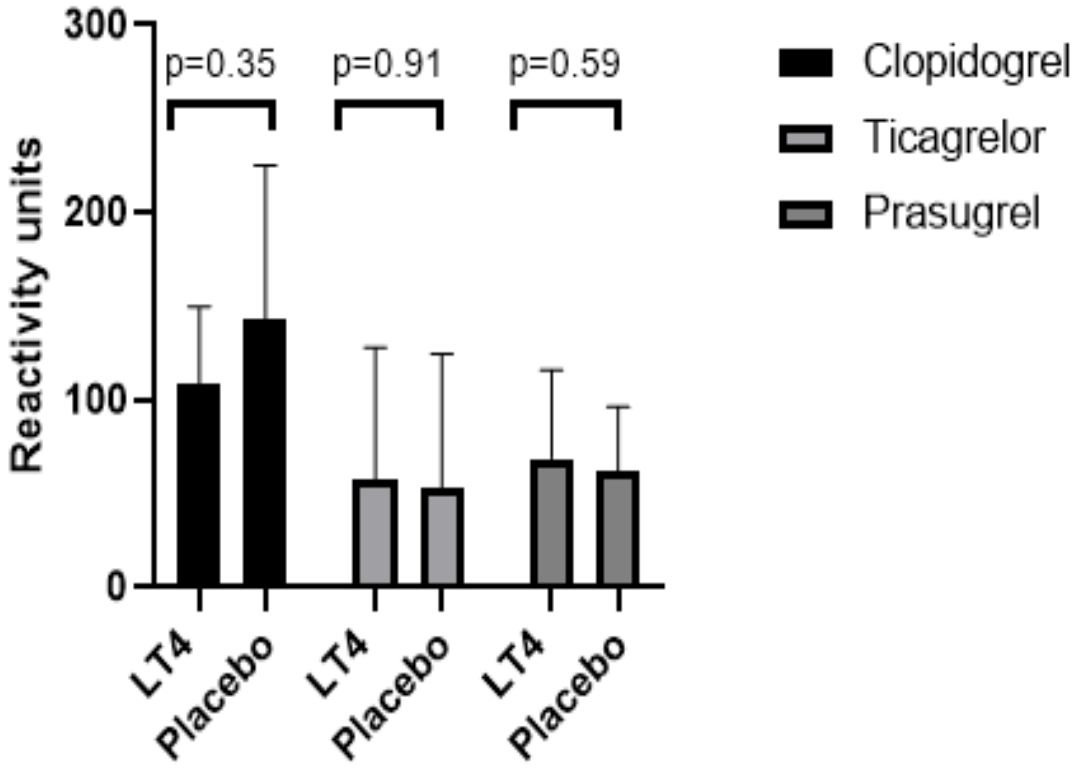


Figure 4-15 A difference in median PRUz between patients on LT4 and placebo at 52 weeks after treatment determined by the specific P2Y12 antiplatelet agent. The solid bars represent the IQR.

Multiple regression was used to ascertain the relationship between on treatment platelet reactivity to aspirin and P2Y12 inhibition at visit 2, as measured by ARU and PRUz, and allocation to either LT4 or placebo. ARU and PRUz at visit 2 were used as the dependent variable in separate analyses whereas the various independent variables included gender, age, type of AMI, smoking status, ARU or PRUz at visit 1 and finally allocation to either LT4 and placebo (allocation code). For on treatment platelet reactivity to P2Y12 inhibition, the type of P2Y12 inhibitor was also used as an independent variable.

For on treatment platelet reactivity to aspirin, ARU, allocation to LT4/placebo was a non-significant predictor (p=0.719) with the adjusted difference (95% confidence interval) of 7.3 ARU units (-47.4-32.9) lower in the LT4 group (Table 4-26). For on treatment platelet reactivity to P2Y12 inhibition, PRUz, allocation to LT4/placebo was a non-significant predictor with p=0.395 and a beta coefficient showing a difference of 9.9 (-13.2-33) PRUz units (Table 4-27). The only positive predictors of platelet inhibition were PRUz at V1 (P<0.001) and type of P2Y12 inhibitor (p=0.003).

	Beta coefficient	95% confidence interval		P-value
Age (years)	1.319	-.921	3.559	0.244
Male gender	-47.304	-95.776	1.168	0.056
STEMI	7.505	-40.388	55.398	0.755
Smokers	-2.615	-27.286	22.056	0.833
ARU V1	0.139	-0.120	0.398	0.288
LT4 vs placebo	-7.265	-47.390	32.861	0.719

Table 4-26 Variables associated with on treatment platelet reactivity to aspirin inhibition after treatment.

	Beta coefficient	95% confidence interval		P-value
Age (years)	0.197	-1.082	1.477	0.759
Male gender	-19.798	-47.738	8.142	0.162
STEMI	-11.594	-42.591	19.403	0.457
Smokers	-11.369	-25.436	2.699	0.111
P2Y12 inhibitor	-25.034	-41.185	-8.884	0.003
PRUz V1	0.619	0.390	0.848	0.000
LT4 vs placebo	9.890	-13.210	32.991	0.395

Table 4-27 Variables associated with on treatment platelet reactivity to P2Y12 inhibition after treatment.

4.3.3 The effect of levothyroxine on viscoelastography and clot kinetics

Standard TEG parameters (e.g. R, K, MA to kaolin and CI) were compared in both groups. The maximum amplitude to kaolin (MA-CK) and clot index (CI), both a measure of clot strength, significantly decreased in both the LT4 and placebo groups after 1 year of treatment (Table 4-28). However when the MA-CK and CI parameters were compared between both groups after 1 year, there was no significant difference (Table 4-29). At baseline the MA-CK was 67.8±5.4 vs. 66.7±4.9 in the placebo group, p=0.68 whereas after 1 year of treatment the MA-CK was 61.5±5.4 vs. 58.9±12.4 in the placebo group, p=0.24. After 1 year of treatment, there were no significant differences in the other TEG parameters between both groups (Table 4-29). The % of clot lysis at 30 minutes did not significantly decrease in each group between both visits and furthermore there was no significant difference in clot lysis between both groups at the end of the study.

Platelet mapping studies showed no significant reduction of maximum viscoelastic strength of thrombus after treatment with a P2Y12 inhibitor, in both the LT4 group and placebo groups, as measured by maximum amplitude (MA-ADP, in mm) upon stimulation by 10µl of ADP (30±14.2 vs. 26.1±11.4, p=0.07 in LT4 and 29.8±16.9 vs. 26±15.9, p=0.17 in placebo) (Table 4-28). Response to aspirin improved significantly in both group of patients with a reduction in maximum amplitude of the thrombus (MA-AA, in mm) upon stimulation with 10µl of arachidonic acid (21.7±16.4 vs. 13.5±12.2, p=0.002 in LT4 and 18.9±13.8 vs. 13.4±11.8, p=0.0014 in placebo). However when the MA-ADP and MA-AA were compared between both groups at visit 2, there was no significant difference (Table 4-29).

	LT4 n= 32			Placebo n= 32		
	Baseline	1 year after therapy	P value	Baseline	1 year after therapy	P value
R, min	5.5±1.2	5.6±1.0	0.73	5.2±1.5	5.4±1.7	0.55
K, min	1.2±0.3	1.4±0.3	0.002	1.1±0.3	1.2±0.9	0.59
Maximum Amplitude (MA-CK), min	67.8±5.4	61.5±5.4	<0.001	66.7±4.9	58.9±12.4	0.001
Clot Lysis 30min, %	95.9±2.7	97.1±3.4	0.096	95.8±3.5	95.7±4.4	0.91
Clot Index (CI) *	1.5 (0.6-2.5)	0.65 (-0.18-1.9)	0.001	1.7 (0.7-3.3)	0.7 (-0.5-1.7)	0.001
MA-AA, mm*	18.4 (8.6-34.5)	9.1 (6-14.4)	0.002	15 (8.3-26.2)	9 (4.8-18.1)	0.014
MA-ADP, mm	30±14.2	26.1±11.4	0.07	29.8±16.9	26±15.9	0.17
% platelet inhibition to AA	86.2±15.3	88.1±18.5	0.51	85.6±16.2	86.5±19.2	0.79
% platelet inhibition to ADP	69.8±18.7	65.3±18.7	0.13	63.4±18.3	64.3±17.1	0.76

Table 4-28 Standard TEG measurements with citrated kaolin in both LT4 and placebo groups at baseline and after therapy. Within each group, values were compared using the paired t-test.

Values are shown as mean (±SD) or median (IQR).

*Non-normally distributed data, analysed after log-transformation with Student t-testing.

MA – Maximum amplitude; AA – Arachidonic acid; ADP - Adenosine diphosphate.

R: Time taken till initial fibrin formation.

K: Time taken to reach a fixed level of clot strength.

MA-CK: Maximum viscoelastic strength of the thrombus by kaolin.

MA-AA: Maximum viscoelastic strength of the thrombus by arachidonic acid.

MA-ADP: Maximum viscoelastic strength of the thrombus by adenosine diphosphate.

	LT4 (n=32)	Placebo (n=32)	P value
R, min	5.6±1.0	5.4±1.7	0.51
K, min	1.4±0.3	1.2±0.9	0.37
Maximum Amplitude, min	61.5±5.4	58.9±12.4	0.24
Clot Lysis 30min, %	97.1±3.4	95.7±4.4	0.14
Clot Index *	0.65 (-0.18-1.9)	0.7 (-0.5-1.7)	0.99
MA –AA, mm *	9.1 (6-14.4)	9 (4.8-18.1)	0.97
MA-ADP, mm	26.1±11.4	26±10.6	0.97
% platelet inhibition to AA	88.1±18.4	86.5±19.2	0.72
% platelet inhibition to ADP	65.3±18.7	64.3±17.3	0.83

Table 4-29 A direct comparison of TEG and platelet mapping parameters between both LT4 and placebo groups after treatment.

Values are shown as mean (±SD) or median (IQR).

*Non-normally distributed data, analysed after log-transformation with Student t-testing.

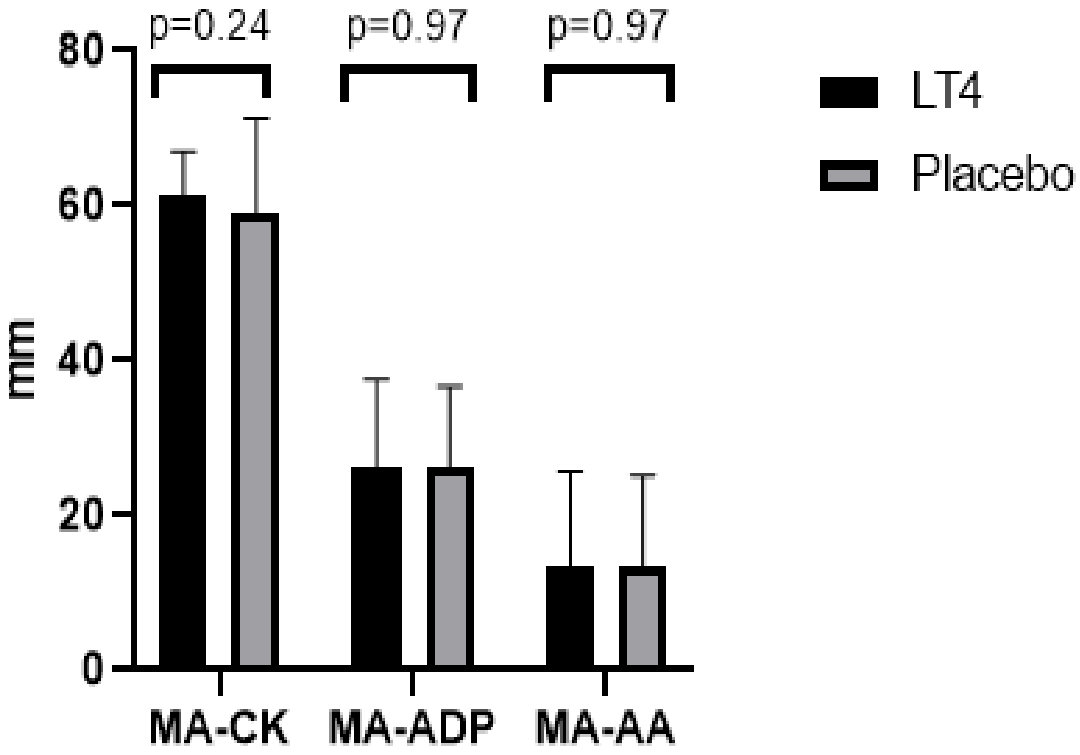
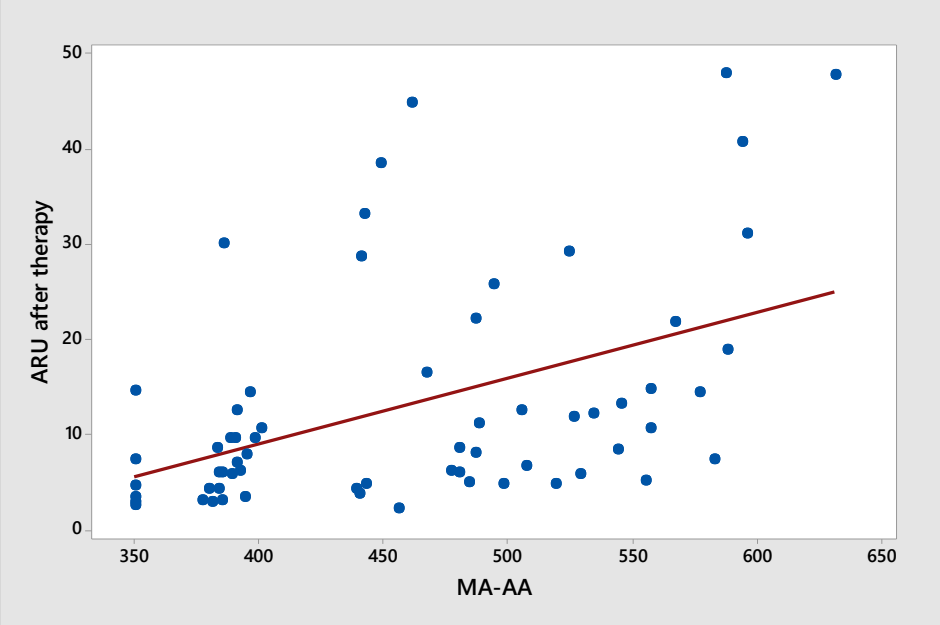


Figure 4-16 A comparison of mean TEG and Platelet Mapping platelet aggregation indices after treatment. Maximum amplitude of thrombus upon stimulation with Kaolin (MA-CK), arachidonic acid (MA-AA) and ADP (MA-ADP) was not significantly different in both LT4 and placebo groups after treatment. The solid bars represent 1X standard deviation.

The correlation between VerifyNow assay and TEG Platelet mapping was assessed in the cohort of patients with both groups combined. There was no correlation between ARU and PRUz indices of VerifyNow and maximum amplitude of the thrombus formed in TEG upon stimulation by kaolin (rho 0.119, p=0.321 for ARU and rho -0.022, p=0.856 for PRUz) (Table 4-30). However, there was a significant positive correlation seen between ARU and maximum amplitude of the thrombus formed in TEG upon stimulation by AA (0.357, p=0.002) (Figure 4-17). Interestingly, ARU also correlated with the maximum amplitude of thrombus formation by ADP (0.305, p=0.010). PRUz (which measured P2Y12 response) correlated positively with maximum amplitude of thrombus formation in TEG upon stimulation by ADP (0.524, P<0.001) as well as upon stimulation by AA (0.255, p=0.032). In summary, these results demonstrate that both ARU and PRUz did not predict total clot strength on stimulation by kaolin but were a good marker for assessing clot strength on stimulation by AA and ADP. Therefore VerifyNow is a good alternative to TEG, as a point of care test, to assess platelet inhibition to antiplatelets.

Rho, (2-tailed p value)	ARU	PRUz
R, min	0.069	-0.074
	(0.562)	(0.540)
K	0.046	0.176
	(0.699)	(0.141)
MA for kaolin, mm	0.119	-0.022
	(0.321)	0.856
CI	0.035	0.010
	(0.775)	0.934
MA for ADP, mm	0.305	0.524
	*(0.010)	*(<0.001)
MA for AA, mm	0.357	0.255
	*(0.002)	*(0.032)

Table 4-30 Correlations between VerifyNow and thromboelastography in the whole cohort of patients after treatment. For each measurement, values represent Rho followed by the p-value.



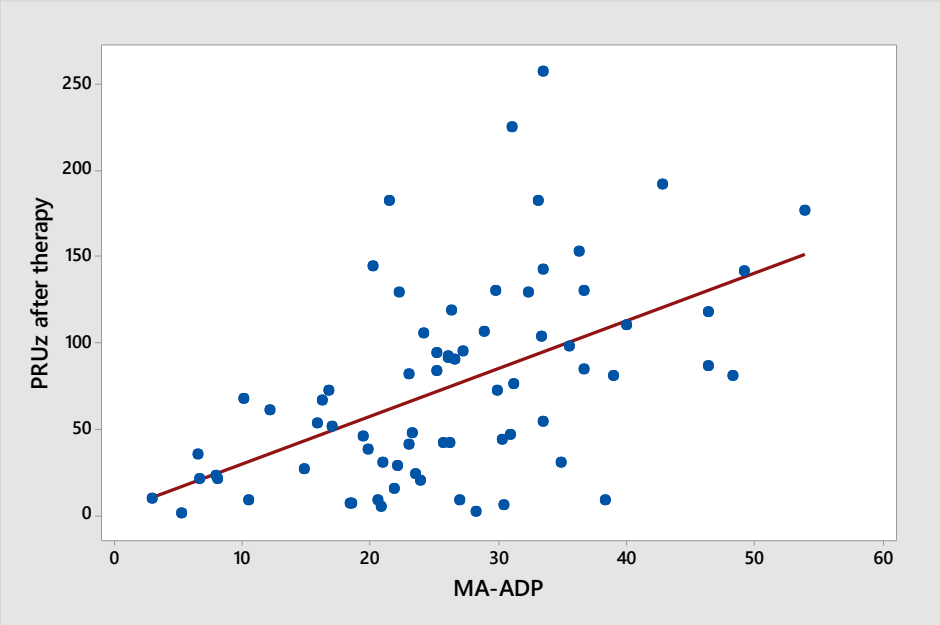


Figure 4-17 Correlation between VeifyNow indices and TEG Platelet Mapping indices at the end of the study. There was a significant correlation between MA-AA and ARU (0.357, p=0.002) as well as between MA-ADP and PRUz (rho 0.524, P<0.001).

Multiple regression was used to ascertain the relationship between MA to kaolin, considered the most important parameter in TEG, and allocation to either LT4 or placebo. MA to kaolin at visit 2 were used as the dependent variable in whereas the various independent variables included gender, age, type of AMI, smoking status, MA at visit 1 and finally allocation to either LT4 and placebo (Table 4-31). The analysis showed that allocation to LT4/placebo was a non-significant predictor with p=0.312 and a beta coefficient showing the mean MA to be only 2.36 lower in the LT4 compared to placebo corrected for all other variables.

	Beta coefficient	95% confidence interval		P-value
Male gender	2.894	-2.820	8.608	0.315
Age (years)	-0.130	-0.395	0.135	0.332
STEMI	-4.790	-10.509	0.928	0.099
Smokers	-1.615	-4.438	1.207	0.257
MA(mm) V1	0.305	-0.187	0.796	0.220
LT4 vs placebo	-2.369	-7.011	2.274	0.312

Table 4-31 Variables associated with MA to kaolin when comparing both the LT4 and placebo groups.

4.3.4 The effect of levothyroxine on endothelial function

Endothelial function was assessed with EndoPAT which measured the reactive hyperaemia index (RHI). An increase in RHI corresponded with an improvement in endothelial function. 32 patients in the LT4 group and 39 patients in the placebo group underwent EndoPAT. At baseline, the RHI in the LT4 group was 1.86±0.44 vs. 1.86±0.73 in the placebo group, p=0.995. After 1 year of treatment, the RHI in the LT4 group was 2.06±0.69 vs. 1.96±0.61 in the placebo group, p=0.51. Although the RHI increased more in the LT4 group, this did not reach statistical significance. Multiple regression was used to correct for each baseline variable with the dependent variable being the RHI at the end of the study whereas the independent variables included: age, gender, type of AMI, smoking status, RHI at the study start and finally allocation to LT4/placebo (Table 4-32). Allocation to LT4 was not a significant predictor (p=0.41) with the adjusted difference (95% confidence interval) RHI being only 0.119 (-0.17-0.4) higher in the LT4 group compared to placebo as shown from the beta coefficient. Gender and RHI at V1 were the only positive predictors of RHI at visit 2, with females having a higher RHI than males.

	Beta coefficient	95% confidence interval		P-value
Age	0.002	-0.014	0.017	0.841
Male gender	-0.577	-0.946	-0.208	0.003
Type of AMI	-0.198	-0.509	0.112	0.206
Smokers	0.081	-0.094	0.257	0.358
RHI V1	0.400	0.171	0.630	0.001
LT4/placebo	0.119	-0.165	0.403	0.405

Table 4-32 Multiple regression to assess the relationship of different variables with RHI at end of study.

4.3.5 Summary of results

Outcome	LT4		Placebo		Adjusted difference (95% confidence intervals)	P value
	Baseline	V6	Baseline	V6		
Badimon Chamber	(n=32)		(n=32)			
Mean thrombus area	11221 (±5688)	10677 (±4997)	11329 (±5033)	10410 (±4113)	711 (1748-3170)	0.56
VerifyNow	(n=32)		(n=32)			
ARU	436 (±85.4)	452±85.1	411 (±72.3)	441±79.4	-7.3 (-47.3-32.9)	0.72
PRU	59.8 (±52.1)	72.9 (±52.3)	47.1 (±46)	76.1±63.8	9.9 (-13.2-33)	0.39
TEG	(n= 32)		(n=32)			
MA-CK	67.8±5.4	61.5±5.4	66.7±4.9	58.9±12.4	-2.4 (-7-2.3)	0.31
Clot index	1.7±1.6	0.7±1.3	1.9±1.7	0.7±1.6	0.65 (-0.63-0.76)	0.85
MA-AA	21.7±16.4	13.5±12.2	18.9±13.8	13.4±11.8	0.85 (-4.3-5.9)	0.74
MA-ADP	30±14.2	26.1±11.4	29.8±16.9	26±15.9	-0.1 (-5.1-4.9)	0.97
EndoPAT	(n=32)		(n=39)			
RHI	1.86±0.44	2.06±0.69	1.86±0.73	1.96±0.61	0.12 (-0.17-0.4)	0.40

Table 4-33 Summary of the effect of LT4 on platelet dependent thrombosis, platelet reactivity, clot kinetics and endothelial function.

4.3.6 Discussion

This study shows that treating mild SCH patients post AMI does not affect thrombus burden, viscoelastic properties of clot formation and lysis, platelet reactivity or endothelial function. Therefore poorer outcomes in SCH patients with cardiovascular disease are unlikely to be attributed to platelet reactivity or an increase in the coagulation cascade. A recent study using the Badimon chamber at our institution showed thrombus area in patients with SCH 7-10 days post non-ST elevation myocardial infarction to be larger than in euthyroid patients despite the use of dual antiplatelets in the form of aspirin and clopidogrel (Viswanathan *et al.*, 2014a). In the current study all patients were on dual antiplatelet therapy and secondary preventive therapy post AMI, and there was no difference in median thrombus area between the LT4 and placebo groups after 1 year. An important difference in the current study was the use of different P2Y12 inhibitors, with not all patients taking clopidogrel as the second antiplatelet in addition to aspirin. As per updated ACS guidelines, the different P2Y12 inhibitors included ticagrelor and prasugrel as the second antiplatelet. The Plato study showed ticagrelor reduces the rate of death from vascular causes and AMI compared to clopidogrel whereas in the TRITON-TIMI study prasugrel therapy compared to clopidogrel reduced the rate of ischaemic events including stent thrombosis (Wiviott *et al.*, 2007; Wallentin *et al.*, 2009). Therefore the use of more potent antiplatelet therapies and optimal secondary prevention measures post AMI may be an explanation for the negative findings and a reason for why LT4 may not further affect thrombus burden.

Previous small studies have demonstrated TH deficiency to not only affect the coagulation pathway but to also inhibit the fibrinolytic pathway. Such studies have shown increased VII activity, increased factor VII activity:factor VII antigen ratio, increased levels of fibrinogen and decreased antithrombin III activity in subjects with SCH (Muller *et al.*, 2001; Canturk *et al.*, 2003; Guldiken *et al.*, 2005). From these studies, TH may activate the fibrinolytic pathway leading to increased thrombus breakdown. Although individual clotting factors were not measured as in these studies, the use of TEG in this study measured the rate and strength of clot formation induced by thrombin which activates platelets and the coagulation cascade. By assessing thrombus formation continuously over a period of time, TEG reflects whole blood thrombosis by showing the interaction between platelets leading to platelet aggregation; and the interaction of platelets with fibrin and the coagulation cascade. This involved measuring each cycle of clot formation and the susceptibility of the clot to autolyse. The current study demonstrated no effect of LT4 on the time taken to form a stabilised thrombus, as well as no

effect on the maximum tensile strength of the thrombus. Furthermore there was no effect of LT4 on clot autolysis suggesting no difference in fibrinolysis between both groups. The likely reason for differences between previous studies and the current study includes differences in patient risk profile and baseline characteristics. Firstly, the current study was performed in patients post AMI in whom thrombotic risk was being targeted and treated by means of antiplatelet medications to reduce further cardiovascular events whereas subjects in previous studies had no history of cardiovascular disease. The current patients were on dual antiplatelet therapy whereas subjects in previous studies were not even on a single antiplatelet agent in the form of aspirin. Thirdly, increases in individual clotting factors in SCH patients likely make no difference to thrombus formation as we know clot formation is dependent on multiple factors including the interaction of the clotting cascade, platelets and fibrin. Therefore even if patients with SCH have increased clotting factors as shown in previous studies, the use of antiplatelets in platelet inhibition reduces the interaction of platelets with the coagulation cascade preventing an increase in thrombus burden and clot kinetics.

The current study also shows no effect of LT4 on improving endothelial function in SCH as previous studies have shown. A RCT by Nagasaki et al showed restoring euthyroidism with LT4 in SCH subjects significantly decreased brachial-ankle pulse wave velocity, a measure of arterial stiffness (Nagasaki *et al.*, 2009). Two RCTs showed LT4 treatment to improve endothelial function in SCH patients (Taddei *et al.*, 2003; Razvi *et al.*, 2007). The RCT by Razvi et al showed LT4 to reduce LDL levels, improve quality of life and improve endothelial function as measured by FMD in SCH patients (Razvi *et al.*, 2007). These studies were performed in stable SCH patients in whom many subjects had no other cardiovascular risk factors and were not on medications which improved endothelial function. In the current study, all patients were on an ace-inhibitor, a beta blocker and the maximum dose of atorvastatin post AMI. A number of clinical and experimental studies have shown an improvement in endothelial function with the use of statins and ace-inhibitors in patients with CAD (Dupuis *et al.*, 1999; Schiffrin *et al.*, 2000; Prasad *et al.*, 2001). Ace inhibitors inhibit the production of angiotensin which is a potent vasoconstrictor and increases the production of bradykinin leading to increase NO production whereas statins increase the production of NO synthase. Therefore subjects being on optimal doses of secondary preventive medications may have resulted in minimal improvements in endothelial function with thyroxine therapy despite previous studies showing significant improvements. Furthermore, it is likely that the dose of thyroxine in our study may have been suboptimal although serum TSH levels in the LT4 group,

at the end of the study, were within the target range (0.4 – 2.5 mU/L). Razvi et al used a starting dose of 100mcg from the start whereas in the current study, the initial dose was 25mcg and the median dose by visit 5 (6 months post randomisation) was 50mcg. Additionally, patients in the study may not have had a low enough thyroid function to warrant treatment in the first place with the median TSH being below 6.0 mU/L in both the LT4 and placebo groups.

In summary, the present study shows no effect of LT4 therapy on the different mechanisms which are associated with increased cardiovascular disease, although these results are not applicable to all patients with SCH.

4.4 Thyroid dysfunction and patient reported outcomes

4.4.1 Results

Patient reported outcomes were assessed using the Centre for epidemiologic studies depression scale (CES-D), Minnesota living with heart failure questionnaire (MLWHF), and the Short form mental and physical score (SF12-M and SF12-P). The CES depression score did not significantly improve in both groups between the start and end of the study (Table 4-34). When both groups were directly compared at both study visits, the CES-D score was not significantly different at V1 with median scores (IQR) of 9 (4-16) vs. 8 (2.5-15), p=0.67, and V6 with median scores of 10 (2-18) vs. 5 (2-14), p=0.16, in the LT4 and placebo groups respectively (Table 4-35). Overall the median scores of the participants in both groups suggested a low risk of clinical depression (using the accepted clinical cut-off score of 16.0).

The Minnesota scores showed a significant decrease in quality of life (QOL) scores in both groups between the start and end of the study but there was no significant difference in the median (IQR) scores between both groups at V1 of 27 (12.3-40) vs. 23 (11.5-37.5), p=0.64, and V6 scores of 13 (7-24) vs. 15 (6-30), p=0.58, in the LT4 and placebo groups respectively. These results demonstrated that the median self-rated scores of trial participants at baseline in both groups were suggestive of moderate QOL from heart failure but improved to being rated as 'good' over the subsequent 52 weeks.

The mean (± s.d) SF12 scores showed a minimal increase in the mental scores in both groups by the end of study whereas the mean physical scores significantly increased in both groups. There was no significant difference in both SF12 scores between both groups at V6 with scores of 50.4 (±11.7) vs. 52.8 (7.9), p=0.27, for SF12-M and 46 (±10.1) vs. 45.6 (±10.6), p=0.86, for SF12-P in the LT4 and placebo groups respectively. The mean mental score of approximately 50 suggests that the average participant in this trial rated their mental health as being good or very good whereas for physical health the mean score of approximately 40 suggests that the average participant in this trial scored their physical health as being good or very good.

These results show that heart failure QOL improved whereas physical health increased in both groups despite no significant differences when both groups were directly compared. Furthermore, mood and mental status did not improve in each group due to low symptom scores at the study start. The results overall showed that LT4 treatment did not make any significant difference to patient reported outcomes and QOL post AMI when compared to placebo.

Multiple regression was used to ascertain the relationship between each individual score at the end of study and allocation to either LT4 or placebo. For each questionnaire, the end of the study score was the dependent variable whereas the various independent variables included gender, age, type of AMI, the score at visit 1 and allocation to either LT4 or placebo. For all 4 questionnaires, allocation to LT4 was a non-significant predictor for each questionnaire score at visit 2 (CES-D p=0.05, Minnesota p=0.28, SF-12 physical 0.95, SF-12 mental 0.96) (Table 4-36, Table 4-37, Table 4-38, Table 4-39).

	LT4 n= 46			Placebo n= 49		
	Baseline	1 year after therapy	P value	Baseline	1 year after therapy	P value
CES depression*	9 (4-16)	10 (2-18)	0.99	8 (2.5-15)	5 (2-14)	0.08
MLWHF *	27 (12.3-40)	13 (6.8-24.3)	<0.001	23 (11.5-37.5)	15 (6-30)	<0.001
SF-12 mental	49.6 (±10.5)	50.4 (±11.7)	0.87	51.6 (±10.8)	52.8 (7.9)	0.21
SF-12 physical	39.9 (±10.6)	46 (±10.1)	0.004	38.5 (±10.9)	45.6 (±10.6)	<0.001

Table 4-34 A comparison of patient reported outcomes in both groups at visit 1 and visit 6.

Variables are shown as mean (±SD) and median (IQR).

*Non-normally distributed data, analysed after log-transformation with Paired t-test.

CESD: scores range from 0 to 60, with higher scores indicating greater depressive symptoms. MLWHF: scores range from 0 to 105, with higher scores indicating increase in symptoms of heart failure. SF12 Mental: scores range from 41 to 61, with higher scores indicate perceived better mental health. SF12 Physical: scores range from 20 to 56, with higher scores indicating better physical health.

	LT4 (n=46)	Placebo (n=49)	P value
CES depression V1*	9 (4-16)	8 (2.5-15)	0.67
MLWHF V1*	27 (12.3-40)	23 (11.5-37.5)	0.64
SF-12 mental V1	49.6 (±10.5)	51.6 (±10.8)	0.41
SF-12 physical V1	39.9 (±10.6)	38.5 (±10.9)	0.56
CES depression V6*	10 (2-18)	5 (2-14)	0.16
MLWHF V6*	13 (6.8-24.3)	15 (6-30)	0.58
SF-12 mental V6	50.4 (±11.7)	52.8 (7.9)	0.27
SF-12 physical V6	46 (±10.1)	45.6 (±10.6)	0.86

Table 4-35 A comparison of patient reported outcomes between both groups at visit 1 and visit 6.

Variables are shown as mean (±SD) and median (IQR).

*Non-normally distributed data, analysed after log-transformation with Student t-testing.

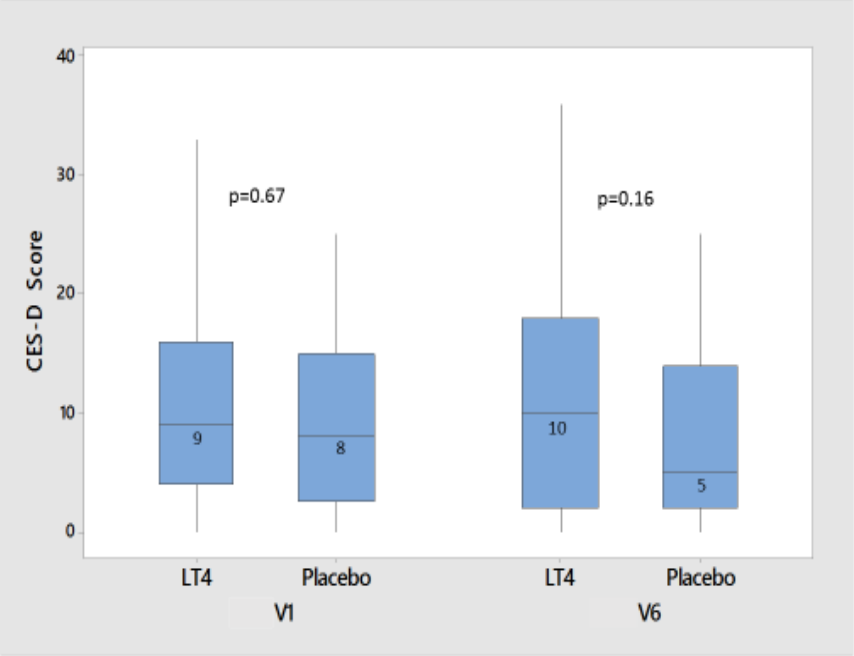


Figure 4-18 Box plot of CES-D scores in both LT4 and placebo groups at visits 1 and 6.

The box represents the 25th to 75th centile. The transverse line with numbers inside the box represents the median and "whiskers" above and below the box show the locations of the minimum and maximum.

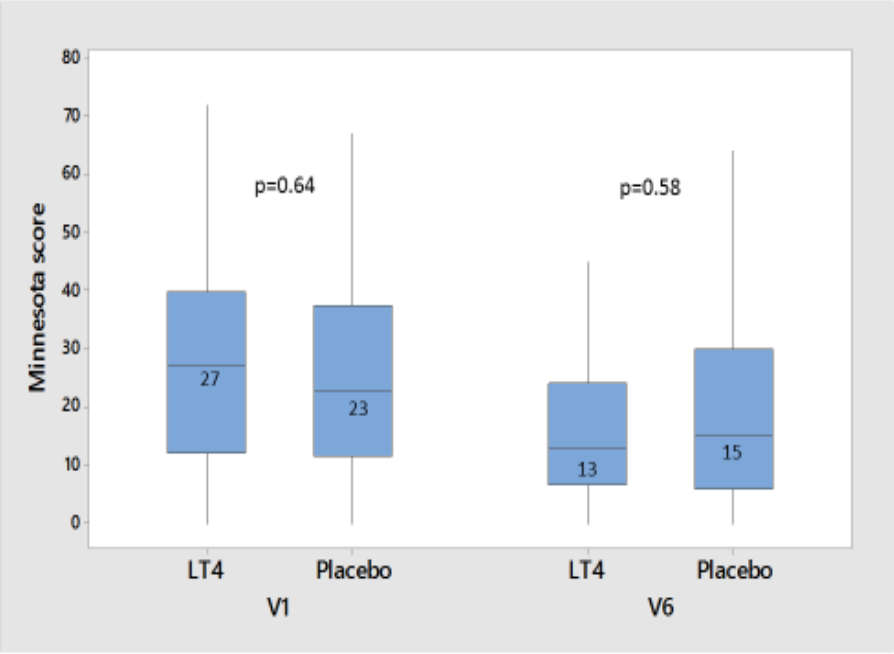


Figure 4-19 Box plot of Minnesota scores in both LT4 and placebo groups at visits 1 and 6.

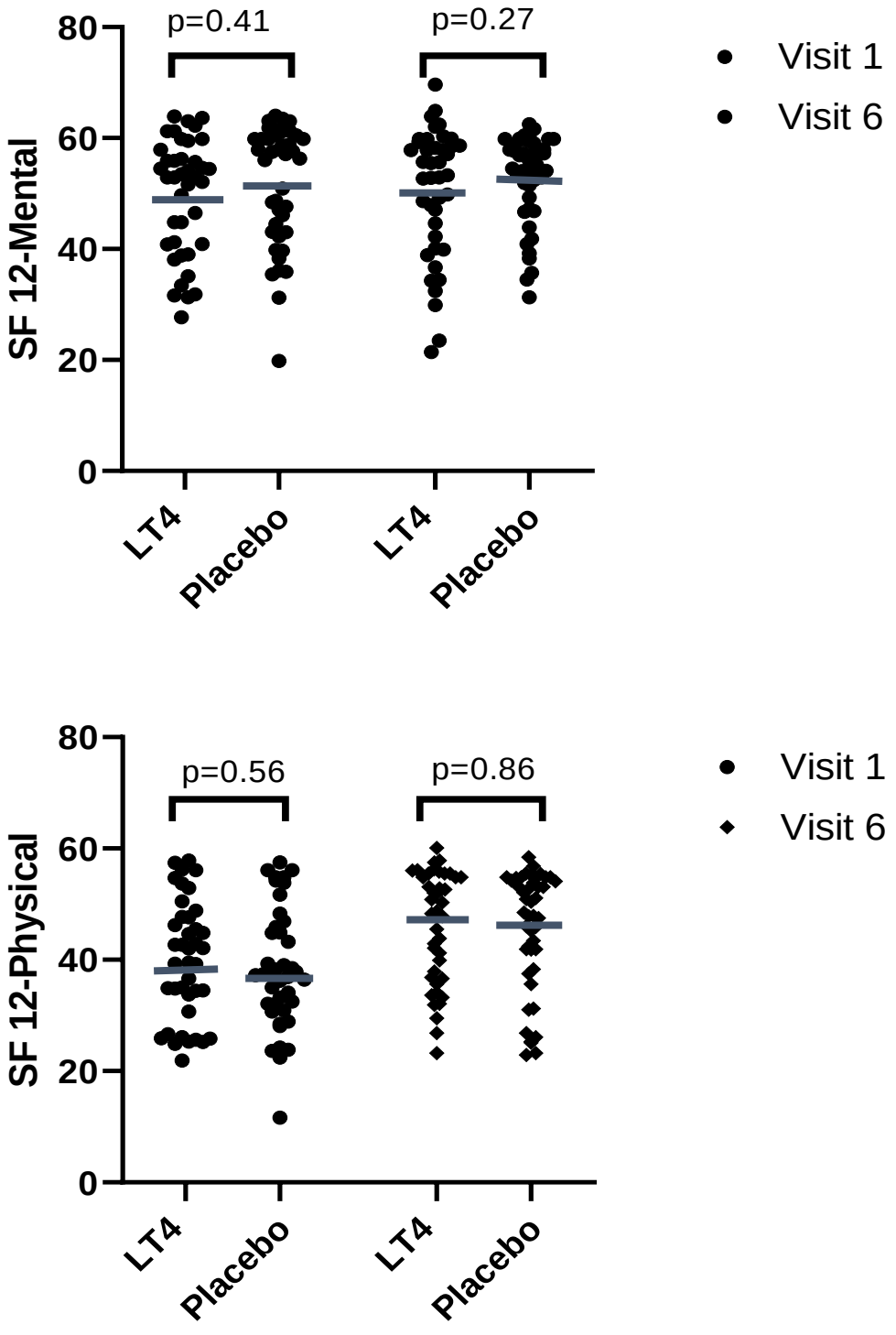


Figure 4-20 A comparison of the mental and physical scores between LT4 and placebo groups at baseline and after 1 year of treatment**.** The bars represent mean scores.

	Beta coefficient	95% confidence interval		P-value
Age	-0.061	-0.228	0.106	0.470
Gender	0.038	-3.821	3.896	0.985
Type of MI	0.960	-2.493	4.412	0.582
CES-D V1	0.626	0.447	0.805	0.000
LT4 vs placebo	3.130	-0.005	6.265	0.050

Table 4-36 Multiple regression to assess the relationship between the allocation to LT4/placebo and CES-D questionnaire independent of other variables.

	Beta coefficient	95% confidence interval		P-value
Age	-0.287	-0.657	0.083	0.126
Gender	3.277	-4.864	11.417	0.425
Type of MI	-1.072	-8.626	6.483	0.778
Minnesota V1	0.517	0.320	0.715	0.000
LT4 vs placebo	-3.741	-10.560	3.078	0.278

Table 4-37 Multiple regression to assess the relationship between the allocation to LT4/placebo and the Minnesota questionnaire independent of other variables.

	Beta coefficient	95% confidence interval		P-value
Age	-0.076	-0.320	0.169	0.539
Gender	3.853	-1.397	9.103	0.148
Type of MI	1.033	-3.816	5.882	0.672
SF-12 physical V1	0.357	0.130	0.584	0.003
LT4 vs placebo	-0.141	-4.542	4.259	0.949

Table 4-38 Multiple regression to assess the relationship between the allocation to LT4/placebo and the SF-12 physical questionnaire independent of other variables.

	Beta coefficient	95% confidence interval		P-value
Age	0.052	-0.209	0.312	0.693
Gender	2.252	-3.109	7.612	0.405
Type of MI	0.775	-4.298	5.848	0.761
SF-12 Mental V2	0.489	0.254	0.724	0.000
LT4 vs placebo	0.134	-4.551	4.818	0.955

Table 4-39 Multiple regression to assess the relationship between the allocation to LT4/placebo and the SF-12 mental questionnaire independent of other variables.

Summary of results

Patient reported outcomes	LT4		Placebo		Adjusted difference (95% confidence intervals)	P value
	Baseline	V6	Baseline	V6		
	(n=45)		(n=45)			
CESD	9 (4-16)	10.0 (2.0-18.0)	8 (2.5-15)	5.0 (2.0-14.0)	3.13 (-0.005-6.27)	0.05
MLWHF	27 (12.3-40)	13.0 (6.8-24.3)	23 (11.5-37.5)	15.0 (6.0-30)	-3.74 (-10.6-3.08)	0.28
SF12 Physical	39.9 (±10.6)	46.0 (+- 10.1)	38.5 (±10.9)	45.6 (+- 10.6)	-0.14 (-4.54-4.23)	0.95
SF12 Mental	49.6 (±10.5)	50.4 (+- 11.7)	51.6 (±10.8)	52.8 (+- 7.9)	0.13 (-4.6-4.8)	0.95

Table 4-40 Summary of results to show patient reported outcomes.

Patient reported outcomes were compared using linear regression adjusting for age, sex, type of AMI, and baseline values.

4.4.2 Discussion

In the present study, LT4 therapy in SCH patients post AMI was not associated with improved patient reported outcomes and quality of life after 1 year of treatment. Furthermore, although the heart failure related QOL did improve in both groups after 1 year, there was no significant difference between both groups respectively. This is the first study to report such outcomes in SCH patients post AMI.

The current study does not support the use of LT4 therapy in improving QOL in SCH patients post AMI. These findings support a recent meta-analysis and systematic review of 21 randomised controlled studies which showed no improvement in quality of life or thyroid reported symptoms in patients with SCH treated with LT4 therapy (Feller *et al.,* 2018). This meta-analysis be Feller et al included 2 of the latest randomised studies recently published which are the largest studies to date assessing LT4 treatment and quality of life (Stott *et al.*, 2017; Mooijaart *et al.*, 2019). However, the largest contribution to the meta-analysis was from the TRUST Study which recruited older asymptomatic individuals treated with low dose LT4 therapy (Stott *et al.*, 2017). Therefore the analysis may not be applicable to younger or symptomatic patients. The current results are also consistent with a Cochrane review of 22 studies showing no benefit of LT4 treatment on quality of life, physical and mental scores and daily living scores (Reyes Domingo *et al.*, 2019). The results of the present study support current European and US guidelines which do not advocate the treatment of patients with SCH unless they have persistent symptoms or have TSH levels above 10 mU/L irrespective of age (Garber *et al.*, 2012; Pearce *et al.*, 2013). However the European Thyroid Association does recommend a trial of LT4 therapy in younger symptomatic patients irrespective of their TSH levels (Pearce *et al.*, 2013).

Despite the negative findings in the current study, there are limitations which should be considered. Firstly, the median TSH of all participants in the LT4 group was 5.75 mU/L, indicating mild SCH, and this combined with the low burden of symptoms at the start of the study and a low median dose of LT4 use may be a reason as to why LT4 treatment did not make a significant difference in the interventional group. In the meta-analysis by Feller et al, the mean TSH levels were below 7 mU/L in half of the studies included in the analysis. When the RCTs from the meta-analysis are assessed separately, we find that in studies with participants with a mean TSH above 7 mU/L or the use of high dose LT4 therapy, there was a significant improvement in symptoms with LT4 treatment (Meier *et al.*, 2001; Razvi *et al.*,

2007; Aghili *et al.*, 2012). In the Basel Thyroid Study by Meier et al, the mean TSH levels prior to treatment was 11.7 and administering a mean LT4 dose of 85.5mcg/day resulted in improvement in hypothyroid symptoms after 48 weeks (Meier *et al.*, 2001). In the study by Aghili et al, the mean TSH level was 8.25 and administering LT4 for 3 months resulted in a significant improvement in cognitive scores (Aghili *et al.*, 2012). In the study by Razvi et al, although the mean TSH was 6.6, LT4 treatment at a higher dose of 100mcg lead to a significant improvement in tiredness (Razvi *et al.*, 2007). Therefore a subgroup of patients with higher TSH levels and a higher burden of symptoms may benefit from treatment irrespective of the current data. Furthermore subjects with SCH enrolled to the present study as well as previous studies are likely to be of low symptom burden from their SCH, as patients with more severe symptoms are likely to be prescribed treatment and therefore be unrepresented in clinical trials. No study to date has recruited patients with only severe symptoms secondary to SCH and seven studies (1263 adults) in the meta-analysis by Feller et al recruited patients with only mild-moderate symptoms.

TSH levels increase with age meaning some older patients enrolled to the present study may not have had true SCH, resulting in no improvement in symptoms to treatment, although international guidelines do not define SCH according to age and TSH cutoff values. Vadiveloo et al demonstrated that using age specific TSH reference ranges in patients above 70 would prevent the overestimation and unnecessary treatment of SCH (Vadiveloo *et al.*, 2013). This is further supported by studies which have shown no benefit of LT4 therapy on QOL in patients above the age of 70 (Stott *et al.*, 2017; Mooijaart *et al.*, 2019). In the TRUST Study, the mean age of participants was 74.4 and LT4 treatment provided no symptomatic benefit to participants with a mean TSH of 6.4 at the start of the study (Stott *et al.*, 2017). In another recent study by Mooijaart et al, LT4 treatment in patients above the age of 80 with a mean TSH of 6.4 resulted in no significant improvement in quality of life (Mooijaart *et al.*, 2019). Furthermore, in a secondary analysis of the TRUST Study which analysed 638 persons with the highest symptom burden, LT4 treatment failed to improve QOL or symptoms of tiredness (de Montmollin *et al.*, 2020). The findings of these two studies support the current European and US guidelines in not initiating treatment to SCH patients above the age of 80 and adopting a more conservative approach (Garber *et al.*, 2012; Pearce *et al.*, 2013).

Meyerovitch et al found that TSH levels fluctuate and returned to normal in 62% of healthy subjects with a TSH between 5.5 and 10 mIU/L who were followed up over 5 years (Meyerovitch *et al.*, 2007). Transient illness, environmental and stress factors are likely to

explain this variation. Other than in the TRUST Study and the studies by Mooijaart et al and Razvi et al which recruited subjects with a TSH measured on atleast two occasions to diagnose SCH, other studies assessing QOL in SCH patients have not stated whether subjects had repeat thyroid testing, to suggest true SCH, prior to commencing treatment with LT4 therapy. Finally, the type of symptoms linked to hypothyroidism and SCH are very non-specific and the probability of diagnosing SCH based on one of these symptoms is 10% (Bekkering *et al.*, 2019). The Colorado Study showed that 20-25% of patients with normal thyroid function report atleast one of these symptoms (Canaris *et al.*, 2000). Therefore in the current study, it is difficult to ascertain an improvement in symptiology and QOL with LT4 treatment when such symptoms were only mild prior to therapy, and not necessarily related to the thyroid dysfunction but rather to recovery post AMI.

In summary, LT4 treatment did not improve patient reported outcomes and QOL in SCH patients post AMI, but there may be a selective group of patients who may still benefit depending the severity of their SCH as well as symptom burden irrespective of TSH levels. It is essential for clinicians to decide whether specific symptoms impacting on QOL are likely related to their thyroid status or are symptoms of another systemic process.

4.5 The association of thyroid dysfunction with mortality

4.5.1 Subclinical hypothyroidism and mortality

The relationship between thyroid parameters and all-cause mortality was evaluated using Cox proportional hazards multivariable analysis. Survival times were calculated from the date of the AMI till the date of death or date of being known to be alive. The median (IQR) follow up period was 42 (37-49) months. We used restricted cubic splines to account for nonlinearity of the associations, but no evidence of nonlinearity was observed. All analyses were adjusted for relevant variables such as gender, age, smoking status (current, ex- or non- smoker), body mass index (BMI), type of AMI (STEMI or NSTEMI), serum creatinine, history of ischemic heart disease (yes/no), hypertension (yes/no), diabetes mellitus (yes/no), cerebrovascular disease (yes/no), atrial fibrillation (yes/no) and hypothyroidism (yes/no), standardised troponin and CRP.

312 SCH patients were compared with 1437 euthyroid patients. During the follow up period, there were 43 (13.8%) deaths in the SCH group whereas the number of deaths in the euthyroid group were 156 (10.9%), with p=0.167 using the Chi-squared test. Using Cox proportional hazards multivariable analysis, patients in the SCH group had a similar all cause mortality with an adjusted hazard ratio (95% CI) of 1.05 (0.73-1.50), p=0.814. Figure 4-21 demonstrates the cumulative survival curves for both the SCH and euthyroid groups.

As observed in chapter 4.1.2, the diagnosis of SCH and the proportion of patients who normalised their thyroid function was independently and significantly associated with time of sampling. All-cause mortality in the SCH participants was not different across the various time periods (p for interaction between SCH and time-period = 0.34). When time-period specific TSH ranges were utilised (Table 4-41), the prevalence of SCH reduced dramatically (n=45; 2.5%) and the prevalence of SCH was similar across all time-periods when time-period specific TSH reference intervals were utilised (p=0.79). In contrast to the findings of the SCH group identified using a uniform TSH reference interval, mortality was significantly higher in SCH patients that were categorised using a time-period specific reference interval with HR of 2.07 (1.05-4.10), p=0.036 (Figure 4-22). The number of deaths in the SCH group were 9 (20%) compared to 189 (10.9%) in the euthyroid group. Figure 4-22 shows a steep drop in the survival curve in the SCH group, compared to the euthyroid group, representing a higher mortality from the study start. This is followed by further divergence of the curves throughout the follow up. Table *4-42* shows other predictors of long-term mortality when SCH and euthyroid patients

were compared using the Cox proportional hazard model, with SCH diagnosed using time-period specific TSH reference intervals.

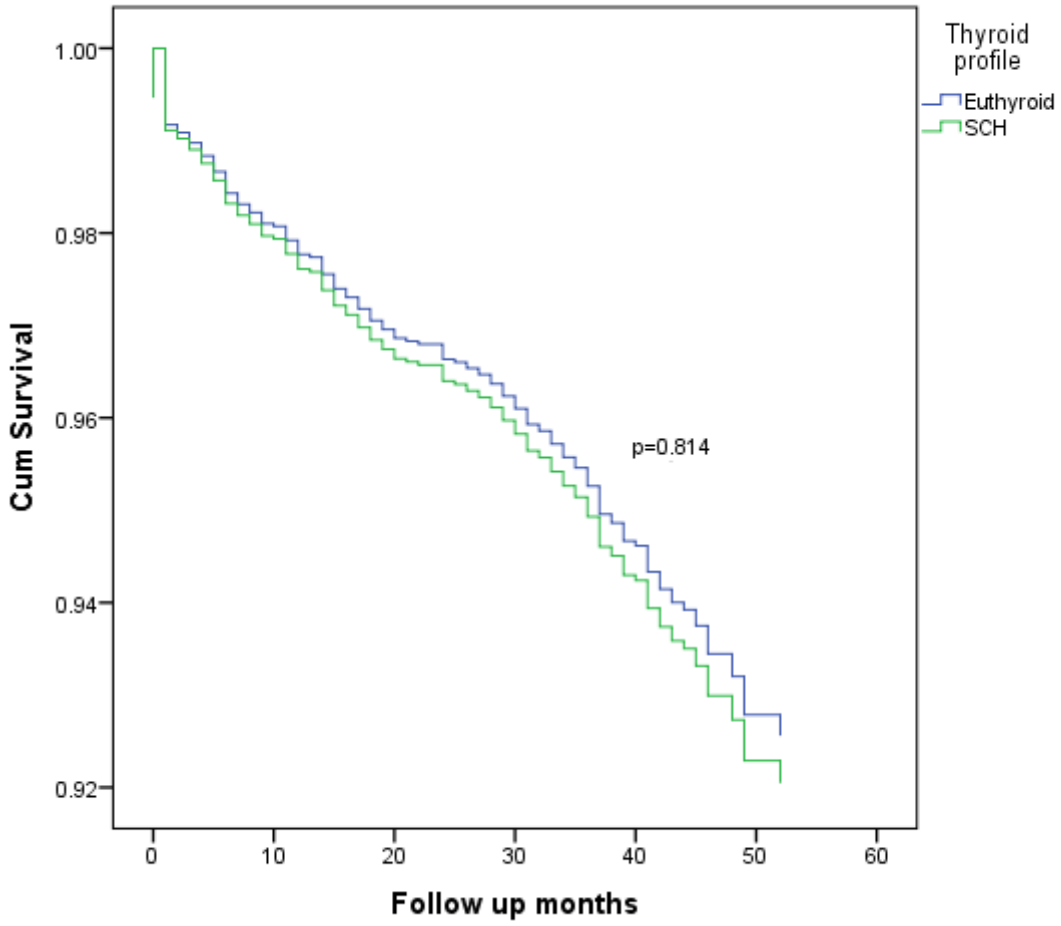


Figure 4-21 Hazard plot of all-cause mortality demonstrating cumulative survival when comparing SCH patients with euthyroid using a uniform TSH reference range. There was no difference in mortality, p=0.814.

	00:01 – 06:00	06:01 – 12:00	12:01 – 18:00	18:01 – 00:00
Median TSH	2.62	1.94	1.80	2.09
(2.5th – 97.5th centile)	(0.59 – 9.26)	(0.57 – 8.21)	(0.37 – 6.38)	(0.52 – 8.23)
Mean FT4	16.3	16.1	15.9	16.3
(2.5th – 97.5th centile)	(11.6 – 24.7)	(11.1 – 22.6)	(11.4 – 23.2)	(11.3 – 25.5)
Mean FT3	4.9	4.8	4.7	4.8
(2.5th – 97.5th centile)	(3.0 – 7.0)	(3.3 – 6.6)	(3.3 – 6.2)	(3.1 – 6.6)
Euthyroid n (%)	323 (96.7)	506 (95.1)	518 (95.6)	373 (94.9)
SCH n (%)*	8 (2.4)	13 (2.4)	14 (2.4)	10 (2.5)
SHyper n (%)*	3 (0.9)	13 (2.4)	11 (2.0)	10 (2.5)

Table 4-41 Reference ranges for TSH, FT4 and FT3, and prevalence of thyroid status calculated according to the time of sampling.

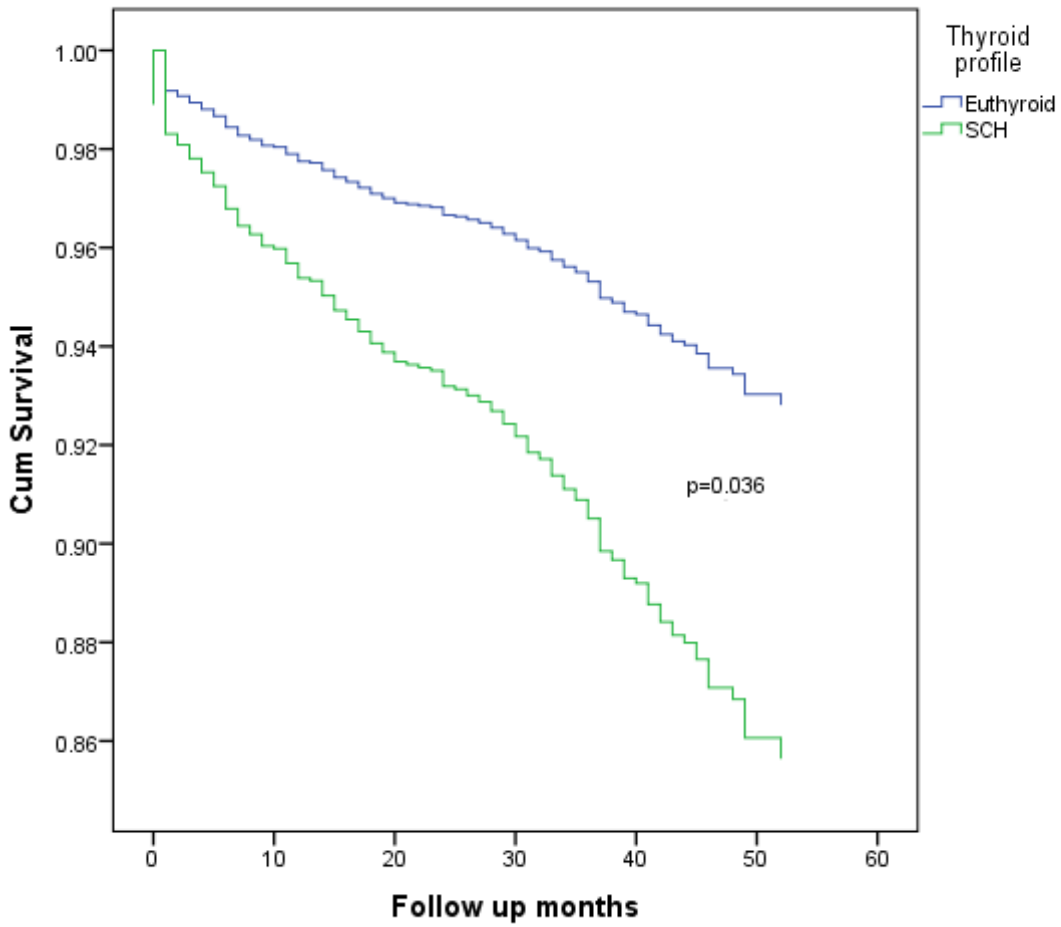


Figure 4-22 Hazard plot of all-cause mortality, comparing SCH patients with euthyroid, when time-period specific TSH reference intervals were applied. SCH patients had a higher mortality, p=0.036.

Variable	HR (95% CI)	P-value
Female gender	0.85 (0.61-1.20)	0.37
Age (per year increase)	1.09 (1.07-1.12)	<0.001*
BMI (per unit increase)	0.97 (0.94-1.01)	0.16
Smoking Never smoked Current smokers Ex-smokers	 1.0 (Reference) 1.65 (1.09-2.49) 1.51 (1.02-2.23)	0.004*
Type of MI NSTEMI	 1.51 (1.02-2.23)	 0.038*
SCH	2.07 (1.05-4.10)	0.036*
TPOAb (per unit increase)	1.01 (0.99-1.02)	0.29
Creatinine (per µmol/dL increase)	1.04 (1.02-1.06)	<0.001*
Ischaemic heart disease	1.19 (0.86-1.65)	0.29
Diabetes mellitus	1.93 (1.38-2.70)	<0.001*
Hypertension	1.01 (0.73-1.39)	0.97
Hypercholesterolaemia	0.74 (0.51-1.06)	0.11
Cerebrovascular disease	1.13 (0.68-1.86)	0.64
Peripheral vascular disease	2.02 (1.20-3.40)	0.009*
Standardised troponin (per unit increase)	1.24 (1.03-1.49)	0.022*
CRP (per mg/L increase)	1.01 (1.005-1.013)	<0.001*

Table 4-42 Predictors of long-term mortality in SCH and euthyroid patients using the Cox proportional hazard model, with SCH diagnosed using time-period specific TSH reference intervals. BMI – body mass index, MI – myocardial infarction, NSTEMI - Non-ST elevation myocardial infarction, SCH- subclinical hypothyroidism, TPOAb – TPO antibodies, CRP – C-reactive protein. *p<0.05 indicating statistical significance.

4.5.2 LT3 and mortality

When the Cox proportional hazard model was used in the whole patient cohort of the ThyrAMI 1 Study, T3 levels at baseline significantly predicted subsequent mortality with an increase in T3 levels associated with a reduction in mortality; adjusted hazard ratio (95% CI) of 0.63 (0.50-0.77), p<0.001 (Table *4-43*). Other variables associated with increased mortality included: age 1.08 (1.06-1.10), current smoking status 1.83 (1.19-2.81), NSTEMI 1.73 (1.16-2.58), creatinine 1.01 (1.01-1.02), PVD 3.13 (1.95-5.02), diabetes mellitus 1.64 (1.18-2.3), standardised troponin 1.29 (1.07-1.54) and CRP 1.01 (1.00-1.01). Baseline serum TSH or FT4 were not associated with increased mortality 1.01 (0.98-1.05), p=0.32 and 1.06 (0.97-1.13), p=0.08, respectively.

Variable	HR (95% CI)	P-value
Female gender	0.92 (0.66-1.28)	0.61
Age (per year increase)	1.08 (1.06-1.10)	<0.001*
BMI (per unit increase)	0.98 (0.95-1.02)	0.43
Smoking Never smoked Current smokers Ex-smokers	 1.0 (Reference) 1.83 (1.19-2.81) 0.82 (0.58-1.15)	0.003*
Type of MI NSTEMI	 1.73 (1.16-2.58)	 0.007*
TSH (per mU/L increase)	1.01 (0.98-1.05)	0.32
FT4 (per pmol/L increase)	1.06 (0.97-1.13)	0.08
FT3 (per pmol/L increase)	0.63 (0.50-0.77)	<0.001*
Creatinine (per µmol/dL increase)	1.01 (1.01-1.02)	<0.001*
Ischaemic heart disease	1.17 (0.85-1.62)	0.34
Diabetes mellitus	1.64 (1.18-2.3)	0.003*
Hypertension	1.01 (0.74-1.38)	0.97
Hypercholestrolaemia	0.78 (0.53-1.12)	0.18
Cerbrovascular disease	0.98 (0.60-1.60)	0.94
Peripheral vascular disease	3.13 (1.95-5.02)	<0.001*
LT4 use	1.48 (0.83-2.62)	0.18
Standardised troponin (per unit increase)	1.29 (1.07-1.54)	0.007*
CRP (per mg/L increase)	1.01 (1.00-1.01)	0.01*

Table 4-43 Predictors of long-term mortality in all the patients in the ThyrAMI 1 study using the Cox proportional hazard model. BMI – body mass index, MI – myocardial infarction,

NSTEMI - Non-ST elevation myocardial infarction, TSH – thyrotropin, FT4 – free thyroxine 4, FT3 – free triiodothyronine, LT4 – levothyroxine, CRP – C-reactive protein.

*p<0.05 indicating statistical significance.

T3 levels were inversely associated with increased mortality in a linear manner as a continuous variable (Table 4-43 and Figure 4-23). Further analysis was conducted by comparing mortality in patients with a normal and low T3, using a T3 cut-off value of 3.5. 1263 patients in the normal T3 group and 63 patients in the low T3 group were compared. During a follow up period of 55 months, there were 147 deaths (11.1%) in total. In the normal T3 group there were 125 (9.89%) deaths whereas the number of deaths in the LT3 group was 22 (34.9%), with a p<0.001 using the Chi-squared test. Using Cox proportional hazards multivariable analysis, patients in the low T3 group had a higher all-cause mortality with an adjusted hazard ratio (95% CI) of 2.15 (1.30-3.55), p=0.002. Figure 4-24 demonstrates the cumulative survival curves for both the normal and low T3 groups using the Kaplan–Meier analysis and log-rank test.

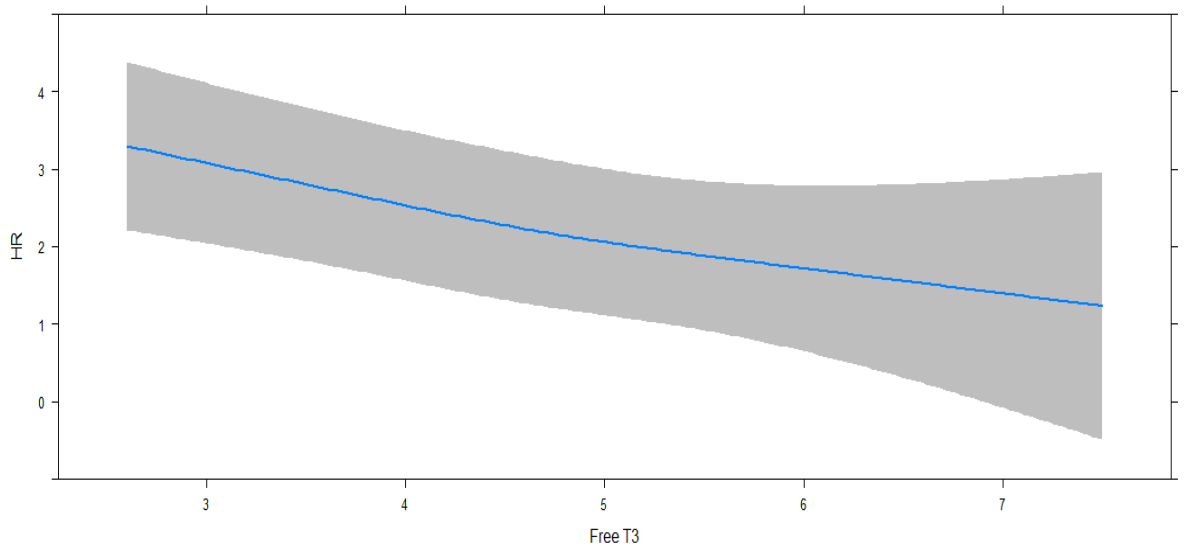
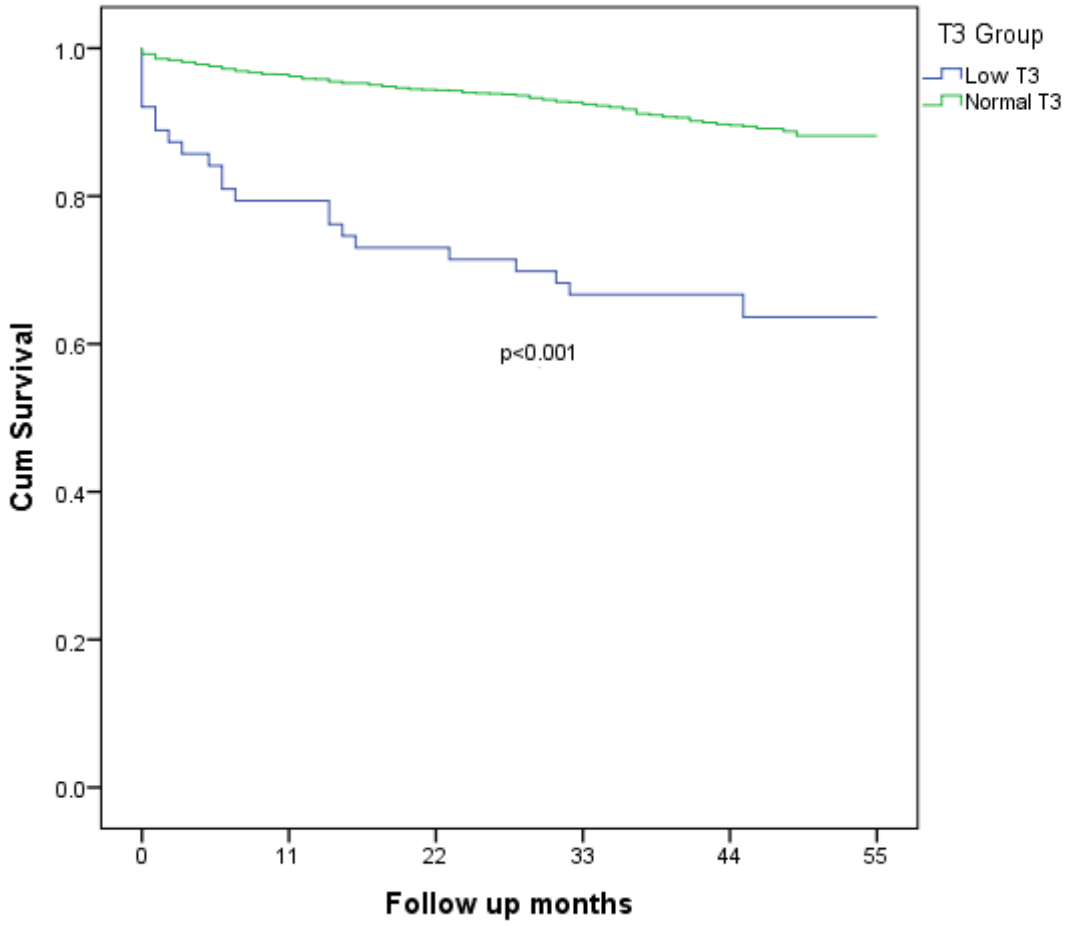


Figure 4-23 Relationship of T3 with all-cause mortality in the ThyrAMI-1 cohort.

Adjusted for age, gender, BMI, smoking status, type of acute myocardial infarction (STEMI or NSTEMI), TSH, T4, T3, creatinine, ischemic heart disease, diabetes mellitus, hypertension, hypercholesterolaemia, cerebrovascular disease, peripheral vascular disease, atrial fibrillation, hypothyroidism, serum creatinine, standardised troponin and CRP.



Number at risk					
Normal T3: 1263	1218	1194	1172	1147	1138
Low T3: 63	50	46	42	42	41

Figure 4-24 Kaplan–Meier curves demonstrating cumulative survival in the low T3 and normal T3 groups. The log rank test was significant, p<0.001.

4.5.3 Relationship between thyroid function parameters and CRP at baseline

There was a significant negative linear relationship between serum T3 levels and CRP with $p<0.0001$ (Figure 4-25). However, there was no significant relationship between serum T4 or TSH levels with CRP. Higher CRP levels at baseline were significantly associated with higher risk of subsequent mortality using Cox proportional hazards multivariable analysis (Table 4-43) with an adjusted hazard ratio of 1.01 (1.00 – 1.01), $p=0.01$. As CRP may not just be a confounder but also be a mediator in the relationship between T3 and mortality, an additional Cox proportional hazards multivariable analysis in which the separate variables of T3 and CRP were replaced by the combination groups of FT3 and CRP were performed (Figure 4-26). The same covariates for the multivariable models, as per previous analyses, were selected. This demonstrated that compared to patients in the high FT3 and low CRP group, all other groups had higher adjusted mortality risk (p for trend <0.001). Patients in the low T3 and high CRP group had more than 3-fold risk of subsequent mortality with HR 3.2 (1.62-6.82) (Figure 4-26). Even patients in the low T3 and low CRP group had a nearly 2-fold risk of subsequent mortality with HR 1.97 (1.03-4.23). Interestingly, patients in the group in whom both the T3 and CRP levels were high had a similar risk of subsequent death as the high FT3 and low CRP group, suggesting that the increased risk of an amplified inflammatory response may be mitigated by higher serum T3 levels.

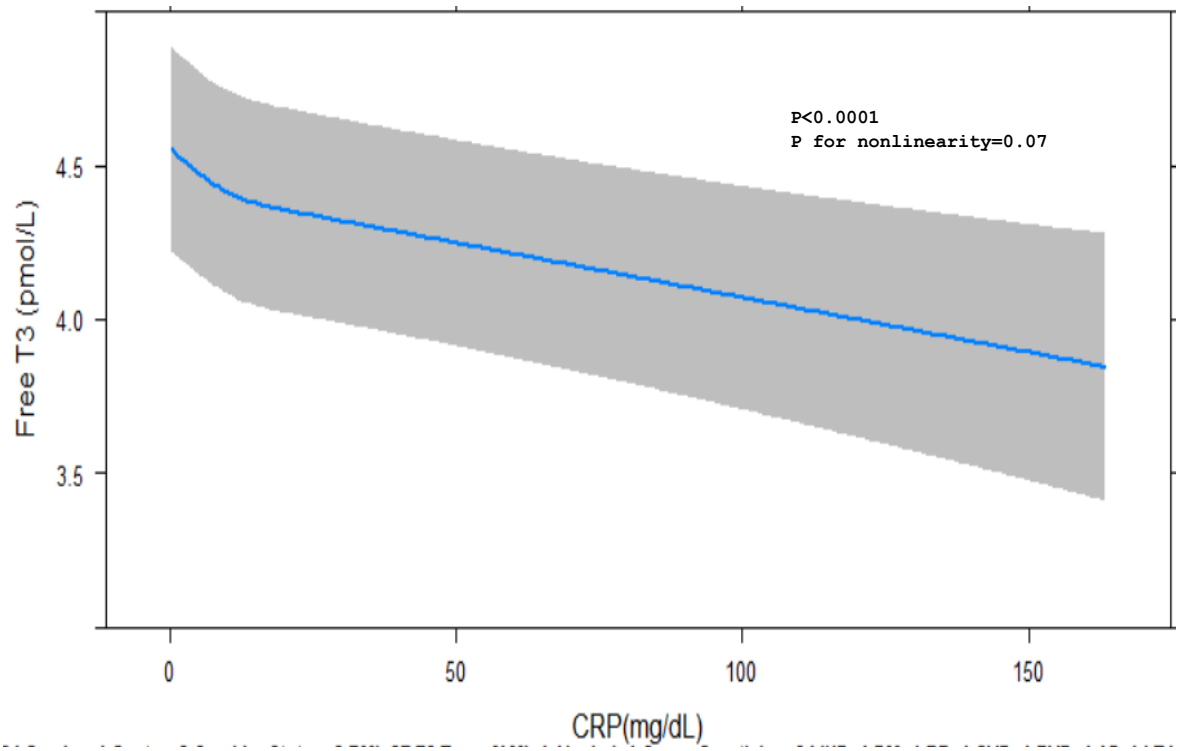


Figure 4-25 Relationship of T3 with CRP.

Adjusted for age, sex, body mass index, smoking status, type of acute myocardial infarction (STEMI or NSTEMI), ischemic heart disease, type 2 diabetes mellitus, hypertension, cerebrovascular disease, peripheral vascular disease, atrial fibrillation, hypothyroidism, serum creatinine, and standardised troponin.

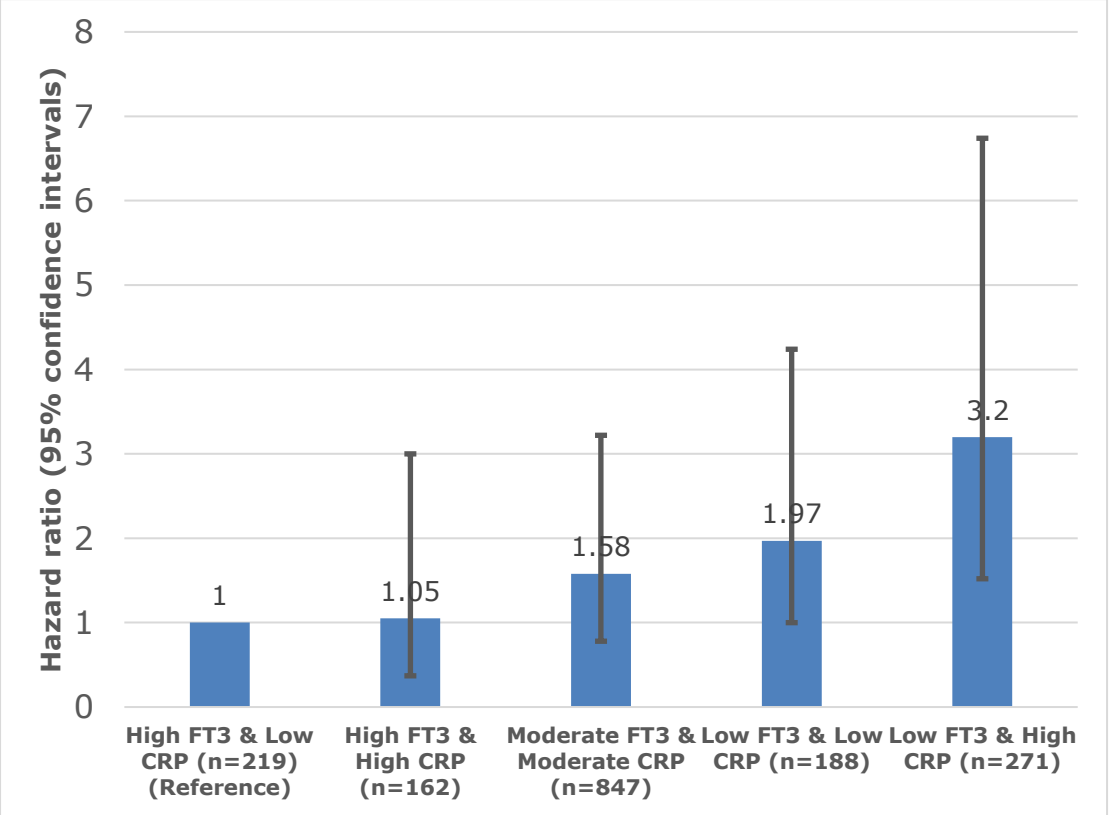


Figure 4-26 T3 and CRP levels and risk of mortality.

Adjusted for age, sex, body mass index, smoking status, type of acute myocardial infarction (STEMI or NSTEMI), ischemic heart disease, type 2 diabetes mellitus, hypertension, cerebrovascular disease, peripheral vascular disease, atrial fibrillation, hypothyroidism, serum creatinine, and z-troponin.

4.5.4 Discussion

This study has demonstrated that SCH diagnosed with TSH levels higher than the time-period specific reference range, rather than diagnosed using a uniform TSH reference range as per current practice, and low serum T3 levels are associated with increased mortality in patients with AMI. Furthermore, the predictive role of T3 was more pronounced in AMI patients with an elevated CRP which is a marker of inflammation.

The results of this study support previous studies which have demonstrated worse mortality outcomes in patients with SCH and low T3 who have cardiovascular disease. Iervasi and colleagues found cardiac patients with SCH (6.7% of the total cohort) and low circulating T3 levels (29.2%) to have a higher risk of cardiovascular and all-cause mortality than euthyroid individuals within a large cohort of patients admitted with cardiac events (Iervasi *et al.*, 2007). However, patients with acute MI were excluded. Other studies have also demonstrated worse outcomes in patients with SCH and acute cardiovascular disease (McQuade *et al.*, 2011; Molinaro *et al.*, 2012; Seo *et al.*, 2018). In another study, Iervasi et al investigated 573 patients admitted with acute cardiac events of which 173 patients with low T3 were compared with 400 patients with normal T3. Patients presented with various cardiac conditions including AMI, heart failure, arrhythmias and cardiomyoapthies with the number of cumulative and cardiac deaths being significantly higher in the low T3 group during a 1 year follow up period (Iervasi *et al.*, 2003). Zhang et al demonstrated similar results in 501 patients of which 171 patients had a low T3 (Zhang *et al.*, 2012). A further interventional study showed a low T3 in up to 20% of 641 consecutive patients below the age of 75 admitted with a STEMI whom were shown to have a lower survival rate despite percutaneous coronary intervention, further supporting the role of TH in myocardial recovery (Lazzeri *et al.*, 2012).

Clinical data strongly suggest that inflammation has a role in the progression of atherosclerosis with studies demonstrating elevated biomarkers of inflammation such as CRP and IL-6 to be associated with increased cardiovascular events (Ridker *et al.*, 1997; Ridker *et al.*, 2000). Treatment with canakinumab, a monoclonal antibody which reduces IL-6 by inhibiting interleukin 1β, reduces recurrent cardiovascular events (Ridker *et al.*, 2017). However, there is paucity of data on the relation between low FT3, inflammation and subsequent mortality after acute AMI. Our findings however demonstrate that a combination of low T3 levels and elevated CRP augment mortality risk compared to either biomarker alone. Patients with low FT3 and high CRP had a more than three-fold subsequent risk of mortality compared to those with high T3 and low CRP. The present study not only confirms that low T3 levels are

associated with higher mortality but importantly this association may in part be via the inflammatory process. It is unknown if inflammation is one of the mechanisms by which low T3 levels and subsequent mortality are linked or whether T3 and CRP levels are independently related to mortality. Evidence supporting the former view include previous experimental studies showing the association between deiodinase enzymes, markers of inflammation and TH levels. Markers of inflammation such as IL-1 and IL-6 have been shown to decrease TH experimentally by inhibiting D1 activity and competing for receptor coactivators leading to decrease conversion of T4 to T3 (Yu and Koenig, 2000). The hypoxia and the inflammatory response reduce deiodinase activity in the cardiomyocyte, resulting in a decrease of intracellular T3 bioavailability (Gereben *et al.*, 2008). This relative lack of T3 is further compounded by an increase in D3 deiodinase expression, leading to degradation of T3 into inactive iodothyronines (Simonides *et al.*, 2008). On the contrary, T3 and CRP levels could be linked independently as shown from the present data in which patients with low T3 and low CRP levels still had a nearly 2-fold increase in mortality.

A consensus amongst clinicians and scientists is the low T3 encountered post AMI is likely due to the stress response of the myocardial insult, and hence a biomarker of disease severity, rather than an exacerbating factor which could influence injury. However, as seen in chapter 4.1, there was no association between low TH levels and troponin on multiple regression analysis. Therefore the low TH levels post AMI could be maladaptive irrespective of troponin levels. A subsequent reduction in bioavailable thyroid hormone in the acutely affected tissue has been considered a protective mechanism by reducing metabolic demand (Simonides *et al.*, 2008). However Rajabi et al have suggested the low T3 in the injured myocardium could be a benefit in conserving energy after an MI, by changing myocyte expression, however this is also considered to be a maladaptive state in the long term if not reversed which has poor prognostic benefits (Rajabi *et al.*, 2007). It is therefore plausible that the post-AMI reduction in circulating T3 is maladaptive with T3 therapy having a potential role in improving outcomes.

The protective effect of TH observed in AMI experimentally include improved LV function, reduced apoptosis, induced angiogenesis and mitochondrial preservation (Jabbar *et al.*, 2017). TH has also been shown to limit the extent of infarct size and apoptosis in the border area of the infarcted tissue experimentally leading to an improvement in LV function (Chen *et al.*, 2008; Kalofoutis *et al.*, 2010). This limitation of infarct extension is through the activation of the cellular pro-survival pathways PI3K/Akt and PKC and suppression of the p38 MAPK pathway shown experimentally in rodent models of AMI treated with T3 (Kuzman *et al.*, 2005;

Pantos *et al.*, 2009a). In a randomized controlled trial of T3 therapy in chromic heart failure subjects, oral liothyronine administration for 6 weeks was associated with a reduction in CRP levels further supporting the potential benefit of T3 replacement in inflammation (Amin *et al.*, 2015). In other clinical studies T3 therapy has been shown to improve left ventricular function in patients with heart failure (Pingitore *et al.*, 2008; Amin *et al.*, 2015) and those undergoing surgical revascularization (Klemperer *et al.*, 1995; Sirlak *et al.*, 2004; Ranasinghe *et al.*, 2006). In a recent trial of T3 in a small number of AMI patients has demonstrated safety (Pingitore *et al.*, 2019).

In summary, patients diagnosed with SCH on time specific reference ranges have a higher mortality than when a uniform TSH reference range is used, as per current practice. This demonstrates TSH reference ranges should take time of sampling into account when diagnosing SCH to prevent a potential over diagnosis. The present data also advances the literature and shows low T3 and elevated CRP levels to augment mortality risk compared to either biomarker alone. This identifies a group of individuals who may potentially benefit from T3 therapy - AMI patients with low serum FT3 and high CRP. The ideal dose, safety and efficacy of T3 will need to be tested in future therapeutic trials in this high-risk population.

5 Chapter: Conclusion

5.1 General discussion

This study demonstrated a high prevalence of thyroid dysfunction in patients admitted with AMI with SCH being observed in 1 in 6 patients. Other forms of thyroid dysfunction including LT3 and SHyper had a lower prevalence. Older individuals, females, those with higher TPOAb levels or higher creatinine concentrations and patients who had their thyroid function samples obtained in the early hours of the morning had a higher risk of being classed as having SCH. The results of this analysis have shown the diagnosis of subclinical thyroid disease and subsequent rates of normalisation of serum TSH levels in patients with AMI are significantly influenced by the time of sampling. Furthermore, SCH patients diagnosed when time-specific reference ranges are applied, and patients with LT3 had an increased mortality compared to those who were euthyroid. The interventional trial of LT4 therapy in those with sustained SCH demonstrated no improvement in markers of LV function and, similarly, secondary endpoints such as thrombus area, viscoelastography and clot kinetics, platelet reactivity, endothelial function and patient reported outcomes, showed no significant difference with treatment. Therefore the results of this study suggest the higher observed mortality risk in AMI patients with mild SCH may not be attenuated by LT4 treatment and that rather a raised serum TSH may simply be a biomarker of worse prognosis.

Previous observational studies have assessed the prevalence of SCH in patients with AMI (Ertugrul *et al.*, 2011; Molinaro *et al.*, 2012; Zahler *et al.*, 2019) and this study adds to this by identifying risk factors for the common thyroid dysfunction states which may be useful for clinicians managing patients with AMI . The prospective nature of this study meant data could be collected in a uniform manner and therefore assessed other important variables such as TPO antibodies and time of sampling on the prevalence of thyroid dysfunction and the rates of normalisation. The time of blood sampling from participants was a strong predictor of SCH with samples taken between 00:01 and 06:00 followed by samples between 18:01 and 24:00 being the strongest predictors of SCH. However, patients who had SCH diagnosed between 12:01 and 18:00 were more likely to remain in SCH on repeat testing with only 28% of patients normalising their TSH compared to 50% of patients who were initially diagnosed with SCH between 00:01 and 06:00. The present data suggests the time of sampling should be considered when devising the reference range for TSH to avoid the inappropriate classification and treatment of individuals with thyroid dysfunction. This is further supported by the mortality

data which found patients diagnosed with SCH solely based on the time-specific reference ranges to have higher mortality than when SCH was diagnosed using a uniform TSH reference range as per current practice. The current study therefore supports previous observational studies which have shown SCH to be associated with worse outcomes including mortality in patients with cardiovascular disease (Iervasi *et al.*, 2007; Rodondi *et al.*, 2010; McQuade *et al.*, 2011; Molinaro *et al.*, 2012; Rhee *et al.*, 2017; Seo *et al.*, 2018).

The prevalence of LT3 syndrome in this study was 1.3% and such patients had an increased mortality. This prevalence is lower than previous observational studies demonstrating a prevalence between 20-30% in patients with acute cardiac events including AMI (Iervasi *et al.*, 2003; Iervasi *et al.*, 2007; Lazzeri *et al.*, 2012; Molinaro *et al.*, 2012; Zhang *et al.*, 2012). However, these studies included patients with cardiac diseases other than AMI (Iervasi *et al.*, 2007; Molinaro *et al.*, 2012), whereas one study did not include patients with AMI (Iervasi *et al.*, 2003). A meta-analysis using a strict diagnostic criteria showed the pooled prevalence of LT3 syndrome to be 17.6% in patients with acute cardiovascular events (Wang *et al.*, 2017). These studies also demonstrated an increased long-term mortality in LT3 patients which is supported by the meta-analysis by Wang et al. A likely explanation for the low prevalence of LT3 in the current study may be due to all thyroid function tests being performed on admission to hospital, and no later than 24 hours post hospital admission. Furthermore, thyroid function was evaluated prior to coronary angiography, whereas in previous studies thyroid tests were performed beyond 1 week of admission. Therefore the impact from non-thyroidal illness and coronary angiographic dyes may likely explain the higher prevalence in previous studies as contrast media doubles the risk of thyroid dysfunction (Rhee *et al.*, 2012). In addition, the inclusion of patients with chronic heart diseases may have increased the prevalence of LT3 in these studies.

There was no significant association between FT3 and troponin levels. There was also no association between SCH and troponin levels meaning the increased mortality in SCH and LT3 may not be related to troponin levels or potential infarct size. This may explain that low TH levels post AMI are not due to the stress response of the myocardial insult but rather may be an exacerbating factor which could influence myocardial injury and hence mortality. Elevated inflammatory markers also augmented the increased mortality observed in LT3 patients with the presence of LT3 and an elevated CRP resulting in a three-fold increase in mortality compared to a two-fold increase in patients with a low FT3 and CRP. However, despite the current study and previous studies demonstrating adverse outcomes in patients with LT3 and

SCH post AMI, we cannot be sure of a casual association between the two. Therefore clinical studies involving T3 replacement are necessary to identify this link. The current data advances the literature and identifies a group of individuals who may potentially benefit from T3 therapy - AMI patients with low serum FT3 and high CRP levels.

The RCT showed no benefit of LT4 therapy on improving LV function. However, nearly 40% of patients recruited to the trial had evidence of preserved LV function and the effect of treatment in patients with worse LVEF remains unknown. It is interesting that the pre-specified subgroup analysis in patients with LVEF <55% suggested that LT4 may be associated with improvement in LVEF. There may be a treatment benefit in this specific group of patients with previous studies relating to SCH patients with heart failure demonstrating higher hospitalisation rates and worse outcomes compared to euthyroid patients with heart failure (Iacoviello *et al.*, 2008; Rodondi *et al.*, 2008; Silva-Tinoco *et al.*, 2011). Furthermore, other markers of LV contractility (such as global longitudinal strain) may offer greater prognostic information (Park *et al.*, 2018a). It is interesting that LT4 treatment showed a trend towards a reduction in LV diastolic and systolic volumes suggesting that irrespective of no significant improvement in LV function, TH treatment may ameliorate pathological cardiac remodelling.

Previous studies have demonstrated LT4 therapy to improve cardiac and vascular function (Biondi *et al.*, 1999; Monzani *et al.*, 2001; Taddei *et al.*, 2003; Ripoli *et al.*, 2005; Razvi *et al.*, 2007). This study showed no benefit of LT4 treatment on surrogate markers of vascular function such as endothelial dysfunction, thrombus burden and clot kinetics. Therefore any future trial that does show a benefit of LT4 therapy in reducing the cardiovascular events in SCH patients could be by alternative means other than ameliorating common cardiovascular risk factors.

5.2 Strengths and limitations

The strengths of this study include its study design in being a double-blinded RCT, being well powered to detect a significant difference between both groups and participants being on all recommended secondary prevention therapy and chosen based on a strict eligibility criteria to minimise confounding variables. Another strength was that cardiac MRI, considered as the reference-standard technique, was utilised to assess cardiac volumes and function (Pohost *et al.*, 2003). Despite the negative findings, a greater risk of mortality in patients with SCH after AMI highlights the need for on-going studies to reduce vascular event rates to levels seen in

euthyroid patients. One of the reasons for the discrepancy detected in the previous observational studies of poor prognosis in individuals with SCH and the lack of efficacy of LT4 shown in this trial could be because many observational studies diagnosed SCH based on a single blood test. In this trial, only patients with sustained subclinical hypothyroidism were recruited and all baseline thyroid function were assessed prior to coronary angiography. In addition, patients with mild subclinical hypothyroidism (at least one TSH value below 10.0 mU/L) were recruited, as this group constitutes the majority of subclinical hypothyroidism patients and where the greatest uncertainty of treatment efficacy prevails (Bekkering *et al.*, 2019). It remains unknown if targeting treatment in individuals with more severe disease (TSH >10.0 mU/L) may or may not be beneficial.

There are limitations to the study: a potential delay in the initiation of LT4 treatment, a median dose of 50mcg at the end of the study being lower than in other trials demonstrating a benefit on surrogate markers of vascular function, and the bioavailability of cardiac T3 potentially being low due to impaired conversion of T4 to T3 from changes in the activating and inactivating thyroid hormone enzymes (deiodinases). Furthermore, nearly 40% of patients recruited in this trial had evidence of preserved LV function and the effect of treatment in patients with worse LVEF remains unknown. Additional research might be useful to evaluate efficacy in this group of patients. Individuals with chronic persistent subclinical hypothyroidism may be a different population to the population studied here and it is not possible to exclude whether levothyroxine might improve LV function in this population outside the situation of a recent acute myocardial infarction. Finally, the TSH reference range used to diagnose SCH is not applicable to everyone with large studies such as the TRUST Study and a follow up study of over 400,000 patients demonstrating a large number of individuals diagnosed with SCH normalised their thyroid function during follow up (Meyerovitch *et al.*, 2007; Stott *et al.*, 2017). In the present study, the median TSH decreased from 5.7 (4.7-7.3) to 3.2 (2.7-4.2) at the end of the study in the placebo group indicating some patients normalised their TSH over the course of the study and may not have had true SCH despite the strict eligibility criteria.

5.3 Future directions

This study shows that treatment of SCH in AMI patients is not associated with an improvement in cardiac function or surrogate markers of vascular function. Further studies are needed to

investigate the worse outcomes observed in these patients which will help clarify whether SCH has a direct casual cause or is just a biomarker of a worse prognosis. One specific approach could include having stricter inclusion criteria to diagnose SCH based on time or age specific reference ranges rather than utilising a uniform TSH reference range as per current practice. An alternative approach would be to recruit patients with more severe SCH (with a TSH>10 mIU/L) as this group has been associated with worse mortality outcomes (Rodondi *et al.*, 2010), and the development of heart failure (Rodondi *et al.*, 2008; Nanchen *et al.*, 2012). This could set more clarity on treating such patients as per current guidelines where current evidence is lacking (Pearce *et al.*, 2013).

Further studies assessing LV function should assess SCH patients with an impaired LV function; as a preliminary analysis in the current study demonstrated a trend towards improvement in this group of patients. Additionally, the use of T3 could be an alternative to LT4 treatment in future studies investigating both SCH and LT3 syndrome which bypasses the activating thyroid hormone enzymes (deiodinases) used to convert T4 to T3, resulting in a higher therapeutic effect of TH treatment. An open-label trial of oral T3 in 37 patients with STEMI and low serum T3 levels demonstrated 6 months of oral T3 therapy to be safe although there was no improvement in LV function and scar size as measured by CMR (Pingitore *et al.*, 2019). In other clinical studies T3 therapy has been shown to improve left ventricular function in patients with heart failure (Pingitore *et al.*, 2008; Amin *et al.*, 2015) and those undergoing surgical revascularization (Klemperer *et al.*, 1995; Sirlak *et al.*, 2004; Ranasinghe *et al.*, 2006).

Further studies should assess the effect of T3 replacement on markers of inflammation in patients with AMI. This is important as the worse outcomes in patients with cardiovascular disease and inflammation, as shown in previous studies (Ridker *et al.*, 1997; Ridker *et al.*, 2017), could be mediated via low TH levels. Finally, irrespective of effects on cardiac function and surrogate markers vascular function, large RCTs should also be conducted to assess the role of TH replacement therapy on long term patient outcomes post AMI with such studies being adequately powered to detect any difference in mortality.

Appendices

Appendix 1: Questionnaires

Center for Epidemiologic Studies Depression Scale (CES-D), NIMH

Below is a list of the ways you might have felt or behaved. Please tell me how often you have felt this way during the **past week**. Circle **one** number on each line.

	During the Past Week			
	Rarely or none of the time (less than 1 day)	Some or a little of the time (1–2 days)	Occasionally or a moderate amount of time (3–4 days)	All of the time (5–7 days)
1. I was bothered by things that usually don't bother me	0	1	2	3
2. I did not feel like eating; my appetite was poor	0	1	2	3
3. I felt that I could not shake off the blues even with help from my family or friends	0	1	2	3
4. I felt I was just as good as other people	0	1	2	3
5. I had trouble keeping my mind on what I was doing	0	1	2	3
6. I felt depressed	0	1	2	3
7. I felt that everything I did was an effort	0	1	2	3
8. I felt hopeful about the future	0	1	2	3
9. I thought my life had been a failure	0	1	2	3
10. I felt fearful	0	1	2	3
11. My sleep was restless	0	1	2	3
12. I was happy	0	1	2	3
13. I talked less than usual	0	1	2	3
14. I felt lonely	0	1	2	3
15. People were unfriendly	0	1	2	3
16. I enjoyed life	0	1	2	3
17. I had crying spells	0	1	2	3
18. I felt sad	0	1	2	3
19. I felt that people dislike me	0	1	2	3
20. I could not get "going"	0	1	2	3

MINNESOTA LIVING WITH HEART FAILURE® QUESTIONNAIRE

The following questions ask how much your heart failure (heart condition) affected your life during the past month (4 weeks). After each question, circle the 0, 1, 2, 3, 4 or 5 to show how much your life was affected. If a question does not apply to you, circle the 0 after that question.

Did your heart failure prevent you from living as you wanted during the past month (4 weeks) by -	**No**	**Very Little**				**Very Much**
1. causing swelling in your ankles or legs?	0	1	2	3	4	5
2. making you sit or lie down to rest during the day?	0	1	2	3	4	5
3. making your walking about or climbing stairs difficult?	0	1	2	3	4	5
4. making your working around the house or yard difficult?	0	1	2	3	4	5
5. making your going places away from home difficult?	0	1	2	3	4	5
6. making your sleeping well at night difficult?	0	1	2	3	4	5
7. making your relating to or doing things with your friends or family difficult?	0	1	2	3	4	5
8. making your working to earn a living difficult?	0	1	2	3	4	5
9. making your recreational pastimes, sports or hobbies difficult?	0	1	2	3	4	5
10. making your sexual activities difficult?	0	1	2	3	4	5
11. making you eat less of the foods you like?	0	1	2	3	4	5
12. making you short of breath?	0	1	2	3	4	5
13. making you tired, fatigued, or low on energy?	0	1	2	3	4	5
14. making you stay in a hospital?	0	1	2	3	4	5
15. costing you money for medical care?	0	1	2	3	4	5
16. giving you side effects from treatments?	0	1	2	3	4	5
17. making you feel you are a burden to your family or friends?	0	1	2	3	4	5
18. making you feel a loss of self-control in your life?	0	1	2	3	4	5
19. making you worry?	0	1	2	3	4	5
20. making it difficult for you to concentrate or remember things?	0	1	2	3	4	5
21. making you feel depressed?	0	1	2	3	4	5

Name_____ Date _____

SF-12v.2 HEALTH SURVEY

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.

Answer every question by selecting the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

1. In general, would you say your health is:

Excellent	Very good	Good	Fair	Poor
○	○	○	○	○

2. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	○	○	○
b. Climbing several flights of stairs	○	○	○

3. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Accomplished less than you would like	○	○	○	○	○
b. Were limited in the kind of work or other activities.	○	○	○	○	○

4. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Accomplished less than you would like	○	○	○	○	○
b. Did work or activities less carefully than usual	○	○	○	○	○

Appendix 2: Publications and presentations

A. Peer reviewed publications

1. Jabbar A, Ingoe L, Junejo S, Carey P, Addison C, Thomas H, Parikh J, Austin D, Hollingsworth K, Stocken D, Pearce S, Greenwood J, Zaman A, Razvi S.
   Effect of levothyroxine on left ventricular ejection fraction in patients with subclinical hypothyroidism and acute myocardial infarction: a randomized clinical trial.
   JAMA 2020 Jul 21;324(3):249-258.
2. A Jabbar, L Ingoe, H Thomas, P Carey, S Junejo, C Addison, J Vernazza, D Austin, J P Greenwood, A Zaman, S Razvi.
   Prevalence, predictors and outcomes of thyroid dysfunction in patients with acute myocardial infarction: the ThyrAMI-1 study.
   J Endocrinol Invest 2020 Sep 8. doi: 10.1007/s40618-020-01408-0.
3. Salman Razvi, Owain Leng , Avais Jabbar, Arjola Bano, Lorna Ingoe, Caroline Addison, Honey Thomas, Peter Carey, Shahid Junejo, David Austin, John P Greenwood, Azfar Zaman. Sample Timing, Diagnosis of Subclinical Thyroid Dysfunction and Mortality in Acute Myocardial Infarction: ThyrAMI1 Study.
   J Clin Endocrinol Metab 2020 Apr 1;105(4)
4. Razvi S, Jabbar A, Pingitore A, Danzi S, Biondi B, Klein I, Peeters R, Zaman A, Iervasi G. Thyroid Hormones and Cardiovascular Function and Diseases.
   J Am Coll Cardiol. 2018 Apr 24;71(16):1781-1796.
5. Jabbar A, Pingitore A, Pearce SH, Zaman A, Iervasi G, Razvi S.
   Thyroid hormones and cardiovascular disease.
   Nature Reviews Cardiology 2017 Jan;14(1):39-55.
6. Jabbar A, Ingoe L, Pearce S, Zaman A, Razvi S.
   Thyroxine in acute myocardial infarction (ThyrAMI) - levothyroxine in subclinical hypothyroidism post-acute myocardial infarction: study protocol for a randomised controlled trial.
   Trials 2015 Mar 25;16:115.
7. Jabbar A, Razvi S.
   Thyroid disease and vascular risk.
   Clinical Medicine 2014 Dec;14 Suppl 6:s29-32.

B. Posters and Regional Meetings

1. Prevalence of and factors predicting thyroid dysfunction at the time of ST- and non-ST- elevation myocardial infarction – the ThyrAMI 1 study. Newcastle Cardiovascular Research Meeting 2017.
2. Prevalence of and factors predicting thyroid dysfunction at the time of ST- and non-ST- elevation myocardial infarction – the ThyrAMI 1 study. The British Thyroid Association Meeting 2018.
3. Prevalence of and factors predicting thyroid dysfunction at the time of ST- and non-ST- elevation myocardial infarction – the ThyrAMI 1 study. NEERAG (Northern Endocrine Regional Research and Audit Group) 2017.

C. Book Chapter

1. Jabbar A, Razvi S. (2020) 'Blood Pressure in Thyroid Dysfunction', in Thyroid and Heart – a comprehensive translational essay. Eds: Iervasi G, Pingitore A, Gerdes AM, Razvi S. Springer, pp. 239-244.

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