# The role of Vitamin D and Osteocalcin on Diabetic Macrovascular and Microvascular Events in the FIELD Study

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August, 2013

<u>**Response to thesis examiners remarks:**</u>

**The first examiner had two main issues which required addressing before the thesis could be accepted.**

**The first issue was that the examiner has queried the reasoning behind reporting 25OH Vitamin D results per 50nmol/L decrease in vitamin D. Their assertion being that the standard practise is to report results per 1SD change, which would equate to roughly 25nmol/L.**

When we presented a preliminary analysis at the EAS in 2012 the analysis, at that stage, showed:  Eight percent, 52% and 89% of subjects had serum vitamin D levels <25, <50 and <75nmol/L respectively.  Serum 25OH-D was an independent predictor of both macrovascular and microvascular events, and remained so in multivariate analysis stratified for study treatment allocation and adjusted for potential confounders. Each 25nmol/L decrease in serum 25OH-D was associated with a relative increase of 11.4% (95% CI 3-20%, P<0.01) in macrovascular complications and 10.1% (95%CI 4-17%, P<0.01) in microvascular complications. Compared with those in the top quartile of serum 25OH-D, the lowest group showed 21% (*P*<0.02) and 23% (*P*=0.001) greater macrovascular and microvascular event risks respectively. Subsequent adjustments removed the independence of the relationship between 25OH-D and microvascular complications. Additionally, changing to a broader interval of 50 nmol/L (approximately 25% of the range) does not alter the slope or significance of the association, but it does increase the incremental odds ratio. The authors of this study have prepared a paper for publication using the 50nmol/L cut point because this is commonly used in metanalyses and clinical studies where it approximates to the threshold for adequacy. It is with this in mind, and with the support of my supervisor, that I stand by the use of 50nmol/L.

Included below are graphs showing this comparison, with Table 1 being the analysis per 50nmol/L decrease and Table 2 being analysis per 25nmol/L decrease.

**The second remark made by the examiner was that no data was presented to support the statement "exploratory further adjustment for physical activity score did not materially change these relationships". The examiner asks for the data to be presented, or a reference made to the original publication, as well as a reference to this method of physical activity score being used to validate its inclusion.**

I agree with the examiner's remarks, and so a new table, "Table 16", has been included to this manuscript on p87 and shows the effect of physical activity on vitamin D levels. This is linked with the text on p74 with the statement:

*"Exploratory further adjustment for physical activity score or hs-CRP level did not materially change these relationships (table 16)."*

Additionally, a reference to a study by Rankin *et al*. has been included on p57 under the heading "assessment of other clinical variables" to justify the use of this method of scoring.

**The examiner has also suggested a number of minor changes and alterations that, while good to address, are not required for the completion of this thesis. Among these comments is the statement that the graphs and tables are too difficult to read and that they require adjustment. There are also a small number of formatting and spelling inconsistencies to rectify to enable smooth reading of the document.**

Graphs and tables have been changed and are far easier to read. Spelling and grammar has been inspected again with minor edits. I have fixed the in-text hyperlinking formatting that resulted in an error message being displayed instead of a figure or table number.

**The second examiner raised the issue that it was not clear what tests were performed on which autoanalyser in the section heading 2.5. Sample Testing.**

While it is outlined in sections 2.6, 2.7 and 2.8 what tests were performed, I have included the following paragraphs at the end of sections 2.5.1 and 2.5.2 for the sake of clarity:

Section 2.5.1, p50: *Tests performed on the DiaSorin Liaison included 25OH Vitamin D and Osteocalcin assays.*

Section 2.5.2, p52: *Tests performed on the Roche module included basic biochemistries, liver and kidney biomarkers, which are outlined in section 2.8. Of particular focus in this project, the Roche module was used to measure the inflammatory marker, C - reactive protein.*

**Both examiners expressed a desire to see more detail into additional discoveries or potential from the results of this study.**

While we are thrilled at the interest this thesis is generating, my supervisor and I feel that it's not within the scope of the research thesis, at this particular time, to explore these concepts in full. Additional themes of this thesis will be explored and made available in publication at a later date once more data is collected and analysed.

**Table 1: Comparison of 50nmol/L decrease of 25OH Vitamin D**

| | n | Events | % | Unadjusted model | | Adjusted model | |
|---|---|---|---|---|---|---|---|
| | | | | OR or HR (95% CI) | P | OR or HR (95% CI) | P |
| **Macrovascular** | | | | | | | |
| 25OH-D (nmol/L) (per 50 nmol/L decrease) | 9524 | 1250 | 13.12 | 1.20 (1.05–1.39)† | 0.01 | 1.23 (1.06–1.42) † | 0.007 |
| **Microvascular** | | | | | | | |
| 25OH-D (nmol/L) (per 50 nmol/L decrease) | 9524 | 2395 | 25.15 | 1.18 (1.05–1.32)† | 0.006 | 1.11 (0.98–1.26) † | 0.11 |

**Table 2: 25nmol/L decrease of 25OH Vitamin D**

| | n | Events | % | Unadjusted model | | Adjusted model | |
|---|---|---|---|---|---|---|---|
| | | | | OR or HR (95% CI) | P | OR or HR (95% CI) | P |
| **Macrovascular** | | | | | | | |
| 25OH-D (nmol/L) (per 25 nmol/L decrease) | 9524 | 1250 | 13.12 | 1.10 (1.02–1.18)† | 0.01 | 1.11 (1.03–1.19) † | 0.007 |
| **Microvascular** | | | | | | | |
| 25OH-D (nmol/L) (per 25 nmol/L decrease) | 9524 | 2395 | 25.15 | 1.08 (1.02–1.15)† | 0.006 | 1.05 (0.99–1.12) † | 0.11 |

## Abstract

**Objective**

People with diabetes frequently develop vascular disease, and accumulating evidence suggests a coupled interaction between bone turnover and glycaemic control. We investigated the relationship between blood 25-hydroxy vitamin D (25OH-D) and osteocalcin (OCN) concentration on vascular disease risk in type 2 diabetes.

**Research design and methods**

The relationships between blood 25OH-D and OCN concentration at baseline and the incidence of macrovascular (including myocardial infarction, stroke) and microvascular (retinopathy, nephropathy, neuropathy, and amputation) disease were analysed with Cox proportional-hazards models and logistic regression in an observational study of patients in the 5-year Fenofibrate Intervention and Event Lowering in Diabetes trial.

**Results**

50% of the patients low 25OH-D concentrations, as indicated by median blood 25OH-D concentration of 49nmol/L. These patients with a blood 25OH-D concentration < 50nmol/L had a higher cumulative incidence of macrovascular and microvascular events than those with levels ≥ 50nmol/L. Patients with OCN levels below the population mean of 9.1 ng/mL show a significantly higher cumulative incidence of microvascular events than those with ≥ 9.1 ng/mL. Higher baseline serum OCN was associated with a reduction in risk of microvascular events of 8% per 5ng/mL. Undercarboxylated OCN was not a significant predictor of micro- or macrovascular events. Multivariate analysis, stratified by treatment and adjusted for relevant confounders, identified blood 25OH-D and OCN concentration as an independent predictor of macrovascular and microvascular events.

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**Statement of Originality:**

I declare that studies presented in this thesis are the result of original research conducted during my enrolment as a candidate for the degree of Master of Philosophy in Medicine at the University of Sydney.

The experimental work on the novel biomarkers is an extension to the FIELD study, with laboratory measurements performed on samples collected during the course of the study. The material presented in this thesis has not been published elsewhere, except where due acknowledgement has been made.

**List of Abbreviations:**

| | |
|---|---|
| BMI | Body Mass Index |
| $Ca^{2+}$ | Calcium |
| cAMP | Cyclic Adenosine Monophosphate |
| CHD | Coronary Heart Disease |
| CRP | C-Reactive Protein |
| CVD | Cardiovascular Disease |
| DM | Diabetes Mellitus |
| FIELD | Fenofibrate Intervention and Event Lowering in Diabetes |
| HA | Hydroxyapatite |
| HbA1c | Glycated Haemoglobin A1c |
| HDL | High-Density Lipid |
| HOMA-IR | Homeostatic Model Assessment – Insulin Resistance |
| Hs-CRP | High Sensitive C-Reactive Protein |
| IQR | Interquartile Range |
| LDL | Low-Density Lipid |
| MI | Myocardial Infarction |
| NHMRC | National Health and Medical Research Council |
| NHS | National Health Survey |
| OCN | Osteocalcin |
| OR | Odds Ratio |
| PTH | Parathyroid Hormone |
| PVD | Peripheral Vascular Disease |
| RAS | Renin-Angiotensin System |

| | |
|---|---|
| **RECORD** | Rosiglitazone Evaluated for Cardiovascular Outcomes in Oral Agent Combination Therapy for Type 2 Diabetes |
| **T1DM** | Type 1 Diabetes Mellitus |
| **T2DM** | Type 2 Diabetes Mellitus |
| **UKPDS** | U.K. Prospective Diabetes Study |
| **UV** | Ultraviolet light |
| **VDR** | Vitamin D |
| **VIDAL** | Vitamin D and Longevity Trial |
| **VITAL** | Vitamin D and Omega-3 Trial |
| **WHO** | World Health Organisation |

**Table of Contents**

**Chapter 1: Introduction**

**Section 1.1: Cardiovascular Disease**

Cardiovascular disease (CVD) is the leading cause of mortality, accounting for an estimated 17.3 million deaths worldwide in 2008. The World Health Organisation (WHO) estimates that by 2030 this figure will rise to 23.3 million deaths. More people die of heart disease each year than any other cause[1]. In Australia CVD accounted for 34% of deaths; this is consistent with the global trend. Based on self-reported data in the 2007-08 National Health Survey (NHS) an estimated 3.4 million people (17% of the total Australian population) reported to have one or more cardiovascular diseases. CVD directly contributed to 475,000 hospitalisations and indirectly contributed to a further 797,000. It remains one of the most expensive disease groups, costing $5.9 billion in 2004-2005 [2].

**Section 1.1.1: Types of Cardiovascular disease**

 CVD refers to a group of disorders that affect the heart and blood vessels. They include:

- *coronary heart disease* – disease of the blood vessels supplying the heart muscle;

- *cerebrovascular disease* – disease of the blood vessels supplying the brain (stroke);

- *peripheral arterial disease* – disease of blood vessels supplying the arms and legs;

- *rheumatic heart disease* – damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria;

- *congenital heart disease* – a normal heart structure existing at birth;

- *deep vein thrombosis* – blood clots in the veins, which can dislodge and move to the heart and lungs.

The predominant types of CVD that occur are coronary heart disease, stroke and, particularly when type 2 diabetes mellitus (T2DM) is a co-morbidity, peripheral arterial disease.

Coronary Heart Disease (CHD), also known as ischaemic heart disease, is the most common form of CVD in Australia, with approximately 3% of the population (around 685,000 people) having an event at some point in their life [2]. There are two forms that CHD commonly presents as – myocardial infarction (MI) and angina. MI is an acute event where blood supply to the heart is blocked, causing damage to the heart muscle and often resulting in fatality. Angina is a chronic condition where blood flow to the heart is temporarily deficient. It is generally not life-threatening on its own; however patients with angina are more likely to suffer an MI or similar events than those without it.

The risk of CHD is greatly increased with age. In 2007-2008, 7% of Australians aged 55-65years were estimated to have CHD. This number jumps to 24% in those aged 85 years and older. The rates are higher for males than females in both groups [2].


Cerebrovascular disease is a broad category used to describe diseases concerning the condition of blood vessels leading to the brain. One of the most common forms of cerebrovascular disease is a stroke, which occurs when an artery supplying blood to the brain becomes blocked or begins to bleed. Strokes are divided into two categories – ischaemic and haemorrhagic. The former is caused by a blockage of a blood vessel by a blood clot or particle of some kind. The latter defines the rupturing and subsequent bleeding of a blood vessel. Both forms of stroke are often fatal.

A 2003 survey reported that an estimated 1.7% of females (168,400 people) and 1.8% of males (178,300) in the Australian population had suffered a stroke in their lifetime. The prevalence of stroke increased dramatically with age for both sexes, with 8.1% of males and 5.3% of females aged 65-74 years having an event. This prevalence increased to 14.7% and 11.4% respectively in persons aged 75-84 years, and increased again to 15.1% and 17.1% respectively in persons aged 85 years and over [2, 3].

Peripheral vascular disease (PVD) occurs when there is an obstruction in blood supply to the peripheral systems, such as hands and feet. The two common ways PVD presents are atherosclerosis of the peripheral arteries (APA) and abdominal aortic aneurysm (AAA). APA most commonly affects the arteries supplying blood to the legs and feet, due to atherosclerotic conditions causing the narrowing and potential occlusion of blood vessels. In extreme cases, patients with APA may require amputation of the affected area. AAA refers to the abnormal widening of the aorta in the abdomen below the diaphragm. This can become life-threatening as it increases the risk that the aortic wall will rupture and bleed, and may require surgical intervention to resolve.

The risk factors for PVD increase with tobacco use, high levels of cholesterol, high blood pressure and obesity – all of which are the most common risk factors for CVD. There is limited data on the percentage of the population who reported having PVD in one of its forms. Data from the 2007-08 NHS showed that there were 25,796 hospitalisations due to PVD, or about 5% of total CVD hospitalisations. The majority of these were for APA, 56%, with AAA accounting for a further 18%. The remainder were comprised primarily of other kinds of aneurysms and embolisms [2].

**Section 1.1.2: Risk factors for CVD**

There are some non-modifiable risk factors for CVD such as age, sex, family history and ethnicity that can affect the incidence and severity of CVD. The WHO identifies aging as the most powerful risk factor for CVD. Many of the risk factors for CVD, however, are largely avoidable or able to be managed. A 2003 analysis of the burden of disease in Australia highlighted that there were twelve modifiable risks associated with CVD (Figure 1). High blood pressure and high blood cholesterol were the largest contributors, followed by physical inactivity, high body mass, tobacco use, alcohol consumption, and low fruit and vegetable consumption [3].

**Figure 1: Broad cause groups for burden of disease in Australia [3]**

| | Broad cause group | | | | | | | All causes |
|---|---|---|---|---|---|---|---|---|
| | Cancer | CVD | Mental | Neuro-logical | Injury | Diabetes | Other | |
| Total burden ('000) | 499.4 | 473.8 | 350.5 | 312.8 | 185.1 | 143.8 | 667.4 | 2,632.8 |
| Attributable burden (%)[a] | | | | | | | | |
| Tobacco | 20.1 | 9.7 | — | −0.6 | 0.5 | — | 8.9 | 7.8 |
| High blood pressure | — | 42.1 | — | — | — | — | — | 7.6 |
| High body mass | 3.9 | 19.5 | — | — | — | 54.7 | 1.1 | 7.5 |
| Physical inactivity | 5.6 | 23.7 | — | — | — | 23.7 | >−0.1 | 6.6 |
| High blood cholesterol | — | 34.5 | — | — | — | — | — | 6.2 |
| Alcohol | | | | | | | | |
|   Harmful effects | 3.1 | 0.9 | 9.7 | — | 18.1 | — | <0.1 | 3.3 |
|   Beneficial effects | — | −5.6 | — | — | — | — | >−0.1 | −1.0 |
|   Net effects | 3.1 | −4.7 | 9.7 | — | 18.1 | — | <0.1 | 2.3 |
| Low fruit & vegetable consumption | 2.0 | 9.6 | — | — | — | — | >−0.1 | 2.1 |
| Illicit drugs | — | <0.1 | 8.0 | — | 3.6 | — | 2.5 | 2.0 |
| Occupational exposures & hazards | 3.1 | 0.4 | — | 0.8 | 4.7 | — | 3.4 | 2.0 |
| Intimate partner violence | 0.5 | 0.3 | 5.5 | 0.1 | 2.5 | — | 0.2 | 1.1 |
| Child sexual abuse | <0.1 | <0.1 | 5.8 | — | 1.4 | — | <0.1 | 0.9 |
| Urban air pollution | 0.8 | 2.7 | — | — | — | — | 0.4 | 0.7 |
| Unsafe sex | 1.0 | — | — | — | — | — | 1.4 | 0.6 |
| Osteoporosis | — | — | — | — | 2.4 | — | — | 0.2 |
| Joint effect[b] | 32.9 | 69.3 | 26.9 | 0.2 | 31.7 | 60.1 | 17.2 | 32.2 |

(a)   Attributable burden within each column is expressed as a percentage of total burden for that column.
(b)   Figures for joint effects are not column totals. See Section 4.1 for further details.

**High Blood Pressure and CVD**

Also known as hypertension, high blood pressure is a major risk factor for nearly all forms of CVD. It is also considered to be a form of CVD on its own. On a global scale, hypertension has been found to be responsible for more deaths and disease than any other biomedical factor. High blood pressure is accountable for 42% of the burden of CVD, making it the largest contributor to this disease. Four-fifths of this burden related to premature death and the remainder to disability [3].

The WHO defines high blood pressure as any of the following [1]:

- systolic blood pressure of 140 mmHg or more
- diastolic blood pressure of 90 mmHg or more
- receiving medication for high blood pressure.

High blood pressure affects CVD by exerting pressure on arterial walls. High blood pressure is a major risk factor for stroke, coronary heart disease, heart failure, peripheral vascular disease and kidney failure. The risk factors for high blood pressure are largely the same as those for other forms of CVD. They include age, poor diet (particularly a high salt intake), obesity, excessive alcohol consumption, and insufficient physical activity. Studies have shown there is a relationship between high blood pressure and risk of CVD, chronic kidney disease and death [4]. When high blood pressure is controlled, the risk is reduced, but not necessarily to the level of an unaffected person [5].

**High blood cholesterol and CVD**

Cholesterol is a fatty substance produced by the liver and carried by the blood to supply the rest of the body. Its natural function is to supply material for cell walls and hormones, however,

excessive levels in the blood can lead to atherosclerosis and heart disease. Two of the components of cholesterol can play an important role in CVD: low-density lipoprotein (LDL) cholesterol, often known as 'bad' cholesterol, and high-density lipoprotein (HDL) cholesterol, often known as 'good' cholesterol.

High levels of LDL can contribute to atherosclerosis. In contrast, high levels of HDL can have a protective effect and help reduce atherosclerosis. Although LDL and HDL have opposing effects, the total cholesterol level is often used as an indicator of CVD risk and, as a general principle, the lower it is, the better. For clinical and population monitoring purposes a level of 5.5 mmol/L is often labelled as 'increased' and one of 6.5 mmol/L or more as 'high', however these are arbitrary levels and desirable levels may be below 4 mmol/L.

For most people, saturated fat in the diet is the most important factor associated with elevated blood cholesterol levels, although genetic factors can also affect blood cholesterol levels in some individuals. Sufficient physical activity and a healthy diet play an important role in maintaining healthy blood cholesterol levels. High blood cholesterol was the second highest contributor, with 35% of the burden of CVD after high blood pressure [3].

**Smoking and CVD**

As its components are absorbed into the bloodstream, tobacco smoke increases the risk of CVD through many mechanisms. It damages blood vessels, increases the risk of plaques, increases the risk of clots at the site of plaques and reduces blood oxygen levels. Smoking cessation is associated with substantially improved cardiovascular function and reduced risk of cardiovascular morbidity and mortality [6]. The risk of a coronary event among smokers declines rapidly after quitting. One year after cessation, the risk of CHD is halved when compared to those who continue to smoke [7] and within 2–6 years, the risk is similar to that of non-smokers, although some studies have found there is a residual increased risk for up to 10 years [8, 9].

Based on the 2007 National Drug Strategy Household Survey (NDSHS), 17% of Australians aged 14 years and over smoked tobacco daily in 2007, equating to 2.9 million people. A further 1% smoked tobacco weekly and 2% smoked less than weekly. In total, just over 19% of Australians aged 14 years and over were current smokers in 2007 [3].

**Physical activity and CVD**

Physical activity is any bodily movement that the muscles produce which results in energy expenditure. This activity can be in the form of deliberate activity in leisure time, such as exercise or sport, or other forms of non-leisure activity, such as walking or cycling for transport. Occupational activity, activity associated with a person's job, and incidental activity, the activity associated with everyday tasks such as shopping and housework can contribute to overall physical activity and its associated health benefits [3]. If the food energy going into the body is not balanced by energy expenditure over a sustained period of time, the excess food energy is stored as body fat resulting in weight gain and the possibility of becoming overweight or obese. The National Physical Activity Guidelines for Australians [10] recommend at least 30 minutes of moderate-intensity physical activity on most, preferably all, days of the week. Physical inactivity is associated with an increased risk of ill health and death, particularly relating to CVD. People who do not participate in regular physical activity are almost twice as likely to die from coronary heart disease as those who do participate [11]. Regular physical activity, whether deliberate or incidental, has a protective effect, lowering the risk of developing CVD and other CVD risk factors. Based on NHS definitions, in 2007–08 about 72% of Australians aged 15 years and over did not undertake sufficient physical activity, being either sedentary or having low levels of activity.

**Diet and CVD**

A poor diet refers to a diet which does not support good health outcomes, or which could contribute to poor health. Dietary behaviour plays an important role in health and wellbeing and can either reduce or increase the risk of various diseases, including the development of CVD. Poor dietary behaviour contributes to several biomedical risk factors, including high blood pressure and blood cholesterol levels, as well as obesity and T2DM. The National Health and Medical Research Council (NHMRC) dietary guidelines for all Australians recommend that people eat a wide variety of nutritious foods and limit the intake of sugar, salt, alcohol and fat, especially saturated fat [12].

There are several different types of fats, including saturated, polyunsaturated, monounsaturated and trans fats. Diets high in saturated or trans fat increase the risk of CHD by raising blood cholesterol levels, notably the LDL form of cholesterol.

Consumption of fruit, vegetables and legumes reduces the risk of CVD, as well as other diseases such as T2DM and some cancers [2]. The beneficial components of fruit and vegetables are not yet fully understood, but there is evidence that antioxidant phytochemicals, antioxidants and other vitamins, minerals and fibre found in fruit and vegetables may contribute to lower levels of blood cholesterol, blood pressure and atherosclerosis.


**Alcohol and CVD**

The risk of alcohol-related harm can be measured as either short-term or long-term risk. Long-term excessive drinking is associated with CVDs such as stroke, heart disease, hypertension, heart failure and congenital heart disease [4, 13]. The lifetime risk of harm from drinking alcohol increases with the amount consumed. Additionally, alcohol is a source of energy and therefore must be considered for its potential to increase body mass and lead to overweight and obesity. The 10 grams of alcohol in a standard drink contains 290 kilojoules - around the same amount of energy as found in 8 grams of fat. Alcohol can also affect blood triglyceride levels, complicating

the effects of high blood cholesterol [2]. Low levels of alcohol have been thought to provide some protection against CVD. However, the recent Australian Burden of Disease and Injury Study reported that the only group for whom the benefits of small amounts of alcohol outweighed the harmful effects were females over the age of 65 years [3] and suggested that any benefits from alcohol consumption are restricted to middle-aged and older adults in countries with high rates of CVD [14]. Other research advises that benefits from alcohol consumption only occur at very low levels of drinking, or that there is no protective factor at all [14].

**Obesity and CVD**

Overweight, body mass and obesity are conditions in which a person is considered to carry an unhealthy amount of excess body weight through consuming greater than the required amount of energy that the body uses. This results in weight gain over time as the unused food energy is stored as body fat. Obesity is a severe form of being overweight and is a well-established risk factor for CVD [15]. Being overweight, and in particular being obese, is a risk factor for diseases and conditions that include CHD, high blood pressure, high blood cholesterol, T2DM, certain cancers, psychosocial problems and musculoskeletal conditions. As the amount of excess weight increases, so does the risk of developing these conditions. In addition, being overweight can hamper the ability to control or manage chronic disorders. Weight loss reduces the incidence and severity of the majority of these disorders.

The two main methods for monitoring body weight at the population level are the body mass index (BMI) and waist circumference. BMI is the most commonly used measure for monitoring overweight and obesity in a population. It is calculated by dividing a person's weight in kilograms by the square of their height in metres (kg/m2).

**Table 1: Body Mass Index**

| BMI Classifications: |
| --- |
| *Underweight = <18.5* |
| *Normal weight = 18.5 – 24.9* |
| *Overweight = 25 – 29.9* |
| *Obesity = >30* |

The classifications of overweight body mass and obesity are based primarily on the association between BMI and mortality and are the standards recommended by WHO and outlined in the National Health Data Dictionary (NHDD). These classifications may not be suitable for all ethnic groups, or for children and adolescents [16].

Rates of overweight body mass and obesity continue to increase in Australia and overseas. Based on data measured in the 2007–08 NHS, the majority of adults (60%) had a BMI indicating they were overweight or obese; just under a quarter of these fell into the obese category [3].

About 8% of the burden of disease and injury in Australia in 2003 was attributed to high body mass, placing it third in the 14 risk factors studied. After high blood pressure, high blood cholesterol and physical inactivity, it was the fourth largest contributor to the burden of CVD, accounting for 20% [3].

**Section 1.2: Diabetes**

Diabetes mellitus (DM) is one of the fastest growing causes of mortality, with the WHO projecting that by 2030 DM will be the 7th leading cause of death worldwide. DM currently affects over 347million people globally [17]. In Australia, the 2007-2008 NHS reported that an estimated 898,800 people (4.4% of the total population) had been told by a health care professional that they had DM at some point in their lives. Additionally, data from the 2004-2005 National Aboriginal and Torres Strait Islander Health Survey reports that an estimated 6.3% of the total Indigenous population have DM. In both surveys, non-insulin dependent diabetes, or T2DM, accounted for almost 90% of all diabetic cases [18].

Type 2 diabetes mellitus (T2DM) is a chronic condition in which the body cannot properly use its main energy source, the sugar glucose. Insulin is the hormone responsible for regulating levels of glucose in the blood, and is produced by pancreatic β-cells. If there is an increase in the resistance to insulin, then increased levels are required to overcome this. Diabetes can then arise due to either the pancreas not producing enough insulin to meet demand, or the body is unable to effectively use the insulin produced. Unlike conditions in Type 1 DM (T1DM), there is no autoimmune destruction of insulin-producing beta-cells in the pancreas. Instead, T2DM arises from a number of contributing factors, namely obesity, impaired insulin action, impaired insulin secretion, and increased endogenous glucose output [19]. The exact way in which these factors influence the development and progression of T2DM is not yet fully understood. What is well established, however, is that overweight body mass and obesity are strongly associated with development of T2DM and may be responsible for the majority of the growing diabetes pandemic. Furthermore, weight loss is strongly associated in prospective studies with decreased progression from impaired glucose action to T2DM [20].

DM is often characterised by hyperglycaemia, that is, excess blood sugar levels. As mentioned, one of the earliest detectable signs of T2DM onset is the body's impaired ability to respond to insulin. The pancreas responds by increasing the output of insulin secretion to offset resistance. For reasons that are still unclear, β-cell insulin secretory capacity declines and hyperglycemia becomes more severe [21]. Excessively high levels of blood sugar can, over time, cause significant damage to a number of bodily systems. Effects on the vascular system are one of the major sources of morbidity and mortality in patients with T2DM, with many complications involving either macrovascular (large vessel) or microvascular (small vessel) pathologies.

**Section 1.2.1: Macrovascular Disease**

Macrovascular disorders are those which involve an array of complications brought about through the many conditions, such as MI and stroke, caused by CVD. The predominant process involved in macrovascular complications is atherosclerosis [22, 23]. Atherosclerosis is particularly prevalent in persons with T2DM, as damage to blood vessel walls due to hyperglycaemia causes an inflammatory response. In response to endothelial injury and inflammation, oxidized lipids from LDL particles accumulate in the endothelial wall of arteries. Monocytes infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes, which in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation. This process results in the development of an atheroma; a lipid-rich atherosclerotic lesion with a fibrous cap which results in the narrowing the affected vessel [22]. Rupture or fragmentation of the atheroma can result in a number of acute CVD conditions including MI, stroke or pulmonary embolism [24]. In addition to atheroma formation, there is strong evidence of increased platelet adhesion and hypercoagulability in patients with T2DM. Impaired nitric oxide generation and increased free radical formation in platelets, as well as altered calcium regulation, may promote the aggregation of platelets, therefore contributing to the formation of clots [22]. Increased levels

of plasminogen activator inhibitor type 1 may also impair the ability to break down clots in patients with T2DM. The combination of increased blood coagulability and impaired fibrinolysis further increases the risk of vascular occlusion and therefore major cardiovascular events, such as coronary heart disease (CHD).

**Section 1.2.2: Microvascular disease**

Microvascular complications are those which involve the myriad of small vessels throughout the body. They include damage to vision (retinopathy), kidneys (nephropathy) and nerves (neuropathy). The risk of developing microvascular disease as a result of DM depends on the severity and duration of hyperglycaemia.

**Section 1.2.3: Diabetic Retinopathy**

Diabetic retinopathy is one of the most common microvascular complications of DM. It is responsible for approximately 10,000 new cases of blindness every year in the United States alone [25]. The risk of developing diabetic retinopathy or other microvascular complications of DM depends on both the duration and the severity of hyperglycemia. The U.K. Prospective Diabetes Study (UKPDS) showed that development of diabetic retinopathy in patients with T2DM was related to both severity of hyperglycemia and presence of hypertension in, and most patients with T1DM develop evidence of retinopathy within 20 years of diagnosis[26]. Retinopathy may begin to develop as early as 7 years before diagnosis in patients with T2DM. The exact pathological process leading to retinopathy is still unclear, however there are several proposed pathological mechanisms by which retinopathy may occur in diabetes.

The first of these mechanisms involves the enzyme aldose reductase, which is the initial enzyme in the intracellular polyol pathway. This pathway involves the conversion of glucose into glucose

alcohol, or sorbitol. High glucose levels increase the flow of sugar molecules through the polyol

pathway, which causes sorbitol accumulation in cells. It is thought that osmotic stress from this

influx of sugar and resulting sorbitol accumulation could be an underlying mechanism in the

development of diabetic microvascular complications. This theory is supported by animal models,

in which sugar alcohol accumulation has been linked to microaneurysm formation, thickening of

basement membranes, and loss of pericytes [27, 28]. Studies with aldose reductase inhibitors as a

treatment method, however, have been unsuccessful [27]. Growth factors, including vascular

endothelial growth factor (VEGF), growth hormone, and transforming growth factor β, have also

been proposed as possible mechanisms which play important roles in the development of

diabetic retinopathy. VEGF production is increased in diabetic retinopathy, possibly in response to

hypoxia. In animal models, suppressing VEGF production is associated with lower incidence and

slower progression of retinopathy [22].


Diabetic retinopathy is generally classified as either background or proliferative. Background

retinopathy includes features such as small hemorrhages in the middle layers of the retina.

Clinically, they appear as small dots across the retina and thus are generally referred to as 'dot

hemorrhages'. Microaneurysms are small vascular dilatations that occur in the retina, often the

first sign of retinopathy, and appear clinically as red dots during retinal examination [29]. Retinal

oedema may result from microvascular leakage and is indicative of compromise of the blood-

retinal barrier and may require intervention because of an associated with visual deterioration.

Proliferative retinopathy is characterised by the formation of new blood vessels on the surface of

the retina and can lead to vitreous hemorrhage. White areas on the retina, referred to as "cotton

wool spots", can be a sign of impending proliferative retinopathy [29]. If proliferation continues,

blindness can occur through vitreous hemorrhage and subsequent traction retinal detachment.

Without intervention, visual loss may occur. Laser photocoagulation can often prevent

proliferative retinopathy from progressing to blindness and therefore, close surveillance for the existence or progression of retinopathy in patients with diabetes is crucial[25].

**Section 1.2.4: Diabetic Nephropathy**

Nephropathy is the leading cause of renal failure in the United States. It is defined by proteinuria of more than 500 mg in 24 hours in the setting of DM, however this is preceded by lower degrees of proteinuria, or microalbuminuria. Microalbuminuria is defined as albumin excretion of 30–299 mg in 24 hours. Without intervention, diabetic patients with microalbuminuria typically progress to proteinuria and overt diabetic nephropathy. This progression occurs in both T1DM and T2DM. As many as 7% of patients with T2DM may already have microalbuminuria at the time they are diagnosed [30]. In the European Diabetes Prospective Complications Study, the cumulative incidence of microalbuminuria in patients with T1DM was approximately 12% during a period of 7 years [30, 31]. In the UKPDS, the incidence of microalbuminuria was 2% per year in patients with T2DM, and the prevalence 10-years after diagnosis was 25% [30, 32]. Pathological changes to the kidney include increased glomerular basement membrane thickness, microaneurysm formation, mesangial nodule formation, and others. The underlying mechanism of injury may also involve some or all of the same mechanisms as diabetic retinopathy. Screening for diabetic nephropathy or microalbuminuria may be accomplished by either a 24 hour urine collection or a spot urinalysis for microalbumin. Measurement of the microalbumin-to-creatinine ratio may help account for concentration or dilution of urine, and spot urinalysis may be more convenient for patients than 24 hour urine collections. It is important to note that falsely elevated urine protein levels may be produced in conditions such as urinary tract infections, exercise, and haematuria.

The initial treatment of diabetic nephropathy, as for other complications of DM, is prevention. As for other microvascular complications of DM, there are strong associations between glucose control (as measured by haemoglobin A1c [A1C]) and the risk of developing diabetic nephropathy.

Patients should be treated to obtain the lowest safe glucose level to prevent or control diabetic nephropathy [30, 32, 33]. Treatment with angiotensin-converting enzyme (ACE) inhibitors has not been shown to prevent the development of microalbuminuria in patients with T1DM but has been shown to decrease the risk of developing nephropathy and cardiovascular events in patients with T2DM [30, 34].

In addition to aggressive treatment of elevated blood glucose, patients with diabetic nephropathy may benefit from treatment with antihypertensive drugs. Renin-angiotensin system (RAS) blockade has been demonstrated to yield benefits beyond simply lowering blood pressure, and several studies have demonstrated effects of treatment with ACE inhibitors and angiotensin receptor blockers (ARBs) on renal protection. Both ACE inhibitors and ARBs have been shown to decrease the risk of progression to macroalbuminuria in patients with microalbuminuria by as much as 60–70%. These drugs are recommended as the first-line pharmacological treatment of microalbuminuria, even in patients without hypertension [30]. Similarly, patients with macroalbuminuria benefit from control of hypertension. Hypertension control in patients with macroalbuminuria resulting from diabetic kidney disease slows the decline in glomerular filtration rate (GFR). Treatment with ACE inhibitors or ARBs has been shown to further decrease the risk of progression of kidney disease, also independent of the blood pressure–lowering effect. Combination treatment with an ACE inhibitor and an ARB has been shown to have additional effects on renal protection. It should be noted that patients treated with these drugs (especially in combination) may experience an initial increase in creatinine and must be monitored for hyperkalaemia [29, 35].

**Section 1.2.5: Diabetic Neuropathy**

Diabetic neuropathy is recognised as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes [36]. As with other microvascular complications, the risk of developing diabetic neuropathy is proportional to both the magnitude and duration of hyperglycemia. The precise nature of injury to the peripheral nerves from hyperglycemia is unknown but is likely related to mechanisms such as polyol accumulation, injury from AGEs, and oxidative stress. Peripheral neuropathy in diabetes may manifest in several different forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations occur after foot ulceration or injury, which can result from diabetic neuropathy [37]. Due to the considerable morbidity and mortality that can result from diabetic neuropathy, it is important for clinicians to understand its manifestations, prevention, and treatment. The most common form of diabetic neuropathy is chronic sensorimotor distal symmetric polyneuropathy. Typically, patients with this condition experience burning, tingling, and "electrical" pain in their distal extremities, but sometimes they may experience simple numbness. In patients who experience pain, it may be worse at night [20]. As patients with simple numbness can present with a painless foot ulceration it is important to realize that lack of symptoms does not exclude the presence of neuropathy. Patients experiencing peripheral neuropathies also typically experience loss of ankle reflex [37]. Pure sensory neuropathy is relatively rare and is associated with periods of poor glycaemic control or considerable fluctuation in diabetic control. It is characterised by isolated sensory findings without signs of motor neuropathy. Symptoms are typically most prominent at night [37]. Mononeuropathies typically have a more sudden onset and involve virtually any nerve, but most commonly the median, ulnar, and radial nerves are affected. Cranial neuropathies have been described but are rare. It should be noted that nerve entrapment occurs frequently in the setting of DM. Electrophysiological evaluation in diabetic neuropathy demonstrates decreases in both amplitude of nerve impulse and conduction but may be useful in identifying the location of nerve

entrapment. Diabetic amyotrophy may be a manifestation of diabetic mononeuropathy and is characterised by severe pain, muscle weakness and atrophy, usually in large thigh muscles [37]. Several other forms of neuropathy may mimic the findings in diabetic sensory neuropathy and mononeuropathy. Chronic inflammatory polyneuropathy, vitamin B12 deficiency, hypothyroidism, and uremia should be ruled out in the process of evaluating diabetic peripheral neuropathy [37]. Diabetic autonomic neuropathy also causes significant morbidity and occasionally mortality in patients with DM. Neurological dysfunction may occur in most organ systems and can by manifest by gastroparesis, constipation, diarrhea, anhidrosis, bladder dysfunction, erectile dysfunction, exercise intolerance, resting tachycardia, silent ischemia, and even sudden cardiac death [37].

There is no specific treatment for diabetic neuropathy, although many drugs are available to treat its symptoms. The primary goal of therapy is to control symptoms and prevent worsening of neuropathy through improved glycaemic control. Some studies have suggested that control of hyperglycemia and avoidance of glycaemic excursions may improve symptoms of peripheral neuropathy. Many drugs, including duloxetine, paroxetine, citalopram, pregablin, carbamazepine, topiramate, tramadol, and oxycodone have all been used to treat painful symptoms, but only duloxetine and pregablin possess official indications for the treatment of painful peripheral diabetic neuropathy [37]. Treatment with some of these medications may be limited by side effects of the medication, and no one drug is universally effective.

**Section 1.3: Vitamin D**

**Section 1.3.1: Synthesis and Metabolism of Vitamin D**

Vitamin D refers to a group of secosteroids used by the body to assist in the absorption and utilization of calcium and phosphate. Vitamin D is different from other vitamins in that humans are able to produce it themselves, provided with adequate exposure to ultraviolet (UV) radiation, therefore it is not considered a true essential vitamin. Vitamin D is also found naturally in some foods, mainly animal products such as fatty salt-water fish, including salmon and herring. Some foods, particularly in the United States of America (USA), are fortified with chemically synthesised vitamin D.

The skin of humans and some animals has a high concentration of sterol 7-dehydrocholesterol. Regular exposure of skin to the ultraviolet radiation found in sunlight results in the photochemical conversion of 7-dehydrocholesterol to previtamin D3. This skin synthesis generally contributes 80-90% of total vitamin D supply. [38] UV synthesis of Vitamin D is particularly effective in people with lower levels of melanin. The darker the skin pigment, the more UV is absorbed by the melanin and less vitamin D is produced.

Previtamin D3 exerts no significant biological effect. Instead, two hydroxylation steps are required to produce the most active vitamin D metabolite, 1,25-dihydroxyvitamin D (1,25(OH)2D). First, vitamin D is hydroxylated to 25(OH)D, a process occurring mainly in the liver. Then, the enzyme 1α-hydroxylase further hydoxylates 25(OH)D to 1,25(OH)2D in the kidney. As the kidney is the major site for production of circulating 1,25(OH)2D, serum levels of 1,25(OH)2D are therefore significantly determined by renal 1α-hydroxylase activity, which is tightly regulated by factors related to calcium and phosphorus metabolism[39].

### Section 1.3.2: Vitamin D and Cardiovascular Disease

An increasing body of evidence indicates that there is a consistent relationship between plasma vitamin D status and several important aspects of health. Prominent amongst these is mounting evidence that plasma vitamin D level has a strong inverse relationship to several major cardiovascular risk factors. Consequently, vitamin D status is negatively associated with several cardiovascular outcomes. This indicates an increased risk of cardiovascular and total mortality amongst adults with inadequate vitamin D levels.

Vitamin D receptors (VDR) have previously been located in myocardium and arterial smooth muscle [40, 41], and is consistent with the possibility that vitamin D may directly influence cardiac contractility, vascular resistance and atherosclerosis. VDRs are also evident in kidney tissue, pancreatic beta cells, T and β-cells, enterocytes and hepatocytes. This has led to the supposition that vitamin D may also influence major cardiovascular risk factors such as blood pressure, renal function, inflammation, obesity and insulin resistance [41-43] . The activation of VDR in tissue mediates a range of responses that are likely to protect against several forms of CVD. These changes are consistent with protection against the atherothrombotic processes that mediate coronary artery disease and stroke. Conversely, inadequacy of vitamin D is theoretically likely to be associated with promotion of atherosclerosis, thrombosis and risk factors such as hypertension, diabetes, inflammation and metabolic disturbance.

### Section 1.3.3: Vitamin D on Blood Pressure and Renal Function

Evidence from vitamin D receptor-null mice [44] suggests that vitamin D is an important homeostatic regulator of the renin angiotensin system (RAS). 1,25(OH)2D inhibits renin gene transcription by modulation of the cyclic adenosine monophosphate (cAMP) signalling system [45]. This is likely to explain the association between vitamin D levels and hypertension [46-48]. The strength and consistency of the association between low levels of vitamin D and measures of RAS activity  has prompted clinical assessments of the impact of vitamin D or vitamin D receptor

agonists on blood pressure, RAS activity and related outcomes such as renal impairment and cardiac failure [49, 50].

The beneficial effects of vitamin D on RAS activity and blood pressure imply that it might protect renal function by direct and indirect mechanisms. Renal impairment is a potent risk factor for CVD, so this protection could be expanded to include cardiovascular endpoints. Additionally, impaired renal hydroxylation of 25 hydroxyvitamin D may initiate a vicious cycle of reduced vitamin D activity, reduced renal function and associated CVD risk [51]. The homeostatic responses to phosphate retention, which include parathyroid hormone (PTH) secretion, may even exacerbate the severity of CVD outcomes; such as cardiac failure in their own right [52]. Any independent effect of PTH may be further modulated by other vitamin D sensitive mediators of bone and mineral homeostasis including fibroblast growth factor 23 [53], which is thought to influence tissue calcification, PTH secretion, ventricular hypertrophy and endothelial dysfunction. Vitamin D has been shown to be directly associated with endothelial function [54] but a trial of a single oral high dose of vitamin D supplementation did not show any impact on endothelial function in patients with peripheral artery disease, the majority of whom were vitamin D deficient [55].

**Section 1.3.4: Vitamin D on Immunity and Inflammation**

Vitamin D deficiency has been linked to altered immune status. Particular attention has been paid to the role of vitamin D in T-cell responses. Whilst this is relevant to autoimmune CVD risk factors such as T1DM and the cellular responses in the chronic inflammatory process of atherosclerosis, the pro-inflammatory effect of vitamin D deficiency is thought to be of greatest relevance to CVD risk. The detrimental role of inflammation in many forms of CVD is well established. It has been determined that the inverse relationship between vitamin D and the widely studied inflammatory marker, C-reactive protein (CRP) was only present when vitamin D was below the median of the National Health and Nutrition Examination Survey (NHANES) population [56]. This relationship

was independent of co-variables known to affect CRP. C-reactive protein (CRP) is the most sensitive of the acute phase reactants, and its concentration increases rapidly during inflammatory processes. It is synthesized by the liver and consists of five identical polypeptide chains that form a five-membered ring having a molecular weight of 105 kD. The main function of CRP is to bind and detoxify endogenous toxic substances produced as a result of tissue damage. CRP also helps the body to remove dead, dying or foreign cells [57].

The complexity of interactions between inflammatory mediators infers that similar inverse relationships are to be expected between other inflammatory markers and vitamin D levels, particularly when vitamin D is at similarly low levels. Inflammatory responses also influence other biological processes that may contribute to CVD. For example, an inverse relationship has been described between vitamin D and haemostatic markers (tissue plasminogen, and to a lesser extent fibrinogen and d-dimer) [58], which may have separate pro-coagulant effects that independently contribute to the risk of CVD and stroke. Nevertheless, other studies has failed to show a beneficial effect of vitamin D plus calcium replacement on the level of inflammatory mediators such as CRP or interleukin-6 (IL-6) [59].

Adjustment for important co-variates such as obesity may diminish the strength of the relationship between vitamin D and inflammatory mediators [58]. Conversely, the inverse inter-correlation between vitamin D and the level of inflammatory mediators may infer a shared mechanism of action. A study of new onset T2DM showed that the risk was associated with low levels of vitamin D, but this risk was significantly attenuated by adjustment for the level of inflammatory mediators such as CRP, IL-6 and soluble intercellular adhesion molecule-1 [60]. This suggests that vitamin D status may mediate its effect on the risk of development of diabetes via its effect on inflammation.

**Section 1.3.5: Vitamin D on Insulin Resistance in Obesity**

Accumulated evidence suggests that vitamin D may support pancreatic β-cell function, affecting metabolic risk factors for CVD such as T2DM. Strengthening evidence in support of a relationship between vitamin D status and carbohydrate metabolism is one of the most recent developments in the role of vitamin D in CVD. While studies have varied in design, most [61-68] but not all [69] have reported an inverse relationship between vitamin D status and T2DM. Overall, metanalyses conclude that such a relationship is significant [70, 71]. Supporting evidence includes the observation that the expected lower prevalence of T2DM in subjects with adequate vitamin D levels is accompanied by a longer femoral bone length, consistent with preservation of bone mass [45].

Central to the issue is the observation that vitamin D status is associated with both subcutaneous, and more particularly, visceral obesity [72]. A positive association exists between vitamin D and adiponectin levels, independent of BMI [73]. Studies of the association between vitamin D and clinical features of insulin resistance [74] resemble those studies described for T2DM. Low levels of vitamin D are associated with measures of insulin sensitivity [75] and the risk of future development of manifestations of the metabolic syndrome in non-diabetic subjects [76]. Metanalyses suggests that the pattern of association of vitamin D and insulin resistance is significant, independent of the features of insulin resistance that have been used in the different studies. Furthermore, the typical clinical features of insulin resistance, other than impaired glucose metabolism, constitute a cluster of significant cardiovascular risk factors in their own right. Hypertension has previously been mentioned as a potent risk factor for CVD, but the dyslipidaemic pattern that is frequently encountered in insulin resistance (elevated triglyceride with reduced high-density lipoprotein cholesterol and small, dense low-density lipoprotein particles) is also associated with low vitamin D levels [77]. Furthermore, low vitamin D status may adversely affect the atherogenic process of foam cell formation in diabetics via an effect on scavenger receptors [78]. Authors of systematic reviews identify limitations such as small size and

short duration of the intervention studies that have examined the effect of vitamin D

supplements on insulin resistance and related metabolic disturbances. Studies conducted thus far

have used different interventions (with or without calcium supplements) and, overall, provide

insufficient power and consistency to conclude that the role of vitamin D in insulin resistance and

subsequent diabetes is causative.

Whilst intervention studies have been insufficient to establish independent causality, there is

substantial evidence to support further trials of the use of vitamin D supplementation to improve

aspects of insulin resistance. Plasma insulin increased in two studies [43, 79], but reduced insulin

sensitivity in the latter prevented the overall improvement in glucose disposal noted in the

former. Previous studies have suggested that the combination of vitamin D and calcium might be

effective for the preservation of insulin sensitivity [59]. A recent trial of vitamin D

supplementation in the form of fortified yoghurt achieved improvements in glucose control and

lipid metabolism in T2DM [80]. It also improved endothelial markers, whilst another well

designed trial showed that a VDR agonist potentiated protection against progression of

proteinuria in diabetic patients who were already receiving an RAS antagonist [50].


**Section 1.3.6: Vitamin D and Clinical Cardiovascular Manifestations**

The molecular mechanisms described previously support clinical studies that describe

associations between vitamin D inadequacy and CVD risk factors such as hypertension [45, 48],

hyperlipidaemia [81] and thrombophyllia [82].  However, vitamin D levels do not seem to be

related to some risk factors that are the cause of particular types of cardiovascular disease.

Vitamin D levels are not a risk factor for atrial fibrillation [83], thus the documented association

between vitamin D and stroke [84]  is likely to depend on other mechanisms. The effect of vitamin

D status on ventricular arrhythmia, and its potential to affect the incidence of sudden death is less

clear-cut [85]. In one study, a combination of low 25-hydroxy vitamin D and elevated parathyroid

hormone has been reported to be associated with more than two-fold increase in risk in sudden

cardiac death in otherwise healthy subjects, which might be of particular importance in patients

with renal impairment due to of the role of PTH in renal function [86, 87]. Although CVD consists

of a diverse range of conditions with multifactorial causes, it is equally evident that vitamin D

metabolism influences a wide range of biological processes, therefore it is plausible that vitamin

D status could be associated with virtually any form of CVD. For example, the functional status of

heart failure patients is proportional to vitamin D status [67, 88], as might be expected on the

basis of direct effects of vitamin D on the myocardium [89]. This effect is amplified in the setting

of chronic renal impairment where vitamin D metabolism is further impaired [90].


**Section 1.3.7: Metanalyses of Vitamin D and CVD**

The quality of the evidence supporting an association between vitamin D status and CVD ranges

from cross sectional to prospective epidemiological studies [91-100]. The consistency of the

findings has been maintained, and is illustrated by the most recently reported cohort from the

Copenhagen City Heart study [101]. Consequently, metanalyses have consistently revealed an

inverse relationship between vitamin D status and CVD events [89, 102-105]. A small number of

studies have considered possible confounding factors such as health disparities [106] and

ethnicity [107].  This possibility is supported by the contrast between the association of dietary

vitamin D status with stroke [108], with the example that plasma 25-hyroxyvitamin D levels are

associated with fatal stroke in whites but not blacks [109]. Alternatively, this may reflect loss of

statistical power due to subgroup analysis because most evidence favours a consistent inverse

relationship between vitamin D levels and CVD mortality [93]

CVD is the leading cause of death in many of the conditions associated with inadequate vitamin D

levels and as a result, total mortality is inversely proportional to vitamin D status in several

situations including hypertension [100], insulin resistance [110], diabetes [111],  and established

heart failure [112]. A non-specific association with mortality has also been widely reported [39,

91, 92, 96, 113, 114]. Indeed, this has led to speculation with regards to the reduction in mortality that might be achieved by the correction of vitamin D levels in the population [115].


**Section 1.3.8: Vitamin D therapy and CVD**

Unfortunately, randomised controlled trials of vitamin D replacement have not lived up to expectations. Lipids showed little change in response to vitamin D [116-119] and endothelial function was not improved [120]. CVD outcomes studies have been difficult to interpret because the intervention regime frequently included calcium supplementation. This led to concern about possible arterial calcification [121] and other detrimental effects. Some trials have suggested that calcium supplements could exacerbate the risk of CVD [122]. Systematic review suggests that vitamin D in high doses may exert a mild protective effect on CVD [123] whilst calcium supplements are probably neutral. The results of recent randomised trials include new data from the Rosiglitazone Evaluated for Cardiovascular Outcomes in Oral Agent Combination Therapy for Type 2 Diabetes (RECORD) study which revealed a non-significant trend towards fewer CVD deaths [124]. The pressing need for more data from randomised controlled trials of vitamin D supplementation [125] is unlikely to be fulfilled until the results of the vitamin D and longevity trial (VIDAL) and vitamin D and omega-3 trial (VITAL) are available. In the interim, currently available data poses a clinical dilemma. The association between low vitamin D status and CVD risk and its risk factors is well established. On the other hand, a causative role cannot be established because vitamin D supplements have only a neutral to mild effect on the severity of CVD risk factors and CVD itself. This perhaps reflects the great diversity of biological effects that are mediated by vitamin D. A similar pattern has been observed with other risk factors with a nutritional component such as plasma homocysteine levels.  It is possible that vitamin D and other metabolic markers may reflect metabolic risk of CVD without providing a treatment target. Nevertheless, the question concerning the use of vitamin D for CVD prevention requires a

conclusive answer because vitamin D represents a safe, affordable therapy that may also provide health benefits in other organ systems.

**Section 1.4: Osteocalcin**

**Section 1.4.1: Synthesis and Metabolism of Osteocalcin**

Osteocalcin is a calcium-binding bone matrix protein that contains the amino acid, γ-carboxyglutamic acid [126]. Osteocalcin (OCN), also called bone γ-carboxyglutamic acid (Gla) protein or BGP, is a calcium (Ca2+) binding protein available in the organic matrix of bone, dentin, and possibly other mineralised tissues [127]. OCN is a 49 amino acid protein produced exclusively by bone-forming osteoblasts and osteocytes [127-129] to aid in the binding of free calcium ions and hydroxyapatite (HA), and is one of the most abundant non-collagen proteins in bone. It undergoes γ -carboxylation, catalyzed by γ -carboxylase, which requires vitamin K as a coenzyme [130]. Low-levels of OCN has been reported in bone marrow megakaryocytes, in peripheral blood platelets, in endothelial progenitor cells, in vascular smooth muscle cell, as well as in the brain, intestine and kidney.

**Figure 2: γ –carboxylation of osteocalcin with vitamin K as a coenzyme [126]**
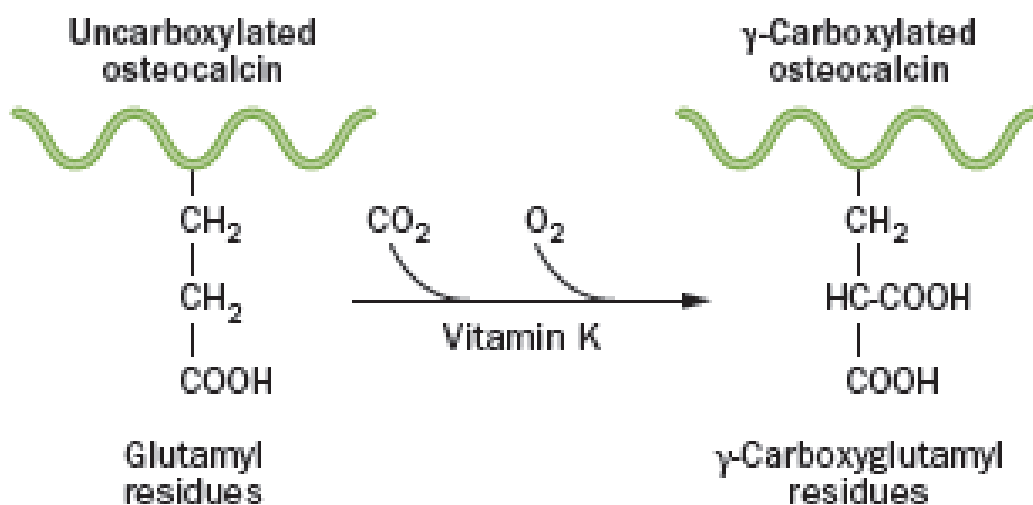
The major structural feature in an OCN molecule is the presence of 3 Gla residues. These typically confer proteins with high affinity for mineral ions, including $Ca^{2+}$, and play an important role in calcium binding. Two dimensional nuclear magnetic resonance (^{1}H 2D NMR) and X-ray crystallography studies have defined OCN's 3-dimensional structure containing 3 α-helical regions, a C-terminal, hydrophobic core, and an unstructured N terminus. Posttranslational $\gamma$ - carboxylation of Gla residues in position 17, 21, and 24 results in 3 Gla residues found in the first helical region. These residues interact with the inter-calcium spacing in the HA lattice in cortical bone and, when bound to free $Ca^{2+}$, facilitate a conformational change that aligns them in a complementary fashion to the $Ca^{2+}$ ions on the C-axis of the HA crystal lattice. The C terminus of the OCN molecule extends outward and would be accessible to neighbouring cells as well as endogenous proteinases [131].

**Figure 3: Structural model of an Osteocalcin molecule [132]**
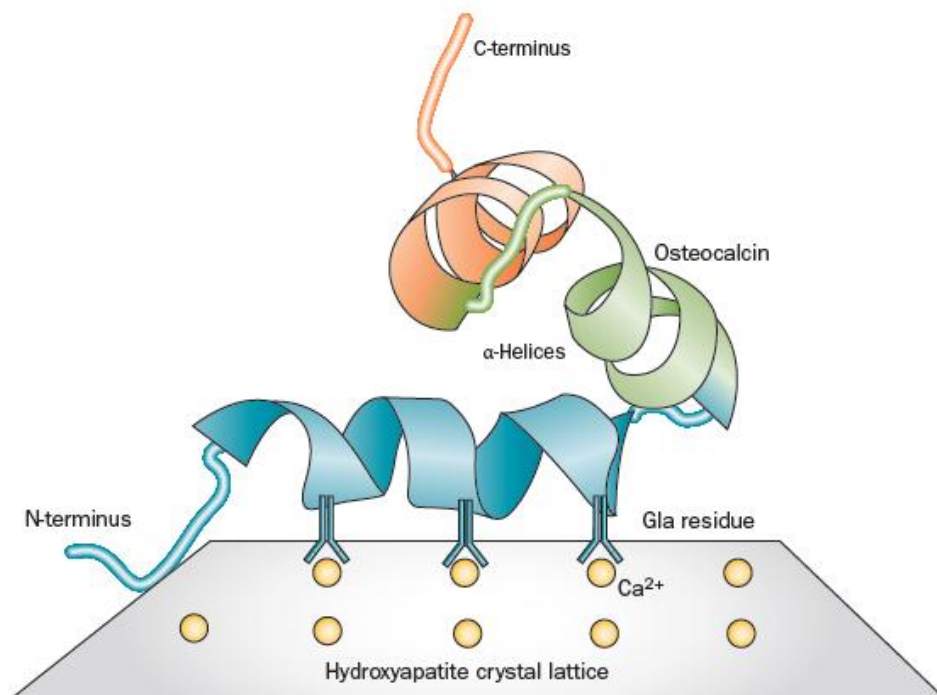
The structure of OCN is consistent with many reports of the C-terminal peptides having chemotactic activity to osteoclast precursors and supports a role for OCN in bone remodelling [133-135]. Data from animal studies suggest that undercarboxylated OCN (ucOCN) represents the metabolically active fraction of OCN that links bone turnover and glucose metabolism [136-138]. ucOCN comprises all forms with carboxylation of fewer than three Gla residues, ranging from zero to two [127, 129, 139]. Both carboxylated and undercarboxylated OCN can be detected in bone and in blood circulation [139].

While the specific role of OCN is still unclear, its role in regulating bone makes it an ideal biomarker in determining bone turnover. Serum OCN levels directly reflect the level of synthesis of osteoblasts and consequently, osteoblast activity. This correlation may explain the reduced serum OCN levels in patients treated with cortisteroids [140], well known as one of the major causes of osteoporosis [141] and an inhibitor of osteoblast activity [142].

### Section 1.4.2: Osteocalcin and Bone Metabolism

The link between OCN and bone metabolism is historically well documented, although not completely understood. The primary function of the skeleton is to provide the mechanical support of the body, respond to outside mechanical forces or molecular signals, and function as a reservoir for normal mineral metabolism. The skeleton undergoes a constant turnover of bone, with removal of old bone by osteoclasts and replacement with new bone by osteoblasts. This coupling of bone formation and resorption maintains steady bone mass. After growth ceases, any imbalance can lead to bone loss. Bone tissue is a specialised connective tissue, composed of cells and mineralised extracellular matrix. The latter consists of HA crystals that form an organic matrix of collagen fibres and non-collagenous proteins, such as OCN, osteopontin, osteonectin and bone sialoproteins [143]. OCN regulates bone turnover by modulating osteoblast and osteoclast activity and by acting as a regulator of bone mineralization [143].

The literature on the exact role of OCN in bone metabolism is inconclusive, likely due to the diversity of the experimental systems used. Several studies demonstrated that OCN inhibits HA crystal growth [132, 144-147]. Moreover, in the steady-state gel system, OCN acts at the early stage of mineralisation by inhibiting the nucleation of HA [147]. In contrast OCN had no effects when attached to agarose beads [148] and loss of function of the OCN gene in mice demonstrated that OCN did not affect bone mineralisation. OCN deficient bone did not show differences in mineral apposition rates and bone mineral content when compared with wild-type mice [149]. A more detailed analysis of OCN deficient mice, obtained using a Fourier transform infrared microspectroscopy, demonstrated that thin sections of femora isolated from knockout mice had lower carbonate substitutions and consequently, smaller and less perfect crystals than wild-type, providing evidence that OCN regulates bone mineral maturation [145]. More recently, the role of OCN in influencing mechanical and chemical properties of cortical bone from mouse femora has been reported. OCN deficiency induced the reduction of the degree of carbonate substitution and this appeared to have a greater effect on bone hardness than elasticity [150]

### Section 1.4.3: Osteocalcin and Glucose Metabolism

While the relationship between OCN and bone turnover is well documented, accumulating evidence suggests a coupled interaction between bone turnover and glucose metabolism [136-138]. Bone-derived OCN appears to play a key role in the crosstalk between bone and glucose metabolism. Early studies showed a correlation between oral glucose tolerance test (GTT) and bone mineral density (BMD) [151, 152]. Conversely, a study where obese, postmenopausal women were subjected to an energy-restricted diet showed increased bone turnover and reduced BMD [153]. Likewise, another study in which obese patients surgically treated with vertical banded gastroplasty showed prominent bone loss [154]. This suggests that obesity, and therefore energy metabolism, somehow offers protection to bone mass. If, then, OCN is involved in the regulation of glucose and insulin metabolism, it would be expected that circulating levels

would be correlated with indicators of glycaemic control including fasting plasma glucose (FPG), glycated haemoglobin A1c (HbA1c), and serum insulin. Murine studies have identified two potential genes for metabolism control in which protein biosynthesis is restricted to bone [138]. The first of these, Esp (also known as Ptprv), is a gene expressed only in osteoblasts and Sertoli cells and encodes a transmembrane protein, tyrosine phosphatase (OST-PTP, also known as R-PTP-V). Deletion of Esp produced animals that were lean, hypoglycaemic, and had increased β-cell proliferation, insulin secretion and insulin sensitivity. When adipocytes from normal mice were grown in the presence of osteoblast-conditioned media from either wild-type mice or mice with the deletion of Esp, expression of adiponectin was increased by 40% and 100%, respectively. Likewise, insulin expression was increased in wild-type islets grown in the presence of osteoblast-conditioned media from wild-type mice (40%) or from mice with deletion of Esp (100%) [138]. These results suggest that osteoblasts secrete one or many factors that affect β-cells and adipocytes, and that OST-PTP regulates the activity of this factor. Because of its osteoblast-specific expression, a logical candidate for this factor was OCN. When examined further, it was found that OCN -knockout mice were obese, with elevated glucose and lipid concentrations, reduced insulin levels, reduced numbers of β cells, and were both glucose-intolerant and insulin-insensitive. However, in the original description of the OCN -knockout mouse [149], bone formation was elevated compared with wild-type mice, a finding that conflicts with other animal models of DM in which bone formation is reduced. In contrast to observations in humans with T2DM, both insulin secretion and sensitivity were decreased in these mice, effects that were attributed to a decrease in adiponectin expression in adipose tissue [136]. Overall, the phenotype was the exact opposite of that observed in the Esp knockout (−/−) mice. Islets and adipocytes from wild-type mice, cultured with osteoblast-conditioned media derived from OCN -knockout mice, showed decreases in insulin and adiponectin. Furthermore, the metabolic phenotype was normalised in Esp−/− mice lacking one allele of OCN, further supporting the notion that OST-PTP and OCN are in the same pathway. Circulating levels and expression of OCN were normal in Esp−

/− mice, which suggests that OST-PTP does not regulate the biosynthesis of osteocalcin, but rather regulates its metabolic function. Given that the only known modifiable aspect of OCN is its γ-carboxyglutamic acid residues, Lee et al. [138] showed that ucOCN, but not carboxylated OCN, induces expression of both adiponectin in adipocytes and insulin in islets. This finding presents a major paradigm shift, given that all known vitamin-K-dependent proteins require the presence of γ-carboxyglutamic acid for function, including the carboxylating enzyme itself [155].

To establish the role of OCN in glucose metabolism, Ferron et al. implanted mice with osmotic mini pumps containing ucOCN [136]. Doses that delivered up to 3 ng/ml to the circulation were given for 4 weeks, and resulted in low blood glucose levels and an increase in serum insulin levels. In these mice, the circulating level of ucOCN was 7 ng/ml, approximately 10% of total circulating OCN levels measured in adult mice fed standard rodent chows. At the doses given to the mice, the proportion of ucOCN increased to only 14%, which is within the intraindividual variation that is normal in humans consuming a varied diet. This observation highlights the need to compare uncarboxylated or undercarboxylated OCN to total OCN in both human and animal studies to understand any relevant changes that are related to metabolism or vitamin K intake.

Animal experiments have shown that OCN stimulates pancreatic insulin production and the pertussis toxin-sensitive G protein-coupled receptor GPRC6A is a likely candidate that mediates OCN effects on pancreatic β-cells [126]. Evidence exists for the involvement of GPRC6A in the regulation of biological processes in humans. GPRC6A is a seven-transmembrane receptor that mediates signaling of a wide range of l-α-amino acids, predominantly the basic amino acids, arginine, lysine and orthinine [156]. GPRC6A is widely expressed in brain and peripheral tissues of humans, including kidney, skeletal muscle, testis and leucocytes [157]. It is directly activated by high concentrations of $Ca^{2+}$, a response that is augmented by carboxylated OCN [158]. Mice lacking GPRC6A have been produced by two separate laboratories. Wellendorph et al. found that knockout mice were viable and fertile, developed normally and exhibited no significant differences in body weight or skeletal manifestations compared with their wild-type littermates

[159]. In contrast, Pi et al. reported a complex metabolic phenotype, decreased BMD and impaired mineralisation [160]. Given the wide expression of this receptor, the question remains how OCN functions as a cell-specific ligand. It is suggested that potentially a co-receptor is required for tissue specificity, as seen for FGF-23 (another bone-derived factor) and its co-receptor, Klotho, however this supposition remains largely unexplored [161].

**Section 1.4.4: Osteocalcin and Diabetes Mellitus**

While the existence of altered bone metabolism among patients with DM is a well-characterised phenomenon, the mechanisms behind this phenomenon are not well understood [162]. Bone mass is low in patients with T1DM but higher than normal in those with T2DM. Yet, in both patients with T1DM and those with T2DM, increased fracture risk is observed at a given BMD as compared to that in individuals without diabetes mellitus [163-165].

Most of the evidence linking bone-derived OCN with energy metabolism is derived from animal studies. At present there are only a few human studies reporting correlations between circulating OCN levels and indices of glycaemic control in humans [166-169]. These studies are small and have significant methodological limitations. Prospective studies relating serum OCN levels to clinical outcomes, such as the development of diabetes and diabetic complications, have not been performed yet. Furthermore, existing studies in humans do not support the concept of undercarboxylated OCN being the metabolically active fraction [168, 170]. It therefore remains unclear if the functions of OCN observed in mice are similar in humans, and, if so, what the clinical implications may be.

**Section 1.5: Fibrates in the FIELD study**

The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) was a large-scale, randomised, international clinical trial investigating fibrate therapy in diabetes [171]. Although fibrates have been used in the treatment of dyslipidaemia associated with T2DM for over 20 years, their role in reducing risk factors of CVD in diabetic patients was still largely unexplored. FIELD anticipated that fenofibrate therapy would have a greater effect on non-apolipoprotein B and HDL-cholesterol levels than statin therapy among the patient group.

Fenofibrate is a fibric acid derivative used in the treatment of severe hypertriglyceridaemia and mixed dyslipidaemia by improving the lipid profile, particularly triglyceride and HDL levels. Compared with statin monotherapy, fenofibrate monotherapy tends to improve triglyceride and HDL levels to a significantly greater extent, whereas statins improve LDL and total cholesterol levels to a significantly greater extent. Fenofibrate is also associated with promoting a shift from small, dense, atherogenic LDL particles to larger, less dense LDL particles [172, 173]. In the FIELD study, Fenofibrate typically lowered total cholesterol by 15%, however changes in low density lipids (LDL) were variable over time while high density lipids (HDL) increased by 10-15% and plasma triglycerides fell by 30-40% [173].

**Section 1.5.1: FIELD and large scale clinical trials in CVD and diabetes**

Prior to the start of the FIELD study, the role of lipid modification in diabetes was largely unknown. Previous studies, such as the Scandinavian Simvastatin Survival Study (4S), the Cholesterol and Recurrent Events (CARE) and West of Scotland (WOSCOPS) had examined benefits of statin use in hypercholesterolaemic patients and reported reductions in CHD mortality [174-176]. None of these studies, however, included a sufficient number of diabetic patients. Since then, a number of studies of statin treatment have been reported that included larger numbers of diabetic patients. Some of these, such as the Long Term Intervention with Pravastatin

in Ischemic Disease (LIPID) study, the Heart Protection study, the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT), Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT-LLT) demonstrated various improvements in diabetic outcomes [177-179]. The results of the Collaborative Atorvastatin Diabetes Study (CARDS) showed a particularly remarkable reduction of cardiac and stroke events in high CVD risk diabetic patients following atorvastatin use [180].

The Helsinki Heart Study examined the long term effects of the fibrate use using the fibrate gemfibrozil in patients suffering from hypercholesterolaemia with no prior CHD. The results showed a significant reduction in coronary events, greater than sole reliance on lowering LDL levels. These results were even stronger for diabetic patients [181].

Other large trials, such as the Veterans Low HDL-Cholesterol Intervention Trial (VA-HIT) and the Bezafibrate Infarct Prevention (BIP) study examined fibrate usage in patients with prior MI. Both studies reported reduction in major cardiovascular events among patients with low HDL and high triglycerides at baseline, higher than patients receiving the same treatment minus the dislipidaemia symptoms [182, 183]. Additionally the VA-HIT study reported lower coronary mortality and reduced cardiovascular events in diabetic subjects receiving fibrate treatment.

**Section 1.5.2: FIELD Trial and Outcomes**

Using fenofibrate was a logical, yet unproven, method of treatment for dyslipidaemia associated with T2DM, through increasing HDL whilst lowering triglyceride levels [184]. The FIELD study was set up to evaluate the effect of fenofibrate against a placebo on a background of usual care. FIELD found that fenofibrate was associated with a non-significant reduction of 11% for CHD and non fatal MI [185]. This result was less than the expected outcome for fenofibrate on CHD and can be explained by subjects starting other lipid therapies on study, 17% of placebo and 8% of fenofibrate groups, and by the relatively low-risk study population of the FIELD trial. The effect of fenofibrate was highest in the subject group with marked dyslipidaemia when compared with other groups, with a reduction of 27% and 6% respectively. The absolute risk reduction was also highest in these patients (4.3% and 0.8% respectively).  The effect of fenofibrate treatment varied according to the presence of dyslipidaemia when only subjects with metabolic syndrome were included in the analysis. Subjects with low HDL cholesterol levels had a 22% higher risk of CVD, while subjects with high triglyceride levels had a 24% risk. Elevated blood pressure, particularly systolic pressure, was significantly stronger in primary prevention. An increase in waist circumference appeared to have no effect on CVD risk.

Additionally, fenofibrate use was associated with significant increases in creatinine and serum homocystine levels. Somewhat contrarily, those with the greatest increase in these adverse risk markers were also those who gained the greatest CVD protection. Fenofibrate improved FIELD tertiary microvascular outcomes, such as slowing the progression of nephropathy and neuropathy. It was associated with a reduced need for laser therapy for treatment of retinopathy. The mechanics behind these benefits are currently unknown, and are the focus of further inquiry by the FIELD Study Investigators into the aetiology, clinical assessment and treatment of macro- and microvascular complications of CVD and diabetes [173].

**Section 1.6: Aims**

The aim of the project is to investigate the role novel biomarkers in the large-scale FIELD study, involving over 9,000 subjects with type 2 diabetes mellitus followed for 5 years. FIELD provides a unique opportunity to examine the relationship of vitamin D and OCN levels with both macro-vascular and micro-vascular events. The study recorded over 2067 cardiovascular events and 3186 micro-vascular events at baseline with a further 1250 and 2395 events respectively during follow-up. Here, this paper reports the relationships between baseline serum biomarker levels and prospective macro-vascular and micro-vascular events in the FIELD cohort.

**Chapter 2: METHODS**

**Section 2.1: Study Design**

The FIELD trial is a multinational, double-blind, placebo-controlled trial in 63 centers in Australia, New Zealand, and Finland. The study population consisted of 9795 participants aged 50–75 years with type 2 diabetes mellitus diagnosed according to WHO criteria, and who were considered to be at increased risk of coronary heart disease.

After being screened for eligibility and completing informed consent, all patients entered a run-in phase followed by the randomisation process. The run-in phase involved a 4 week period of diet only, followed by 6 weeks of single-blind placebo, followed by 6 weeks of single-blind active run-in phase which administered 200mg of micronized fenofibrate for all patients. The randomisation process assigned each patient to either a placebo, or 200mg micronized fenofibrate. Randomisation used a dynamic allocation method, whereby stratification for key factors such as age, sex, history of MI, lipid and urinary albumin levels. All patients were monitored through regular visits to clinic and primary care physicians. Patients were recruited from hospital clinics and community sources. Complete medical records were available from 9515 participants over a 5 year follow-up period.

**Table 2: Eligibility criteria for FIELD study participation**

| Individuals were eligible for FIELD study provided following characteristics: |
| :---: |
| male or female, aged 50–75 years inclusive |
| T2DM with age at diagnosis >35 years |
| considered to be high risk for coronary heart |
| the patient was not already taking any cholesterol-lowering drug |
| total cholesterol level 3 to 6.5 mmol/L, |
| either a total cholesterol:HDL ratio of ≥ 4.0 a blood triglyceride level >1.0 mmol/L |
| no other predominant medical problem that might limit compliance with 5 years of study treatment or compromise long-term participation and clinic attendance in the trial |

**Table 3: Ineligibility criteria for FIELD study participation**

| Individuals were not eligible for FIELD study provided any of the following characteristics: |
| :---: |
| serum triglyceride >5 mmol/L in the baseline visit fasting blood sample |
| concurrent treatment with any other lipid-lowering agent |
| serum creatinine >130 µmol/L |
| known chronic liver disease, transaminases > twice upper limit of normal or symptomatic gall-bladder disease |
| myocardial infarction or hospital admission for unstable angina within 3 months |
| female, of child-bearing potential, unless sterilized or on reliable approved methods of contraception, including oral contraceptives |
| concurrent cyclosporin treatment (or a condition likely to result in organ transplantation and the need for cyclosporin during the next 5 years) |
| known allergy to any fibrate drug or known photosensitivity |

Samples were originally stored and tested at one of two sites; Adelaide, Australia and Helsinki, Finland. Samples were later transferred to facilities in Sydney, Australia for storage and further analyses.

All patients gave written informed consent. The study protocol was approved by local and national ethics committees and was undertaken in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines.

The role of the candidate during the course of this study was to set up the vitamin D and osteocalcin assays on the DiaSorin Liaison (DiaSorin Spa, Italy), and other assays on the Roche Modular (Roche Diagnostics, Australia) auto-analysers. This was subsequently followed by the measurement of 25 OH vitamin D, OCN and hs-CRP in all baseline samples over the course of two years. In a subset of 1902 patients, total and undercarboxylated OCN were measured at baseline and after 2 years of treatment

**Section 2.2: Sample Size**

The original FIELD sample size was estimated to be approximately 8000 with fatal MI as a primary outcome, however this number was expanded to a final figure of 9795 to retain a power of 80% over the 5 year duration.

**Section 2.3: Safety and Event monitoring**

The trial had an independent Safety and Data Monitoring Committee to safeguard the patients' interests and for the formal evaluation of the study. Patients were monitored regularly by way of lipid profiles, liver function tests, creatine phosphokinase, fasting glucose, HbA1c, and urinary microalbumin. This committee had the right to recommend that the study should be modified or stopped if it met pre-specified stopping rules for adverse events or efficacy.

The Management Committee, the collaborators, the study sponsor and all the central administrative staff, with the exception of the unblinded statistician, remained blinded to interim results. The study was approved by local ethics committees at each participating institution.

**Section 2.4: Study Sponsorship**

The principal sponsor of the FIELD trial was Laboratoires Fournier S.A., Dijon, France, who supplied micronized fenofibrate and matching placebo. The company had no involvement in the collection or analysis of the data accrued during the trial. The study was also supported by the National Health and Medical Research Council (NHMRC) of Australia. The study was coordinated independently by the NHMRC Clinical Trials Centre, University of Sydney, Australia, and overseen by the study management committee. The study has been endorsed by the National Heart Foundation of Australia, Diabetes Australia, the New Zealand Society for the Study of Diabetes, and the Finnish Diabetes Association.

**Section 2.5: Sample Testing**

All samples were measured on DiaSorin Liaison (DiaSorin Spa, Italy) or Roche Modular (Roche Diagnostics, Australia) auto-analysers.

**Section 2.5.1: DiaSorin Liaison**

The operating principle of the DiaSorin Liaison (DiaSorin Spa, Italy) is based on a chemiluminescence immunoassay system. This assay system operates on the principle of a one, two, or three step incubation sequence.

A one-step assay is where the sample, tracer reagent and magnetic particles are added to the disposable reaction module in the one step, followed by a 10-30 minute incubation period. After this period, the reaction module is washed to remove any free material. When the last wash has been completed, the reaction module is transported to the measuring chamber where starter reagents are added to initiate the chemiluminescence reaction.

A two-step assay is similar to the one-step, but with an added step of incubation and washing. Sample material and magnetic particles are added for the first incubation period. This is then washed, and tracer material is added. There is then an additional period of incubation and washing before the starter reagent is added and chemiluminescent reaction takes place. The chemically emitted light from this reaction is measured by a high-sensitive, low-noise photo multiplier, which can measure a range from 300 to 650nm. It operates as an ultra-fast photon counter, which are in turn used as units of measurement for raw data, are expressed as a relative light unit (RLU).

Tests performed on the DiaSorin Liaison included 25OH Vitamin D and Osteocalcin assays.
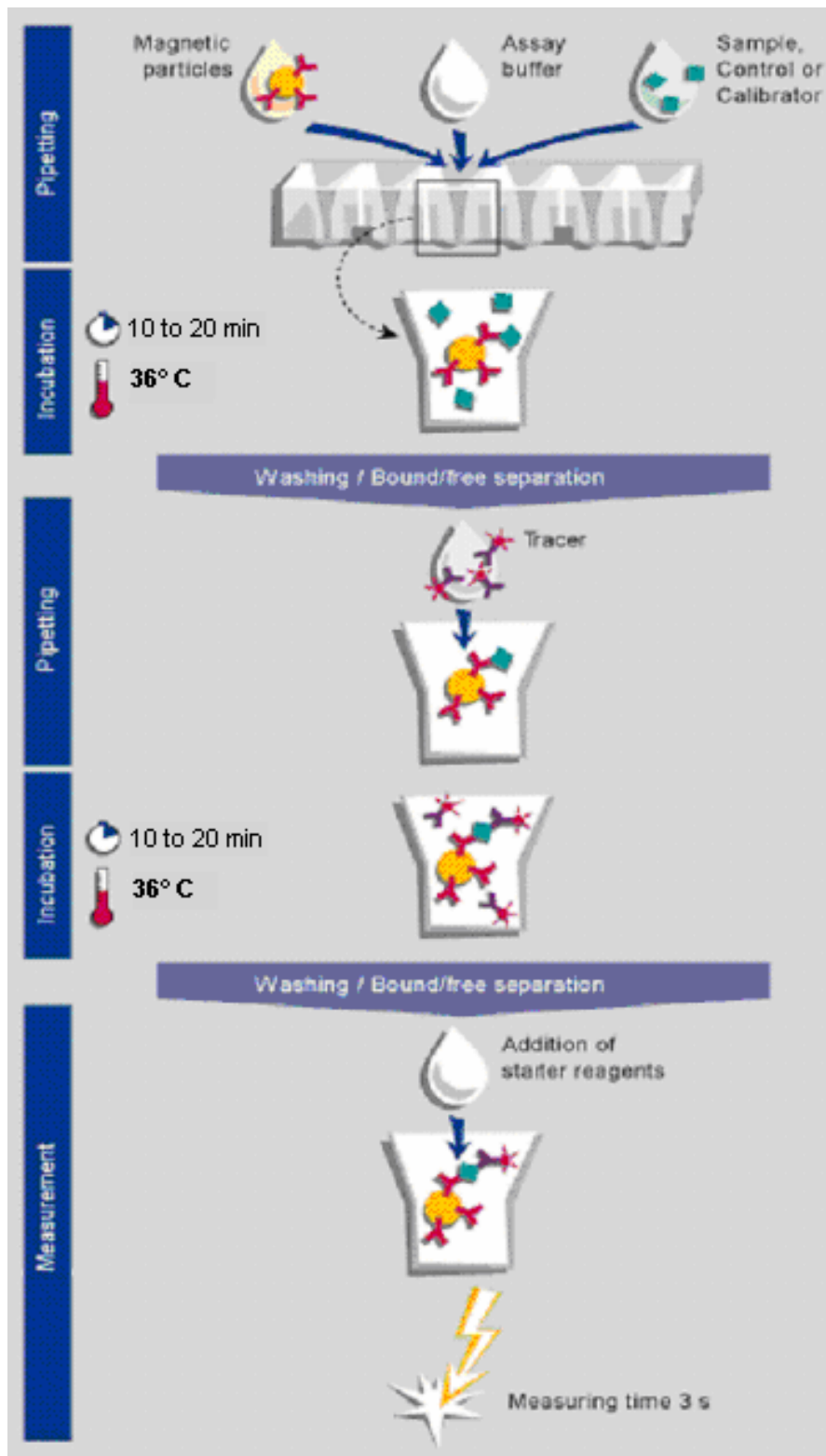
**Figure 4: Example of steps involved in a two-step chemiluminescence assay**

**Section 2.5.2: Roche Diagnostics**

The operating principle of the Roche P800 module is based on a photometric measuring system. It uses multiple wavelength spectro-photometer with wavelengths ranging from 340 to 800nm. The tungsten halogen lamp operates in mono- and bi-chromatic optical modes with a lightpath of 5 ±0.02mm.

Analysis of a sample occurs through a series of operations. Prior to testing the P800 module resets itself, cleaning, rinsing and drying reaction cuvettes. This is to ensure reaction cells are clear of previous matter to avoid contamination. After reaction cuvettes have been cleaned the cells undergo a blank absorption measurement, using water as the zeroing reagent. This forms the zero baseline for subsequent measurements.

Roche assays utilise the principle of immunoturbidimetrics. These assays can be used for quantitative measurement of drugs or biomarkers in body fluids like serum, plasma, or urine. The assays are based on an agglutination reaction induced by the antigen-antibody binding. When a light is directed to the sample mixture, absorbance change measured photometrically is proportional to the rate of agglutination of the microparticles.

Tests performed on the Roche module included basic biochemistries, liver and kidney biomarkers, which are outlined in section 2.8. Of particular focus in this project, the Roche module was used to measure the inflammatory marker, C - reactive protein.

**Section 2.6: Vitamin D**

**Blood sampling and measurement of 25OH Vitamin D**

Total serum 25 OH vitamin D was measured at baseline in all patients. Fasting blood samples were collected into serum tubes. After collection, samples were allowed to clot and then separated immediately and frozen on site. Frozen samples were transported to one of two central laboratories in Adelaide, Australia, and Helsinki, Finland, where they were aliquoted and stored at -80°C until analysis. Serum 25OH vitamin D was assayed by a chemiluminescence immunoassay (DiaSorin Spa, Italy) on a LIAISON automated analyser (DiaSorin Spa, Italy). This assay detects 25 OH-$D_2$ and 25 OH-$D_3$ in an equimolar fashion. The assay is a two-step chemiluminescence immunoassay for quantitative determination of total 25 OH vitamin D in serum. During the first incubation, vitamin D is dissociated from its binding protein and binds to the specific antibody on the reaction module. After 10 minutes the tracer, vitamin D linked to an isoluminol derivative, is added. When this second 10 minute incubation has completed, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured and expressed as an RLU, which is inversely proportional to the concentration of vitamin D present in calibrators, controls, or samples.

The lower limit of detection with this assay was 10nmol/L; undetectable levels were assigned a value midway between this and zero (5nmol/L). The assay used in this study was a pre-market version that has been shown to compare closely with higher order methods including liquid chromatography tandem mass spectrometry (LCMS) and radioimmunoassay (RIA). In addition, this pre-market assay has been shown to have an identical performance to the current market version of the DiaSorin Liaison assay [186]. Assay performance was monitored over time by internal quality-control procedures including the use of high and low concentration pooled sera. For the purposes of this study, vitamin D levels < 50nmol/L were deemed to be deficient. Only 271 (2.8%) samples were unavailable for analysis.

**Section 2.7: Osteocalcin**

**Blood sampling and measurement of osteocalcin:**

Total serum OCN was measured at baseline in all patients. Fasting blood samples were collected into serum tubes. After collection, samples were allowed to clot and then separated immediately and frozen on site. Frozen samples were transported to one of two central laboratories in Adelaide, Australia, and Helsinki, Finland, where they were aliquoted and stored at -80°C until analysis.

For the analysis of OCN, frozen samples were transported to the Department of Biochemistry at the Royal Prince Alfred Hospital (RPAH) in Sydney, Australia. Serum OCN was assayed by a chemiluminescence immunoassay (DiaSorin Spa, Italy) on a Liaison automated analyser (DiaSorin Spa, Italy). The method for quantitative determination of OCN is a one-step, sandwich type chemiluminescence immunoassay. Affinity-purified mouse antibody to synthetic human OCN is coated to the reaction module. The second affinity-purified mouse antibody is conjugated to an isoluminol derivative. During the incubation, OCN binds to the reaction module and is subsequently bound by isoluminol conjugated antibody. After the incubation, the unbound material is removed with a wash cycle. The starter reagents are then added and a flash chemiluminescent reaction is initiated. The light signal is measured as RLU and is proportional to the concentration of OCN present in calibrators, controls, or samples. This assay detects intact OCN.

In a subset of 1902 patients total and undercarboxylated OCN were measured at baseline and after 2 years of treatment. UcOCN was measured using the same assay preceded by a sample pre-treatment with HA as described by Gundberg et al. [139]. The pre-treatment exploits the decreasing affinity of ucOCN for HA as the number of Gla residues declines. UcOCN results were expressed as both a percentage of total OCN, and in absolute values. Assay performance was monitored over time by internal quality-control procedures including the use of pooled sera.

**Section 2.8: Assessment of other biomarkers**

Measurement of high sensitive C-reactive protein (hs-CRP) used an automated immune turbidometric assay from Roche Diagnostics run on a Modular E170 analyser (Roche Diagnostics, Australia). Measurements of CRP in blood are used to detect systemic inflammatory processes to assess treatment of bacterial infections with antibiotics, to differentiate between active and inactive forms of disease with concurrent infections, to monitor rheumatic disease, to determine the presence of post-operative complications at an early stage, such as infected wounds, thrombosis and pneumonia, and to distinguish between infection and bone marrow transplant rejection. Hs-CRP measurements have been used for early detection of infection in pediatric patients and risk assessment of coronary heart disease. Studies indicated that the highly sensitive measurement of CRP could be used to predict the risk of coronary heart disease in apparently otherwise healthy persons and to aid in the prognosis of recurrent events [57].

There are two different tests for CRP. The standard test measures a much wider range of CRP levels but is less sensitivein the lower ranges. The hs-CRP test is more sensitive and can detect lower concentrations of CRP with greater accuracy. After randomisation had occurred, fasting blood samples were taken at baseline, at 4, 8, and 12 months, then yearly until the study close, for plasma lipids plasma creatinine, and alanine aminotransferase. A range of biomarkers, including Plasma glucose, haemoglobin A1c (HbA1c), apolipoproteins A1, A2, and B, lipoprotein A, insulin, C-peptide, fibrinogen and homocysteine (HOMA), urine albumin, and urine creatinine, were measured periodically during the follow-up process. All blood and urine samples were analysed in one of two central laboratories in Adelaide, Australia, and Helsinki, Finland. Both laboratories participated in national quality assurance schemes for all analytes and were aligned for lipid and HbA1c analyses through the Canadian External Quality Assurance Laboratory in Vancouver. Methods used to measure lipids were accredited by the Centers for Disease Control Lipid Standardisation Program [185]. All data was made available for this project.

**Section 2.9: Calibration and Quality Control**

Calibration of the autoanalysers is performed using a master curve with 2-point recalibration. The measuring signals of the calibrators allow the shift of all master curve points to a working curve, corresponding with the actual conditions during measurement. The master curve is defined with 10 curve base points. Two calibrators, included with the assay kits, with defined concentration values are measured on a weekly basis. These measured signals (expressed in RLU) are compared with the master curve signal of the corresponding calibrator concentrations. The relative difference between the measured RLU and the master RLU of the calibrators is calculated and a linear extrapolation is performed between the recalculated RLU (Y-axis) and the logarithmic (Log) concentrations (X-axis). Based on appropriate compensation factors, a re-adjustment of the master curve points is made in order to achieve, by a "cubic spline function", the working curve [187].
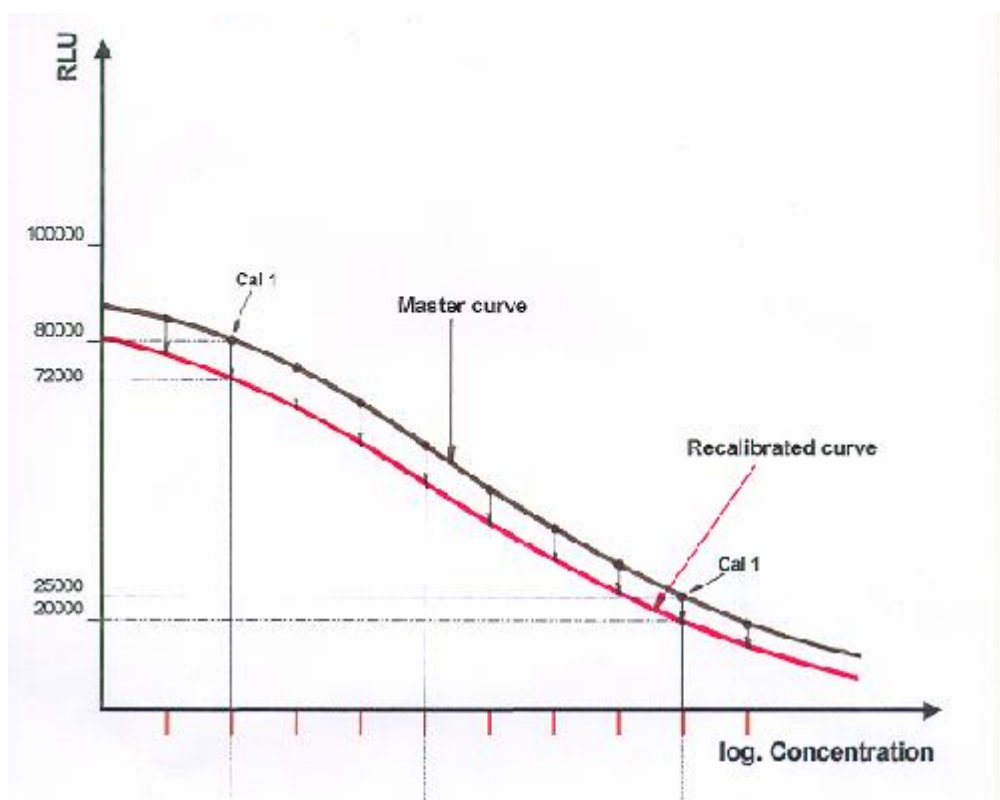


**Figure 5: Master curve for assay calibration [187]**

Strict quality control measures were established to ensure consistency in testing over the two year period. External controls were provided by both DiaSorin and Roche for vitamin D, OCN, and hs-CRP, and included a high and low control. These controls were run daily and results were deemed to be acceptable if they maintained a CV% of <2.5.

In addition to the external controls, internal controls were created using pooled serum made available from the Department of Biochemistry at RPAH. These serum samples were tested individually and divided into two groups based on their result. Aliquots of these two high and low groups were made and frozen at -80°C until use.

**Section 2.10: Assessment of other clinical variables**

Baseline physical activity levels were recorded in response to a self-reported questionnaire including the categories self-care/home (e.g. house work, lawn mowing), occupational (type of work, e.g. sitting, home building), recreation (sports) and physical conditioning (aspects of general fitness). All categories were recorded on a scale from 1 (very light) to 5 (very heavy) (Table 4: Baseline physical activity levels). This is an established method of scoring as validated in previous studies [188].

**Table 4: Baseline physical activity levels**

| | SELF CARE/HOME | OCCUPATIONAL | RECREATIONAL | PHYSICAL CONDITIONING |
|---|---|---|---|---|
| **VERY LIGHT = 1** | Washing, shaving, dressing, desk work, writing, washing dishes, driving car | Sitting, standing, driving truck, operating crane | Bowls | Walking 4 km/hour |
| **LIGHT = 2** | Light house work, weeding, raking leaves, lawn mowing, waxing floors (slowly), painting, carrying objects (7-14kg) | Stocking shelves, light carpentry, light weldi\ng, machine assembly | Dancing, golf, tennis (doubles) | Walking 6-8km/hour, swimming (breaststroke), bicycling |
| **MODERATE = 3** | Light digging, level lawn mowing, climbing stairs slowly, carrying objects < 30kg | Carpentry (exterior home building), shovelling dirt, using pneumatic tools | Tennis (singles), hiking, skiing, light backpacking, skating, basketball, horseback riding | Walking 9-10 km/hour, swimming (freestyle) |
| **HEAVY = 4** | Carrying objects < 45kg, climbing stairs (moderate speed) | Labouring | Canoeing, mountain climbing, touch football | Jogging 10km/hour, swimming (crawl stroke) |
| **VERY HEAVY = 5** | Carrying loads upstairs >90kg, climbing stairs quickly, shovelling heavy snow | Heavy labouring | Handball, squash, cross-country skiing | Running > 15km/hr, bicycling > 28km/hr |

**Section 2.11: Verification of macro- and microvascular disease**

All major CVD events and all other deaths were adjudicated by an outcomes assessment committee, who were unaware of treatment allocation, with definitions specified prior to study onset. A diagnosis of MI required at least two of three criteria: ECG changes, ischaemic symptoms, or raised cardiac enzymes. A stroke required evidence of sudden onset of focal neurological deficits, lasting at least 24 hours, with cerebral imaging excluding haemorrhage to confirm an ischaemic stroke. Cause-specific mortality was classified into major categories of coronary, other vascular, cancer, and other non-CVD subtypes.

Incident microvascular events were classified as follows:

- peripheral neuropathy, abnormal monofilament test;

- nephropathy, urinary albumin:creatinine ratio ≥2.5mg/mmol for men and ≥3.5mg/mmol for women;

- retinopathy, on-study laser treatment for diabetic retinopathy (including macular oedema);

- amputation, minor amputation without known peripheral vascular disease in the same limb.

Measurement of urinary albumin excretion and the monofilament test were performed at baseline, at 2 and 5 years, and at study close.

**Section 2.11: Statistical analysis**

Normally distributed variables were summarised as means and standard deviations, and non-normally distributed variables as medians and interquartile ranges. Chi-square tests were used to compare categorical variables. For continuous variables, groups were compared using t-tests or Wilcoxon rank–sum tests if the variables were non-normally distributed.  Spearman correlations were used for bivariate analysis of continuous variables.

The effect of 25 nmol/L increment in serum vitamin D, and 5 ng/mL increment in serum OCN, on total cardiovascular events was assessed in non-adjusted and adjusted Cox proportional-hazards

analysis. The adjusted model included  gender, age, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking, baseline alcohol use, baseline insulin use, and total vitamin D and OCN was stratified on treatment. Logistic regression was used to assess the association of vitamin D, total OCN and ucOCN with on-study microvascular events. As well as considering vitamin D and OCN in the models as a continuous variable (linear effect only considered), quartiles of vitamin D and OCN were also examined. Adjustments for seasonal variation of vitamin D were made and had an insignificant effect on the outcome.

Cumulative risk curves of cardiovascular events were derived from the Kaplan–Meier method and the interval-censored data method was used to plot the cumulative risk curves of microvascular events.

P values less than 0.05 were considered statistically significant.  All analyses were performed with SAS (version 9.2) and ACCorD (Analysis of Censored and Correlated Data) software (version 1.6.3).


The effect of incremental differences in serum vitamin D and OCN levels was assessed in two ways:

- cross-sectional associations with existent events at baseline used logistic regression analysis unadjusted, and adjusted for potential confounders shown below
-  prospective associations with incident events during follow-up used unadjusted and adjusted Cox proportional-hazards analysis for macro-vascular events, and logistic regression for micro-vascular events (as some of the latter were only assessed at specific time-points).


In each case, the prospective analyses were stratified by study treatment allocation and adjusted for baseline event status. Adjusted models also included gender, age, diabetes duration, HbA1C, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking, baseline use of oral hypoglycaemics and insulin.


In exploratory analyses, additional adjustments were undertaken for physical activity score and hsCRP. As well as considering vitamin D and OCN in the models as a continuous variable (with results

reported for increments of 50 nmol/L), quartiles of vitamin D and OCN were also examined.

Cumulative risk curves of macro-vascular and micro-vascular events were derived from the Kaplan–

Meier method.

Two sided P values less than 0.05 were considered statistically significant.  All analyses were

performed with SAS (version 9.2) and ACCorD (Analysis of Censored and Correlated Data) software

(version 1.6.3).

**Chapter 3: Results**

**Section 3.1: Baseline characteristics**

The study treatment groups were well matched for baseline characteristics, such as age, body mass index, smoking habits, blood pressure and duration of diabetes. Both groups had similar cardiovascular histories. The use of glucose-lowering and cardiovascular medication was as expected for a cohort of people with diabetes and did not differ between the two groups.

**Section 3.2: Description of events**

During the 5-year follow-up period, 1247 patients (13.1% of the entire cohort) had at least one macrovascular event and 2395 (25.1% of the entire cohort) had at least one microvascular event. Most of the affected patients had one or two events during the 5-year follow-up. New or worsening nephropathy (1632 or 17.2% of the entire cohort) and new neuropathy (650 or 6.8% of the entire cohort) were the two most common microvascular complications. Retinopathy requiring laser surgery and amputations occurred in less than 5% of the entire cohort (Table 5). Previous publications from the FIELD Trial have reported that fenofibrate treatment lowers microvascular events, such as nephropathy, retinopathy and amputations [185].

**Table 5: Description of all events**

|  | Total CVD events | Microvascular events | Amputation | Neuropathy | Nephropathy | Retinopathy |
|---|---|---|---|---|---|---|
| Total number of patients with a prior event | 2065 (21.7%) | 3183 (33.5%) | 17 (0.2%) | 544 (5.7%) | 2441 (25.7%) | 791 (8.3%) |
| Number of patients with at least one on-study event | 1247 (13.1%) | 2393 (25.1%) | 49 (0.5%) | 650 (6.8%) | 1632 (17.2%) | 390 (4.1%) |
|     Without a prior event | 728 | 1483 | 47 | 50 | 1322 | 198 |
|     With a prior event | 519 | 910 | 2 |  | 310* | 192 |

* Worsening (from microalbuminuria to macroalbuminuria)

**Figure 6: FIELD study flow chart**

```
┌─────────────────────┐
│  13900 screened     │
└─────────────────────┘
           │                    2625 ineligible
           │                          10 died
           ├───────────────────►      93 compliance problems
           │                          85 suspected drug reaction
           │                    1334 withdrew consent
           ▼
┌─────────────────────┐
│  9795 randomised    │
└─────────────────────┘
           │
           │
           ├───────────────────► 280 samples not available
           │
           │
           ▼
┌──────────────────────────────────────────┐
│  9524 measured for Vitamin D at baseline  │
│  9515 measured for Osteocalcin at baseline│
└──────────────────────────────────────────┘
           │
           │
           │        5206 without prior macrovascular or microvascular disease
           │        1132 with prior macrovascular disease only
           ├──────► 2251 with prior microvascular disease only
           │        935 with prior macrovascular and microvascular disease
           │
           │
           ▼
┌──────────────────────────────────────────┐
│  1250 on-study macrovascular events       │
│  2395 on-study microvascular events       │
└──────────────────────────────────────────┘
```

**Section 3.3: Vitamin D**

**Table 6: Baseline characteristics by serum vitamin D concentration (≥50 vs <50)**

|  | Total Vitamin D ≥ 50 nmol/L (N=4560) | Total Vitamin D < 50 nmol/L (N=4964) |
|---|---|---|
| **General characteristics** | | |
| Assigned Fenofibrate | 2266 (49.7%) | 2495 (50.3%) |
| Male | 3235 (70.9%) | 2732 (55.0%)* |
| Age at visit 1 (years, mean[SD]) | 62.5 (6.8) | 62.0 (7.0)* |
| Diabetes duration (years, median[IQR]) | 5 (2-9) | 5 (2-10)* |
| Body-mass index (kg/m2, median[IQR]) | 29.2 (26.5-32.6) | 30.4 (27.1-34.5)* |
| Blood pressure - systolic (mmHg, mean[SD]) | 139.8 (15.0) | 141.2 (15.7)* |
| Blood pressure - diastolic (mmHg, mean[SD]) | 81.7 (8.3) | 82.4 (8.7)* |
| Ex/current smoker | 2849 (62.5%) | 2860 (57.6%)* |
| **Clinical History** | | |
| Previous cardiovascular disease | 947 (20.8%) | 1120 (22.6%)* |
| Peripheral Vascular Disease | 341 (7.5%) | 400 (8.1%) |
| Coronary revascularisation (CABG or PTCA) | 195 (4.3%) | 154 (3.1%)* |
| History of hypertension | 2498 (54.8%) | 2907 (58.6%)* |
| History of diabetic retinopathy | 345 (7.6%) | 448 (9.0%)* |
| Neuropathy (self-reported) | 574 (12.6%) | 789 (15.9%)* |
| **Laboratory data** | | |
| Total cholesterol (mmol/L, mean[SD]) | 4.98 (0.71) | 5.09 (0.70)* |
| LDL cholesterol (mmol/L, mean[SD]) | 3.05 (0.66) | 3.07 (0.64) |
| HDL cholesterol (mmol/L, mean[SD]) | 1.08 (0.25) | 1.11 (0.27)* |
| Triglycerides (mmol/L, median[IQR]) | 1.68 (1.31-2.24) | 1.79 (1.38-2.40)* |
| HbA1c (median[IQR]) | 6.7 (6.0-7.6) | 7.0 (6.2-8.0)* |
| Plasma creatinine (umol/L, mean[SD]) | 79.1 (15.5) | 76.3 (15.9)* |
| Dyslipidaemia | 1624 (35.6%) | 1982 (39.9%)* |
| Urine ACR - microalbuminuria | 921 (20.3%) | 1128 (22.8%)* |
| Urine ACR - macroalbuminuria | 185 (4.1%) | 210 (4.2%) |
| Hight sensitive C-reactive protein (mg/L, median[IQR]) | 2.6 (1.3-5.5) | 2.9 (1.3-6.3)* |

|  | Total Vitamin D ≥ 50 nmol/L (N=4560) | Total Vitamin D < 50 nmol/L (N=4964) |
|---|---|---|
| **Baseline cardiovascular medication** | | |
| Any Antithrombotic | 1432 (31.4%) | 1547 (31.2%) |
|     Any warfarin | 119 (2.6%) | 114 (2.3%) |
|     Aspirin or other antithrombotic without warfarin | 1313 (28.8%) | 1433 (28.9%) |
| Angiotensin II receptor antagonist | 241 (5.3%) | 273 (5.5%) |
| ACE inhibitor | 1460 (32.0%) | 1741 (35.1%)* |
| Beta blockers | 602 (13.2%) | 784 (15.8%)* |
| Calcium antagonist | 878 (19.3%) | 956 (19.3%) |
| Diuretic | 658 (14.4%) | 795 (16.0%)* |
| Nitrate | 230 (5.0%) | 303 (6.1%)* |
| **Baseline blood-glucose-lowering medication** | | |
| Diet | 1309 (28.7%) | 1220 (24.6%)* |
| Oral hypoglycaemic agent | 2724 (59.7%) | 2955 (59.5%) |
| Any insulin | 527 (11.6%) | 789 (15.9%)* |
| **Vitamin D supplementation** | | |
| Vitamin D supplements | 3 (0.1%) | 4 (0.1%) |
|     Vitamin D <700 IU daily | 3 (0.1%) | 3 (0.1%) |
|     Vitamin D >=700 IU daily | 0 (0.0%) | 1 (0.0%) |
| Calcium supplements | 123 (2.7%) | 137 (2.8%) |

* $p < 0.05$

**Section 3.3.1: Baseline Characteristics**

Vitamin D supplements were used by 0.1% but only one patient used a dose >700 IU daily.

In patients who were vitamin D deficient at baseline (serum 25OH vitamin D <50 nmol/L), cardiovascular disease, hypertension, retinopathy, and nephropathy were more prevalent (Table 6). In addition, these patients had a higher baseline prevalence of dyslipidaemia and microalbuminuria. On average, vitamin D deficient patients also had 0.3% higher HbA1c, 0.3mg/L higher hs-CRP, and 1.2 kg/m2 higher BMI. There were significantly fewer former and current smokers amongst the vitamin D deficient participants.

**Table 7: Summarier of Total Vitamin D by treatment group**

| Visit | Placebo | | | | | | | | Fenofibrate | | | | | | | | All patients | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Mean | SD | Median | Q1 | Q3 | Min | Max | N | Mean | SD | Median | Q1 | Q3 | Min | Max | N | Mean | SD | Median | Q1 | Q3 | Min | Max |
| Visit 1 | 4763 | 50.79 | 19.8 | 49 | 36 | 63 | 5 | 157 | 4761 | 50.77 | 20.4 | 48.8 | 37 | 63 | 5 | 196 | 9524 | 50.78 | 20.1 | 49 | 37 | 63 | 5 | 196 |
| Visit 9 | 962 | 53.74 | 21.6 | 52.8 | 38 | 68 | 5 | 144 | 959 | 50.37 | 20.4 | 49 | 36 | 62 | 5 | 168 | 1921 | 52.06 | 21.1 | 50.6 | 37 | 66 | 5 | 168 |
| Visit 20 | 881 | 62.77 | 23.6 | 60.9 | 47 | 78 | 5 | 171 | 876 | 58.68 | 20.9 | 56.25 | 44 | 70 | 12.4 | 179 | 1757 | 60.73 | 22.4 | 58.4 | 45 | 74 | 5 | 179 |

Note: Values below the limit of detection imputed to half of the smallest non-zero measurement.



**Figure 7: Baseline distribution of serum vitamin D concentrations in the FIELD study**
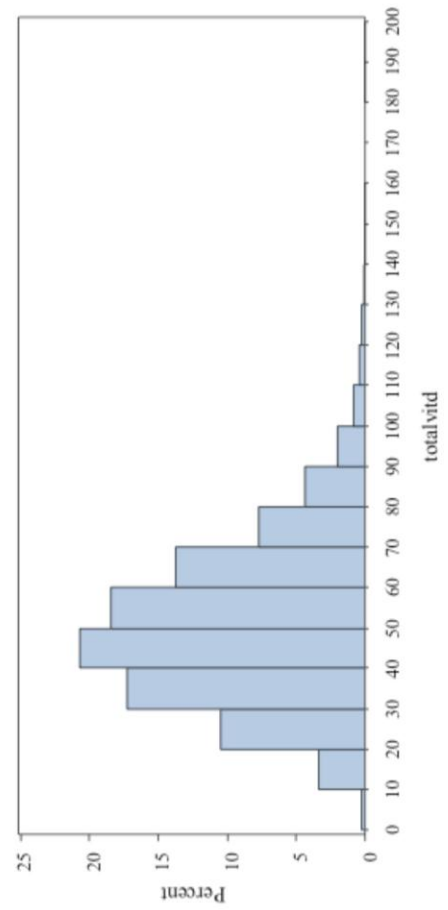
**Figure 7: Distribution of serum vitamin D concentrations in the FIELD study after two years**
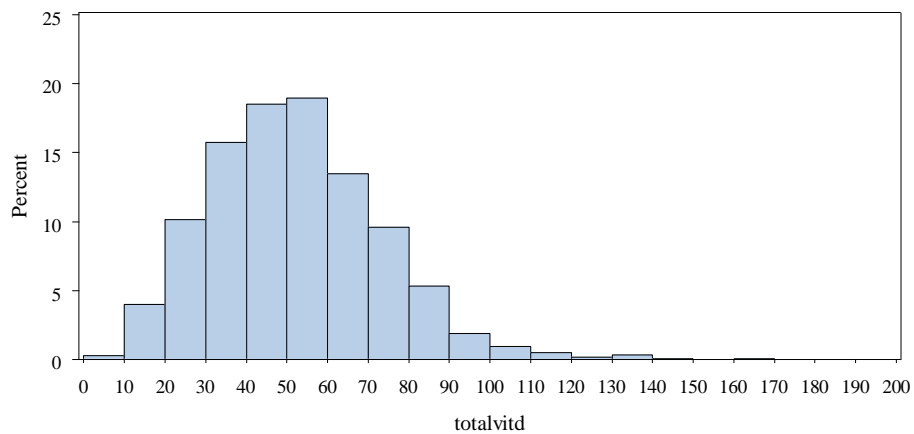


**Figure 8: Distribution of serum vitamin D concentrations in the FIELD study after five years**
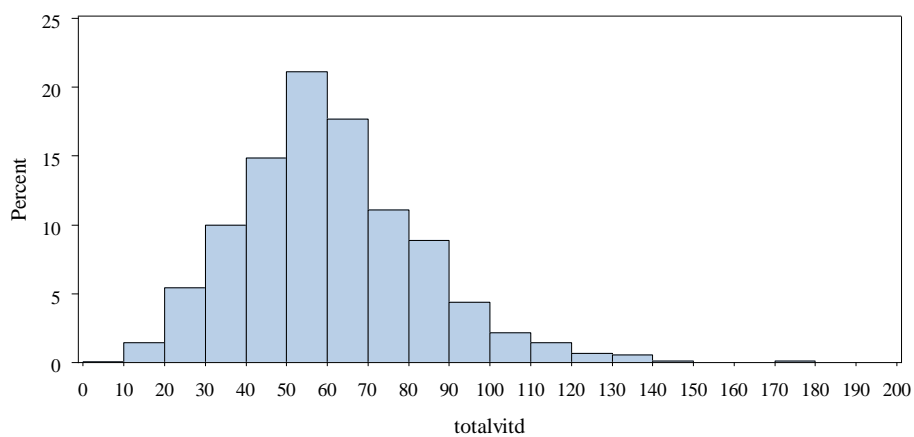


The median vitamin D level in the entire cohort at baseline was 49 nmol/L (range 5-196 nmol/L) (Table 7) and there was no significant difference between the two patient groups. This median rose in visits 9 and 20 to 50.6 nmol/L (range 5-168 nmol/L) and 58.4 nmol/L (range 5-179nmol/L) respectively. Eight percent, 52% and 89 % had serum vitamin D levels <25, <50, and <75 nmol/L, respectively.

**Table 7: Association of baseline Vitamin D (continuous) with on-study total CVD events**

| | Non adjusted model | | | Adjusted model* | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P | HR | 95% CI | P |
| Total Vitamin D (per 50 nmol/L decrease) | 1.2043 | (1.046 - 1.387) | 0.010 | 1.2254 | (1.058 - 1.420) | 0.007 |

*Cox model is stratified on treatment (no biomarker-treatment interactions were present) and adjusted for gender, age, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking, baseline OH use, baseline insulin use and prior CVD.



Figure 10: Total CVD events rate by quartiles of baseline Vitamin D
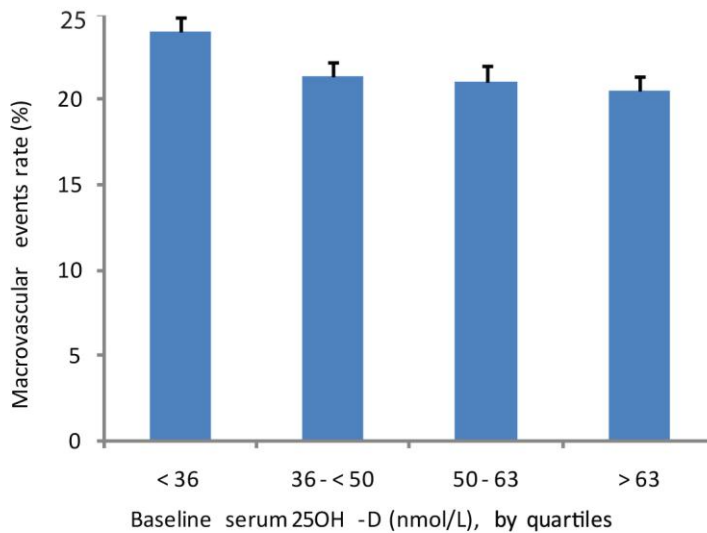


Figure 11: Total Microvascular events rate by quartiles of baseline Vitamin D
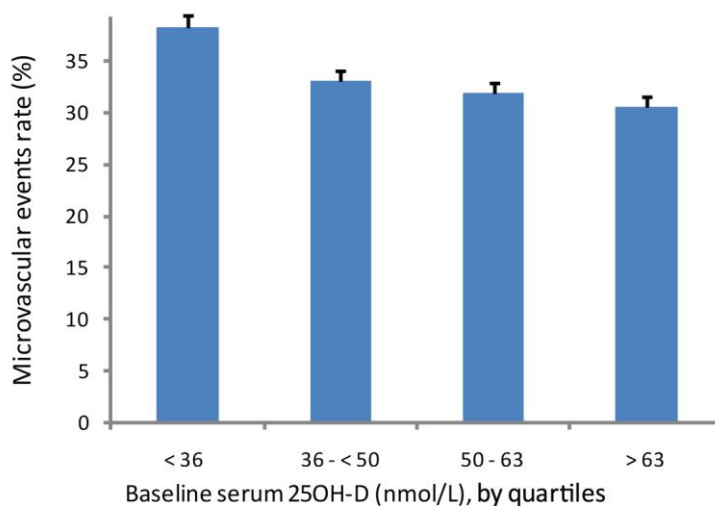
**Figure 9: Cumulative risk curves of time to first CVD events**



| Numbers at risk | | | | | | | |
|---|---|---|---|---|---|---|---|
| < 50 nmol/L | 4964 | 4817 | 4638 | 4481 | 4321 | 2430 | 779 |
| ≥ 50 nmol/L | 4560 | 4453 | 4300 | 4166 | 4016 | 2097 | 734 |
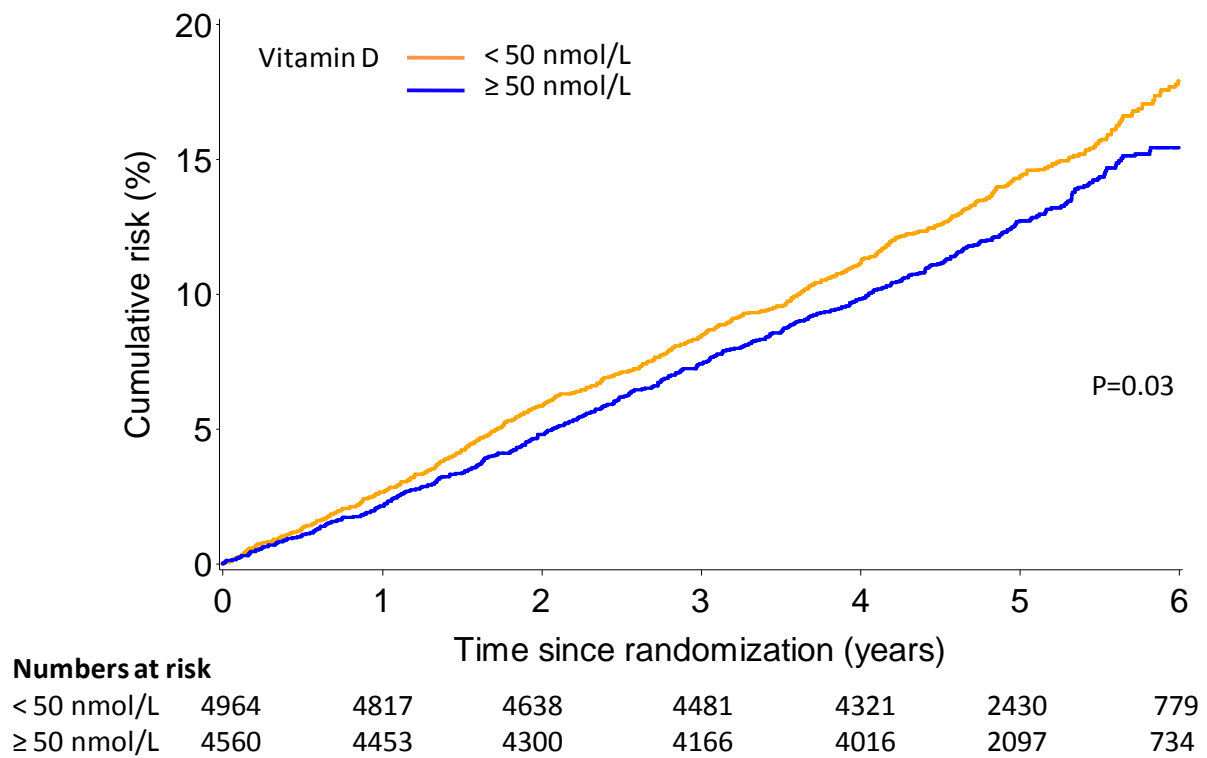
**Figure 10: Cumulative risk curves of time to first microvascular events**



| Numbers at risk | | | | | | | |
|---|---|---|---|---|---|---|---|
| < 50 nmol/L | 4964 | 4830 | 4224 | 3475 | 3416 | 1855 | 534 |
| ≥ 50 nmol/L | 4560 | 4478 | 3950 | 3346 | 3280 | 1610 | 529 |
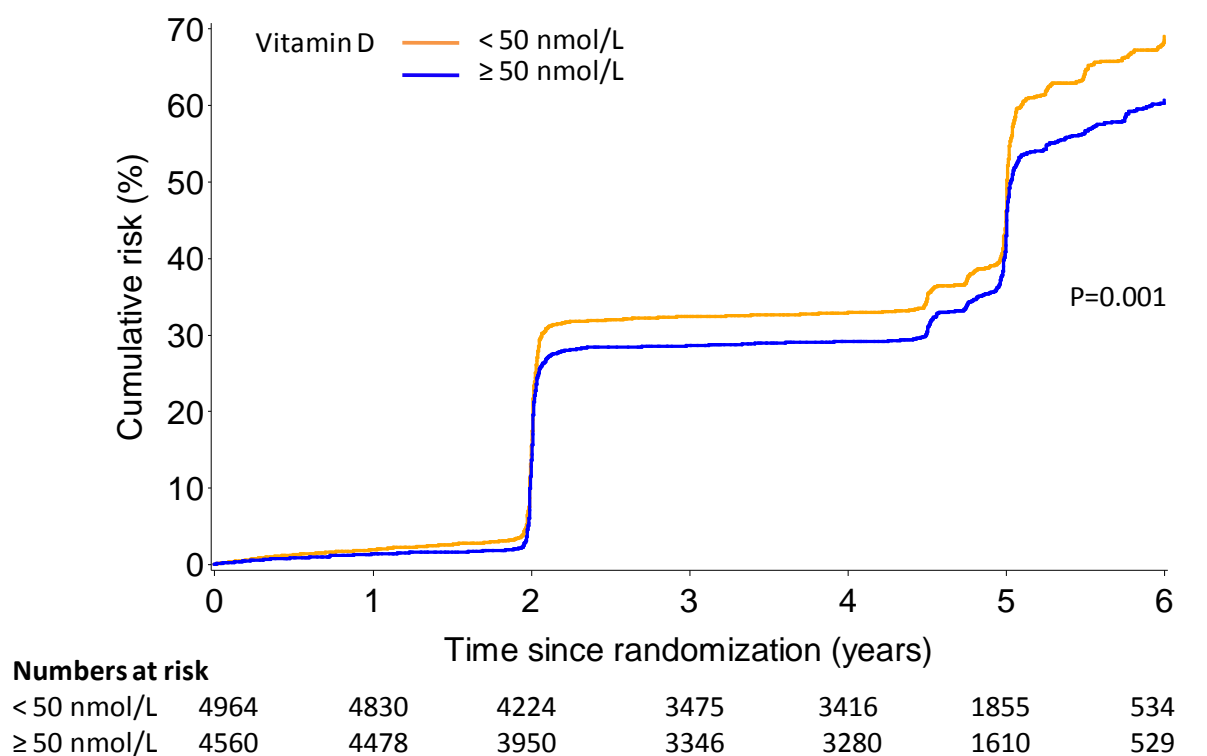
**Table 8: Association of the serum concentration of vitamin D at baseline with previous and new events macrovascular disease events**

### Association between vitamin D and <u>macrovascular</u> disease events

| | | | | | Non adjusted model | | | Adjusted model | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | N | n | % | OR (95% CI) | P | | OR (95% CI)* | P | |
| *Only previous events recorded at baseline* | | | | | | | | | | |
| Vitamin D (per 50 nmol/L decrease) | | 9524 | 2067 | 21.70 | 1.20 (1.06 – 1.36) | 0.004 | | 1.19 (1.04 – 1.35) | 0.013 | |
| | *Quartile* | N | n | % | HR (95% CI) | P | *P value for trend* | HR (95% CI)** | P | *P value for trend* |
| *New events during follow-up period* Vitamin D (per 50 nmol/L decrease) | | 9524 | 1250 | 13.12 | 1.20 (1.05 - 1.39) | 0.010 | | 1.23 (1.06 - 1.42) | 0.007 | |
| Vitamin D (nmol/L) | >63 | 2354 | 293 | 12.45 | 1.00 | | 0.017 | 1.00 | | 0.011 |
| | 50 - 63 | 2206 | 269 | 12.19 | 0.99 (0.84 - 1.17) | 0.89 | | 1.00 (0.84 - 1.18) | 0.97 | |
| | 36 – <50 | 2680 | 353 | 13.17 | 1.07 (0.91 - 1.24) | 0.42 | | 1.10 (0.94 - 1.28) | 0.25 | |
| | < 36 | 2284 | 335 | 14.67 | 1.19 (1.02 - 1.40) | 0.026 | | 1.21 (1.03 - 1.43) | 0.020 | |

*Odds ratio is adjusted for age, gender, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking and baseline use of oral hypoglycaemics and insulin.

**Hazard ratios are stratified by treatment and adjusted for prior macrovascular disease, age, gender, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking and baseline use of oral hypoglycaemics and insulin.

**Table 9: Association of the serum concentration of Vitamin D at baseline with previous and new events microvascular disease events**

| | | | | | **Association between Vitamin D and new <u>microvascular</u> disease events** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **Non adjusted model** | | | **Adjusted model** | | |
| | | | | | *OR (95% CI)* | *P* | | *OR (95% CI)\** | *P* | |
| *Only previous events recorded at baseline*<br>Vitamin D (per 50 nmol/L decrease) | | | | | 1.32 (1.19 – 1.47) | <0.001 | | 1.19 (1.05 – 1.34) | 0.005 | |
| | *Quartile* | *N* | *n* | *%* | *OR (95% CI)* | *P* | *P value for trend* | *OR (95% CI)\*\** | *P* | *P value for trend* |
| *New events during follow-up period,*<br>Vitamin D (per 50 nmol/L decrease) | | | | | 1.18(1.05 - 1.32) | 0.006 | | 1.11 (0.98 - 1.26) | 0.11 | |
| Vitamin D (nmol/L) | >63 | 2354 | 568 | 24.13 | 1.00 | | <.001 | 1.00 | | 0.13 |
| | 50 - 63 | 2206 | 526 | 23.84 | 0.98 (0.86 - 1.13) | 0.82 | | 0.95 (0.82 - 1.09) | 0.46 | |
| | 36 – <50 | 2680 | 679 | 25.34 | 1.07 (0.94 - 1.21) | 0.32 | | 1.02 (0.89 - 1.16) | 0.81 | |
| | < 36 | 2284 | 622 | 27.23 | 1.18 (1.03 - 1.34) | 0.016 | | 1.10 (0.95 - 1.26) | 0.19 | |

*Odds ratio is adjusted for age, gender, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking and baseline use of oral hypoglycaemics and insulin.

**Odds ratios are stratified by treatment and adjusted for prior microvascular events, age, gender, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking and baseline use of oral hypoglycaemics and insulin.

**Section 3.3.2: Prediction by baseline serum Vitamin D of macrovascular events**

Serum 25OH Vitamin D was an independent predictor of macrovascular events, both existent events at baseline and incident events over 5 years of follow-up. This relationship remained unchanged in multivariable analyses (Table 8). For each 50 nmol/L lower baseline serum vitamin D level, the unadjusted odds of having existent cardiovascular disease at baseline was 20% (95 % CI, 6-36 %; $P$ = 0.004) higher, and 19% (95% CI, 4-35%; $P$=0.01) higher after adjustment for confounders. Similar associations were seen with incident events during follow-up, with a 20% (95% CI, 5-39%; $P$=0.01) higher risk of a cardiovascular event for each 50 nmol/L lower serum level of vitamin D, and a 23% (95% CI, 6-42%; $P$=0.007) higher risk after multivariable adjustment. Exploratory further adjustment for physical activity score or hs-CRP level did not materially change these relationships (table 16). The association between quartiles of serum vitamin D and incident macrovascular events is shown in Table 8. In the adjusted model, patients in the lowest quartile of serum vitamin D (<36 nmol/L) showed 21% higher risk of macrovascular disease than those in the highest quartile (>63 nmol/L).

Levels of vitamin D in the lowest quartile (<36 nmol/L) were associated with an absolute excess rate of existent macrovascular events at baseline of approximately 3% more than those in quartiles 2 (36-50 nmol/L) and 3 (50-63 nmol/L), and approximately 4% more than those in the highest quartile (>63 nmol/L; Figure 10).

Vitamin D deficient patients with a serum vitamin D concentration less than the median (50 nmol/L) had a higher cumulative incidence of macrovascular events than those whose levels fell above the median (Figure 9).

**Section 3.3.3: Prediction by baseline serum vitamin D of microvascular complications during follow-**up

Serum vitamin D was an independent predictor of existent microvascular events at baseline in multivariable analysis adjusted for potential confounders (Table 9). Serum vitamin D was also significantly associated with incident events over 5 years of follow-up in multivariable analysis, unless HbA1C was included in the model. For each 50 nmol/L lower baseline serum vitamin D level, the unadjusted odds of having existent microvascular disease at baseline was 32% (95% CI, 19-47 %; $P<0.001$) higher, and 19% (95% CI, 5-34%; $P=0.005$) higher after adjustment for confounders. Prospective analysis of incident events during follow-up revealed 18% (95% CI, 5-32%; $P=0.006$) higher unadjusted risk of a microvascular event for each 50 nmol/L lower level of serum vitamin D, and a 15% (95% CI, 1-30%; $P=0.03$) higher risk after multivariable adjustment which included all variables other than HbA1C. Exploratory further adjustment for hs-CRP level did not materially change the higher level of risk (14%; 95% CI 1-29; $P=0.04$), but adjustment for HbA1C or physical activity score reduced the incremental risk to 11% and abolished its significance in both cases ($P=0.11$). Adjustment of vitamin D levels for seasonal effects did not strengthen the associations (table 16). Individuals with vitamin D levels in the lowest quartile (<36 nmol/L) had an absolute excess rate of existent microvascular events at baseline approximately 5-7% higher than those in quartiles 2 (36-50 nmol/L), 3 (50-63 nmol/L), and 4 (>63 nmol/L, Figure 11). Compared with those whose levels fell above the median (50 nmol/L), the lower group had an absolute excess of events of about 3% during follow-up (Figure 13).

**Section 3.4: Osteocalcin**

**Table 10: Baseline characteristics by serum osteocalcin concentration (≥9.1 vs <9.1)**

| | Total Osteocalcin >= 9.1 ng/mL (N=4758) | Total Osteocalcin < 9.1 ng/mL (N=4757) | p-value* |
|---|---|---|---|
| **General characteristics** | | | |
| Fenofibrate | 2376 (49.9%) | 2380 (50.0%) | 0.9265 |
| Male | 3035 (63.8%) | 2924 (61.5%) | 0.0193 |
| Age at visit 1 (years, mean[SD]) | 63.2 (6.8) | 61.2 (6.8) | <.0001 |
| Diabetes duration (years, median[IQR]) | 4 (2-9) | 5 (3-10) | <.0001 |
| Body-mass index (kg/m2, median[IQR]) | 28.9 (26.2-32.3) | 30.7 (27.6-34.9) | <.0001 |
| Waist-to-hip ratio (mean[SD]) | 0.93 (0.08) | 0.94 (0.08) | <.0001 |
| Blood pressure - systolic (mmHg, mean[SD]) | 140.3 (15.7) | 140.8 (15.1) | 0.1585 |
| Blood pressure - diastolic (mmHg, mean[SD]) | 81.7 (8.5) | 82.5 (8.5) | <.0001 |
| Current/Ex smoker | 2758 (58.0%) | 2946 (61.9%) | <.0001 |
| **Clinical History** | | | |
| Previous cardiovascular disease | 1023 (21.5%) | 1042 (21.9%) | 0.6327 |
| Prior myocardial infarction | 232 (4.9%) | 229 (4.8%) | 0.8879 |
| Prior stroke/TIA | 296 (6.2%) | 290 (6.1%) | 0.8001 |
| Prior Angina | 603 (12.7%) | 545 (11.5%) | 0.0685 |
| Peripheral Vascular Disease | 333 (7.0%) | 408 (8.6%) | 0.0041 |
| Coronary revascularisation (CABG or PTCA) | 179 (3.8%) | 169 (3.6%) | 0.5863 |
| History of hypertension | 2571 (54.0%) | 2827 (59.4%) | <.0001 |
| History of diabetic retinopathy | 384 (8.1%) | 407 (8.6%) | 0.3914 |
| Neuropathy (self-reported or monofil) | 665 (14.0%) | 698 (14.7%) | 0.3321 |
| Nephropathy (self-reported or ACR measurement) | 123 (2.6%) | 149 (3.1%) | 0.1093 |
| **Laboratory data** | | | |
| Total cholesterol (mmol/L, mean[SD]) | 5.04 (0.70) | 5.03 (0.70) | 0.3006 |
| LDL cholesterol (mmol/L, mean[SD]) | 3.12 (0.63) | 3.01 (0.66) | <.0001 |
| HDL cholesterol (mmol/L, mean[SD]) | 1.10 (0.25) | 1.10 (0.27) | 0.8559 |
| Triglycerides (mmol/L, median[IQR]) | 1.65 (1.28-2.17) | 1.83 (1.41-2.46) | <.0001 |
| HbA1c (median[IQR]) | 6.6 (6.0-7.5) | 7.1 (6.3-8.1) | <.0001 |
| Plasma creatinine (umol/L, mean[SD]) | 80.5 (16.2) | 74.7 (14.8) | <.0001 |

|  | Total Osteocalcin >= 9.1 ng/mL (N=4758) | Total Osteocalcin < 9.1 ng/mL (N=4757) | p-value* |
|---|---|---|---|
| Homocysteine (umol/L, median[IQR]) | 9.7 (8.1-11.7) | 9.4 (7.8-11.3) | <.0001 |
| Dyslipidaemia | 1644 (34.6%) | 1961 (41.2%) | <.0001 |
| Urine ACR - microalbuminuria | 906 (19.1%) | 1142 (24.1%) | <.0001 |
| Urine ACR - macroalbuminuria | 197 (4.2%) | 196 (4.1%) | |
| Hight sensitive C-reactive protein (mg/L, median[IQR]) | 2.4 (1.1-5.0) | 3.2 (1.6-6.9) | <.0001 |
| **Baseline cardiovascular medication** | | | |
| Any Antithrombotic | 1467 (30.8%) | 1508 (31.7%) | 0.3609 |
|    Any warfarin | 46 (1.0%) | 187 (3.9%) | |
|    Aspirin or other antithrombotic without warfarin | 1421 (29.9%) | 1321 (27.8%) | |
| Angiotensin II receptor antagonist | 211 (4.4%) | 302 (6.3%) | <.0001 |
| ACE inhibitor | 1436 (30.2%) | 1759 (37.0%) | <.0001 |
| Beta blockers | 643 (13.5%) | 741 (15.6%) | 0.0043 |
| Calcium antagonist | 936 (19.7%) | 897 (18.9%) | 0.3131 |
| Diuretic | 643 (13.5%) | 808 (17.0%) | <.0001 |
| Nitrate | 282 (5.9%) | 250 (5.3%) | 0.1540 |
| **Baseline blood-glucose-lowering medication** | | | |
| Diet | 1616 (34.0%) | 913 (19.2%) | |
| Oral hypoglycaemic agent | 2490 (52.3%) | 3181 (66.9%) | <.0001 |
| Any insulin | 652 (13.7%) | 663 (13.9%) | |
| **Calcium supplementation** | | | |
| Calcium supplements | 120 (2.5%) | 140 (2.9%) | 0.2079 |

\* P-value from chi square test for categorical variables, t-test for normally distributed continuous variables or Wilcoxon Rank Sum test for non-normally distributed continuous variables. P-valuesfor ACR measurement (normal, micro or macroalbuminuria) and for baseline blood-glucose-lowering medication are from a 3 x 2 chi-square test.

**Table 11: Summaries of Total Osteocalcin by treatment group**

| Visit | Placebo | | | | | | | | Fenofibrate | | | | | | | | All patients | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Mean | SD | Median | Q1 | Q3 | Min | Max | N | Mean | SD | Median | Q1 | Q3 | Min | Max | N | Mean | SD | Median | Q1 | Q3 | Min | Max |
| Visit 1 | 4759 | 10.16 | 4.42 | 9.10 | 7.10 | 12.10 | 1.20 | 46.90 | 4756 | 10.29 | 5.07 | 9.00 | 7.10 | 12.30 | 0.15 | 168.00 | 9515 | 10.22 | 4.76 | 9.10 | 7.10 | 12.20 | 0.15 | 168.00 |
| Visit 9 | 951 | 11.69 | 5.49 | 10.50 | 8.00 | 13.80 | 0.15 | 57.10 | 950 | 12.17 | 5.28 | 10.85 | 8.50 | 14.50 | 0.15 | 45.20 | 1901 | 11.93 | 5.39 | 10.60 | 8.20 | 14.10 | 0.15 | 57.10 |

Note: Values below the limit of detection imputed to half of the smallest non-zero measurement

**Figure 14: Baseline distribution of serum Osteocalcin concentrations in the FIELD study**
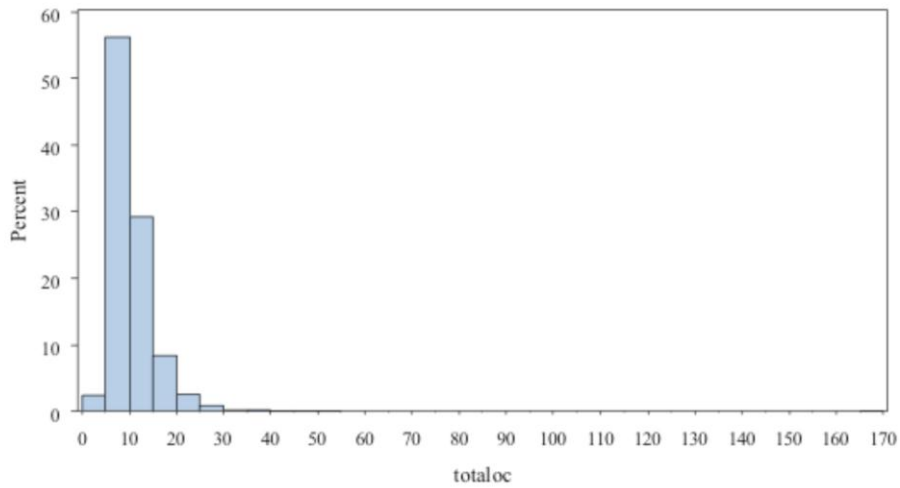


**Figure 15: Baseline percentage distribution of serum Undercarboxylated Osteocalcin concentrations in the FIELD study**
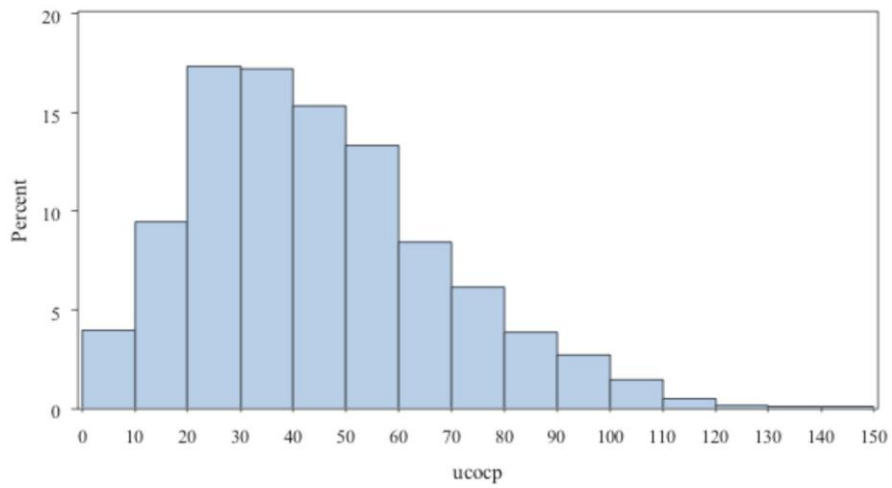


**Figure 16: Baseline distribution of total serum Undercarboxylated Osteocalcin concentrations in the FIELD study**
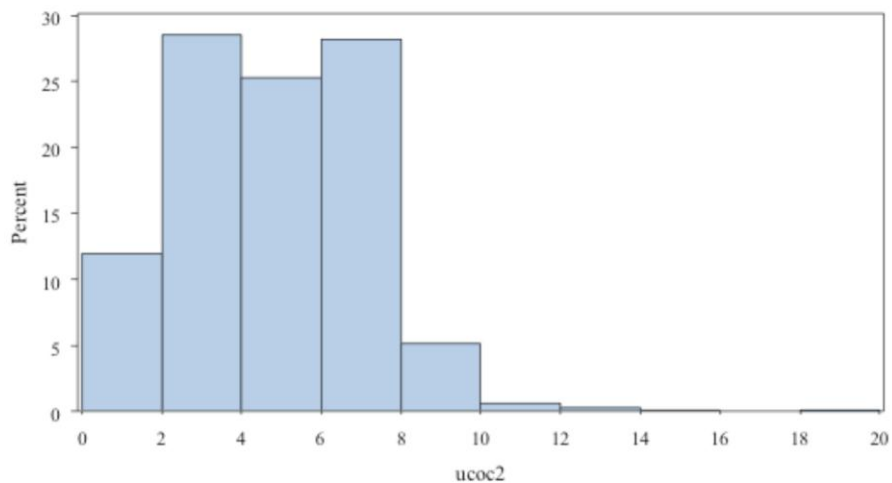
**Figure 17: Total CVD events rate by quartiles of baseline Osteocalcin**
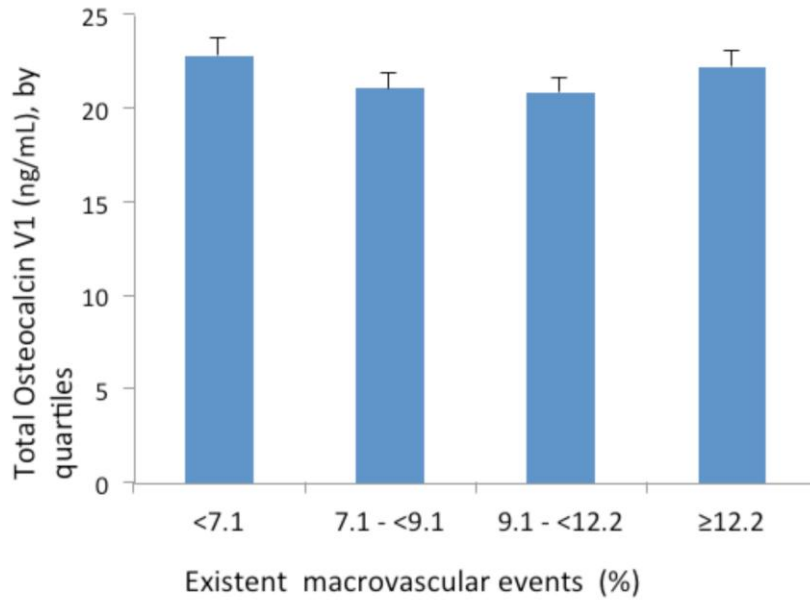


**Figure 18: Total microvascular events rate by quartiles of baseline Osteocalcin**
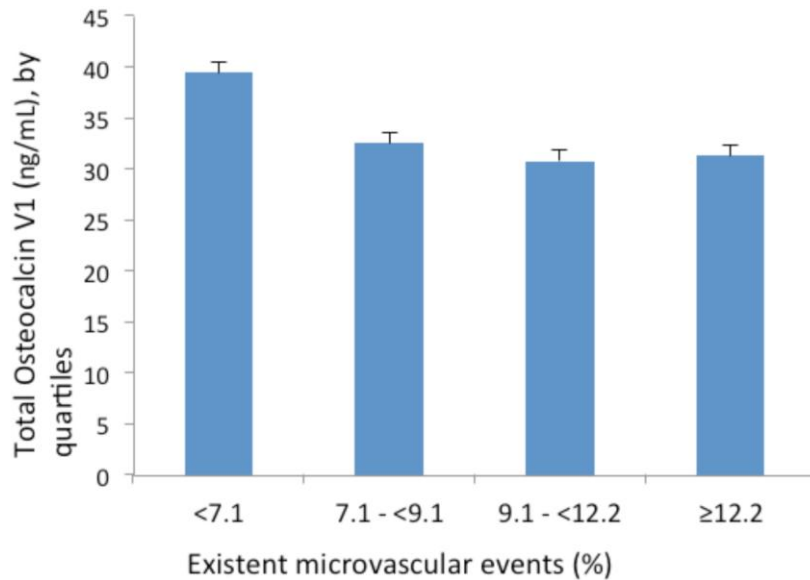
**Table 12: Association of baseline Osteocalcin by quartiles with on-study total CVD events**

| Biomarker | Quartile | N | n | % | Non adjusted model | | | Adjusted model* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | HR (95% CI) | P value | P value for trend | HR (95% CI) | P | P value for trend |
| Total Osteocalcin | >= 12.2 | 2387 | 323 | 13.53 | 1.00 | | 0.91 | 1.00 | | 0.81 |
| (ng/mL) | [9.1 - 12.2) | 2371 | 307 | 12.95 | 0.95 (0.82 - 1.11) | 0.54 | | 0.93 (0.79 - 1.08) | 0.34 | |
| | [7.1 - 9.1) | 2460 | 302 | 12.28 | 0.91 (0.77 - 1.06) | 0.22 | | 0.88 (0.74 - 1.03) | 0.11 | |
| | < 7.1 | 2297 | 315 | 13.71 | 1.03 (0.88 - 1.20) | 0.73 | | 1.00 (0.85 - 1.18) | 0.97 | |
| Undercarboxylated Osteocalcin | >= 6.6 | 509 | 65 | 12.77 | 1.00 | | 0.76 | 1.00 | | 0.27 |
| (ng/mL) | [4.8 - 6.6) | 457 | 54 | 11.82 | 0.94 (0.66 - 1.35) | 0.75 | | 0.90 (0.62 - 1.29) | 0.55 | |
| | [2.8 - 4.8) | 496 | 55 | 11.09 | 0.87 (0.61 - 1.25) | 0.45 | | 0.74 (0.51 - 1.07) | 0.11 | |
| | < 2.8 | 443 | 62 | 14.00 | 1.09 (0.77 - 1.55) | 0.61 | | 0.85 (0.60 - 1.22) | 0.39 | |
| Undercarboxylated Osteocalcin Percent | >= 58.3 | 480 | 51 | 10.63 | 1.00 | | 0.07 | 1.00 | | 0.50 |
| (%) | [41.2 - 58.3) | 473 | 58 | 12.26 | 1.18 (0.81 - 1.72) | 0.39 | | 1.12 (0.76 - 1.63) | 0.57 | |
| | [26.9 - 41.2) | 477 | 57 | 11.95 | 1.13 (0.77 - 1.64) | 0.54 | | 1.01 (0.69 - 1.48) | 0.96 | |
| | < 26.9 | 474 | 70 | 14.77 | 1.44 (1.00 - 2.06) | 0.048 | | 1.18 (0.81 - 1.70) | 0.39 | |

* Cox model is stratified by treatment arm (no biomarker-treatment interactions were present) and adjusted for prior CVD, gender, age, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking, baseline OH use, baseline insulin use.

Table 13: Association of baseline Osteocalcin by quartiles with on-study Microvascular events

| Biomarker | Quartile | N | n | % | Non adjusted model | | | Adjusted model* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | OR (95% CI) | P value | P value for trend | OR (95% CI) | P value | P value for trend |
| Total Osteocalcin | >= 12.2 | 2387 | 552 | 23.13 | 1.00 | | <.001 | 1.00 | | 0.36 |
| (ng/mL) | [9.1 - 12.2) | 2371 | 575 | 24.25 | 1.06 (0.93 - 1.22) | 0.36 | | 0.98 (0.85 - 1.12) | 0.77 | |
| | [7.1 - 9.1) | 2460 | 639 | 25.98 | 1.17 (1.02 - 1.33) | 0.021 | | 1.02 (0.89 - 1.18) | 0.75 | |
| | < 7.1 | 2297 | 627 | 27.30 | 1.25 (1.09 - 1.42) | 0.001 | | 1.06 (0.92 - 1.22) | 0.45 | |
| Undercarboxylated Osteocalcin | >= 6.6 | 509 | 112 | 22.00 | 1.00 | | 0.09 | 1.00 | | 0.29 |
| (ng/mL) | [4.8 - 6.6) | 457 | 110 | 24.07 | 1.12 (0.83 - 1.52) | 0.45 | | 1.07 (0.79 - 1.46) | 0.66 | |
| | [2.8 - 4.8) | 496 | 138 | 27.82 | 1.37 (1.03 - 1.82) | 0.033 | | 1.27 (0.94 - 1.71) | 0.12 | |
| | < 2.8 | 443 | 113 | 25.51 | 1.21 (0.90 - 1.64) | 0.21 | | 1.12 (0.82 - 1.53) | 0.47 | |
| Undercarboxylated Osteocalcin | >= 58.3 | 480 | 116 | 24.17 | 1.00 | | 0.71 | 1.00 | | 0.66 |
| (%) | [41.2 - 58.3) | 473 | 129 | 27.27 | 1.18 (0.88 - 1.57) | 0.27 | | 1.27 (0.94 - 1.72) | 0.11 | |
| | [26.9 - 41.2) | 477 | 113 | 23.69 | 0.97 (0.72 - 1.31) | 0.86 | | 1.02 (0.75 - 1.39) | 0.91 | |
| | < 26.9 | 474 | 115 | 24.26 | 1.01 (0.75 - 1.35) | 0.97 | | 1.00 (0.74 - 1.37) | 0.98 | |

*Odds ratios are stratified by treatment arm (no biomarker-treatment interactions were present) and adjusted for prior microvascular disease, gender, diabetes duration, HbA1c, systolic BP, BMI, lipids (TG, HDLc, LDLc), smoking, baseline OH use, baseline insulin use.

**Figure 11: Cumulative risk curves of time to first CVD event**



| Numbers at risk | | | | | | |
|---|---|---|---|---|---|---|
| < 9.1 ng/mL | 4757 | 4640 | 4465 | 4318 | 4179 | 2209 | 724 |
| ≥ 9.1 ng/mL | 4758 | 4622 | 4465 | 4321 | 4151 | 2313 | 789 |
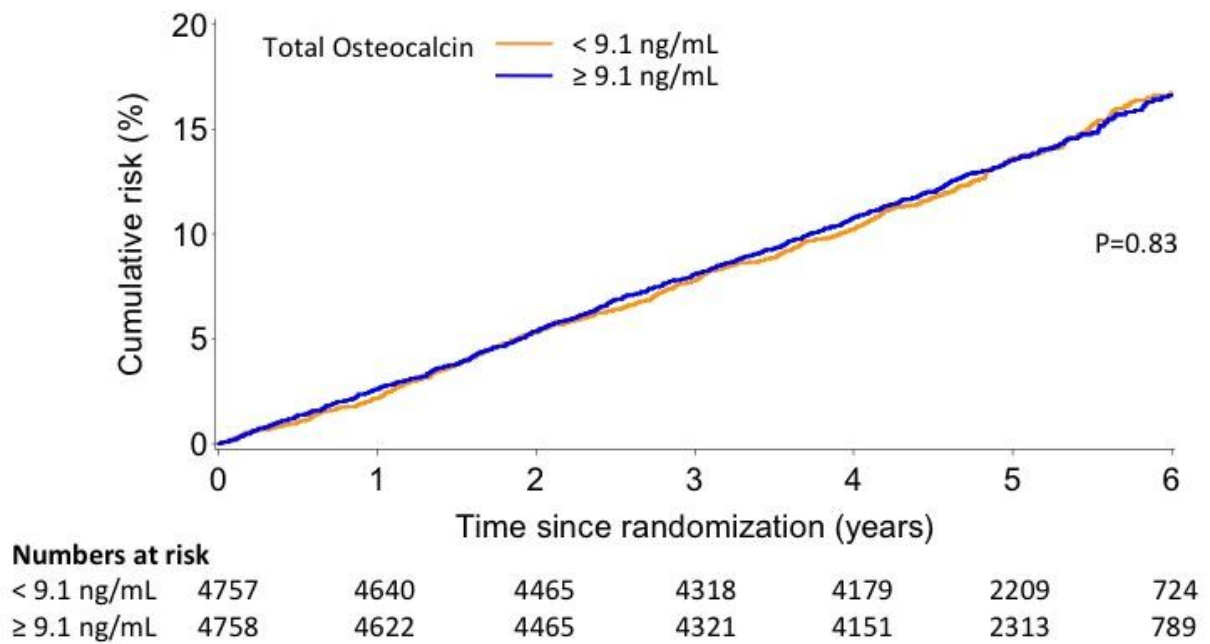
**Figure 12: Cumulative risk curves of time to first microvascular events**



| Numbers at risk | | | | | | |
|---|---|---|---|---|---|---|
| < 9.1 ng/mL | 4757 | 4639 | 4365 | 4008 | 3925 | 2023 | 623 |
| ≥ 9.1 ng/mL | 4758 | 4662 | 4386 | 4085 | 3994 | 2158 | 707 |
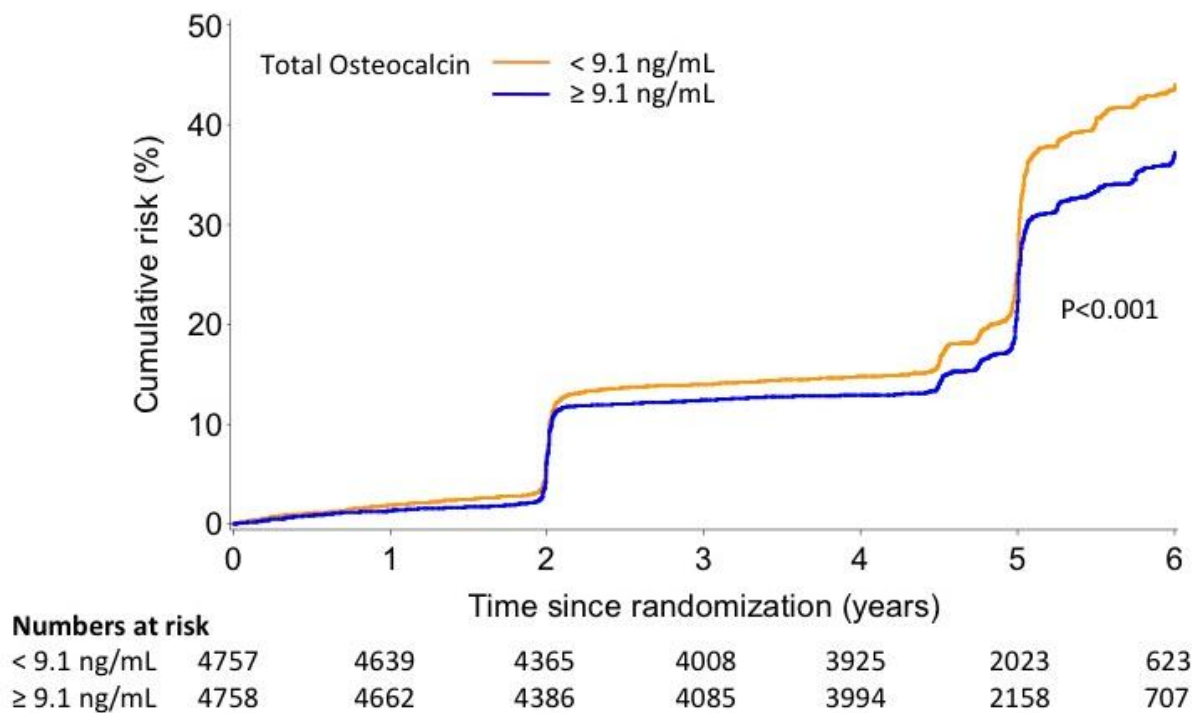
**Section 3.4.1: Baseline Characteristics**

The median serum OCN level in the entire cohort was 9.1 ng/mL and there was no relevant difference between the placebo and the Fenofibrate groups before or during study treatment (Table 11).

At baseline, patients who had a serum OCN concentration below the median had higher body mass index, higher waist-to-hip ratio, longer duration of diabetes, a higher prevalence dyslipidaemia, microalbuminuria and peripheral vascular disease. Furthermore, patients with a baseline OCN level below the median had higher blood levels of glycated haemoglobin; used anti-hypertensive drugs more frequently and were prescribed more intensive anti-diabetic management as judged by a higher prevalence of insulin use or combination oral glucose lowering therapy (Table 10).

**Section 3.4.2: Association between microvascular complications and serum total osteocalcin**

Patients with OCN levels below the population mean of 9.1 ng/mL show a significantly higher cumulative incidence of microvascular events than those with ≥ 9.1 ng/mL (Table 13). When dividing patients into quartiles of serum OCN those in the lowest quartile of serum OCN (< 7.1 ng/mL) showed 25% greater risk of on-study microvascular disease than those in the highest quartile (≥ 12.2 ng/mL) (Figure 18). Serum OCN at baseline was a significant predictor of existing and incident events over 5 years of follow-up in multivariable analysis, unless indices of glycemic control were included in the model. Prospective analysis of incident events during follow-up revealed 9% (95% CI, 5-32%; P=0.006) higher unadjusted risk of a microvascular event for each 5 ng/ml lower level of serum OCN, which remained unchanged after multivariable adjustment which included all variables other than HbA1C, use of glucose lowering medication and duration of diabetes. For each 5 ng/mL increase in serum OCN, the unadjusted odds of having existent microvascular disease at baseline was reduced by 8%. Exploratory further adjustment for indices of glycaemic control (HbA1C, use of glucose lowering medication and duration of diabetes) reduced the incremental risk and abolished its

significance in both cases. Adjustment of OCN for seasonal effects, physical activity or hs-CRP as an indicator of systemic inflammation did not strengthen the associations.

**Section 3.4.3: Relationship between serum osteocalcin, indices of glucose homoeostasis and lipids**

Total serum OCN showed significant inverse correlations with plasma triglycerides and various plasma indices of glucose homoeostasis, such as fasting glucose, glycated haemoglobin, and insulin. OCN also correlated with plasma creatinine. Undercarboxylated OCN did not correlate with plasma triglycerides and insulin. The correlations between undercarboxylated OCN, fasting glucose and glycated haemoglobin were weaker than for total serum OCN.

**Section 3.4.4: Association between vascular complications and undercarboxylated osteocalcin**

The serum concentration of undercarboxylated OCN was not a significant predictor of macro- or microvascular disease, with or without adjustment.  Also, no association with microvascular disease was found when undercarboxylated OCN was expressed as percentage of total serum OCN.

**Table 14: Spearman correlation between continuous variables at baseline**

| | Total Osteocalcin | UC Osteocalcin Calculation | % UC Osteocalcin | Total Vitamin D | HDL | LDL | Triglycerides | BP systolic | Glucose | HbA1c | Insulin | Creatinine | ACR | HOMA2-IR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total Osteocalcin | - | | | | | | | | | | | | | |
| UC Osteocalcin Calculation | 0.358 (p<0.001) | - | | | | | | | | | | | | |
| % UC Osteocalcin | -0.305 (p<0.001) | 0.730 (p<0.001) | - | | | | | | | | | | | |
| Total Vitamin D | 0.028 (p=0.0055) | 0.127 (p<0.001) | 0.094 (p<0.001) | - | | | | | | | | | | |
| HDL | 0.007 (p=0.4941) | 0.106 (p<0.001) | 0.077 (p<0.001) | -0.073 (p<0.001) | - | | | | | | | | | |
| LDL | 0.083 (p<0.001) | 0.008 (p=0.7150) | -0.081 (p<0.001) | -0.018 (p=0.0841) | 0.124 (p<0.001) | - | | | | | | | | |
| Triglycerides | -0.151 (p<0.001) | -0.021 (p=0.3663) | 0.076 (p<0.001) | -0.089 (p<0.001) | -0.364 (p<0.001) | -0.129 (p<0.001) | - | | | | | | | |
| BP Systolic | -0.010 (p=0.3536) | -0.044 (p=0.0544) | -0.052 (p=0.0238) | -0.051 (p<0.001) | 0.054 (p<0.001) | -0.031 (p=0.0019) | 0.041 (p<0.001) | - | | | | | | |
| Glucose | -0.228 (p<0.001) | -0.130 (p<0.001) | 0.040 (p=0.0777) | -0.089 (p<0.001) | -0.037 (p<0.001) | -0.050 (p<0.001) | 0.100 (p<0.001) | 0.086 (p<0.001) | - | | | | | |
| HbA1c | -0.211 (p<0.001) | -0.104 (p<0.001) | 0.067 (p=0.0035) | -0.116 (p<0.001) | -0.003 (p=0.7537) | -0.021 (p=0.0338) | 0.072 (p<0.001) | 0.076 (p<0.001) | 0.765 (p<0.001) | - | | | | |
| Insulin | -0.150 (p<0.001) | -0.039 (p=0.0882) | 0.064 (p=0.0049) | -0.118 (p<0.001) | -0.178 (p<0.001) | -0.132 (p<0.001) | 0.246 (p<0.001) | 0.104 (p<0.001) | 0.048 (p<0.001) | 0.065 (p<0.001) | - | | | |
| Creatinine | 0.210 (p<0.001) | -0.060 (p=0.0090) | -0.184 (p<0.001) | 0.129 (p<0.001) | -0.208 (p<0.001) | -0.030 (p=0.0026) | 0.016 (p=0.1062) | 0.054 (p<0.001) | -0.024 (p=0.0192) | -0.010 (p=0.3254) | -0.043 (p<0.001) | - | | |
| ACR | -0.092 (p<0.001) | 0.001 (p=0.9751) | 0.036 (p=0.1208) | -0.079 (p<0.001) | -0.076 (p<0.001) | -0.048 (p<0.001) | 0.121 (p<0.001) | 0.281 (p<0.001) | 0.194 (p<0.001) | 0.229 (p<0.001) | 0.114 (p<0.001) | 0.019 (p=0.0649) | - | |
| Homa2-IR | -0.175 (p<0.001) | -0.054 (p=0.0206) | 0.069 (p=0.0031) | -0.123 (p<0.001) | -0.170 (p<0.001) | -0.133 (p<0.001) | 0.252 (p<0.001) | 0.110 (p<0.001) | 0.165 (p<0.001) | 0.154 (p<0.001) | 0.987 (p<0.001) | -0.050 (p<0.001) | 0.130 (p<0.001) | - |
| Hs-CRP | -0.174 (p<0.001) | 0.005 (p=0.8135) | 0.143 (p<0.001) | -0.060 (p<0.001) | -0.016 (p=0.1089) | 0.008 (p=0.4382) | 0.123 (p<0.001) | 0.057 (p<0.001) | 0.028 (p=0.0053) | 0.087 (p<0.001) | 0.288 (p<0.001) | -0.163 (p<0.001) | 0.118 (p<0.001) | 0.286 (p<0.001) |

**Table 16: Exploratory models investigating the impact of HbA1c, hs-CRP, physical activity and seasonal variability on the association between serum 25OH-D and new events during follow-up**

| New events during follow-up | | Adjusted model | |
|---|---|---|---|
| | | OR or HR (95% CI) | *P* |
| **Macrovascular** | | | |
| 25OH-D (nmol/L) (per 50 nmol/L decrease) | Adjusted for all* + HbA1c | 1.23 (1.06–1.42) | 0.007 |
| | Adjusted for all* + hs-CRP | 1.26 (1.09-1.46) | 0.002 |
| | Adjusted for all* + physical activity | 1.21 (1.04-1.42) | 0.02 |
| | Adjusted for all* + seasonal variability | 1.24 (1.06-1.44) | 0.006 |
| **Microvascular** | | | |
| 25OH-D (nmol/L) (per 50 nmol/L decrease) | Adjusted for all* + HbA1c | 1.11 (0.98–1.26) | 0.11 |
| | Adjusted for all* + hs-CRP | 1.14 (1.01-1.29) | 0.04 |
| | Adjusted for all* + physical activity | 1.11 (0.98-1.27) | 0.11 |
| | Adjusted for all* + seasonal variability | 1.12 (0.99-1.27) | 0.08 |

**Chapter 4: DISCUSSION**

**Section 4.1: Previous  Vitamin D Studies**

This large-scale prospective study demonstrates that an inverse association exists between the serum concentration of vitamin D and the risk of future macrovascular and microvascular disease. This association exists independent of factors such as treatment and the duration of diabetes. Patients who were vitamin D deficient at baseline also had a higher frequency of self-reported cardiovascular and microvascular disease events before the study. Patients with a higher baseline of serum vitamin D were associated with statistically significant reductions in the risk of macrovascular disease by 20 % and microvascular disease by 18% per 50nmol/L. After adjustment for some classic risk vascular predictors, including HbA1C, these results became 23 % and 11%, respectively, and lost significance.

**Section 4.1.1: Vitamin D in FIELD**

The FIELD results are in keeping with existing cross-sectional [189] and prospective [190] studies showing an inverse relationship between vitamin D and cardiovascular disease. A cross-sectional analysis of data from the third National Health and Nutrition Examination Survey (NHANES) showed a higher prevalence of vitamin D deficiency in patients with cardiovascular disease than patients without (29.3 vs. 21.4 %) [189]. In addition, vitamin D deficient subjects had an odds ratio for pre-existing cardiovascular disease of 1.20 (95 % CI, 1.01-1.36; $P$=0.03). However, the study was limited by its cross-sectional design and lacked a rigorous verification of cardiovascular disease. One nested case-control study, including 454 patients with cardiovascular events and 900 matched controls, found that individuals with vitamin D deficiency at baseline had a 10-year relative risk of nonfatal myocardial infarction or fatal coronary heart disease of 2.09 (95 % CI, 1.24-3.54; $P$=0.02 for trend)[190]. It is not uncommon for case-control studies to overestimate risk, and they are often affected by selection bias and lack of randomisation. In contrast, the FIELD study was a prospective,

randomised study with 1250 cardiovascular events rigorously verified by an independent event review committee.

The results of the FIELD study expand on the existing area of knowledge by showing in a large, prospective cohort that low serum vitamin D is also a risk factor for development of peripheral neuropathy, retinopathy, nephropathy, and amputation. Previous cross-sectional studies reported lower circulating vitamin D levels in diabetic patients with microvascular disease events [191-194], however all of these studies were limited by their size and design. Using the NHANES study as an example, the diagnosis of peripheral neuropathy is based on self-reported symptoms of neuropathy, such as pain, tingling, numbness, or loss of feeling in hands or feet [194]. In contrast, Kaur et al. reported a higher prevalence of vitamin D deficiency in patients with diabetic retinopathy but found no association between vitamin D deficiency and neuropathy or nephropathy [195].

**Section 4.2: Potential Mechanisms of Vitamin D**

The potential mechanisms that explain the relationship between vitamin D deficiency and vascular disease (microvascular and macrovascular disease) include pancreatic β-cell dysfunction, peripheral insulin resistance, chronic inflammation and endothelial dysfunction. In animal models vitamin D deficiency impairs insulin synthesis [196, 197], possibly via a reduced intracellular calcium level [198]. The final statistical models presented in this study were adjusted for indices of glycaemic control, such as glycated haemoglobin, fasting glucose and insulin. With this in mind, β-cell dysfunction and abnormal glucose homoeostasis may explain the increased risk of microvascular disease with decreasing serum levels of vitamin D, but not macrovascular disease. Due to the well established link between immune function and vitamin D [199], chronic inflammation is a recognized mechanism that links vitamin D deficiency with microvascular and macrovascular diseases. In the present study, serum vitamin D remained a significant predictor of vascular events after adjustment for HS-CRP, a biomarker of chronic inflammation. A sedentary lifestyle characterised by insufficient

physical activity is another established risk factor for cardiovascular disease and T2DM. Physical activity may increase vitamin D levels as a consequence of an associated increase in sunlight exposure. Adjustment for activity level affected the inverse association between vitamin D and microvascular, but not macrovascular, outcomes. Direct effects of vitamin D on vessel function are another explanation for the relationship between vitamin D and vascular disease [200-206]. However, Wang and DeLuca recently reported that the VDR is not expressed in cardiac myocytes and vascular smooth muscle cells [207], which challenges the hypothesis of direct vascular effects of vitamin D. In fact, it appears that most of the antibodies used in previous studies to identify VDR detect proteins other than VDR, and may have led to false-positive results. In summary, existing data is insufficient to establish the mechanism responsible for the increased vascular risk in vitamin D deficient individuals.

The median serum vitamin D level in the FIELD cohort was 49 nmol/L, indicating that more than 50 % of the participants were vitamin D deficient. Furthermore, 25% had serum vitamin D levels of 36mol/L or less. This supports previous reports showing a high prevalence of vitamin D deficiency in the general population and in elderly individuals worldwide [208-210]. Considering the relationship of vitamin D with common chronic diseases, the high prevalence of vitamin D deficiency has socioeconomic implications, affecting individual patients and health care systems.

The main strengths of the study are its prospective and randomised design, the large number of participants and the high number of verified microvascular and macrovascular events.
Serum 25OH-D was measured with a well characterised latest generation chemiluminescence immunoassay that compares well with higher-order methods, such as liquid chromatography tandem mass spectrometry. This is important as the performance of vitamin D assays varies substantially [186, 211].

Our study also has some limitations. Fenofibrate treatment is known to reduce cardiovascular and microvascular events and may have biased our results. However, the effect of serum vitamin D on microvascular and macrovascular risk was similar in the placebo and the fenofibrate groups, and no heterogeneity was detected. Vitamin D deficient individuals had a higher prevalence of microvascular and macrovascular disease at baseline. This predisposed them to a higher event rate during the study period, however there was no difference in the association seen among people with and without prior macrovascular and microvascular disease.

These relationships raise the question whether vitamin D supplementation can lower the risk of vascular events. Few prospective intervention studies with sufficient statistical power have addressed this question. In the Womens Health Initiative Study, over 36 000 women were randomised to twice daily 200 IU of vitamin D or placebo. Over an average of 7 years, vitamin D supplementation did not affect cardiovascular or cerebrovascular risk [212]. The recently published RECORD trial also failed to show a reduction in vascular disease mortality [124] in over 5000 patients treated with 800 IU of vitamin D, 1000 mg of calcium carbonate, both, or placebo for 24-62 months. Short-term administration of a supraphysiologic dose of vitamin D does not affect insulin sensitivity [213]. In contrast, supplementation with 2000 IU of cholecalciferol for 16 weeks has been reported to improve ß-cell function [214]. Together with our results, the negative outcomes of vitamin D supplementation studies highlight the importance of endogenous vitamin D synthesis and the need for further research into the role of vitamin D deficiency.

**Section 4.3: Osteocalcin**

This large scale prospective study demonstrated an association between the serum concentration of OCN and future microvascular disease, which was independent of treatment, gender, age, weight and renal function. Higher baseline serum OCN was associated with a reduction in the risk of microvascular disease by 8 % per 5 ng/mL. Our results support the concept of a coupled interaction between bone and energy metabolism and are in keeping with recent animal studies showing a regulatory role of OCN in glucose metabolism [136-138]. After adjustment for parameters of glucose homoeostasis the relationship between OCN and microvascular events was no longer significant, suggesting that the association between osteocalcin and microvascular disease is mainly explained by the interaction between OCN and glucose metabolism. Undercarboxylated OCN was not associated with future vascular disease.

Our results expand existing knowledge by providing human evidence that serum OCN is related to clinical outcomes, such as nephropathy, neuropathy, retinopathy and amputations, in patients with diabetes mellitus. Furthermore, the data suggest a possible regulatory role of OCN in glucose homoeostasis. This is supported by the significant correlations between OCN and various indices of glucose homoeostasis, such as glycated haemoglobin, fasting glucose and insulin. In addition, adjustment for parameters of glucose homoeostasis and the duration of diabetes eliminated the association between OCN and microvascular disease making direct effects of OCN on small blood vessels unlikely. Previous cross-sectional studies in humans showed that people with low serum OCN have significantly higher blood concentrations of glucose, insulin and glycated haemoglobin than those with high levels of OCN [166-168]. Fernandez-Real, for example, reported significant correlations between OCN and insulin secretion ($r = 0.41$; $p = 0.03$) as well as OCN and the HOMA index of insulin resistance ($r = 0.43$; $p = 0.03$) in non-diabetic patients [167]. In acromegalic patients, where the excess of growth hormone causes abnormalities in glucose metabolism, circulating OCN

levels were found to be four times higher than in matched healthy controls [168]. Furthermore, OCN was the best predictor of insulin resistance and β-cell function.

Together with previous cross-sectional studies, the present results raise the question of whether the association between OCN and microvascular events reflects a mechanistic relationship between the serum OCN concentration and glucose metabolism. Several in-vitro and in-vivo studies support this hypothesis [136-138].  For example, when compared to wild-type animals, OCN knock-out mice have higher blood sugar levels, lower circulating insulin and are insulin resistant. Infusion of OCN can improve glucose handling during a glucose loading test in these animals [138]. Furthermore, in wild-type mice fed for 8 weeks with a high fat diet, the continuous administration of OCN improved the development of obesity and improved glucose and lipid metabolism [136]. A potential mechanism that may explain these effects is the regulation of pancreatic insulin production and secretion by OCN. Pancreatic insulin content, the number of β-cells and β-cell proliferation are all reduced in OCN knock-out mice [138]. Furthermore, OCN can stimulate insulin secretion in cultured islet cells from wild type animals. Ueland et al. recently showed that OCN also stimulates the secretion of insulin in human pancreatic islet cells which supports the relevance of this mechanism in humans. Although existing data strongly suggests direct actions of OCN on pancreatic islet cells, the molecular mechanisms are insufficiently understood. In fact, only recently the pertussis toxin–sensitive G protein–coupled receptor GPRC6A has been identified as a candidate receptor that mediates OCN effects in pancreatic β-cells [158].

Latest evidence suggests that the interaction between bone and glucose metabolism is bidirectional rather than unidirectional. In a series of experiments, Ferron et al. showed that insulin can regulate energy metabolism by a bone resorption-dependant mechanism [137]. Binding of insulin to the insulin receptor on osteoblasts leads to a stimulation of bone-resorbing osteoclasts which results in the release of bioactive OCN from the extracellular bone matrix. According to this study, insulin

signalling in osteoblasts is necessary for whole-body glucose homoeostasis. Although in-vitro and in-vivo studies strongly support a coupled interaction between bone and energy metabolism, human evidence is lacking. So far, the most convincing support for this concept comes from weight loss studies. Fernandez-Real, for example, reported that diet combined with weight-bearing exercise increases circulating OCN levels markedly [167]. Post-intervention OCN was associated with both insulin sensitivity and fasting triglycerides. The change in visceral fat was the best predictor of change in OCN after controlling for age, BMI and change in insulin sensitivity. In another study using a hypocaloric diet weight loss of -22 % was accompanied by a doubling of the serum OCN concentration and a substantial improvement in insulin sensitivity as assessed by the HOMA-IR index [215].

Previous animal studies suggest that the undercarboxylated fraction is mainly responsible for the metabolic effects of OCN [136-138]. However, there is little human evidence that supports a particular metabolic function of ucOCN. In the present study ucOCN did not predict future microvascular disease. Furthermore, correlations between ucOCN and various indices of glucose homoeostasis were very weak with correlation coefficients ranging from -0.05 to 0.08. Our observations are consistent with other studies showing that total circulating OCN correlates better with parameters of glucose homoeostasis than ucOCN. For example, in a cross-sectional study with acromegalic patients and matched healthy controls only total OCN was an independent predictor of insulin resistance (as expressed by the HOMA-IR index). Another study showed that one month of intensified glycaemic control increased total OCN in poorly controlled diabetics by 90% while ucOCN exhibited a non-significant rise of only 17% [170]. More importantly, changes in glucose metabolism correlated better with changes in total OCN than with changes in ucOCN. A two year prospective study in elderly men at high cardiovascular risk also failed to show stronger associations between serum ucOCN and indices of glucose metabolism (e.g. HOMA-IR and HOMA-BCF) when compared to

total serum OCN [216]. Consequently, human studies do not support the theory of ucOCN being the metabolically active fraction of OCN in blood.

Most studies that addressed the role of ucOCN in energy metabolism are limited by the methods that have been used to measure ucOCN. UcOCN can be measured by standard OCN assays preceeded by pretreatment steps using HA or barium sulphate or ucOCN-specific enzyme-linked immunoassays (ELISA) [139, 217]. The pretreatment steps exploit the decreasing affinity of ucOCN for HA or barium sulphate as the number of Gla residues declines. The results obtained with this approach depend on the HA concentration that is used, the quality of manufacture of the HA powder and the performance of the OCN immunoassay [139]. In order to overcome the limitations of HA and barium sulphate pre-treatment, an ucOCN-specific immunoassay has been developed and is distributed by Takara Shuzo Co., Ltd. Japan [218]. However, a head-to-head comparison of this enzyme-linked immunosorbent assay (ELISA) with the HA binding method revealed no consistent association and ucOCN measured by ELISA sometimes exceeded the total OCN level [139]. Recently, Ferron and colleagues developed a triple ELISA for the quantitation of total, undercarboxylated and carboxylated OCN [217]. However, this assay has only been validated in murine samples. Higher order methods, such as liquid chromatography tandem mass spectrometry are at least theoretically able to identify various forms of circulating osteocalcin, but a feasible method that can be used in a complex matrix, such as serum or plasma, has not yet been developed.

**Chapter 5: Conclusion**

**Section 5.1: Vitamin D**

This study provides prospective evidence for serum vitamin D as a risk factor for future vascular events. The effect of vitamin D on macrovascular disease was not explained by associated effects on β-cell function and glucose homeostasis, whilst the novel relationship between serum vitamin D and microvascular disease was only partly attributable to long-term glycaemic control. The mechanisms underlying these relationships require further research.

**Section 5.2: Osteocalcin**

Serum concentration of OCN is a predictor for future microvascular disease in patients with DM. This association is largely explained by the relationship between OCN and glucose homoeostasis, which supports the concept of OCN as an endocrine regulator glucose homoeostasis. UcOCN was not associated with future microvascular disease and correlations with various parameters of glycaemic control were weak. More studies are needed to explore the role of OCN as a biomarker and potential therapeutic target in metabolic diseases, such as DM.

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