BIOMARKERS OF OXIDATIVE STRESS IN ~~DIABETES~~

DIABETIC POLY NEURO PATHY.

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Abstract

Aims/Hypothesis: Oxidative stress has been implicated in the development of diabetic neuropathy. The objective of this study was to establish if type 1 or type 2 diabetes in the presence of polyneuropathy (PNP) and/or cardiovascular autonomic neuropathy (CAN) is associated with alterations in plasma and urinary measures of oxidative stress.

Studied population: Diabetic patients (n=185; type1: n=61 and type 2: n=124) were recruited. Of these, 60 patients were without PNP and CAN, 103 patients with PNP but without CAN and 22 patients with PNP and CAN. Non-diabetic subjects (n=70) were employed as controls.

Methods: Plasma and urinary 8-epi-PGF$_{2\alpha}$ and its metabolites were measured by gas-chromatography/mass-spectrometry. Plasma total antioxidant capacity (TAC) was assessed by quenching of peroxiynitrite Pholasin® chemiluminescence (peroxynitrite-QPC), quenching of superoxide anion Pholasin® chemiluminescence (superoxide anion-QPC) and quenching of hypochlorous acid Pholasin® chemiluminescence (hypochlorous acid-QPC). Plasma vitamin C was assayed spectrofluorometrically. Plasma vitamin E was determined by HPLC with fluorometric detection.

Results: Type 1 diabetic patients (PNP-/CAN-) had lower TAC (peroxynitrite-QPC, superoxide anion-QPC), vitamin C levels, vitamin E cholesterol/ratios, and 8-epi-PGF$_{2\alpha}$ concentrations than in control subjects. Lower TAC (superoxide anion-QPC) and increased 8-epi-PGF$_{2\alpha}$ concentrations were seen in the presence PNP. The additional presence of CAN was associated with further reductions in TAC (peroxynitrite-QPC and superoxide

anion-QPC) and vitamin E/cholesterol ratios. Type 2 diabetic patients (PNP-/CAN-) exhibited lower TAC (peroxynitrite-QPC, superoxide anion-QPC), and vitamin E cholesterol/ratios compared to control subjects. Lower TAC (peroxynitrite-QPC and superoxide anion-QPC), vitamin C levels and vitamin E/cholesterol ratios, and increased 8-epi-PGF$_{2\alpha}$ concentrations were observed the presence of PNP. Lower TAC (superoxide anion-QPC) was seen in the presence of additional CAN. Correlations occurred between neurological impairment score of the lower limb (NIS-LL) and peroxynitrite-QPC, superoxide anion-QPC as well as vitamin C levels. Multiple regression analysis revealed that peroxynitrite-QPC was independently associated with the neurological impairment score of the lower limb. Urinary 8-epi-PGF$_{2\alpha}$ and its metabolites levels were lower in diabetic patients than in control subjects. However, no firm conclusion could be drawn concerning urinary 8-epi-PGF$_{2\alpha}$ and its metabolites because the results exhibited substantial variability.

Conclusions: This study has revealed that oxidative stress is enhanced in diabetic patients (PNP-/CAN-). Oxidative stress was more pronounced in patients with PNP and that the additional presence of CAN was without influence. Measurement of TAC, as assessed by peroxynitrite-QPC or superoxide anion-QPC, was superior in the terms of simplicity, cost and diagnostic value to nutrient antioxidants and lipid-oxidation products in assessing oxidative stress. These results indicate improved strategies for patient selection for clinical trials involving antioxidants aimed at the prevention or treatment of diabetic neuropathy.

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Abbreviations

AAPH	2'-Azobis—(2-amidinopropane) hydrochloride
ABEL	Analysis by emitting light
ADA	American Diabetic Association
AFGP	1-Alkyl-2-formyl-3, 4-diglycosyl pyrrole
AMP	Adenosine monphosphate
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSTFA	*N,O-bis*(trimethylsilyl)trifluoroacetamide
C$_{18}$	Octadecylsilane
CAN	Cardiovascular autonomic neuropathy
CML	N$^{\epsilon}$-(carboxymethyl)-Lysine
CoQ10	Coenzyme Q10
COX	Cyclooxygenase
CV	Coefficient of variation
DCF-DA	Dichlorofluorescein-diacetate
DIPEA	Diisopropylethylamine
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme-linked assay
FFI	2(2-furoyl)-4(5)-(2-furanyl)-1H-Imidazole
FRAP	Ferric reducing/antioxidant power
G-6-PD	Glucose-6-phosphate dehydrogenase
GAD	Glutamic acid decarboxylase
GC	Gas chromatography

GPx	Glutathione peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
HbA1c	Haemoglobin glycosylated
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HLA	Human leukocyte antigen
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
IAAs	Insulin autoantibodies
ICAs	Islet cell autoantibodies
ID	Internal diameter
IDDM	Insulin dependent diabetes mellitus
LA	Lipoic acid
LDL	Low-density lipoprotein
LF	Low frequency
LOH	Hydroxylated lipid
LOOH	Lipid hydroperoxide
MCR	Mean circulating resultant
MDA	Malonedialdehyde
MNCV	Slowing motor nerve conduction velocity
MRC	Mean circulating resultant
MS	Mass spectrometry
NAD$^+$	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)

NADP⁺	Nicotinamide adenine dinucleotide phosphate (oxidesed)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NDDG	National diabetes data group
NH₂	Aminopropyl
NICI	Negative ion chemical ionisation
NIDDM	Non-insulin dependent diabetes mellitus
NIS-LL	Neuropathy impairment score of lower limb
NK-κB	Nuclear redox-sensitive transcription factor kappa B
NSS	Neuropathy symptom scores
NSS	Neuropathy symptom scores
O₂·⁻	Superoxide anion
ONOO⁻	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
PFB	Pentafluorobenzyl
PFB-Br	Pentafluorobenzyl-bromide
PG	Prostaglandin
PNP	Polyneuropathy
PUFAs	Polyunsaturated fatty acids
QPC	Quenching of Pholasin® chemiluminscence
RIA	Radioimmunoassay
ROS	Reactive oxygen species
Si	Silica
SIM	Selected ion monitoring
SIN-1	3-Morpholino-sydnonimine
SNCV	Sensory nerve conduction velocity

SOD	Superoxide dismutase
SPE	Solid-phase extraction
TAC	Total antioxidant capacity
TBARs	Thiobarbituric acid reactive substances
TEAC	Total equivalent antioxidant capacity
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TPT	Thermal perception threshold
TRAP	Total radical trapping parameter
UV	Ultraviolet
VLDL	Very low-density lipoprotein
VLF	Very low frequency
VPT	Vibration perception threshold
VPT	Vibration perception threshold
WHO	World Health Organisation

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chemilumenescence (superoxide anion-QPC) and total
neurological impairment score-lower limb (NIS-LL).

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Chapter 1

Introduction

1.1. Diabetes mellitus

Diabetes mellitus is a complex disease characterised by chronic hyperglycaemia, mainly due to insulin deficiency, or cellular resistance to the actions of insulin or a combination of both. Genetic and environmental factors are also implicated in its development. Two conventions for the diagnosis of diabetes mellitus are commonly used: the criteria of the World Health Organisation (WHO) (World Health Organisation, 1985) and the criteria of the National Diabetes Data Group (NDDG) and, later, Report of Expert Committee on the Diagnosis and Classification of Diabetes Mellitus of the American Diabetes Association (ADA) (National Diabetes Data group, 1979; Report of Expert Committee, 2003). According to the criteria established by the ADA an impaired fasting glucose is defined as a fasting plasma glucose concentration of 110-125 mg/dL or 6.1-6.9 mmol/L i.e. a concentration intermediate between that diagnostic of diabetes mellitus ($\geq$ 126 mg/dL or 7.0 mmol/L) and a normal fasting glucose (110 mg/dL or 6.1 mmol/L). An impaired glucose tolerance according to WHO is diagnosed with an oral glucose tolerance test resulting in a 2-hour postload glucose 140-199 mg/dL (7.8-11.0 mmol/L). Therefore, the criteria published by the ADA are considered more accurate regarding the diagnosis of diabetes in younger and more obese subjects than that of the WHO.

1.1.1. Classification

According to the Expert Committee of the ADA, diabetes mellitus can be subdivided into primary and secondary diabetes (Report of Expert Committee, 2003). Primary diabetes comprises the most common forms of diabetes i.e. type 1 or insulin-dependent diabetes mellitus (IDDM) and type 2 or non-insulin dependent diabetes mellitus (NIDDM). Secondary diabetes includes those forms caused through endocrinopathies (drug or chemical-induced),

infections, uncommon forms of immune-mediated diabetes and gestational diabetes mellitus (Report of Expert Committee, 2003).

Type 1 diabetes results from pancreatic β-cell destruction and exists in two forms, an autoimmune form (Atkinson & Maclaren, 1994; Yki-Jarvinen, 1994) and an idiopathic form (Banerji & Lebovitz, 1989; Knip, 1997). The autoimmune form is a chronic disease characterised by cell-mediated autoimmune destruction of the β-cells of the pancreatic islets. The rates and extents of β-cell destruction can be variable (Bottazzo et al., 1989; Bach, 1994; Graves & Eisenbarth, 1999). Markers of autoimmune destruction include islet cell autoantibodies (ICAs), autoantibodies to insulin (IAAs), autoantibodies to glutamic acid decarboxylase (GAD_{65}) and autoantiobodies to the tyrosine phosphatases (IA2 and IA-2β, respectively) (Baekkeskov et al., 1982; Aanstoot et al., 1994; Lan et al., 1996; Bingley et al., 1997; Betterle et al., 2002). 85-90% of newly diagnosed patients exhibit one and usually more of these autoantibodies (Kolb et al., 1988; Bonifacio & Bingley, 1997; Rowley et al., 1992; Tuomilehto et al., 1994; Maclaren et al., 2003). The aetiological agents responsible for the initiation of the autoimmune process and β-cell destruction have not been established. However, it has been suggested that β-cell damage may be triggered by environmental factors such as viruses (Hyoty & Taylor, 2002). By contrast, idiopathic form is strongly inherited, lacks immunological evidence for β-cell autoimmunity and is not human leukocyte antigen (HLA) associated.

Type 2 diabetes is a heterogeneous disorder characterised by insulin resistance, manifesting itself by impaired glucose tolerance in its pre-clinical stages (Zimmet et al., 1992; Unwin et al., 2002). In contrast to type 1 diabetes, type 2 diabetes does not depend on insulin secretion for the prevention of ketonuria and affected patients are not prone to ketosis (Scott et al.,

1997; Neufeld et al., 1998). However, patients may require insulin for the correction of fasting hyperglycaemia if this cannot be achieved with diet or oral anti-diabetic agents.

A major contributing factor to development of type 2 is the metabolic syndrome which consists of a cluster of inter-related risk factors (Reaven, 1988). The condition is also referred to as Syndrom X or the Insulin Resistance Syndrome. The risk factors present in the metabolic syndrome include central obesity, hypertriglyceridemia, lowered high-density lipoprotein (HDL), fasting hyperglycaemia and hypertension (Garber, 2004; Kutschman et al., 2004). Underlying these factors is a decrease sensitivity of the tissues to insulin (insulin resistance). At the onset of disease, insulin resistance may predominate and/or may be accompanied by relative insulin deficiency (Reaven et al., 1976; Reaven, 1993; Lebovitz, 2001; Hauner, 2002). Insulin resistance leads to decompensated hyperglycaemia and subsequent clinical manifestation of the disease (Reaven, 2003). Type 2 diabetes may remain unrecognised for years due to lack of symptoms (Harris, 1993 & 1996).

Other contributing factors to the development of type 2 diabetes include a family history of diabetes mellitus, age, physical inactivity, Western life style, diabetogenic drugs, endocrinophathies and pregnancy (Coonrod et al., 1994; Nijpels, 1998; Sorensen, 2000; Groop, 2000; Hu et al., 2003). Genetic influences in type 2 diabetes play a stronger role than in type 1 diabetes. Prevalence rates in identical twins for type 2 diabetes vary from 50% to 90%, figures that are much higher than among similarly aged non-identical twins, siblings or other first-degree relatives. Genetic influences in type 1 diabetes show lower prevalence rates, between 35% and 50%.

There are two chief definition of the classification of the metabolic syndrome including: 1) The World Health Organisation (WHO) consultation document for the classification of diabetes and its complications (Alberti & Zimmit, 1998); and 2) The National Cholesterol Education Program (NCEP) Expert Panel (NCEP, 2001). Criteria for clinical identification of the metabolic syndrome are shown in Table 1.

Table 1. Guidelines for the diagnosis of the metabolic syndrome.

Risk factor	Defining level
Abdominal obesity (waist circumference) (cm)	
Men	> 102
Women	> 88
Triglycerides (mmol/l)	≥ 1.7
HDL-cholesterol (mmol/l)	
Men	< 1.0
Women	< 1.3
Fasting blood glucose (mmol/l)	≥ 6.1
Blood pressure (mm Hg)	≥ 130/85

There are currently 151 million people worldwide with clinically diagnosed diabetes (Amos et al., 1997; King et al., 1998; Trevisan et al., 1998; Zimmet et al., 1991 & 2001). Of these, 4.9 million people are estimated to be suffering from type 1 diabetes (Amos et al., 1997). Type 1 diabetes normally manifests itself in childhood and young adulthood and is very common in the Nordic countries, particularly Finland (Muntoni & Muntoni, 1999; Karvonen et al., 2000). Macedonia, by contrast, has the lowest incidence with only 2-3 new cases per 100,0000 children. There are not only geographical, but also ethnic variations

accounting for the incidence of type 1 diabetes (Songini et al., 1993; Kocova et al., 1993; Levy-Marchal et al., 1995 & 2001).

Type 2 diabetes affects 5-7% of the population and is most common in middle and old age, occurring only very occasionally in young adults (Harris, 1995; King et al., 1998). The greatest prevalence of type 2 diabetes is found among Pima Indians and Micronesians living on Nauru Island in the Central Pacific (Lillioja, 1996; Trevisan et al., 1998). Again, as with type 1 diabetes, there are considerable geographical, ethnic and socio-economical variations accounting for the prevalence of type 2 diabetes (Ahren et al., 1984; Papoz et al., 1988; Elbagir et al., 1998), being higher in migrants compared to the native populations of countries (King et al., 1993). The numbers of affected individuals are also greater in populations living in urban areas. According to WHO predictions, the prevalence of diabetes will double over the next 25 years (King et al., 1998; Zimmet et al., 2003) with the greatest increases being seen in Africa, Asia, and the Eastern Mediterranean (Muntoni & Muntoni, 1999; Motala, 2002). The main reasons for the expected rise are increasing in childhood and adult obesity, decreasing physical activity and increased urbanisation and ageing (Laron, 1998; Fagot-campagna, 2000; Craig et al., 2000; Rowell et al., 2002; Silink, 2002; Matthews & Wallace, 2002).

1.1.2. Diabetic vascular complications

Diabetes may affect large and small vessels alike (macrovascular and microvascular disease) with different spectrum of clinical manifestations and outcomes. Vascular complications are the major cause of morbidity and mortality in diabetes.

1.1.2.1. Macrovascular disease

Macrovascular disease affects large arterial vessels and is the major cause of myocardial infarction, stroke and peripheral gangrene. It is 2-3 times more common in diabetic patients than in non-diabetics. Macrovascular disease accounts for up to 75% of all deaths in type 2 diabetes (Kereiakes, 1985). The relative risk of macrovascular disease due to diabetes is higher in women than in men and coexisting risk factors such as smoking, hyperlipidaemia and hypertension may be important in determining the course of the disease (Kannel, 2002). Macrovascular disease is largely due to premature atherosclerosis. Initial atherogenic events are endothelium dysfunction and the formation of fatty streaks containing foam cells within the intima of arterial vessels. These lipid-filled cells are derived from smooth muscle cells and macrophages formed from circulating monocytes (Nilsson, 1993; Holvoet, 1998; Ross, 1999).

Oxidation of low-density lipoprotein (LDL) appears to play a major role in the formation of fatty lesions and fibrous plaques in the endothelial lining of blood vessels (Witztum & Steinberg, 1991; Rice-Evans & Bruckdorfer, 1992; Nilsson, 1993; Chisolm & Steinberg, 2000). Oxidation of polyunsaturated fatty acids (PUFAs) present in LDL leads to the release of lipid peroxidation by-products including aldehydes. The latter react with lysine residues on the apo-lipoprotein B of LDL and alter the charge on the molecule. As a result, apo-lipoprotein B is no longer recognised by the cellular LDL receptors. It is, however, recognised by scavenger receptors on macrophages and smooth muscle cells which take up large amounts of lipids. Lipid accumulation, uncontrolled by feedback mechanisms, leads to the formation of foam cells, a hallmark of the developing of atheromatous plaque. In addition, oxidised LDL is cytotoxic leading to the formation of necrotic extra-cellular lipid deposits.

1.1.2.2. Microvascular disease

Diabetic microvascular disease affects small blood vessels, capillaries and precapillary arterioles. Clinical manifestations of diabetic microvascular disease include retinopathy, nephropathy and neuropathy.

1.1.2.2.1. Diabetic retinopathy

Diabetic retinopathy is characterised by retinal vascular damage in the fundus of the eye. Blindness as a result of vitreous haemorrhaging or via fibrosis and retinal detachment can occur. In addition, retinal vessel leakage can occur at any stage of retinopathy producing macular oedema with potentially irreversible loss of central vision. Diabetic retinopathy can be classified into non-proliferative diabetic retinopathy and proliferative diabetic retinopathy (Watkins, 2003; Gardner et al., 2002). The prevalence of retinopathy is highest in early-onset insulin type 1 diabetic patients and lowest in late-onset non-insulin treated diabetics (Porta & Bandello, 2002). It increases with the duration of diabetes and occurs in 75% of patients with diabetes duration for 15 years.

1.1.2.2.2. Diabetic nephropathy

Diabetic nephropathy represents microvascular disease of the glomerulus and is a major cause of end-stage renal disease. Diabetic nephropathy comes in two forms: a diffuse form, and a nodular form representing accelerated disease. Dysfunction of glomerular filtration is manifested by the appearance of microalbuminuria (Raptis & Viberti, 2001). The prevalence of microalbuminuria is as high as 50% in type 1 diabetics at 20 years from diagnosis (Bakris & Sowers, 2002; Tobe et al., 2002). Half of these patients will go on to develop macroalbuminuria whilst a minority develop end-stage renal disease. After 30 years of

diabetes, end-stage renal disease will be present in 20% of type 1 diabetics but only 10% of

type 2 diabetics (Krolewski et al., 1985 & 1996; Ballard et al., 1988; Humphrey et al., 1989).

1.1.2.2.3. Diabetic neuropathy

The term diabetic peripheral neuropathy refers to dysfunction of the peripheral nerves in the

absence of peripheral neuropathy of alternative origin (Greene & Brown, 1987; Vinik et al.,

1992 & 2003; Low, 1994; Sugimoto et al., 2000). Its clinical manifestations include

numbness in the feet, which often results in ulceration and infection, and neuropathic pain.

Autonomic neuropathy also occurs. Thus, diabetic neuropathy is a heterogeneous disorder

that comprises a wide range of abnormalities affecting proximal and distal peripheral sensory

and motor nerves as well as the autonomic nervous system (Thomas, 1973 & 1997; Vinik et

al., 2000). It can be silent or be present with clinical symptoms that are non-specific or subtle

and have a slow progression.

Estimates from clinical studies suggest that diabetic neuropathy occurs in 22-28% of

patients (Pirart et al., 1978; Ziegler, 1994; Tesfaye et al., 1996). The values from population-

based studies vary from 41 to 47% (Dyck et al., 1993, Kumar et al., 1994; Partanen et al.,

1995). Risk factors for the development of diabetic neuropathy are age, height, level of

hyperglycaemia and duration of diabetes (Robinson et al., 1992; Dyck et al., 1997 & 1999;

van de Poll-Franse, 2002; Poncelet, 2003).

Since the term diabetic neuropathy encompasses a range of overlapping clinical syndromes,

a universally accepted system of classification does not exist. The most widely used approach

for the classification of diabetic neuropathy is, however, based on differentiating between

rapidly reversible, peripheral neuropathies and autonomic and focal or multifocal neuropathies (Thomas, 1997; Watkins & Thomas, 1998).

1.1.2.2.3.1. *Hyperglycaemic neuropathy*

The term hyperglycaemic neuropathy defines sensory symptoms present in the lower limbs in newly diagnosed or poorly controlled diabetics that are reversible following the establishment of glycaemic control (Watkins & Thomas, 1998; Thomas, 1999). The syndrome is associated with reduced nerve conduction velocity. The rapidity of the recovery after the establishment of glycaemic control indicates that it is unlikely to depend on nerve fibre regeneration or remyelination (Schneider et al., 1993; Watkins & Thomas, 1998).

1.1.2.2.3.2. *Peripheral neuropathy*

This syndrome represents the most common type of diabetic neuropathy and affects about 20% of diabetic patients (Nathan, 1993; Sima & Cherian, 1997). Risk factors for peripheral neuropathy are duration of diabetes, age, smoking and dyslipidaemia (Thomas, 1999; Christen et al., 1999). The commonest symptoms are numbness and tingling or burning sensations. Diabetic peripheral neuropathy may be asymptomatic despite impairment of conduction velocity, loss of stretch reflexes and the absence of pain and vibratory perception in the feet. When symptomatic it gives rise to symmetric distal neuropathy. Distal sensory neuropathy is usually insidious in its onset and once established is largely irreversible. As neuropathy advances, the sensory loss extends proximally. It can affect the anterior abdominal wall and laterally around the trunk.

1.1.2.2.3.3. *Autonomic neuropathy*

Diabetic autonomic neuropathy can involve any system in the body. It is more common in patients with type 1 diabetes and can occur as early as the first year after diagnosis

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(Airaksinen, 2001; Ziegler, 2001; Schmidt, 2002). Autonomic neuropathy has a gradual onset, progresses slowly and can be classified according to dysfunctions affecting the cardiovascular, gastrointestinal and genitourinary systems (Vinik et al., 2000). Of these, cardiovascular autonomic neuropathy (CAN) is a serious risk factor for sudden death and complications during anaesthesia.

Resting tachycardia and postural hypotension are characteristic of CAN (Vinik et al., 2003). The prevalence of CAN, defined practically in tests as the presence of two abnormal cardiovascular autonomic functions, is around 24% (Ziegler et al., 1992; Stephenson et al., 1996). Prevalence rates of definite and borderline CAN for type 1 diabetics were reported to be 16.8% and 8.5%, respectively whilst the values for type 2 diabetics were 22.2% and 12.2% (Ziegler et al., 1993). Risk factors for CAN are duration of diabetes, degree of glycaemic control and cardiovascular risk factors such as raised lipids, hypertension and/or albuminuria.

1.1.2.2.3.4. *Focal and multifocal neuropathy*

Focal neuropathies are due to vasculitis with subsequent ischaemia and occur predominately in older diabetic patients (Vinik et al., 1992; Dawson, 1993). Nerves affected include the ulnar, median, radial, femoral nerves and lateral cutaneous nerves of the thigh. The third, sixth and seventh cranial nerves are also commonly affected. Symptoms of focal neuropathies include the sudden appearance of wrist or footdrop. The symptoms resolve spontaneously over a period of 6-8 weeks. Focal neuropathy is separated into cranial-, truncal- and proximal motor dysfunctions (Stewart, 1989; Pourmand, 1997; Thomas, 1997; Pestronk, 1998; Pakiam & Parry, 1998, Vinik et al., 2003).

1.1.3. Biochemical changes associated with diabetes

Hyperglycaemia can induce vascular damage via glucose auto-oxidation with subsequent

formation of advanced glycation end products (AGEs), disruption of the polyol pathway,

and ischaemia and reperfusion (Nishikawa et al., 2000; Feldman, 2003). Biochemical events

leading to hyperglycaemia-induced vascular damage is illustrated in Figure 1.

1.1.3.1. Advanced glycation-end products

Proteins become irreversibly modified by glucose leading to tissue browning in the

Maillard reaction. In the reactions, glucose undergoes molecular rearrangement to form an

ene-diol radical anion that is capable of reducing molecular oxygen to a superoxide anion

with the formation of advanced glycation end products (AGEs) (Wolff et al., 1991 & 1993).

Hyperglycaemia may damage cells by AGEs formation on myelin, covalent entrapment of

plasma proteins (albumin and immunoglobulins), cross-linkage of basement membrane

proteins and by glycation of intracellular proteins such as actin and tubulin. (Cullum et al.,

1991; Giardino et al., 1994; Brownlee, 1995; Chibber et al., 1999; McLean, 1997; King,

2001; Sheetz et al., 2002). The interaction between AGEs and their cellular binding

sites/receptors (RAGE) result in the generation of reactive oxygen species (ROS) (Bucala et

al., 1993; Vlassara & Bucala, 1996). Also, AGEs may impair the relaxation of blood vessels

by quenching endogenous nitric oxide.
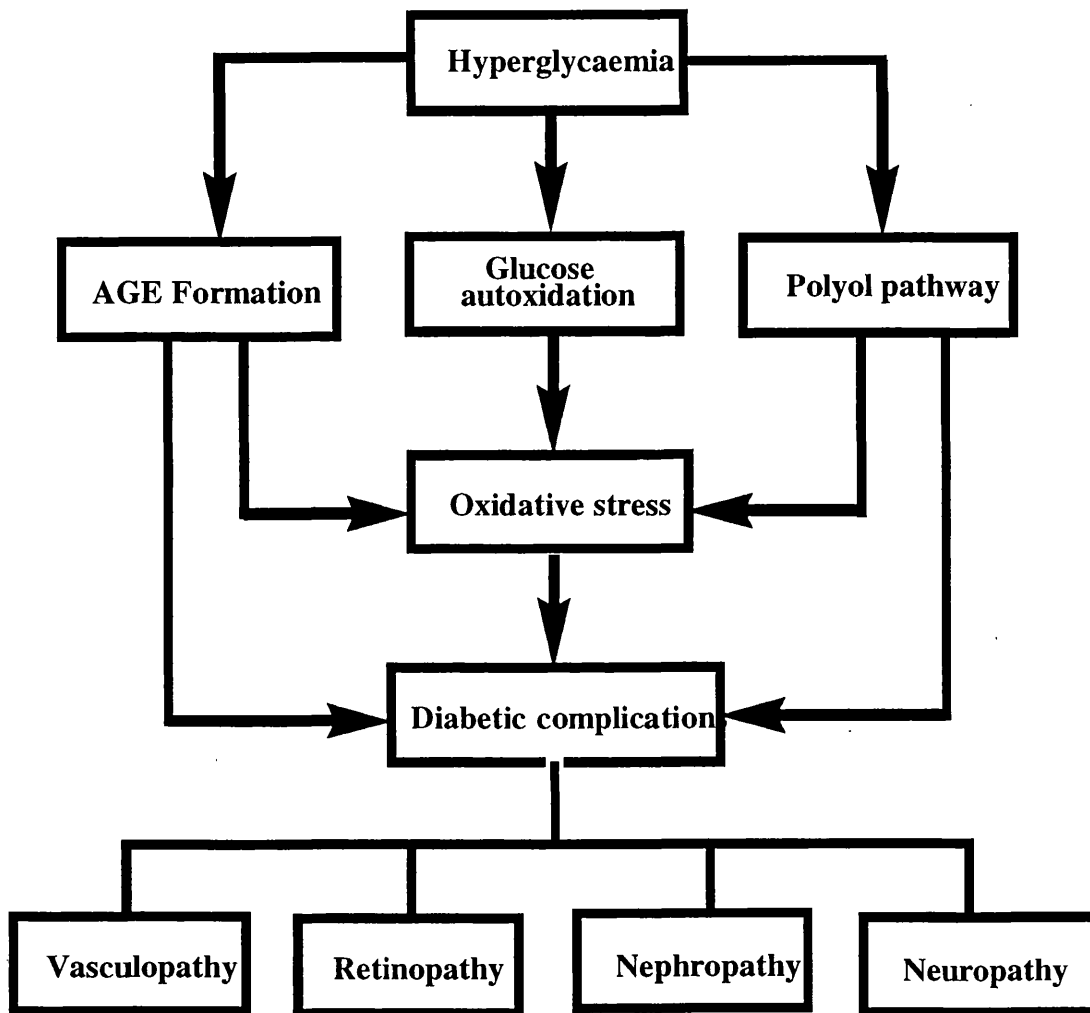
Figure 1: Hyperglycaemia-induced biochemical events leading to diabetic complications.

1.1.3.2. Polyol pathway

The metabolism of glucose by the polylol pathway is achieved by reduction of glucose to sorbitol by aldose reductase (AR), utilising nicotinamide adenine dinucleotide phosphate (NADPH) as the hydrogen donor, followed by oxidation of sorbitol to fructose by the enzyme aldose dehydrogenase which uses NAD as a cofactor. The first reaction favours a decrease in the NADPH/NADP ratio whilst the second reaction uses NAD as the hydrogen acceptor and favours an increase in the NADH /NAD ratio (Figure 2).
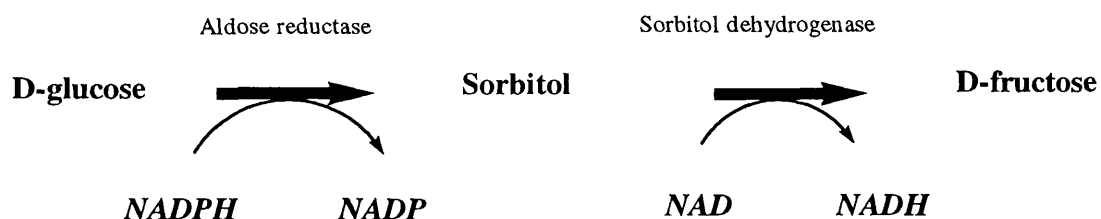
Figure 2: The polyol pathway.

Activation of the polyol pathway during hyperglycaemia enhances the formation of sorbitol and the depletion of NADPH (Gabbay, 1988; Kinoshita et al., 1988; Tesfamariam et al., 1993). A decline in the NADPH/NADP ratio leads to diminished activity of the glutathione-redox-cycle and diminished generation of nitric oxide by endothelial cells. An increase in the NADH/NAD ratio results in enhanced prostaglandin biosynthesis as well as increased superoxide anion production (Williamson et al., 1993a; Tesfamaream, 1994; Obrosova, 2002).

1.1.3.3. Ischaemia and reperfusion

During hyperglycaemia blood glucose levels vary considerably leading to episodes of ischaemia and reperfusion (Williamson et al., 1993b). This process is a considerable source of superoxide anion production through xanthine oxidation. As illustrated in Figure 3, cellular depletion of energy-rich phosphates e.g. adenosine triphosphate (ATP) occurs during ischaemia. Depletion of cellular ATP leads to the accumulation of adenosine monophosphate (AMP), which is subsequently converted through adenine, inosine to hypoxanthine. The latter is utilised by xanthine dehydrogenase and xanthine oxidases (McCord, 1988; Turrens et al., 1991; McCord & Omar, 1993). In healthy tissue, xanthine dehydrogenase accounts for about 90% of total enzyme activity required for the conversion of xanthine to uric acid in cells. This reaction involves the reduction of NAD to NADH and is as follows:

$$\textbf{Xanthine} + \textbf{H}_2\textbf{O} + \textbf{NAD}^+ \rightarrow \textbf{uric acid} + \textbf{NADH} + \textbf{H}^+$$
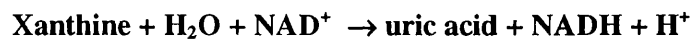
Xanthine oxidase, on the other hand, requires calcium for its activation and utilises xanthine to produce ROS as follows:

$$\textbf{Xanthine} + \textbf{H}_2\textbf{O} + \textbf{O}_2 \rightarrow \textbf{uric acid} + \textbf{O}_2^{\cdot -} + \textbf{2H}^+$$
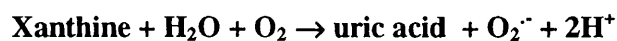
In addition, ischaemia-reperfusion induces the adherence of leukocytes to the endothelium (McCord, 2000; Babior, 2000). The adherent leukocytes migrate through the tissues of the vessel wall and release inflammatory mediators including free radicals. Ischaemia–reperfusion and pseudohypoxia may also produce ROS via enhanced production of prostaglandin H_2 as the enzyme hydroperoxidase uses NADH as a cofactor.
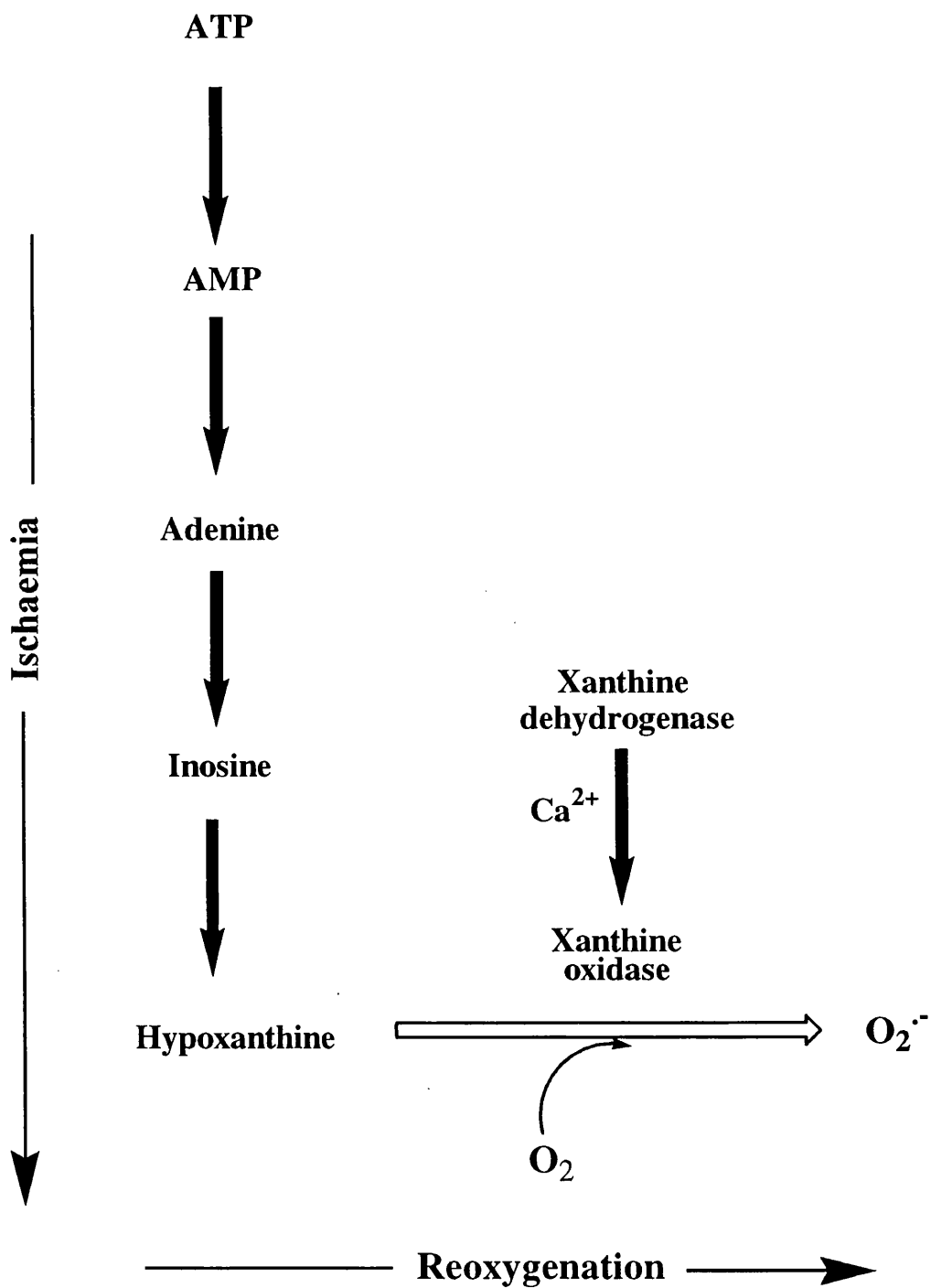
Figure 3: Proposed mechanism for the production of reactive oxygen species during ischaemia-reperfusion.

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Enhanced oxidative stress in turn activates the nuclear redox-sensitive transcription factor kappa B (NK-κB) which up-regulates genes coding for cytokines, adhesion molecules, endothelin-1 and procoagulant tissue factors (Haddad, 2002). These events underlie the development of microvascular diabetic complication (Bierhaus et al., 1996; Tomlinson, 1999; Evans et al., 2003).

1.2. Oxidative stress

The term oxidative stress was first employed as the title of a book edited by Sies entitled "Oxidative stress: Oxidants and antioxidants" (1985). In the introduction to the second edition of this book, oxidative stress was defined as a disturbance in the pro-oxidant/antioxidant balance in favour of the former, which may lead to tissue damage (Sies, 1991). Halliwell (2000) has defined oxidative stress as a serious imbalance between production of reactive oxygen species/ reactive nitrogen species/reactive chlorine species and antioxidant defences. Baynes (2000), on the other hand, has introduced a definition comprising both quantitative aspects of oxidative stress and chemical modification of target molecules. Thus, he defined oxidative stress as a measure of the prevailing levels of ROS in biological systems. Baynes (2000) also emphasized that this definition takes account of the continuous detection of ROS in biological systems determined by the relative rates of their formation and their removal by cellular repair mechanisms.

1.2.1. 1. Reactive oxygen species

Reactive oxygen species (ROS) are atomic or molecular species that contain one or more unpaired electrons. They can act either as a reducing agent by donating electrons or as an oxidising agent by accepting electrons. The most important reactions of ROS in aerobic cells

are molecular oxygen (O_2) and its radical derivatives, including singlet oxygen ($^1O_2$), superoxide anions (O_2^-) and hydroxyl radicals (OH^-) (Halliwell & Gutteridge, 1990).

According to the above definition, O_2 is a bi-radical because it has one unpaired electron in each of its two π^*2p outer anti-binding orbitals (Figure 4). O_2 is not particularly reactive because of the parallel direction of spin of its outer electrons. When O_2 oxidizes another atom or non-radical it has to accept a pair of electrons with opposite parallel spins (Pauli's principle) which fit into the vacant electron orbitals. These reactions occur slowly because electrons are accepted one by one and hence impose a restriction on the oxidation reactions induced by oxygen (Halliwell, 1987). Oxygen reactivity can be increased by either inverting the spin of one of its two outer orbitals or by sequential and univalent reduction to another reactive oxygen species (Halliwell & Gutteridge, 1990).

One possible means of increasing the reactivity of O_2 is to switch the two parallel spinning electrons in the outer anti-binding orbital to the anti-parallel configuration via the input of energy. This process yields singlet oxygen ($^1O_2$) that is highly reactive because spin restriction has been removed. There are two forms of $^1O_2$; delta singlet oxygen ($^1\Delta gO_2$) and sigma singlet oxygen ($^1\Sigma O_2$) (Figure 4). $^1\Delta gO_2$ has both electrons in the anti-parallel configuration in the same orbital. In the case of $^1\Sigma O_2$, the electrons occupy different orbitals. $^1\Sigma O_2$ is very reactive but has a short-life. It rapidly decays after being converted to the $^1\Delta gO_2$ state. $^1\Delta gO_2$ is the most important biologically due to its relatively long half-life (Green & Hill, 1984; Cadenas, 1989; Redmond & Gamlin, 1999).

Superoxide anion is formed by the addition of one electron to ground state oxygen. It is unstable in aqueous solutions, reacting spontaneously with itself to produce hydrogen

peroxide (H_2O_2) and molecular oxygen (Fridovich, 1974, Diguiseppi & Fridovich, 1980; Babior, 1997; Auchere & Rusnak, 2002). The superoxide anion can form a perhydroxyl radical ($HO_2^{\cdot}$), which is more reactive (Bielski et al., 1983; Halliwell & Gutteridge, 1990).

$$O_2 + e^- \rightarrow O_2^{\cdot-}$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$O_2^{\cdot-} + H^+ \rightarrow HO_2^{\cdot}$$
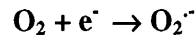
Hydroxyl radical (OH.) is a very reactive chemical species (Halliwell & Gutteridge, 1984; Pryor, 1986). It has a very short half-life and a short radius of action. The main source of OH. in biological systems is the transition metal catalysed Hava-Weiss reaction (Stohs & Bagchi, 1995; Liochev & Fridovich, 1999; Kehrer, 2000; Koppenol, 2001).

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^-$$

	Molecular oxygen	Δ singlet oxygen	Σ singlet oxygen	Superoxide anion
σ* 2p	☐	☐	☐	☐
π* 2p	↑ ↑	↑↓ ☐	↑ ↓	↑↓ ↑
π 2p	↑↓ ↑↓	↑↓ ↑↓	↑↓ ↑↓	↑↓ ↑↓
σ 2p	↑↓	↑↓	↑↓	↑↓
σ* 2s	↑↓	↑↓	↑↓	↑↓
σ 2s	↑↓	↑↓	↑↓	↑↓
σ* 1s	↑↓	↑↓	↑↓	↑↓
σ 1s	↑↓	↑↓	↑↓	↑↓
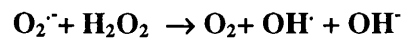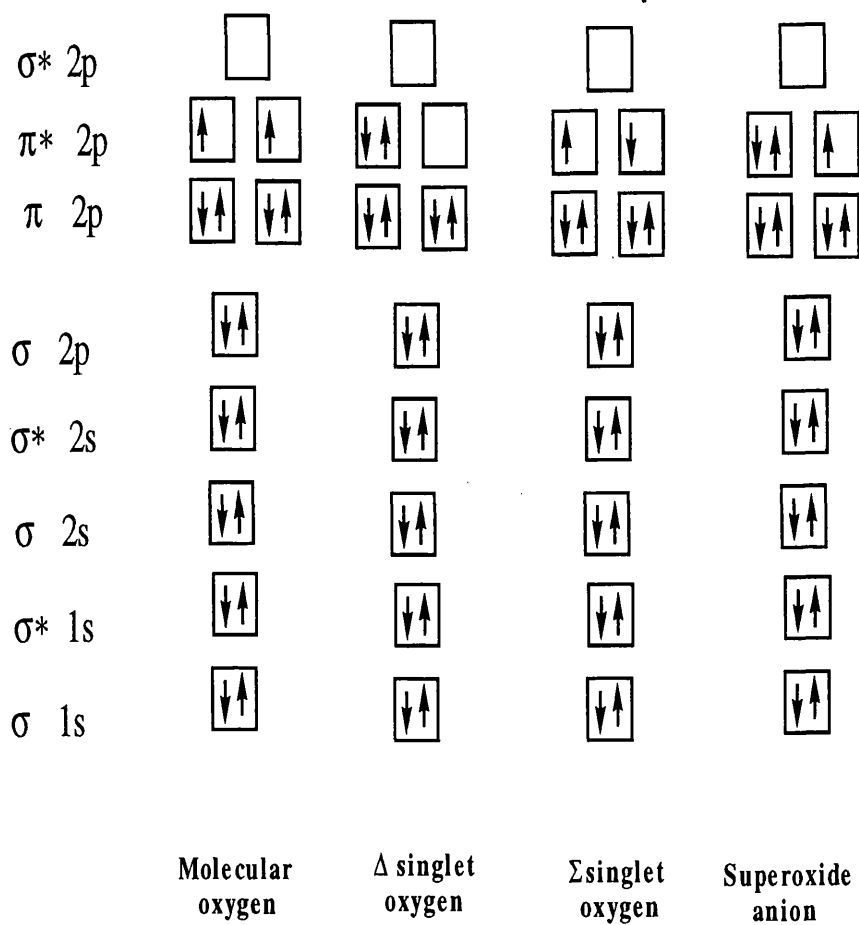
Figure 4: Electronic configuration of ground state oxygen, singlet oxygen and superoxide anion.

1.2.1.2. Reactive nitrogen species

The term reactive nitrogen species (RNS) refers to a variety of products derived from nitric oxide (NO·) (Patel et al., 1999). Nitric oxide, a free radical because the molecule contains an unpaired electron, is a well recognised chemical messenger in mammalian cells (Wink & Mitchell, 1998). In vivo, nitric oxide is synthesised from the amino acid L-arginine in a reaction catalysed by NO synthase (NOS) a family of oxidoreductase (Stuehr, 2004). Nitric oxide itself is a relatively unreactive radical but it is able to form other oxidation and nitration products including nitrosonium ion (NO^+), peroxynitrite ($ONOO^-$) dinitrogen trioxide (N_2O_3) (Beckman & Kappenol, 1996, Squadrito & Pryor, 1998; Augusto et al., 2002). Of these, $ONOO^-$ is the best characterised. $ONOO^-$ is formed by the bi-radical reaction of NO and superoxide anion (O_2^-). The reaction is extremely fast and occurs at a near diffusion-limited rate (Koppenol, 1998). As shown in Figure 5, $ONOO^-$ reacts with tyrosine residues in proteins to form 3-nitrotyrosine (Drew & Leeuwenburgh, 2002). 3-Nitrotyrosine has been shown to be a specific and sensitive marker of nitrosative stress in vivo. It has also been reported that an array of nitroso and/or nitrosylated products are produced during the reaction of $ONOO^-$ with polyunsaturated fatty acids (Rubbo et al., 1994; Ischia et al., 1999; O'Donnell & Freeman, 2001. Coles et al., 2002,; Lim et al., 2002).
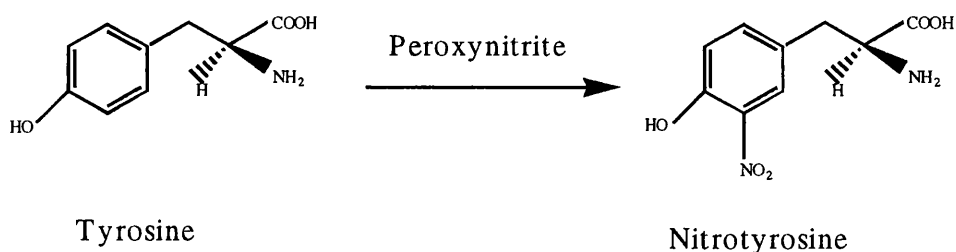
Figure 5: Nitration of L-tyrosine in presence of peroxynitrite.

1.2.1..3. Reactive chlorinated species

The term reactive chlorinated species (RCN) refers to hypochlorous acid (HOCl), hypochlorite (OCl⁻) and nitrylchloride (NO₂Cl). Both HOCl/OCl⁻ are generated by the enzyme myeloperoxidase using hydrogen peroxide and chloride ion as substrates. The main biological target of HOCl is thiol groups and thioethers. The reaction of HOCl with cysteine residues produces oxyacids and cystine whilst that with methionine moieties generates sulphoxides (Carr & Winterbourn, 1997; Winterbourn, 2004). HOCl is also a powerful chlorination agent. It has been shown that it reacts with free amino groups of lysine residues to form chloroamines and also reacts with tyrosine residues yielding 3-chlorotyrosine and 3, 5-dichlorotyrosine (Figure 6). 3-Chlorotyrosine is employed as a marker of HOCl-induced damage (Winterbourn & Kettle, 2000).

In addition, it has been demonstrated that HOCl reacts with unsaturated lipids such as cholesterol and oleic acid to form chlorinated products collectively referred to as chlorohydrin (Winterbourn et al., 1992; Heinecke et al., 1994). Recently, it has been shown that chlorohydrins are formed during the reaction of myeloperoxidase with unsaturated phosphatidylcholine (Arnhold et al., 2001; Panasenko et al., 2003). Nitrylchloride is a chlorinating and nitrating agent that can be formed by the reaction of HOCl with nitrite ion (NO₂⁻) (Bauer, 2000; Halliwell & Whiteman, 2004).
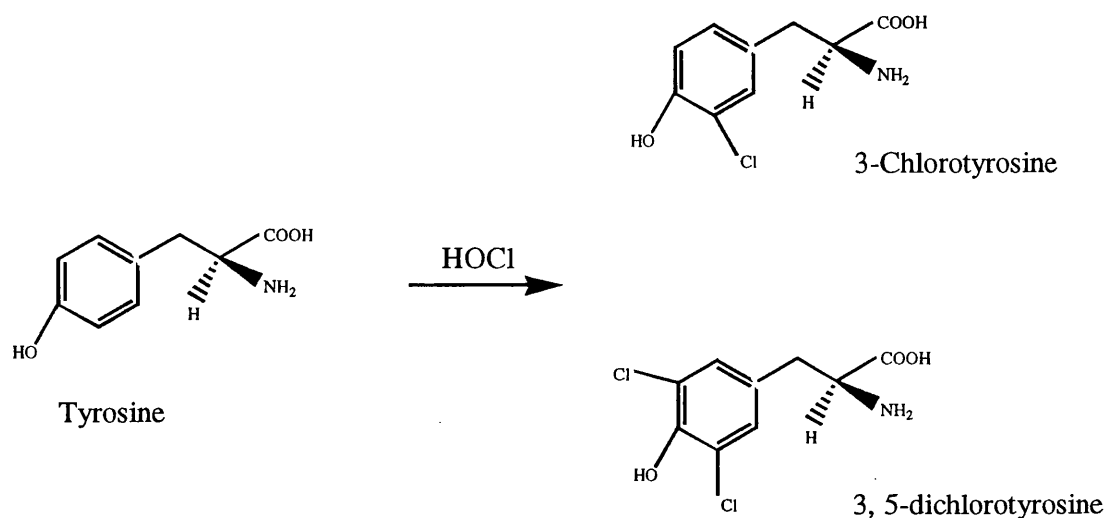
Figure 6: Interaction of L-tyrosine with hypochlorous acid.

1.2.2. Sources of reactive oxygen species
1.2.2.1. Endogenous

Small amounts of ROS are normally produced during biochemical processes in the human body. ROS are produced as part of the body's defence system against invading organisms, for example, during the respiratory burst in phagocytic cells (McCord & Omar, 1993; Darley-Usmar & Halliwell, 1996; Knight, 1995; Harrison, 1997). Endothelial cells and vascular smooth muscle cells are also capable of producing ROS from a variety of enzymatic reactions i.e. cyclooxygenases, lipooxygneases and NADPH-oxidases (Mottley et al., 1982; Riendeau et al., 1982; Channon & Guzik, 2002; Droge, 2002). Auto-oxidation of small soluble molecules such as catecholamines, flavins, tetrahydroproteins and thiols in the cellular cytoplasm may produce ROS by concomitant reduction of molecular oxygen. Some cytoplasmic enzymes generate ROS as by-products of their catalytic actions. Of these enzymes, xanthine oxidase and aldehyde dehydrogenase are the most important examples (Coughlan & Rajagopalan, 1980; Cadenas & Sies, 1985; Bray et al., 1996; Harrison, 2002;

Meneshian & Bulkley, 2002; Garattini et al., 2003). Peroxisomes have a great capacity for generating H_2O_2 because they contain high activities of oxidase enzymes (Reddy et al., 1982; Del Rio et al., 1992). The mitochondrial electron transport chain is another source of ROS generated by biological systems (Naqui et al., 1986; Beyer, 1990; James & Murphy, 2002; Wei & Lee, 2002; Genova et al., 2003). Singlet oxygen can be produced in biological systems via the reaction between hydrogen peroxide and hypochlorite resulting from phagocyte activation (Steinbeck et al., 1993; Uetrecht, 1995; Devasagayam & Kamat, 2002; Tarr & Valenzeno, 2003). ROS is also produced by microsomal cytochrome P-450 during processing of fatty acids and xenobiotics (Hornsby, 1986; Naqui et al., 1986; Bernhardt R, 1996; Goeptar et al., 1995; Massey, 2000).

1.2.2.2. Exogenous

ROS may arise as a result of exposure to environmental factors such as pesticides and pollutions (Duke, 1990; Elstner & Osswald, 1991; Eiserich et al., 1994; Halliwell & Cross, 1994; Kelly et al., 1995). Ionising radiation can convert molecular oxygen into highly reactive singlet oxygen that could cause damage to biomolecules (Kakehashi et al., 1993; Pintar et al., 1994; Kim et al., 2001; Davies, 2003). Many of the damaging effects produced as result of the exposure of tissues to electromagnetic radiation are due to the splitting of water into $OH^.$ and hydrogen ($H^.$) (Cadet et al., 1999; Fink, 2002). Certain drugs and alcohol are metabolised in the body with the generation of ROS through cytochrome P-450 mediated oxidation or when intermediate reaction products escape the normal redox cycle and divert the flow of electrons to oxygen via unknown reactions (Nordmann et al., 1992; Martinez-Cayuela 1995; Coon et al., 1998; Maeda et al., 1999; Fang et al., 2001).

1.2.3. Biological targets for reactive oxygen species

As mentioned above, ROS are essential for the actions of certain enzymes such as cyclooxygenase and host defence mechanisms mediated by neutrophils, macrophages and other cells of the immune system (Warso & Lands, 1983; Moslen, 1994; Darley-Usmar et al., 1995; McCord, 2000; Napoli et al., 2001). Moreover, ROS mediate the activation of transcription factors, gene expression and cell signal transduction (Bierhaus et al., 1996; Sen & Packer, 1996; Schoonbroodt & Piette, 2000; Lum & Roebuck, 2001; Haddad, 2002). ROS generated in the reactions described above can be dealt with by a series of interrelated antioxidant defence systems under normal circumstances, (Darely-Usmar et al., 1995; Bourdon & Blache, 2001). Pathological processes only occur if these antioxidant defence systems are weakened. The targets for ROS action include all the major biomolecules i.e. lipids, proteins, nucleic acid and carbohydrates.

1.2.3.1. Lipids

Lipid peroxidation can occur both enzymatically and non-enzymatically (Cohen, 1994; Spiteller, 1996). Cyclooxygenase, lipooxygenase and cytochrome P-450 are examples of enzymes that catalyse peroxidation of arachidonic acid. The eicosanoids and prostanoids produced during these reactions are both stereo- and regiospecific and are important biological mediators (Maclouf et al., 1989; Capdevila et al., 1992; Oliw, 1994; Brash, 1999; van der Donk et al., 2002).

Non-enzymatic lipid peroxidation is initiated when a reactive chemical species abstracts a hydrogen atom from a methylene moiety of a PUFA (Cosgrove et al., 1987; Halliwell & Chirico, 1993; Porter et al., 1995). Figure 7 illustrates the proposed pathway for the formation of primary and secondary oxidation products during non-enzymatic peroxidation of

arachidonic acid. The greater the number of double bonds in a PUFA molecule the easier it is to remove a hydrogen atom. This explains why PUFAs are prone to auto-oxidation. The hydrogen atom has a single electron and its removal leaves an unpaired electron on the carbon atom. The resulting carbon centred radical is initially stabilised by molecular rearrangement to produce a conjugated diene radical. This in turn reacts with molecular oxygen to form a peroxyl radical that undergoes a rearrangement to form a cyclic endoperoxide radical. The endoperoxide radical is then stabilised by abstracting a hydrogen atom from another molecule such as a PUFA leading to the formation of a prostaglandin (PG) H_2-like compound, which then either decomposes to malonedialdehyde (MDA) or produces a PGF_2-like compound (F_2-isoprostane). As an alternative to endoperoxide radical formation, the peroxyl radical can be converted to a hydroperoxide by abstracting a hydrogen atom from another molecule. Lipid hydroperoxides are labile and are converted to the corresponding alcohols.

Thus, a single initiation reaction can lead to the conversion of PUFAs to a wide-range of primary and secondary oxidation products. The extent to which the propagation chain reaction proceeds depends on many factors including the ratio of lipids to proteins, fatty acid composition, oxygen concentration and the presence of chain-breaking antioxidants (Halliwell & Chirico, 1993; Diplock, 2000).
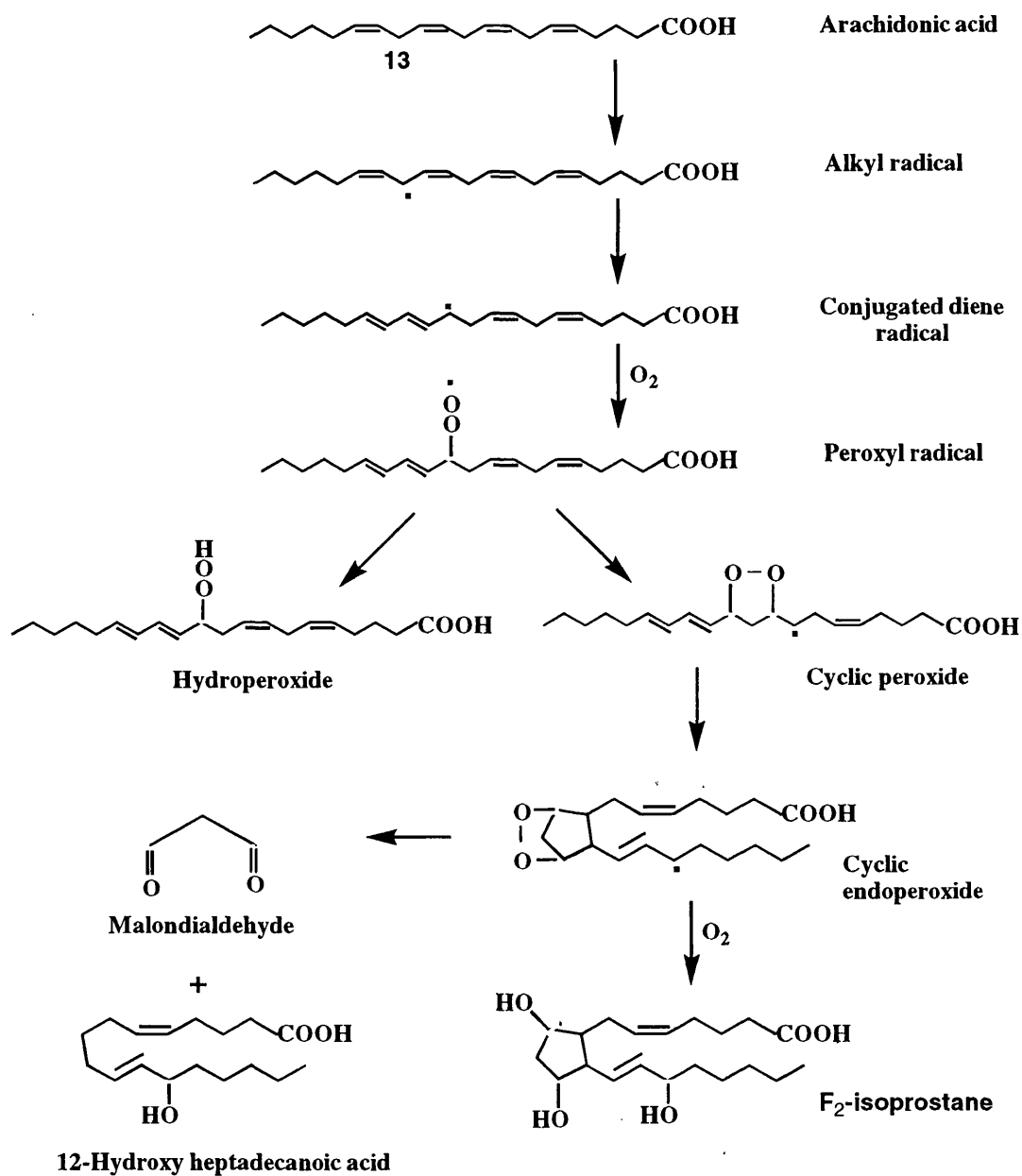
Figure 7: Proposed pathways for the formation of various products during non-enzymatic peroxidation of arachidonic acid.

1.2.3.2. Proteins

ROS attack proteins in a variety of ways. Protein oxidation products and carbonyl derivatives may result from oxidative modification of amino acids containing unsaturated or sulphur groups i.e. tyrosine, phenylalanine, tryptophan, histidine, cysteine and methionine. (Berlett & Stadtman, 1997). The reaction between H_2O_2 and a reduced metal in the metal binding site of an enzyme can lead to the formation of hydroxyl radicals which can attack adjacent amino acid residues (Davies et al., 1999; Davies, 2003).

The oxidation of amino acids can lead to generation of carbonyl compounds produced during the conversion of proline and arginine to glutamic semialdehyde, and histidine to 2-oxo-histidine (Stadtman, 1995; Dean et al., 1997). In addition to the direct oxidation of protein, the introduction of carbonyl groups into protein may occur by reaction of lipid peroxidation products such as 4-hydroxy-2-nonenal with either the ε-amino group of lysine, imidazole moiety of histidine or sulfhydryl group of cysteine residues (Jessup et al., 1986; Kautiainen, 1992). Furthermore, cysteine can be oxidised to the disulfide cystine while methionine is oxidised to methionine sulfoxide (Berlett et al., 1996; Vogt, 1995; Levine et al., 2000; Stadtman et al., 2002).

1.2.3.3. Deoxyribonucleic acid

Hydroxyl radicals (OH$\cdot$) and $^1O_2$ are able to attack deoxyribonucleic acid (DNA), but not $O2^-$ or H_2O_2. Hydroxyl radicals react readily with both purine bases (guanine and adenine) and pyrimidine bases (cytosine, thymine and uracil), while $^1O_2$ only reacts with pyrimidine bases (Piette J, 1991; Halliwell B, 1998; Dizdaroglu et al., 2002; Cooke et al., 2003) (Figure 8).

In the case of guanine, OH˙ can react with C_4, C_5 or C_8 producing the 4-OH˙-, 5-OH˙ - and

8-OH˙ –guanine radicals respectively. These adducts have several possible fates. 8-

hydroxyguanine radicals can be oxidised to form 8-hydroxygaunine (8-OHG) or undergo

reduction followed by ring-opening to yield formamidopyrimidine (FAPy-guanine) (Figure

9). As with guanine base residue, OH˙ can react with C_4, C_5 and C_8 on adenine residues. The

8-OH˙-adenine can then undergo conversion to 8-hydroxyadenine or generate FAPy-adenine

via a ring opening reaction (Halliwell & Aruoma, 1991; Halliwell, 2002).

The action of hydroxyl radicals on cytosine can lead to the formation of several products

including cytosine glycol, 5-hydroxycytosine, 5-OH-6-hydrouracil, 5-OH-uracil, 5,6-OH-

uracil and 5-OH-hydantoin (Dizdaroglu et al., 2002). Thymine can form 5-OH-6-

hydrothymine, cis/trans thymine glycols, 5-OH-5-methylhydantoin, 5,6-dihydrothymine and

5-OH-methyluracil (Halliwell, 2000).
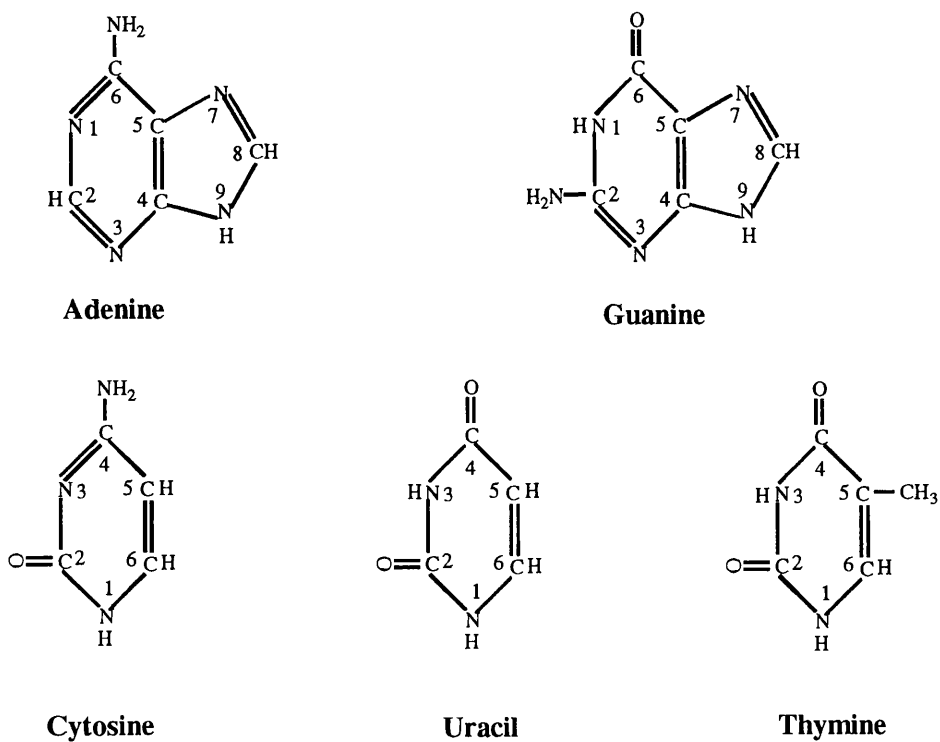
Figure 8: Structure of purine and pyrimidine bases.
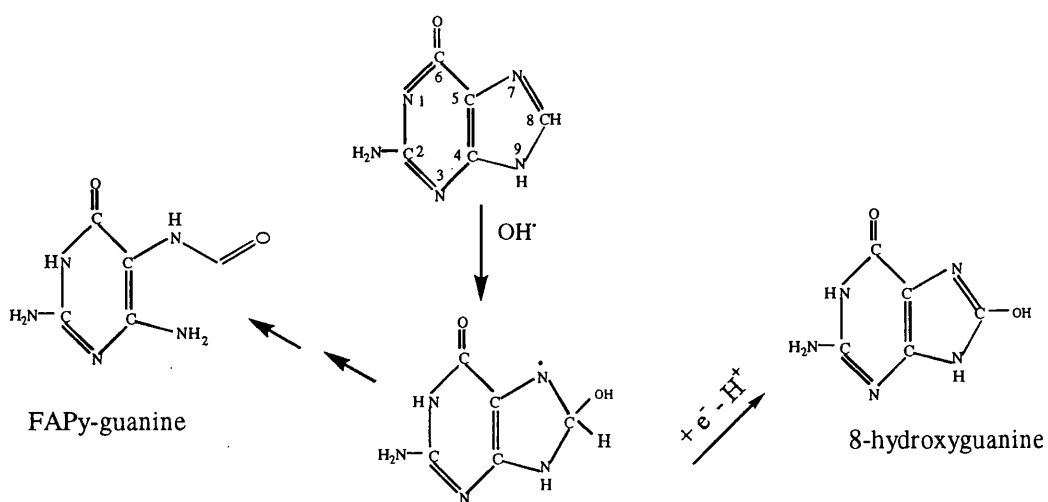
Figure 9: Proposed pathways for the formation of 8-hydroxyguanine and FAPY-guanine during attack by hydroxyl radical on guanine residues.

1.2.3.4. Carbohydrates

Glucose is a reducing sugar and can bind covalently to free amino groups on proteins and aminolipids, such as phosphatidyl ethanolamine, forming an aldimine or a Schiff's base. The Schiff's base may then form more stable Amadori products. Both Schiff's bases and Amadori products are chemically reversible compounds in equilibrium with free glucose. These reactions are referred to as non-enzymatic glycation reactions. The glycation of protein is the first step in the browning process referred to as Maillard browning. Amadori products can undergo further dehydration and condensation to produce advanced glycation end-products (AGEs) (Yaylayan & Huyghues-Despointes, 1994).

Various AGEs can theoretically be formed, including N^ϵ-(carboxymethyl)lysine (CML), pentosidine, 2(2-fyroyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrrole (AFGP), pyrraline, crosslines and imidazolone. Of these, CML and pentosidine are the most frequently analysed AGEs (Bucala et al., 1993; Vlassara & Bucala, 1996; Ulrich & Cerami, 2001). Figure 10 displays the postulated chemistry for the formation of AGEs.

1.3. Antioxidant defences
1.3.1. Antioxidant enzymes

These is a family of metallo-enzymes containing different prosthetic groups and residing in variable intracellular locations that act to remove free radicals or their precursors (Fridovich, 1998; Mates et al., 1999; Nordberg & Arner, 2001).

Protein-NH$_2$ + H-$\overset{\overset{O}{\|}}{C}$-CHOH)$_4$-CH$_2$OH

D-Glucose

$\Updownarrow$ H$_2$O

Protein-HN-CH-(CHOH)$_4$-CH$_2$OH

Schiff's base

$\Updownarrow$ H$^+$

Protein-NH-CH$_2$-$\overset{\overset{O}{\|}}{C}$-(CHOH)$_3$-CH$_2$OH

Amadori-adduct

Enediol

Protein-NH$_2$ + H-$\overset{\overset{O}{\|}}{C}$-$\overset{\overset{O}{\|}}{C}$-(CHOH)$_2$-CH$_2$OH

Ketoaldehydes

Protein-NH-$\underset{OH}{CH}$-$\overset{\overset{O}{\|}}{C}$-(CHOH)$_3$-CH$_2$OH

OH

Ketoaminomethylol

**Protein Crosslinking
AGE formation**
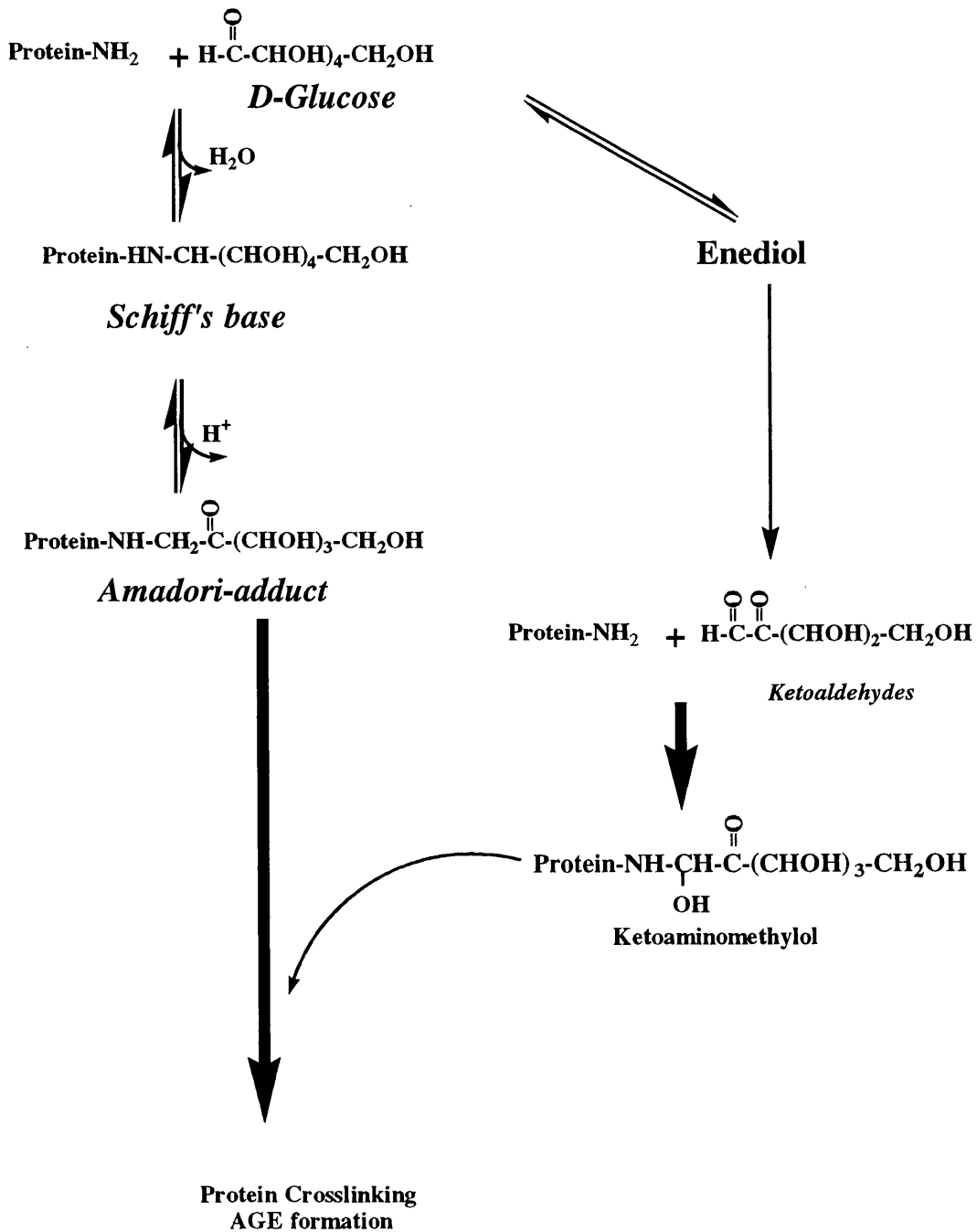
Figure 10: Pathways for the formation of advanced glycosylation end-products (AGEs).

1.3.1.1. Superoxide dismutase

Supeoxide dismutase (SOD) catalyses the conversion of O_2^- to H_2O_2 and O_2 at a rate 4-orders of magnitude higher than that which occurs spontaneously at physiological pH.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
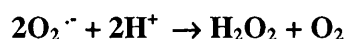
There are three forms of SOD: 1) cytosolic copper/zinc (Cu/Zn)-SOD; 2) mitochondrial manganese (Mn)-SOD and 3) extracellular (EC)-SOD. Cu/Zn-SOD is a homodimer; each subunit containing a Cu and a Zn atom linked to a histamine residue. Cu/Zn-SOD is thought to play a major role as the first line of antioxidant defence (Wiedau-Pazos et al., 1996; McCord & Fridovich, 1969; Ohlemiller et al., 1999). Mn-SOD has four identical subunits containing one manganese atom per subunit. Mn-SOD is thought to protect against oxygen-induced pulmonary injury and adriamycin-induced cardic toxicity (Weisiger & Fridovich, 1973;Yost et al., 1973). EC-SOD is a tetrametric Cu/Zn protein but, unlike Cu/Zn-SOD, is a secretory (Marklund et al., 1982). EC-SOD is present in the interstitial spaces of tissues. It is also found in plasma, lymph and synovial fluid.

1.3.1.2. Catalase

Catalase is responsible for the degradation of hydrogen peroxides before it can damage cellular components (Aebi, 1984).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$
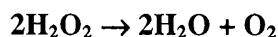
Catalase is a tetrameric enzyme consisting of identical subunits containing a ferriprotoporphyrin group (Reid et al., 1981; Kono & Fridovich, 1983; Kirkman et al., 1984). Catalase is important in dealing with large quantities of H_2O_2 and therefore it is

packaged into peroxisomes along with H_2O_2 producing enzymes (Speranza et al., 1993; Kirkman et al., 1999).

1.3.1.3. Glutathione peroxidases

Glutathione peroxidase (GPx) is responsible for the reduction of lipid hydroperoxides (LOOHs) into alcohols (LOHs). The enzyme requires glutathione as a co-substrate.

$$LOOH + 2GSH \rightarrow LOH + H_2O + GSSG$$

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$
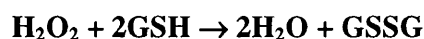
GPx consists of four identical subunits. Each subunit contains a single seleno-cysteine residue, which is essential for catalytic activity (Ursini et al., 1995). Glutathione peroxidase exists as various iso-enzymes (GPx1-4). Each iso-enzyme is responsible for the metabolism of lipid hydroperoxides (LOOHs) in a particular tissue. Phospholipid hydroperoxide glutathione peroxidase (GPx1 and GPx4) is capable of reducing esterified LOOHs that are present in biological membranes and oxidised lipoproteins (Imai et al., 1988; Ursini et al., 1999; Yamamoto, 2000). GPx1 is present in erythrocytes, kidney and liver whilst GPx4 is highly expressed in renal epithelium and testis. GPx2 and GPx3 are located in the gastrointestinal tract (Bjornstedt et al., 1994; Dreher et al., 1997). All GPx iso-enzymes may also be responsible for the dismutation of low intracellular H_2O_2 concentrations.

1.3.2. Low molecular mass compounds
1.3.2.1. Glutathione

Glutathione (γ-glutamylcysteinylglycine, GSH) is a tripeptide, which has many functions in biological systems (Meister & Anderson, 1983; Meister, 1992; Droge & Breitkreutz, 2000). It participates as a co-substrate in the dismutation of both organic and non-organic

hydroperoxides catalysed by glutathione peroxidases. GSH is also involved in the regeneration of α-tocopherol from its radical and in the detoxification of xenobiotics catalysed by glutathione s-transferases (Sies, 1999). Plasma GSH concentration is reported to vary from 0.5-3µM depending on the analytical methods employed (Samiec et al., 1998; Simplicio, 2002).

1.3.2.2. Uric acid

Uric acid is the final product of purine metabolism in primates including humans (Becker, 1993). Uric acid is potent scavenger of $^1O_2$, OH and ·OCl. Its plasma concentration is close to its solubility limit e.g. 200-400 µM (Ames et al., 1981; Kaur & Halliwell, 1996).

1.3.2.3. Ubiquinone

Ubiquinone (2,3-dimethoxy-5-methyl-6-decaprenylbenzoquinone) or coenzyme Q10 (CoQ10) is a lipid soluble quinone. It plays an active role in shuttling electrons between NADH-Q reductase and the cytochrome reductase enzymes of the respiratory chain located in the inner mitochondrial membrane (Crane, 2000; Genova et al., 2003). CoQ10 is involved in the recycling of α-tocopherol from its radical and in preventing lipid peroxidation (Kagan et al., 1990; Ernster & Dallner, 1995; Morre et al., 2000).

1.3.2.4. Lipoic acid

Lipoic acid (LA) or thiotic acid is an essential cofactor (as in its amide forms lipoamide) in the multienzyme complexes that catalyse the decarboxylation of α-keto acids such as pyruvate and α-ketoglutatrate (Marquet et al., 2001). Both the reduced and oxidised forms of lipoic acid are scavengers of ROO, OH, HOCl and ONOO (Kagan et al., 1992; Packer, 1994).

1.3.3. Nutrient antioxidants
1.3.3.1. Vitamin E

The term vitamin E refers to a group of eight naturally occurring lipid-soluble molecules known as the tocopherols (van Acker et al., 1993; Diplock et al., 1998). Dietary vitamin E is absorbed into the lymphatic system from the intestinal tract and enters the blood. It reaches the liver in the form of chylomicron remnants. Vitamin E is incorporated into very low-density lipoprotein (VLDL) in the liver. Redistribution of vitamin E among low-density lipoprotein (LDL) and high-density lipoprotein (HDL) occurs in transfer reactions and during metabolism.

Humans have a dietary requirement for vitamin E because it is not synthesised in the body (Traber & Sies, 1996). Plasma vitamin E concentrations vary from 20 to 60 μM in humans with an adequate vitamin E intake i.e. 15 mg/day (Olmedilla et al., 1997; Pryor, 2000; Polidori et al., 2000 & 2001). Vitamin E is found in vegetable oils, wheat germ, nuts and leafy green vegetables. Figure 11 displays the basic structure of the major isomer i.e. α-tocopherols.
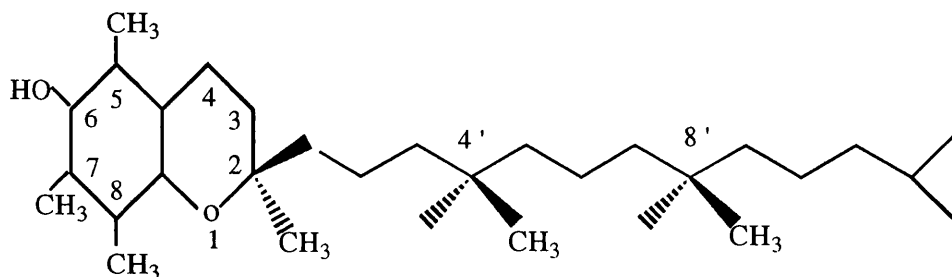
Figure 11: Structure of natural 2'R,4'R,8'R-tocopherol.

1.3.3.2. Vitamin C

The term vitamin C is the generic descriptor for all compounds exhibiting qualitatively the biological activity of ascorbic acid. Therefore, this term refers to both the common biological activity forms i.e. L-ascorbic acid (ascorbic acid) and L-dehydroascorbic acid (dehydroascorbic acid) (Figure 12). Vitamin C is an essential dietary component in humans because it cannot be synthesized in the body (Bode 1997; Wilson, 2002).

Figure 12: Structures of ascorbic acid and dehydroascorbic acid.

Ascorbic acid is the enolic form of an α–ketolactone. The molecular structure contains s two ionisable enolic hydrogen atoms that give the compound its acidic character (pK_{a1} at C_3 = 4.17; pK_{a2} at C_2 =11.57). At low pH levels, the fully protonated form is quite stable and stability decreases as the pH approaches pKa_1. The fully dissociated form of ascorbic acid occurs at pH above 12. Ascorbic acid has strong reducing properties due to its enediol structure, which is conjugated with the carbonyl group in a lactone ring.

Ascorbic acid is essential as cofactor for enzymatic reaction requiring a reduced metal ion i.e. ferrous ion (Fe^{2+}) or coprous ion (Cu^{1+}). Ascorbic acid is required in the procollagen proline hydroxylase reaction which converts proline in procollagen to hydroxyproline in collagen. Ascorbic acid is also essential for the hydroxylation of lysine residues. These reactions ensure the hydroxyproline backbone for normal triple helix formation and the hydroxylysine cross-linkages needed for normal collagen fibre formation (Rose & Bode, 1993; Bode, 1997; Eitenmiller & Landen, 1999). Both prolyl hydroxylase and lysyl hydroxylase are ferric ion (Fe^{3+}) containing enzymes. Ascorbic acid also functions as a cofactor in reaction involving dopamine-β-hydroxylase, a copper-containing enzyme. Dopamine-β-hydroxylase converts dopamine into epinephrine in catecholamine synthesis.

Ascorbic acid can reduce superoxide anion, hydroxyl radicals and reactive oxygen species, which may present in both intracellular and extracellular matrices. Also, it has the power to spare reduced glutathione in the circulation and can restore the antioxidative ability of vitamin E through its recycling via glutathione (Vethanayagam et al., 1999).

Ascorbic acid is easily absorbed from the small intestine in the blood by an active, sodium-dependent process. Intenstinal absorption of ascorbic acid and its entry into cells are facilitated by conversion into dehydroascorbic acid, which is transported across cell membranes more quickly than ascorbic acid (Welch et al., 1995). After entry into the intestinal epithelium or tissue cells, dehydroascrobic acid is readily reduced to ascorbic acid.

Plasma ascorbate concentration ranges from 40-140 μM in humans with an adequate i.e. 100mg/day vitamin C intake (Young, 1996; Frei & Trabe, 2001). Much higher (10 to 100 folds) concentrations have been found in pituitary gland, adrenal glands, leukocytes, eye lens,

brain, liver and kidney gastric juice and lung lining fluid. Plasma ascorbate levels reflect the recent intake of ascorbic acid while the leukocyte content is an index of the body's stores (Rose & Bode, 1993; Bode, 1997; Diplock et. al., 1998). The principle sources of vitamin C are citrus fruits and vegetables.

1.3.4. Metal chelating proteins

Transition metals such as iron and copper are responsible for the generation of OH· by H_2O_2. These metal ions are normally kept safely bound to transport or storage proteins. Free iron concentrations are maintained at low levels through binding to transferrin, lactoferrin or glycoproteins that transport iron in the circulatory system or stored in intracellular ferritin (Aust, 1995; Theil, 2003). Albumin and caeruloplasmin perform similar roles with respect to copper in the circulation (Halliwell, 1988; Linder et al., 1999; Bourdon & Blache, 2001; Hellman & Gitlin, 2002).

1.3.5. Antioxidant network

The antioxidant network is usually initiated by the oxidation of vitamin E by oxidants or lipid free radicals (Figure 13). The vitamin E radical is a slowly reacting reducing agent because the electron is delocalised around the chromanol ring. In another words, the vitamin E radical exists for just sufficient time for it to react with potentially pathological molecules requiring clearance. Abstracting a hydrogen atom from ascorbic acid then regenerates vitamin E. The ascorbate radical can then be regenerated enzymatically with the aid of glutathione. In turn, the oxidised glutathione may be reduced through enzymatic reactions. Other substances that can regenerate vitamin E, though not necessarily enzymatically, include ubiquinol and compounds obtained in the diet like flavonoids, polyphenols and lipoic acid (Packer et al., 1979; McCay, 1985; Guo & Packer, 2000).
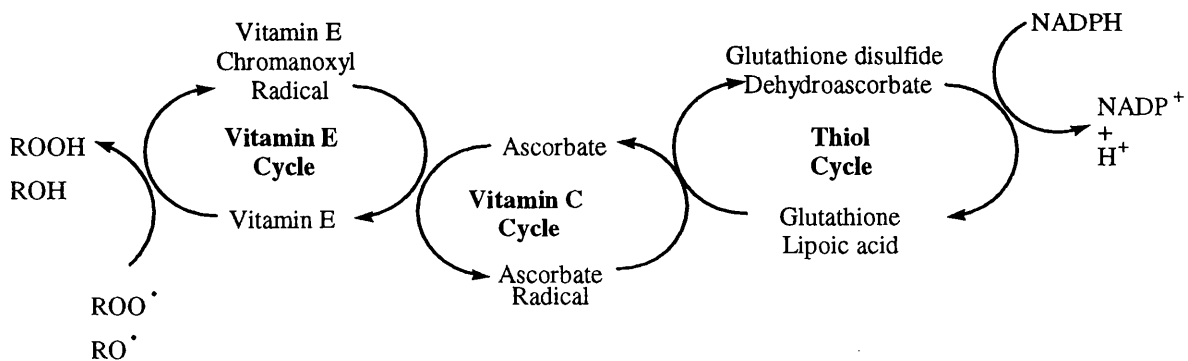
Figure 13: The antioxidant network.

1.4. Methods for the Assessment of oxidative stress
1.4.1. Lipid peroxidation products
1.4.1.1. Conjugated dienes

The conjugated dienes (CD) moiety in a PUFA molecule (Figure 5) has a strong chromophore, which absorbs ultraviolet (UV) light maximally at 233 nm with a molar extinction coefficient of 28000 M^{-1} cm^{-1}. The CD assay is mainly used to analyse liposomes and lipoprotein suspensions (Corongiu & Banni, 1994; Pinchuk & Lichtenberg, 1996; Ahotupa et al., 1998).

1.4.1.2. Lipid Hydroperoxides

Lipid hydroperoxides (LOOHs) are the initial stable product formed during peroxidation of PUFAs (Figure 5). A variety of techniques are currently available for the measurement of individual or total plasma LOOHs. Assessment of individual LOOHs involves total lipid extraction followed by high performance liquid chromatography (HPLC) and the measurement of chemiluminescence produced during decomposition of LOOHs in the presence of luminol or isoluminol (Holley & Cheeseman, 1993; Miyazawa et al., 1994;

Yamamoto et. al., 1998; Handelman, 1999). Total LOOHs are measured using spectrophotometric assays (Hicks & Gebicki, 1979; Cramer et al. 1991; Miller et al., 1991; Nourooz-Zadeh et al., 1994, 1995a, 1997a, 1999 & 2001; Gebicki et al., 1996; Pastorino et al., 1999). Methods for total LOOHs yield values in the µM range whilst HPLC produces values in the nM range.

1.4.1.3. Malondialdehyde

Malondialdehyde (MDA) is a breakdown product of prostaglandin-H_2-like compounds (Figure 5). It accounts for 0.4-0.9% of total hydroperoxides derived from PUFAs with three or more double bonds. The measurement of thiobarbituric acid reactive substances (TBARs) is the most widely used technique for assessing MDA in biological samples. The assay is based upon the reaction of MDA with TBA at high temperature and low pH yielding a TBA-MDA adduct that can be monitored spectrophometrically (532-535 nm) or fluorometrically (Emission 553 nm; Excitation 532 nm) (Gutteridge & Tickner, 1978). A major drawback with the TBA-assay is that TBARS are formed during the decomposition of non-lipid biomolecules such as sialic acid, DNA and carbohydrates, as well as during the enzymatic conversion of prostaglandin H_2 to thromboxane A_2 catalysed by thromboxane synthase. The data can also vary considerably depending on the reaction conditions (Janero, 1990; Esterbauer et al., 1991a). Reported values for TBARS in human plasma range from 0.32 to 35 µM. However, most studies have yielded values for TABRs in human plasma of 2-3 µM.

For a more specific estimation of the TBA-MDA conjugate in plasma, HPLC coupled with fluorometric detection may be used (Conti et al. 1991; Wasowicz et al., 1993; Li & Chow 1994). The HPLC-based MDA assay may improve specificity although it does not adequately

address the question of the source(s) of MDA in biological samples. Typical values for

plasma TBA-MDA conjugates range between 0.3 and 0.6 μM (Halliwell & Chirico, 1993).

1.4.1.4. Other aldehydes

An array of aldehydes, including hydroxyalkenal and alkenals, are formed during the

peroxidation of PUFAs in vivo (Dillard & Tappel, 1989; Esterbauer et al., 1991a; Loidl-

Stahlhofen & Spiteller; 1994; Spiteller et al., 2001). Of these, 4-hydroxynonenal (4-HNE), a

decomposition product of arachidonic acid, has received the most attention. 4-HNE has been

shown to inhibit DNA and RNA synthesis and to be cytotoxic at extremely low

concentrations. The measurement of 4-HNE involves its derivatisation with 2,4-

dinitrophenylhydrazine, lipid extraction, thin layer chromatography and final determination

by HPLC linked to a spectrophotometric detector (Draper et al., 2000). A more sensitive

approach is the use of gas chromatography/mass spectrometry (GC-MS) (Luo et al., 1995;

Liu et al., 1997; Yeo et al., 1999; Spiteller et al., 2001). This procedure involves isolation of

4-HNE derivatives by HPLC or chromatography on a disposable cartridge, conversion of 4-

HNE to a pentafluorobenzyl oxime derivative or the formation of trimethylsilyl (TMS) ether

derivatives, and final analysis by GC-MS using the negative ion chemical ionisation mode.

GC-MS based assays are sensitive and specific but too complex for standard laboratories.

1.4.1.5. Volatile hydrocarbons

Ethane and pentane are produced during peroxidation of PUFAs (n-3 and n-6, respectively).

The process involves the decomposition of fatty-acid hydroperoxides to alkoxyl radicals in

the presence of transition metal ions. The alkoxyl radical then undergoes β–scission to

produce an aldehyde and an ethyl radical or a pentyl radical. Subsequent hydrogen

abstraction yields ethane and pentane. The yield of ethane and pentane per mole of lipid
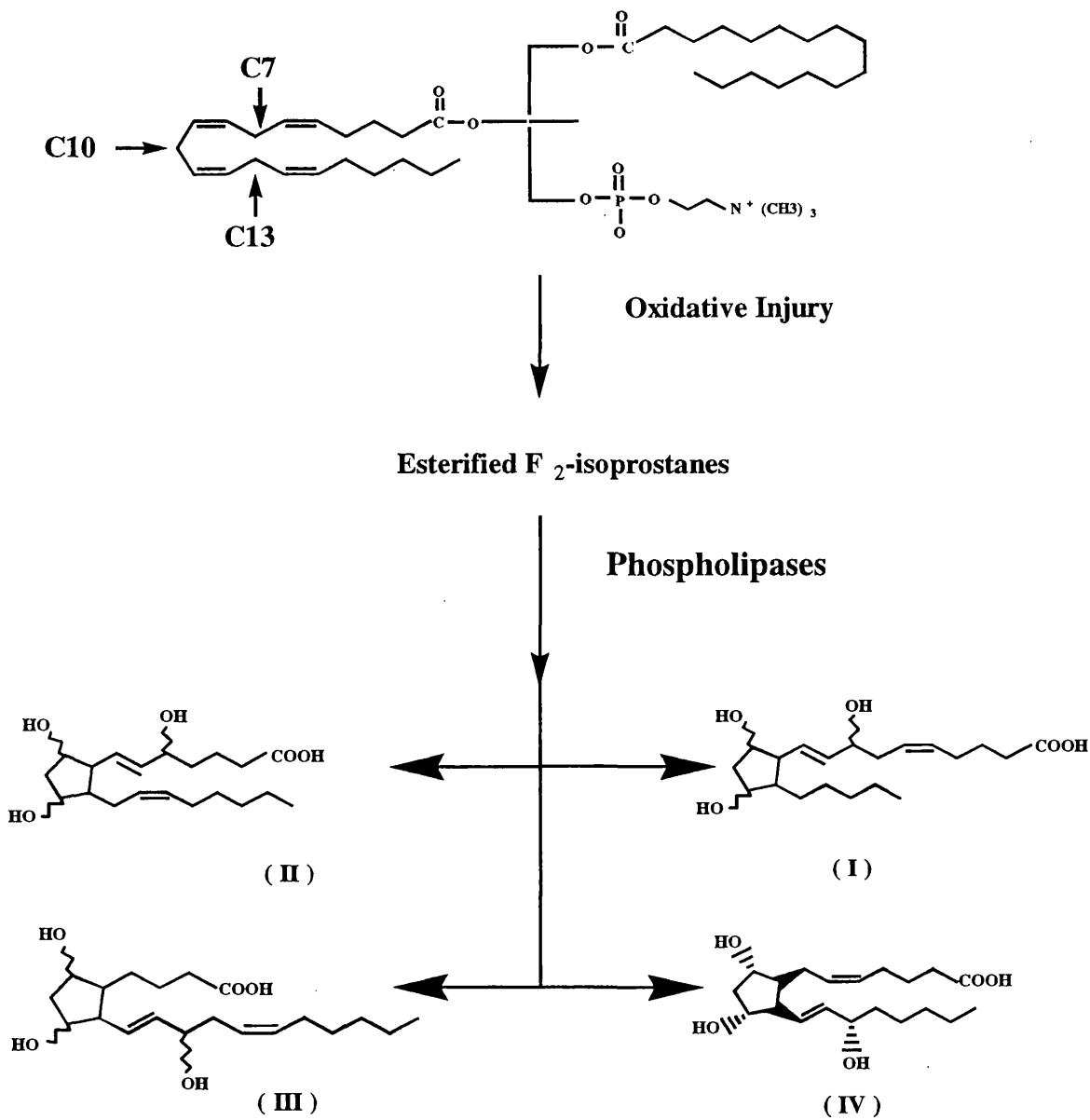
Figure 14: Proposed pathways for the formation of F₂-isoprostanes families (I-IV).

hydroperoxide is estimated to be in the range of 0.5-5%. It is worth noting that metabolic oxidation of ethane and pentane may affect the yield. The measurement of pentane has the unique advantage of being non-invasive and very sensitive. The major drawback of the assay, however, is that a large proportion of exhaled hydrocarbons reflect contamination by inhaled air (Kneepkens et al., 1994; Risby & Sehnert, 1999; Aghdassi & Allard, 2000).

1.4.1.6. F_2-isoprostanes

F_2-isoprostanes are a series of prostaglandin (PG)-like compounds produced by non-enzymatic peroxidation of arachidonic acid via a mechanism independent of the cyclooxygenase (COX) pathway (Morrow et al., 1990ab). The formation of F_2-isoprostanes involves allylic hydrogen abstraction, formation of an arachidonyl radical, insertion of molecular oxygen, endocyclisation to form a bicyclic endoperoxide followed by insertion of a second oxygen molecule (Figure 5). Four possible subfamilies of F_2-isoprostanes are formed as a result of radical attack at positions C_7, C_{10} and C_{13} (Figure 14). Type I F_2-isoprostanes are derived from free radical attack at positions C_7 whilst type II and type III result from free radical attack at position C_{10}. Type IV is derived from free radical attack at position C_{13}. Each subfamily comprises 16 diastereoisomers since the hydroxy group on the cyclopentane ring can be arranged in the 2^3 configuration. In total, sixty-four F_2-isoprostane isomers can be formed during peroxidation of arachidonic acid. Of these 9α-,11α-8-epi-PGF$_2$ (8-epi-PGF$_{2\alpha}$) has received the most attention because it has been shown to produce adverse biological actions (Roberts & Morrow, 1997 & 2000). Circulating F_2-isoprostanes are mainly found esterified to phospholipids and are released by the action of phospholipase A_2.

There are several approaches to the isolation and final determination of F_2-isoprostanes in biological samples. These include chromatographic separation involving solid-phase

extraction (SPE) with or without thin layer chromatography (TLC) and final determination

by gas chromatography-mass spectrometry (GC-MS), radioimmunoassay (RIA) or enzyme

immunoassays (EIA). Gas chromatography-mass spectrometry/negative ion chemical

ionisation (GC-MS/NICI) is the preferred method for the quantification of isoprostanes,

combining the high resolution of GC separation on fused silica capillary columns with the

specificity and sensitivity of mass spectrometry (Moore & Roberts; 1998; Tsikas, 1998;

Nourooz-Zadeh & Smith, 2000).

1.4.2. Oxidatively modified deoxyribonucleic acid

Oxidatively modified nucleobases can be measured by HPLC linked to electron capture

detector (EC) or GC-MS (Loft & Poulsen, 1999). HPLC-EC techniques are the most widely

used for oxidatively modified nucleobase determination because they are selective and

sensitive, and require limited sample preparation (Beckman & Ames, 1999). GC-MS is an

ideal tool for studying DNA because of its ability to provide mass-specific information on

multiple bases in the same sample (Rehaman et al., 2000). Sample preparation is, however,

problematic and the major source of artefact formation due to the presence of a large excess

of undamaged DNA bases compared to damaged DNA bases in cellular DNA.

1.4.3. Total antioxidant capacity

Total antioxidant capacity is a global measure that takes into account all known and

unknown antioxidant activities present in a sample as well as their interactions. Various

assays have been developed for the assessment of plasma antioxidant capacity over the past

three decades. Generally, these can be divided into assays that involve probes that do not act

as prooxidants and those that do. The former include ferric reducing/antioxidant power

(FRAP), total equivalent antioxidant capacity (TEAC) and the current voltammetric assays

(Miller et al., 1993; Benzie & Strain, 1996; Chevion et al., 1997). The latter comprises total radical trapping parameter (TRAP) including luminol, dichlorofluorescin-diacetate (DCFH-DA) and the oxygen radical absorbance capacity (ORAC)-assay (Wayner et al., 1987; Glazer, 1990; Ghiselli et al., 1994; Valkonen & Kuusi, 1997; Tubaro et al., 1998; Alho & Leinonen, 1999). Of these the TRAP and TEAC assays have been the most widely used for the assessment of antioxidant capacity in biological fluids and tissues.

1.5. Evidence for oxidative stress in diabetes
1.5.1. Lipid peroxidation products

Plasma TBARs and/or CD have been found to be increased in diabetics with and without microvascular or macrovascular complications compared to controls, with the largest increases being seen in diabetics with complications (Jennings et al., 1987; Velazquez et al., 1991; MacRury et al., 1993). Griesmacher et al. (1995) reported that plasma TBARS were elevated in type 1 and type 2 diabetic patients compared with controls with the increases being greatest for type 2 diabetics. Armstrong et al. (1996) examined the effect of reducing the intake of energy obtained in the form of dietary fat, on serum lipid peroxidation in newly diagnosed type 2 subjects as well as controls. Serum TBARS were found to be higher in patients than in controls and decreased by about 40% after two months dietary treatment.

Sundaram et al. (1996) measured plasma TBARS in a group of 467 type 2 diabetic patients subjects with and without complications and found that concentrations increased during the 2 years after diagnosis. The changes observed correlated with the duration of disease and increased as complications developed. Plasma LOOH levels were reported to be increased in type 2 diabetic patients but were not influenced by age, sex, smoking, glycaemic control or by the presence of diabetic complications (Nourooz-Zadeh et al., 1995a & 1997a). .

Enhanced formation of plasma 8-epi $PGF_{2\alpha}$ has been observed in type 2 diabetic patients (Gopaul et al., 1995 & 2001; Playford et al., 2003). Davi et al. (1999) detected elevated levels of urinary 8-epi $PGF_{2\alpha}$ in type 1 and type 2 patients compared to controls. No differences, however, were seen between type 2 patients with and without macrovascular complications. It has been shown that improvement in metabolic control, as indicated by a reduction in HbA1c from 9.9% to 7.5%, coincided with a 32% reduction in 8-epi $PGF_{2\alpha}$ levels. Vitamin E supplementation (600 mg daily for 2 weeks) in type 2 was also found to be associated with a 37% reduction in 8-epi $PGF_{2\alpha}$ levels.

1.5.2. Deoxyribonucleic acid oxidation products

Dandona et al. (1996) demonstrated that 8-hydroxydeoxyguanosine (8-OHdG) levels were elevated in mononuclear cells from type 1 and type 2 diabetics compared with controls, the elevation being particularly marked for type 2 diabetics. Rehman et al. (1999) have shown that total DNA-damage was higher in type 2 patients compared with controls. The levels of 8-OHdG, 5-OH uracil, 5-OH methyluracil, thymine glycol, 5-OH methyhydanthoin, 5-OH-hydroxyhydantoin, 5-OH-cytosine, 2-OH-adenine, 8-OH-adenine, FAPy-adenine were also greater. Studies by Leinonen et al. (1997) and Hinokio et al. (1999) have also reported that increased excretion of urinary 8-OHdG occurs in type 2 diabetics.

1.5.3. Antioxidant capacity

Tsai et al (1994) have reported that plasma antioxidant capacity, as assessed by the TRAP assay, was lower in IDDM subjects with poor glycaemic control compared to controls and matched enhanced peroxidation of LDL in diabetics. Haffner et al. (1995) studied the effect of glucose tolerance on the oxidisability of plasma from type 2 diabetic patients, subjects with impaired glucose tolerance (IGT) and individuals with normal glucose tolerance (NGT).

Baseline plasma LOOH levels were similar in all three groups. After incubation with AAPH, a free radical generator, plasma from diabetics had markedly higher plasma LOOH levels compared with IGT and NGT subjects. No differences, however, were found between IGT and NGT subjects regarding the susceptibility of the plasma to AAPH in subjects with NGT or IGT. Santini et al. (1997) studied the relationship between plasma LOOH and antioxidant capacity in type 1 diabetic patients and found that plasma LOOHs were higher in diabetics than in controls. This coincided with decreased antioxidant capacity as measured by the TRAP assay. Increased oxidative stress was influenced by the duration of diabetes, metabolic control and the presence of complications. Others have also reported on decreased plasma total antioxidant capacity in diabetics (Rocic et al., 1997; Aguirre et al, 1998; Ceriello et al., 1997 & 1998; Pinzani et al., 1998; Opara et al. 1999; Valabhji et al., 2001; Pieri et al. 2001; Vessby et al., 2002).

1.5.4. Vitamin E

The published data on plasma α–tocopherol levels in diabetics are contradictory. Several investigators have reported decreased plasma vitamin E in type 1 and type 2 diabetics (Asayama et al., 1993; Sundaram et al., 1996, Ceriello et al. 1997; Olmedilla et al., 1997) By contrast, other investigations have failed to detect differences (Griesmacher et al., 1995; Leonhardt et al., 1996; Osterode et al.,1996; Dyer et al., 1997; Maxwell et al., 1997; VanderJagt et al., 2001). Salonen et al. (1995) have analysed plasma α–tocopherol content in a cohort of 1000 men without clinical evidence of diabetes. They reported a strong independent association between low vitamin E intake before follow up and the risk of developing diabetes. It was suggested that low circulating vitamin E in diabetes could be due to increased consumption or low dietary intake.

1.5.5. Vitamin C

Several investigators have examined plasma ascorbic acid levels in diabetic patients. Som et al. (1981) analysed plasma from control and diabetic subjects for both ascorbic acid and dehydroascorbic acid content. Diabetics had markedly lower levels of plasma ascorbic acid and elevated levels of dehydroascorbic acid, with ascorbic acid being the predominant species in healthy control subjects. These findings were not influenced by age, sex or duration of disease. Moreover, diabetics had a higher turnover of ascorbic acid compared to controls. Reduced plasma vitamin C in diabetes has also been noted by other investigators (Sinclair et al., 1991 & 1994; Sundaram et al., 1996; Dyer et al., 1997; Maxwell et al., 1997a, Armstrong et al., 1996).

1.6. Hypothesis and aims

A growing body of evidence supports the theory that oxidative stress represents a biochemical trigger for neural dysfunction due to reduced endoneural blood flow in diabetic rats. Lipid peroxidation products such as malondialdehyde, 4-hydroxyalkenals and conjugated dienes are elevated in sciatic nerves from diabetic rats (Low & Nickander, 1991; Kihara et al., 1991; Lowitt et al., 1995; Nagamatsu et al., 1996; Nickander et al., 1996; Obrosova et al., 2000). Diminished glutathione and ascorbic acid, and increased oxidised glutathione/ reduced glutathione (GSSG/GSH) as well as dehydroascorbate/ascorbate ratios have been observed in nerves from diabetic animals (Nagamatsu et al., 1995; Obrosova et al., 1999 & 2002; Stevens et al., 2000). Superoxide dismutase (Cu/Zn SOD), calatase, glutathione peroxidase and quinone reductase activities are also reduced in sciatic nerves in diabetic rats (Low & Nickander, 1991; Hermenegildo et al., 1993; Obrosova et al., 2000; Stevens et al., 2000). In addition, nerves of diabetic rats contain lower amounts of vitamin E compared to control animals (Nickander et al., 1994). Treatment of diabetic rats with

insulin or antioxidants is associated with improved neural function (Nagamatsu et al., 1995; Low et al., 1997; Kishi et al., 1999; Stevens et al., 2000; Obrosova et al., 2001; van Dam; 2002).

Despite the aforementioned information on increased oxidative stress in diabetic animal models, data in patients with diabetic neuropathies is lacking. Therefore, it is hypothesised that if oxidative stress is relevant to the pathogenesis of diabetic neuropathies, altered levels of its various markers in plasma and urine should be detected in patients with diabetic neuropathies.

Specific aims:

1) Develop a simpler procedure for the isolation of plasma 8-epi-PGF$_{2\alpha}$.

2) Develop a method for the measurement of urinary 8-epi-PGF$_{2\alpha}$ and its metabolites 2, 3-dinor-8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$.

3) Evaluate Pholasin® as a probe for the assessment of plasma total antioxidant capacity.

4) Investigate the impact of the type of diabetes and its associated neurological complications on plasma total (sum of free and esterified) 8-epi-PGF$_{2\alpha}$, vitamin E, vitamin C and total antioxidant capacity, and urinary 8-epi-PGF$_{2\alpha}$, 2, 3-dinor-8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$.

5) Examine the relationships between plasma and urinary measures of oxidative stress.

Chapter 2

Materials and methods

2.1. Reagents

Prostaglandin F_2 (9,11,15S-trihydroxy-prosta-13E-en-1-oic acid) standards including

9α,11α-PGF$_2$, 9α,11β-PGF$_2$, 9β,11α-PGF$_2$, 9α,11α-8-epi-PGF$_2$, 9α,11α-15R-8-epi-PGF$_2$,

3,3',4,4'-tetradeuterated 9α,11α-PGF$_2$ (PGF$_2$-d$_4$), 3,3',4,4'-tetradeuterated 9α,11α-15S-8-epi

PGF$_2$ (8-epi-PGF$_2$-d$_4$), were obtained from SPI Bio (Cayman Chemical, Ann Arbor, MI, USA).

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), pentafluorobenzyl-bromide (PFB-Br),

diisopropylethylamine (DIPEA), butylated hydroxytoluene (BHT), α-tocopherol, γ-

tocopherol, ascorbic acid, ascorbic oxidase and o-phenylenediamine were purchased from

Sigma-Alderich Chemical Company (Poole, Dorset, U.K). Aminopropyl (NH$_2$), Silica (Si)

and octadecylsilane (C$_{18}$) cartridges (500 mg) were from Waters Corporation (Milford, MA,

USA). 9α,11α-15R-trihydroxy-2, 3-dinor-8-epi-prosta-13E-en-1-oic acid (2, 3-dinor-8-epi

PGF$_2$) 9α,11α-15R-trihydroxy-[5, 6]Δ-dihydro-2, 3-dinor-8-epi-prosta-13E-en-1-oic acid (2, 3-

dinor-5, 6-dihydro-8-epi PGF$_2$) were a gift from Dr Thierry Durand (Department of Pharmacy,

University of Montpellier, Montpellier, France). Pholasin® and Adjuvant K were kindly

provided by Dr Jan Knight (Knight Scientific, Plymouth, UK). All other general-purpose

chemicals and organic solvents were of analytical grade and were from BDH (Poole,

England).

2.2. Sample collection
2.2.1. Blood and plasma preparation

For assessment of plasma nutrient antioxidants and total antioxidant capacity, after a 14 hr

fast, blood was collected by venipuncture into sampling vials (5 ml) containing heparin (10

IU/ml blood) and plasma obtained by centrifugation at 2500xg at room temperature for 10

min. Aliquots (1 ml) of plasma were immediately stored at -85°C until analysis. All analytes

are shown to be stable for at least 4 months at -70 °C (Craft et al., 1988; Lykkesfeldt &

Poulsen, 1995; Miller et al., 1998; Lykkesfeldt et al., 2000).

For assessment of plasma F_2-isoprostanes, after a 14 hr fast, blood was collected by

venipuncture into sampling vials (5 ml) containing ethylenediaminetetraacetic acid (EDTA)

at a final concentration of 0.1% (w/v) and plasma obtained by centrifugation at 2500xg at

room temperature for 10 min. Aliquots (1 ml) of plasma were combined with the chain

breaking antioxidant butylated hydroxytoluene (BHT) at a final concentration of 25 μmol/L

and stored at -85°C until analysis. Plasma F_2-isoprostanes are shown to table for at least 6

month at -70 °C (Morrow et al., 1990a).

2.2.2. Urine

Overnight urine were collected in polyethylene bottles over 12-hrs and aliquots (10 ml)

stored at -85°C until analysis.

2.3. Clinical laboratory measurements

Haemoglobin glycosylated (HbA_{1c}) was measured using the high performance liquid

chromatography (HPLC) technique (Diamat, Bio-Rad, Munich, Germany). Urinary albumin

excretion rate was determined from 12-hour samples using the immuno-nephelometric

technique (Array Protein System, Beckman, Fullerton, CA, USA). Blood glucose was

measured by a hexokinase-based method. Mean fasting blood glucose was computed from at

least 5 values obtained over 2 hours. Uricase based assay was employed for the determination

of plasma uric acid (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). Plasma

and urinary creatinine were measured using a creatininase-based test (Boehringer Mannheim

GmbH; Diagnostica & Biochemicals). Total plasma cholesterol and HDL cholesterol were

measured using the Cholesterol-C high performance CHOD-PAP method (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). Total triglycerides were analysed by a GPO-PAP high-performance enzymatic colorimetric test (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). LDL was calculated from total plasma cholesterol, triglycerides and HDL using the Friedewald formula as follows:

LDL cholesterol = Total cholesterol- (Triglyceride (mmol/l)/2.19)- HDL- cholesterol (mmol/l)

2.4. Measures of oxidative stress
2.4.1. Plasma
2.4.1.1. Vitamin E

Analysis was carried out by HPLC using a Gilson pump model 305 coupled with a Gilson Fluorimeter model 121. Briefly, plasma samples (200 μl) were vortex-mixed with 400 ng of γ-tocopherol in ethanol (500 μl) as the internal standard, water (300 μl) and hexane (1 ml). The samples were centrifuged at 1200xg for 5 min and the upper organic layer was collected into glass vials. Hexane (1ml) was then added and the extraction procedure repeated as described above. The hexane fractions were pooled and evaporated under a stream of nitrogen, the residue then being re-dissolved in acetonitrile (50 μl). Samples (20 μl) were injected onto a Hypersil-ODS column (10 cm x 5 mm, particle size 5 μm, Chrompack, The Netherlands). Tocopherols were separated using acetonitrile/water (80/20; v/v) at a flow rate of 0.7 ml/min and detected at λ_{EX295} nm and λ_{EX340} nm (Nourooz-Zadeh et al., 1995a).

2.4.1.2. Vitamin C

Samples (400 μl) were mixed with 200 μl of metaphosphoric acid and proteins removed by centrifugation at 12000xg for 5 min. For the assessment of total ascorbate (sum of ascorbic acid and dehydroascorbic acid), aliquots (50 μl) of deproteinised plasma were transferred to

microcentrifuge tubes (1.5 ml) and 930 μl of 2 mol/L sodium acetate added. Ten microlitres

of ascorbate oxidase (1000 U/ml) were added and the samples incubated at room temperature

for 30 min. Twenty microliters of o-diphenylenediamine (1mg/ml) were then added and the

samples incubated in dark at room temperature for 30 min. Finally, the samples were

transferred to cuvettes (1 ml) and the fluorescent signal read fluorometrically (λ EX$_{350}$ and

λEM$_{430}$).

For the determination of ascorbic acid in the samples, the protocol was carried out as

described above but in the absence of ascorbate oxidase. The concentrations of ascorbic acid

in the samples were calculated as the differences between dehydroascorbic acid the presence

and absence of ascorbate oxidase

A standard curve was constructed by incubating increasing concentrations of ascorbic acid

(0, 10, 20, 30, 40, 50 60 and 100 μmol/L) with ascorbate oxidase (10 μl) and o-

diphenylenediamine (10 μl) as described above. The concentrations of dehydroascorbate in

the plasma samples were obtained from the standard curve (Obrosova et al., 2000).

2.4.1.3. Total antioxidant capacity
2.4.1.3.1. Quenching of peroxynitrite Pholasin® chemiluminscence

Phosphate buffer (100 μl) (50 mmol/L; pH 7.4) containing Pholasin® (1.7 μg/ml) was

pipetted into microcuvettes (250 μl) and 5 μl plasma or buffer (for basal reading) added. The

reaction was initiated by adding 2 μl of 3-morpholino-sydnonimine HCl (SIN-1) solution (2

mg/ml in water) and the light emission measured at 5 min intervals until the maximum

reading was obtained. Total antioxidant capacity (TAC) was expressed as the time at which

maximum light was emitted. The signal was recorded on a 1250 LKB luminometer.

2.4.1.3.2. Quenching of superoxide anion Pholasin® chemiluminscence

Phosphate buffer (100 μl) (50 mmol/L; pH 7.4) containing Pholasin® (0.5 μg/ml) and adjuvant K (50 μl/ml) were transferred to a microcuvette and plasma or buffer (5 μl) added. Five microlitres of xanthine (50 mmol/L) was then added and the reaction initiated by the addition of 20 μl of xanthine oxidase (0.5 U/ml phosphate buffer). The signal was recorded over a period of 5 min on a 1250 LKB Luminometer. TAC was expressed as the counts (mV) at maximum light emission.

2.4.1.3.3. Quenching of hypochlorous acid Pholasin® chemiluminscence

Phosphate buffer (100 μl) (50 mmol/L; pH 7.4) containing Pholasin® (0.5 μg/ml) and Adjuvant K (50 μl/ml) were transferred to a microcuvette. Plasma or buffer (5 μl) was added and the reaction initiated by adding 40 μl of chloroamine-T (44 mM in water). The light signal generated by the reaction was recorded and TAC expressed as the counts (mV) at maximum light emission. The signal was recorded on a 1250 LKB Luminometer.

2.4.1.4. F$_2$-isoprostanes
2.4.1.4.1. Alkaline hydrolysis

Plasma samples (0.5 ml) were transferred to glass-vials and 4 mol/L aqueous potassium hydroxide (250 μl) added. The samples were incubated for 30 min at 45°C to release esterified lipids and the pH adjusted to 2 by adding 4 mol/L HCl (250 μl). Tetradeutrated prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$-d$_4$) (2.5 ng in 100 μl ethanol) was added as the internal standard and the samples vortex-mixed (Nourooz-Zadeh et al., 1995b; Nourooz-Zadeh, 1999).

2.4.1.4.2. Total lipid extraction

Ethyl acetate (10 ml) and water (1 ml) were added to samples following alkaline hydrolysis. The samples were vortex-mixed for 30 seconds and then centrifuged at 2500xg for 5 min at room temperature.

2.4.1.4.3. Aminopropyl chromatography

Total lipid extracts were applied to aminopropyl (NH_2) cartridges pre-conditioned with hexane (5 ml) and the cartridges washed with ethyl acetate (10 ml). Isoprostanes were eluted by washing the cartridges with 5 ml of ethyl acetate/methanol/acetic acid (10/85/5, v/v/v) (Nourooz-Zadeh et al., 1995b).

2.4.1.4.4. Pentaflurorobenzyl derivatisation

The final extracts from the NH_2 chromatography step were dried under a stream of nitrogen (N_2) at 45°C. Pentafluorobenzyl bromide (PFB-Br)(40 µl, 10% in acetonitrile, v/v) and 20 µl diisopropylethylamine (DIPEA; 10% in acetonitrile, v/v) were added and the samples kept at 45°C for 30 min.

2.4.1.4.5. Trimethylsilyl ether derivatisation

Samples following the PFB derivatisation step were dried under N_2 at 45°C. N,O-*bis*(trimethylsilyl)trifluoroacetamide (50 µl; BSFTA) and DIPEA (5 µl; 10% in acetonitrile, v/v) were added and the samples heated for 30 min 45°C. The samples were then dried under a stream of N_2 at 45°C and the residue redissolved in *iso*-octane (40 µl).

2.4.1.4.6. Gas chromatography-mass spectrometry

The gas chromatographic-mass spectrometric (GC-MS) analysis was carried out on a Hewlett Packard 5890 GC linked to a VG70SEQ MS using the negative ion chemical ionisation (NICI) with ammonia as reagent gas. Separation was carried out on an SPB-1701 column (30m × 0.25mm ID; 0.25 μm film thickness, SUPLECO Dorset, England) using a temperature programme: initial temperature 175°C; initial time: 2 min; rate: 30°C/min; final temperature: 270°C; final time 30 min. Samples (2 μl) were injected into a temperature programmed Gerstel injector. Quantitative analysis was performed using selected ion monitoring (SIM) of the carboxylate anion [M-PBF] or [M-181] at m/z 569, and 573 for 8-epi-PGF$_{2\alpha}$ and PGF$_{2\alpha}$-d$_4$, respectively.

2.4.1.5. Fatty acid analysis

The analysis was carried out on a Fisons gas chromatograph series 8000 linked with a splitless injector and a flame ionisation detector. Plasma samples (200 μl) were transferred into glass vials and heptadecanoic acid (C$_{17:0}$; 10 μg in 250 μl ethanol) added an internal standard. Ethyl acetate (2.5 ml) and water (300 μl) were then added. The samples were vortex mixed for 30 seconds and subsequently centrifuged at 2500xg for 5 min. The upper organic layer was transferred to clean glass vials and the solvent removed under a stream of N$_2$. Fatty acid methyl esters were prepared by adding 14% boron trifluoride in methanol (250 μl) to the dried lipids. The vials were capped and heated at 60°C for 30 min. Water (0.5 ml) and hexane (2 ml) were added and the samples vortex-mixed. After centrifugation at 2500xg for 5 min, the hexane (upper) layers were transferred into new vials and the solvent evaporated under N$_2$ at 45°C. The residue was re-dissolved in hexane (25 μl) of which 1 μl was injected onto a Omegawax® 320 column (0.32 μm X 30 m, film thickness 0.25 μm, SUPLECO Dorset, England) using a temperature gradient of 120-240°C at 10°C / min.

2.4.2. Urine
2.4.2.1. F2-isoprostanes and its metabolites
2.4.2.1.1. Total lipid extraction

Urine samples (2 ml) were transferred to glass vials and acidified by adding 4 mol/L HCl

(50 µl) followed by the addition of 8-epi-PGF$_{2\alpha}$-d$_4$ (2.5 ng in 100 µl ethanol). Ethyl acetate

(10 ml) was added and the samples vortex-mixed for 20 seconds. The samples were

centrifuged at 2500xg for 5 min at room temperature and the organic (upper) phase taken as

the total lipid extract.

2.4.2.1.2. Aminopropyl chromatography

Total lipid extracts were applied to NH$_2$ cartridges and F$_2$-isoprostanes eluted as described

above.

2.4.2.1.3. Pentaflurorobenzyl derivatisation

Final extracts from the NH$_2$ chromatography step were dried and subsequently converted to

PFB-esters as described above.

2.4.2.1.4. Silica chromatography

Following PFB-ester derivatisation, samples were dried under N$_2$ at 45°C. The residues

were re-dissolved in 2 ml of hexane/ethyl acetate (20/80, v/v) and applied to silica (Si)

cartridges which had been pre-washed with hexane (5 ml) and which were subsequently

washed with 3 ml hexane/ethyl acetate (20/80, v/v). Isoprostanes were eluted from the

cartridges by washing with 5 ml of ethyl acetate/methanol (95/5, v/v).

2.4.2.1.5. Trimethylsilyl ether derivatisation

Extracts obtained following Si-chromatography were dried under N$_2$ and were

converted to TMS-ether derivatives as described above.

2.4.2.1.5. Trimethylsilyl ether derivatisation

Extracts obtained following Si-chromatography were dried under N_2 and were converted to TMS-ether derivatives as described above.

2.4.2.1.6. Gas chromatography-mass spectrometry

The instrumentation and conditions for gas chromatographic separation of urinary F_2-isoprostanes were as previously described for plasma isoprostanes. Quantitative analyses were performed using SIM of the carboxylate anion [M-181]⁻ at m/z 541, 543, 569, and 573 for 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$, 2, 3-dinor-8-epi-PGF$_{2\alpha}$, 8-epi-PGF$_{2\alpha}$ and 8-epi-PGF$_{2\alpha}$-d$_4$, respectively.

2.5. Statistical analysis

Continuous data were expressed by the arithmetical mean±SEM. Differences between groups were analyzed using the t-test for two independent samples or the Mann-Whitney U test. Qualitative data were analyzed by the Fisher's exact test. Linear regression analysis was used to study associations between variables. Multiple linear regression analysis using forward stepping with NIS-LL as the dependent variable was performed to examine whether the markers of oxidative stress are independently associated with the severity of polyneuropathy as assessed by the NIS-LL. Independent variables in the original model included age, sex, BMI, diabetes type, diabetes duration, height, HbA$_{1c}$, albuminuria, triglycerides, HDL and LDL cholesterol, retinopathy, and hypertension. Each of the five markers of oxidative stress was added to the original model in five separate models. The level of significance was set at $\alpha=0.05$. All analyses were carried out using the SPSS for Windows (version 11) software package.

Chapter 3

Method Improvement

3.1. Background

A precondition for examining the role of oxidation stress in the development of diabetic complications is the availability of simple, sensitive and reliable assays for the detection of oxidatively modified species and depletion of endogenous antioxidants in biological fluids. The following sections describe improved procedures for the isolation and determination of plasma and urinary F_2-isoprostanes, and methods for assessing plasma antioxidant capacity.

3.2. F_2-isoprostanes
3.2.1. Plasma

There are several approaches to the isolation and/or final determination of plasma F_2-isoprostanes by GC-MS (Morrow et al., 1990ab; Nourooz-Zadeh et al., 1995b & 1999; Mori et al., 1999; Proudfoot et al., 1999; Schwedhelm et al., 2000; Walter et al., 2000). Of these methods, the one combining C_{18}- and NH_2-chromatography has been the procedure of choice in this laboratory because it is less labour intensive than the others and is acceptable with respect to efficiency of extraction (~ 75%) (Nourooz-Zadeh et al., 1995b). Problems may, however, arise with chromatography on C_{18} cartridges e.g. it can be time-consuming and losses may occur depending upon the pH and efficiency of sample loading.

Therefore, it was decided to examine the possibility of replacing the C_{18}-cartridge with a single extraction step of total lipids with ethyl acetate or chloroform/methanol and to simplify the NH_2-chromatographic step by omitting two cartridge washes. The established protocol for the NH_2-chromatograpy procedure involves three sequential washes with ethyl acetate/hexane (30/70, v/v), acetonitrile/water (90/10, v/v) and acetonitrile (10 ml each). PGF_2-like compounds are eluted by washing the cartridges with ethyl acetate/methanol/acetic acid (10/85/5; v/v/v) (Nourooz-Zadeh et al., 1995b).

3.2.1.1. Folch extraction procedure

Folch extraction (chloroform/methanol) is the most commonly used procedure for the partition of total lipids from tissues and biological fluids. No information, however, is available on the recovery of PGF_2-like compounds using the Folch extraction. Therefore, it was decided to evaluate the suitability of this extraction procedure for the partitioning of plasma PGF_2-like compounds.

Plasma samples (0.5 ml) were transferred to glass vials and $PGF_{2\alpha}$ (2.5 ng in 100 μl ethanol) and $[^3H]$-$PGF_{2\alpha}$ as tracer (100 μl containing ~ 10000 cpm) added. Three millilitres of chloroform/methanol (2/1, v/v) followed by water (0.5 ml) were added next and the samples vortex-mixed for 30 seconds. After centrifugation for 5 minutes at 2500xg, the organic layers were transferred to new glass-tubes. Three millilitres of chloroform/methanol (2/1, v/v) were added to the remaining aqueous phases, the extraction procedure repeated as described above and the organic layers pooled. The solvent was dried under a stream of N_2 and the residues re-suspended in ethanol (250 μl) and transferred to beta vials. Scintillation cocktail (3 ml; Pico-Fluor ^{TM} 40) was added and radioactivity monitored using a Beckman LS-6000IC scintillation counter with results expressed as counts per minute (cpm). Radioactivity measurements revealed that only 64 ± 5% (n=3) of the $[^3H]$-$PGF_{2\alpha}$ tracer was recovered from plasma following three sequential extractions with chloroform/methanol.
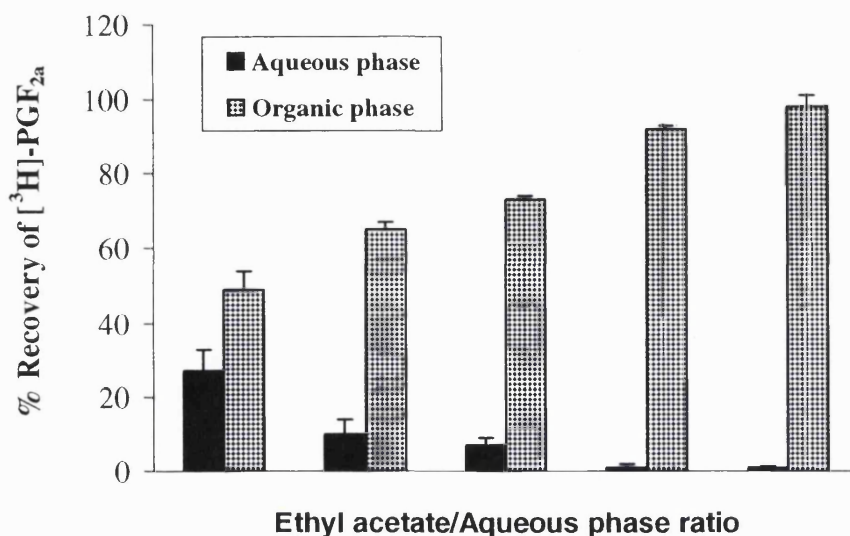
Figure 15: Percentage recovery of $[^{3}H]$-$PGF_{2\alpha}$ added to plasma following ethyl acetate extraction. Plasma (0.5 ml) was spiked with $[^{3}H]$-$PGF_{2\alpha}$ and total lipids were partitioned with ethyl acetate. Aliquots of organic layer were removed and radioactivity counted. Data represent mean of duplicate analysis.

3.2.1.2. Ethyl acetate extraction

Plasma samples (0.5 ml) were transferred to glass vials. $PGF_{2\alpha}$ (2.5 ng in 100 µl ethanol) as carrier and $[^{3}H]$-$PGF_{2\alpha}$ (100 µl containing ~10000 cpm) as tracer were added. Water (1.5 ml) and ethyl acetate (2, 4, 6, 8 or 10 ml) were added and the samples vortex mixed for 30 second. After centrifugation at 2500xg for 5 min, the organic (upper) layers were transferred to glass tubes and the solvent dried under N_2. The residues were re-dissolved in ethanol (250 µl) and subsequently transferred to beta vials. Scintillation cocktail (3 ml) was added and the radioactivity monitored.

Figure 15 shows the percentage recoveries for added radiolabelled $PGF_{2\alpha}$ from plasma following ethyl acetate extraction. The data revealed that the recovery of the tracer was dependent on the ratio of ethyl acetate to aqueous phase. A quantitative extraction (98 ± 3%; n=3) of the tracer added to the plasma was achieved with a ratio of ethyl acetate/aqueous phase of 5/1 (v/v). These data suggest that the ethyl acetate extraction procedure is simpler and more efficient than the Folch extraction for the partitioning of PGF_2-like compounds from biological fluids. The ratio of ethyl acetate/aqueous phase of 5/1 was therefore used in all subsequent experiments.

3.2.1.3. Ethyl acetate extraction / aminopropyl procedure

To evaluate the efficiency of the ethyl acetate/ NH_2-chromatography procedure, plasma (0.5 ml) was spiked with $PGF_{2\alpha}$ (2.5 ng in 100 μl ethanol) as carrier and $[^3H]$-$PGF_{2\alpha}$ (100 μl containing ~10000 cpm) as tracer. Subsequently, water (1.5 ml) and ethyl acetate (10 ml) were added and the samples vortex-mixed for 30 seconds. After centrifugation at 2000xg for 5 min, the organic (upper) layers were applied to NH_2-cartridges, which had been pre-washed with hexane (5 ml). The cartridges were then washed with ethyl acetate (5 ml). Isoprostanes were eluted by washing the cartridges with 5 ml of ethyl acetate/methanol/acetic acid (10/85/5, v/v/v). All fractions following the NH_2-chromatography step were collected and the solvents evaporated under N_2. The residues were re-suspended in ethanol (250 μl), transferred to beta vials and the radioactivity monitored.
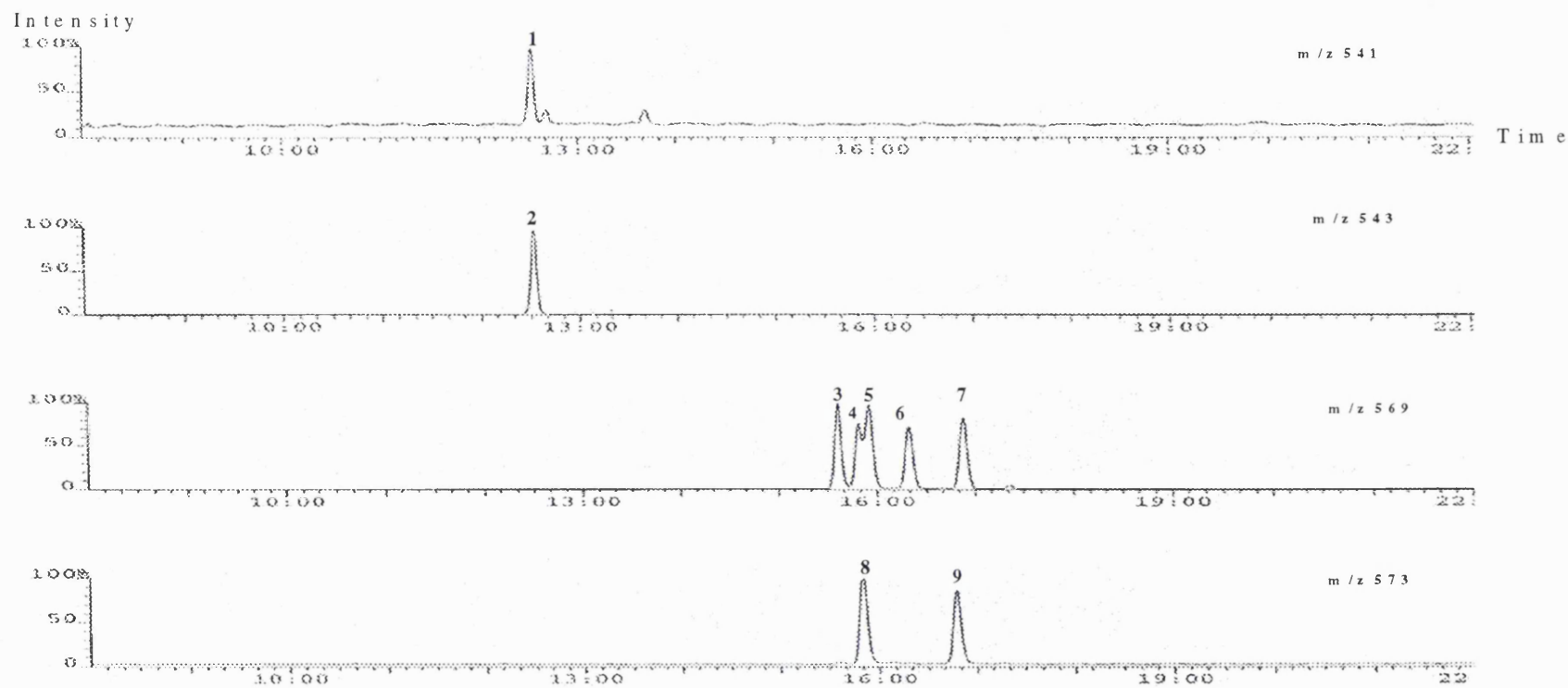
Figure 16: Gas chromatographic separation of PGF$_2$-like compounds. 1) 2,3-dino-5, 6-dihydro-8-epi-PGF$_{2\alpha}$; 2) 2,3-dinor- 8-epi-PGF$_{2\alpha}$; 3) 9β-,11α-PGF$_{2\alpha}$; 4) 9α-,11α-(*15R*)-8-epi-PGF$_{2\alpha}$,; 5) 9α-,11α-(15*S*)-8-epi-PGF$_{2\alpha}$ (8-epi-PGF$_{2\alpha}$); 6) 9α-,11β-PGF$_{2\alpha}$; 7) 9α-,11α-PGF$_{2\alpha}$; 8) tetradeurated 8-epi-PGF$_{2\alpha}$ (8-epi-PGF$_{2\alpha}$-d$_4$) and 9) tetradeutrated 9α-,11α-PGF$_{2\alpha}$ (PGF$_{2\alpha}$-d$_4$). PGF$_2$-like compounds were converted PFB-ester/TMS-ester derivatives and analysed by GC-MS/NICI as described in material and method section. The signals represent selected ion monitoring (SIM) of the carboxylate anion [M-181]$^-$.

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The measurement of [^{3}H]-PGF$_{2\alpha}$ flowing through the cartridges revealed that the tracer was quantitatively retained on the NH$_2$ cartridge during the loading and washing steps. Only traces of radioactivity (<2%) were lost after washing the cartridge with 10 ml of hexane/ethyl acetate (30/70, v/v). The bulk of the [^{3}H]-PGF$_{2\alpha}$ (83 ±11%; n=4) was found to be present in the final ethyl acetate/methanol/acetic acid (10/85/5, v/v/v) eluate. Overall recovery of radiolabel following total lipid extraction and chromatography on NH$_2$-cartridges was 74 ± 4% (n=3). This is in good agreement with the recoveries reported previously for the combined C$_{18}$- and NH$_2$-cartridge chromatographic procedure (Nourooz-Zadeh et al., 1995b).

Figure 16 shows selected ion monitoring (SIM) of the carboxylate anion [M-PFB]$^-$ chromatograms of a mixture PGF$_2$-like compounds. The upper spectrum monitored at m/z 541 represents elution of 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ (dinor-dihydro-8-epi-PGF$_{2\alpha}$). The second chart represents (m/z 543) elution of 2, 3-dinor-8-epi-PGF$_{2\alpha}$ (dinor-8-epi-PGF$_{2\alpha}$). Third trace (m/z 569) represent elution of 9β-,11α-PGF$_{2\alpha}$, 9α-,11α-(15R)-8-epi-PGF$_{2\alpha}$, 9α-,11α-(15S)-8-epi-PGF$_{2\alpha}$ (8-epi-PGF$_{2\alpha}$), 9α-,11β-PGF$_{2\alpha}$, and 9α-,11α-PGF$_{2\alpha}$. The bottom chart (574) represent elution of tetradeurated 9α-,11α-8-epi-PGF$_{2\alpha}$ (8-epi-PGF$_{2\alpha}$-d$_4$) and tetradeutrated 9α-,11α-PGF$_{2\alpha}$ (PGF$_{2\alpha}$-d$_4$).

Figure 17 displays the [M-PFB]$^-$ chromatograms of plasma F$_2$-isoprostanes obtained using the refined ethyl acetate extraction/NH$_2$ cartridge procedure. The upper chromatogram at m/z 569 represents elution of plasma F$_2$-isoprostanes. The bottom chart (m/z 573) represents elution of tetraduetrated PGF$_{2\alpha}$ as the internal standard. Inserted chromatogram represents (m/z 569) represents elution of plasma F$_2$-isoprostanes using the combined C$_{18}$/NH$_2$ cartridge procedure (Nourooz-Zadeh et al., 1995b). The [M-PFB]$^-$ chromatograms

obtained using the two types of extraction procedure were virtually identical, indicating that the combined ethyl acetate extraction followed by a single NH_2-chromatography step is as efficient as the conventional but more time consuming C_{18} cartridge/NH_2-cartridge method for the isolation of plasma F_2-isoprostanes.

In conclusion, an improved method for the isolation of plasma 8-epi-$PGF_{2\alpha}$ prior to final determination by GC-MS/NICI is described. The modifications include replacing chromatography on a C_{18}-cartridge by total lipid extraction with ethyl acetate and omitting two washing steps during subsequent NH_2-chromatography. The improved method is sensitive, simple and less labour-intensive compared to previously used methods.
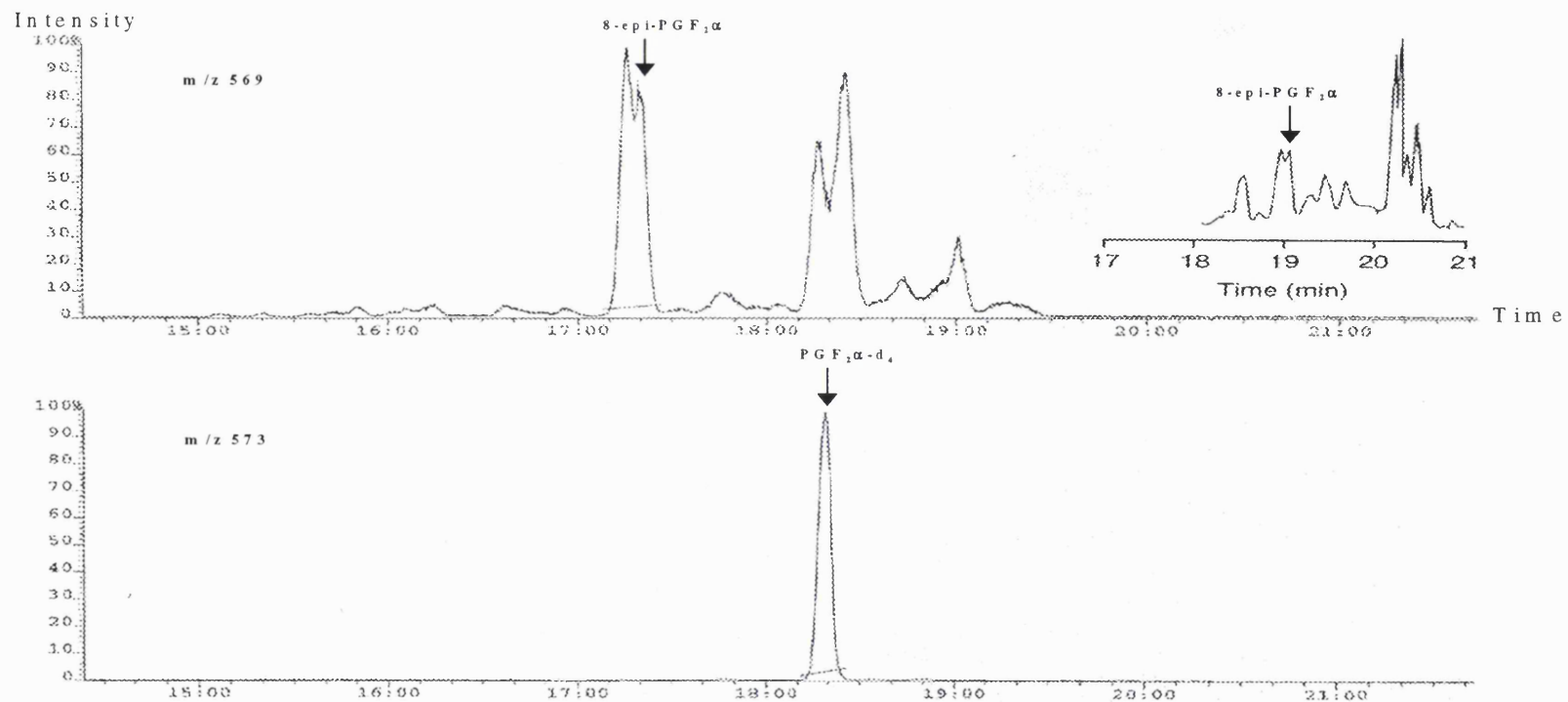
Figure 17: Gas chromatographic separation of plasma F_2-isoprostanes using the combined ethyl acetate extraction/NH_2 chromatograhy procedure. F_2-isoprostanes were converted to PBF-ester/TMS ether derivatives and analysed by GC-MS/NICI. Upper trace [M-PFB]⁻ monitored at m/z 569 represent F_2-isoprostanes while bottom chromatogram represent tetradeutrated PGF $_{2\alpha}$ as the internal standard. Insert displays GC-MS/NICI chromatograph of plasma F_2-isoprostanes using the combined C_{18}/NH_2 enrichment procedure (Nourooz-Zadeh et al., 1995b).

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3.2.2. Urine

Various analytical approaches are available for the isolation of urinary F_2-isoprostanes and/or their metabolites (2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ and 2, 3-dinor-8-epi-$PGF_{2\alpha}$) prior to final determination by GC/MS-NICI. These include: 1) conventional procedures involving chromatography on C_{18}- and Si cartridges followed by thin layer chromatography (TLC) (Morrow et al., 1990ab & 1999); 2) a combination of C_{18} and Si cartridges followed by HPLC (Mori et al., 1999); 3) the inclusion of an HPLC step between C_{18}-chromatography and TLC (Ferretti & Flanagan, 1997); used a combined C_{18} cartridge and high performance liquid chromatography (HPLC) procedure for the simultaneous isolation of 8-epi-$PGF_{2\alpha}$ and dinor-dihydro-8-epi-$PGF_{2\alpha}$ before final analysis of by GC-tandem MS Schwedhelm et al. (2000); and 5) immunoaffinity chromatography (Wang et al., 1995; Davi et al., 1997 & 1999) Of these methods, immunoaffinity chromatography is the method of choice its simplicity and specificity. Unfortunately, its implementation has been limited to leading laboratories in the field because the column material is not available commercially. Therefore, it was decided to explore the possibility of using the combined ethyl acetate/NH_2-chromatography procedure previously validated for plasma as an alternative to the above-mentioned methods.

3.2.2.1. Ethyl acetate extraction /aminopropyl procedure

As seen in Figure 18, the [M-PFB]⁻ traces at m/z 541, 543 and 569 for 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ (dinor-dihydro-8-epi-$PGF_{2\alpha}$), 2, 3-dinor-8-epi-$PGF_{2\alpha}$ (dinor-8-epi-$PGF_{2\alpha}$) and 8-epi-$PGF_{2\alpha}$, respectively, revealed that the combined ethyl acetate extraction/NH_2-chromatography procedure resulted in peaks free of interfering components. However, the [M-PFB]⁻ trace for the tetradeuterated 8-epi-$PGF_{2\alpha}$ as the internal standard was dominated by two major peaks with retention times between 15.20 and 15.80 minutes

which interfered with the accurate determination of 8-PGF$_{2\alpha}$ and its metabolites. The possibility of utilising a single chromatography step on a Si cartridge was, therefore, explored to establish if these interfering components could be removed.

3.2.2.2. Silica chromatography

PGF$_{2\alpha}$ (2.5 ng in 100 µl ethanol) and [^{3}H]-PGF$_{2\alpha}$ (100 µl containing ~ 10000 cpm) were transferred to glass vials (n=3) and dried under N$_2$. The samples were converted to PFB-esters by adding 40 µl of PFB-Br and 20 µl of 10% DIPEA in acetonitrile (v/v). After incubation at 45°C for 30 min the samples were dried down and the residues re-suspended in one millilitre of hexane/ethyl acetate (80/20, v/v). The samples were applied to Si-cartridges (500 mg) which had been pre-washed with 3 ml hexane/ethyl acetate (80/20, v/v). The sample vials were rinsed with another 1 ml aliquot of hexane/ethyl acetate (80/20, v/v) and applied to the cartridges. The cartridges were washed with 5 ml of hexane/ethyl acetate (80/20, v/v) and PGF$_2$-like compounds eluted by washing the columns with 3 ml of ethyl acetate/methanol (5/95, v/v). All fractions following the Si-chromatography step were collected and subsequently dried under N$_2$. The residues were re-dissolved in ethanol (250 µl) and the samples transferred to radioactivity counting vials. Scintillation cocktail (3 ml) was added and the radioactivity was monitored. Only traces of radioactivity (<2%) were lost during sample loading and washing of the cartridges. The majority of the radiolabelled tracer (93 ± 5%; n=3) was recovered in the ethyl acetate/methanol fraction (5/95, v/v).
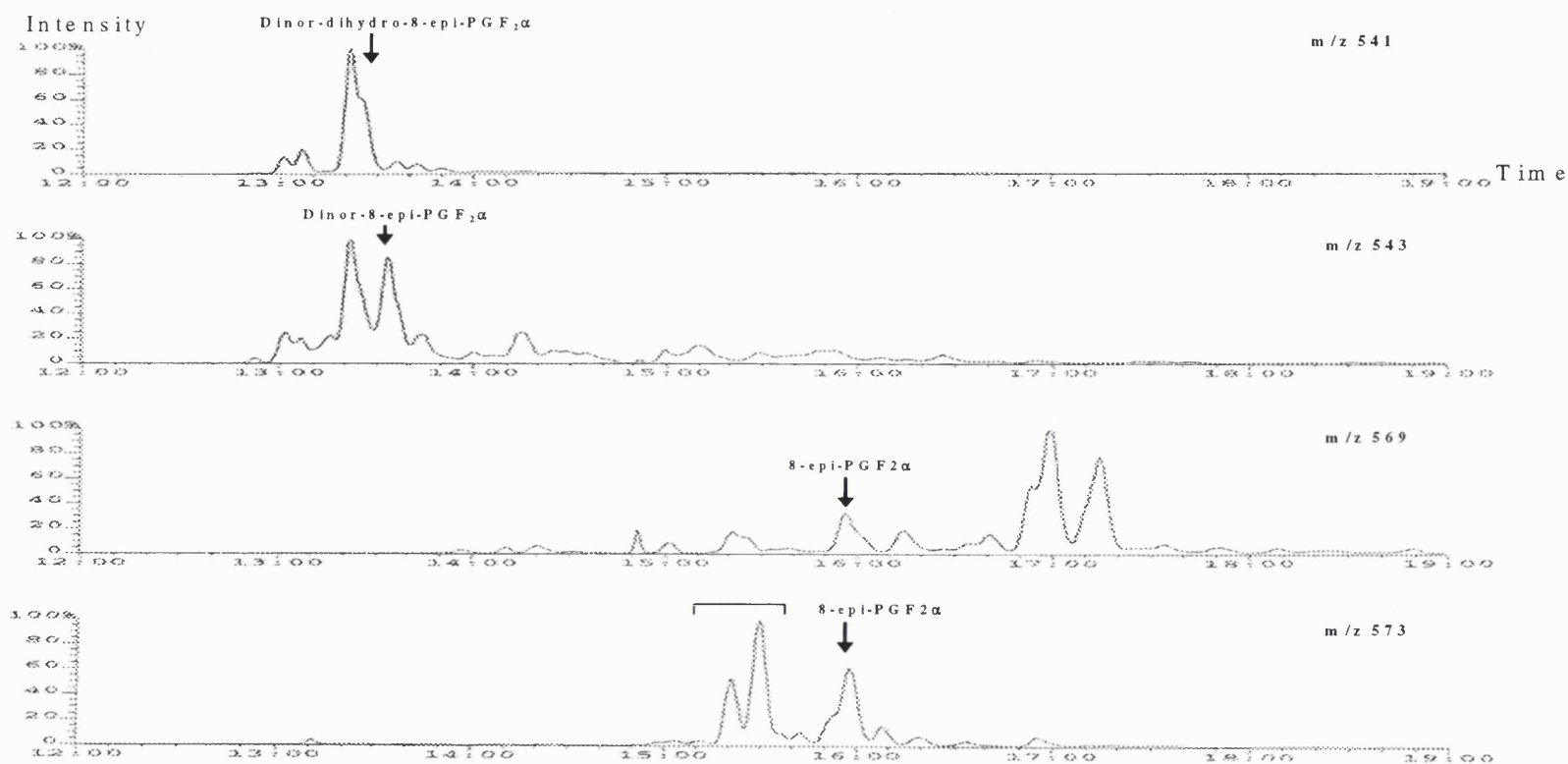
Figure 18: Gas chromatographic separation of urinary PGF$_2$-like compounds using the combined ethyl acetate extraction/NH$_2$ enrichment procedure. PGF$_2$-like compounds were analysed as the PFB-ester/TMS ether derivatives by GC-MS/NICI. First trace [M-PFB]$^-$ at m/z (541), second (m/z 543), third (m/z 569) and fourth (m/573) represent the 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$, 2, 3-dinor-8-epi-PGF$_{2\alpha}$, 8-epi-PGF$_{2\alpha}$ and tetradeutrated 8-epi-PGF$_{2\alpha}$, respectively.

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3.2.2.3. Ethyl acetate/aminopropyl –silica procedure

To evaluate the efficiency of the ethyl acetate/NH_2/Si procedure, aliquots of urine (2 ml) were spiked with PGF$_{2\alpha}$ (2.5 ng in 100 µl ethanol) and [^{3}H]-PGF$_{2\alpha}$ (100 µl containing ~ 10000 cpm) and the pH adjusted to < 2 by adding 50 µl of 4 mol/L HCl. Ethyl acetate (10 ml) was added and the samples were vortex- mixed for 30 seconds. After centrifugation at 2500xg for 5 min, the organic layer was removed and applied to NH_2-cartridges which had been pre-washed with hexane (5 ml). The cartridges were then washed with ethyl acetate (10 ml) and PGF$_2$-like compounds eluted by washing with ethyl acetate/methanol/acetic acid (10/85/5, v/v/v).

Samples were dried under N_2 and PGF$_2$-like species were converted to PFB-ester derivatives as described above. Sample residues were dissolved in 1 ml of hexane/ethyl acetate (80/20; v/v) and applied to Si cartridges, which had been pre-washed with hexane/ethyl acetate (3 ml). The sample vials were rinsed with another aliquot of hexane/ethyl acetate (1 ml) and also applied to the cartridges. The cartridges were subsequently washed with 5 ml of hexane/ethyl acetate (30/70, v/v) and PGF$_2$-like compounds eluted by washing the columns with 3 ml ethyl acetate/methanol (5/95, v/v). The solvent was dried under N_2 and residues were re-suspended in ethanol (250 µl) and the radioactivity monitored. Overall recovery of the radiolabelled tracer following total lipid extraction and chromatography on NH_2- and Si was 90 ± 4 % (n=3).
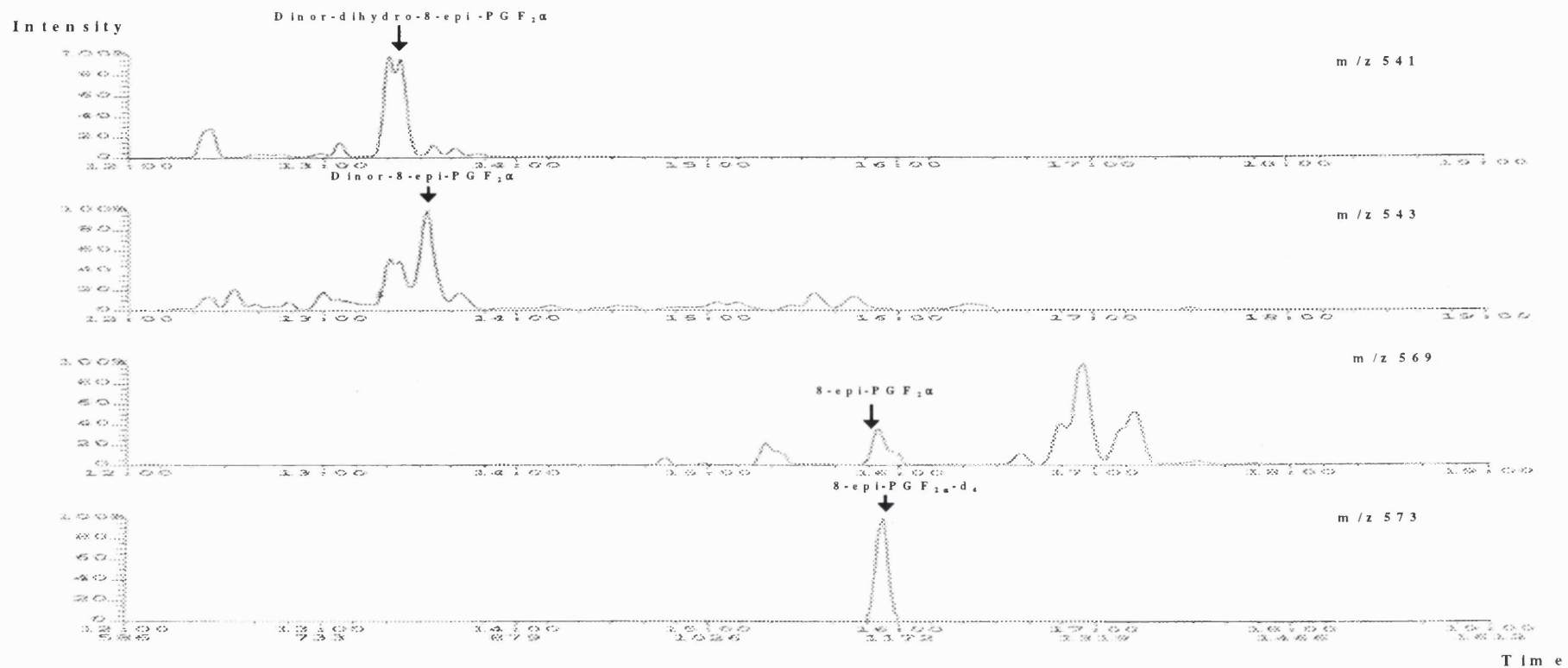
Figure 19: Gas chromatographic separation of urinary PGF$_2$-like compounds as the PBF-ester/TMS ether derivatives using the ethyl acetate extraction NH$_2$/Si chromatography producer. First trace [M-PFB]⁻ at m/z (541), second (m/z 543), third (m/z 569) and fourth (m/573) represent 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$, 2, 3-dinor-8-epi-PGF$_{2\alpha}$, 8-epi-PGF$_{2\alpha}$ and tetradeutrated 8-epi-PGF$_{2\alpha}$ (internal standard) respectively.

As demonstrated in Figure 19, interfering components (retention times between 15.20 and 15.80 minuntes) with the [M-PFB]$^-$ signal at m/z 573 were completely removed by chromatography on Si-cartridges, thus allowing quantitative determination of urinary 8-epi-$PGF_{2\alpha}$ and its metabolites, 2, 3-dinor-5, 6-dihydro-$PGF_{2\alpha}$ and 2, 3-dinor-8-epi-$PGF_{2\alpha}$.

In conclusion, an improved method for the simultaneous isolation of urinary 8-epi-PGF $_{2\alpha}$ and its metabolites 2, 3-dinor-5, 6-dihydro-$PGF_{2\alpha}$ and 2, 3-dinor-8-epi-$PGF_{2\alpha}$ prior to final determination by GC-MS/NICI is developed. The current approach has high sample throughput and is easy to adopt in standard biochemical laboratories, as no HPLC and/or other expensive equipment is required for the initial sample preparation. A schematic outline of the improved analytical procedure for the isolation of plasma and urinary F_2-isoprostanes is shown in Figure 20.
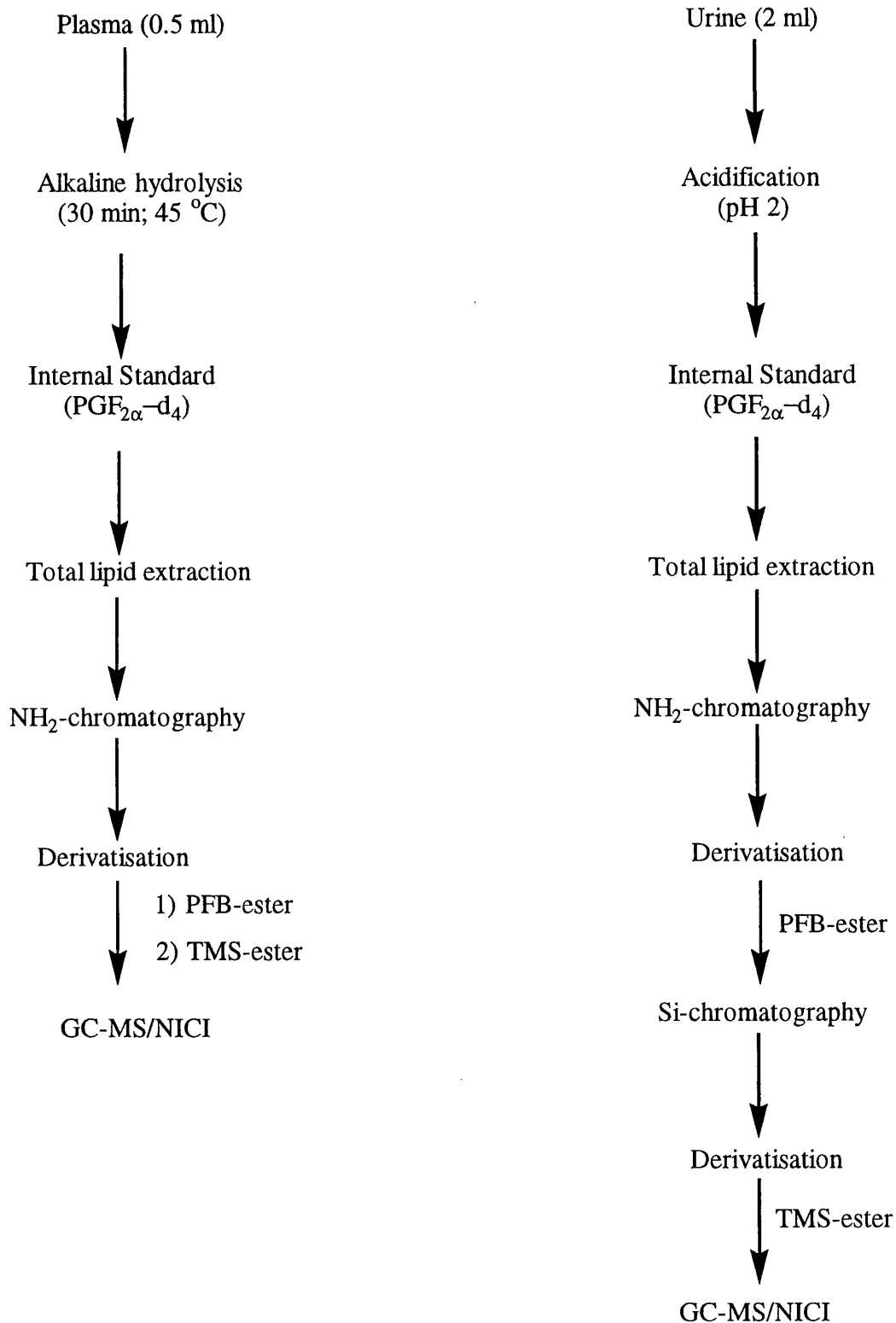
Figure 20: Analytical procedure for the isolation of plasma and urinary F2-isoprostanes.

3.3. Total antioxidant capacity

The techniques commonly used for the assessment of antioxidant capacity fall into two categories. The first category includes assays based on the use of probes that do not act as pro-oxidants. These include ferric reducing/antioxidant power (FRAP)-, total equivalent antioxidant capacity (TEAC)-, and current voltammetric assays. The second category comprises assays based on the use of probes that do act as pro-oxidants. This category includes total radical trapping parameter (TRAP)-, luminol-, dichlorofluorescein-diacetate (DCFH-DA)-, crosin-, phycoerthrin (PE)-based assays, and oxygen radical absorbance capacity (ORAC)-assay. The major disadvantages of the above assays are that they are time consuming and too complicated for the routine screening of large numbers of samples.

In this study, three simple ABEL® (analysis by emiting light) assays were used to measure plasma antioxidant capacity in diabetic patients and control subjects. The assays are based on the capacity of a sample to scavenge free radicals and oxidants in the presence of the unique photoprotein Pholasin® that emits light in the presence of different systems capable of generating free radicals. In the assays used in this study plasma samples were assessed for their antioxidant properties against: superoxide anion (O_2^-), hypochlorous acid (HOCl) and peroxynitrite (ONOO$^-$). Superoxide anion was produced by the oxidation of xanthine by the enzyme xanthine oxidase; HOCl was produced from chloramine-T. Chloramine-T spontaneously degrades to generate chlorine ions that in turn react with hydrogen peroxide produced during dismutation of superoxide anion or by other mechanisms to produce HOCl. Peroxynitrite is formed in the third ABEL® assay by the reaction between superoxide and nitric oxide released simultaneously and continually from a solution of 3-morpholino-sydnonimine HCl (SIN-1).

$$O_2^{\bullet-} \text{ (superoxide)} + NO^{\bullet} \text{ (nitric oxide)} \rightarrow ONOO^{-} \text{ (peroxynitrite)}$$
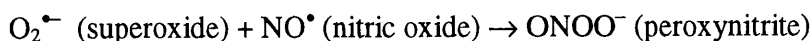
3.3.1. Plasma
3.3.1.1. Oxidation

The suitability of the three ABEL® assays for the assessment of antioxidant capacity was tested in plasma which had been oxidised with 2'-Azobis-(2-amidinopropane) hydrochloride (AAPH), an agent which generates peroxyl radicals capable of initiating lipid peroxidation. Briefly, plasma (9 volumes) was mixed with 100 mmol/L AAPH (1 volume) and heated at 60°C for up to 180 min. As a control, a plasma sample (9 volumes) was mixed with water (1 volume) and treated under the same conditions. Aliquots (200 µl) were removed at different time intervals (0, 60, 120 and 180 min, respectively) and assessed for antioxidant capacity using the different assay systems. Plasma fatty acid composition was also investigated.

3.3.1.2. Changes in fatty acid composition

In this study, attention was focused on linoleic acid ($C_{18:2}$ n-6) and arachidonic acid ($C_{20:4}$ n-6) when examining the effects of AAPH on the peroxidation of plasma fatty acids because they are the major polyunsaturated fatty acids (PUFAs). As shown in Figure 21, there were no changes in the composition of $C_{18:2}$ and $C_{20:4}$ during the incubation of control plasma at 60°C for up to 3 hrs. In the presence of AAPH, a gradual decline in the content of C18:2 was observed over the 3 hrs incubation period.

Thus, 5%, 14% and 17 % decreases in plasma $C_{18:2}$ content were observed after 1, 2 and 3 hr of incubation, respectively. By contrast, no significant changes in $C_{20:4}$ content were evident during the course of the incubation of plasma with AAPH. These observations are in line with a previous study in which the effects of AAPH on PUFA composition during

oxidation of isolated human lipoprotein fractions (VLDL, LDL and HDL) was examined

(Nourooz-Zadeh et al., 1996).
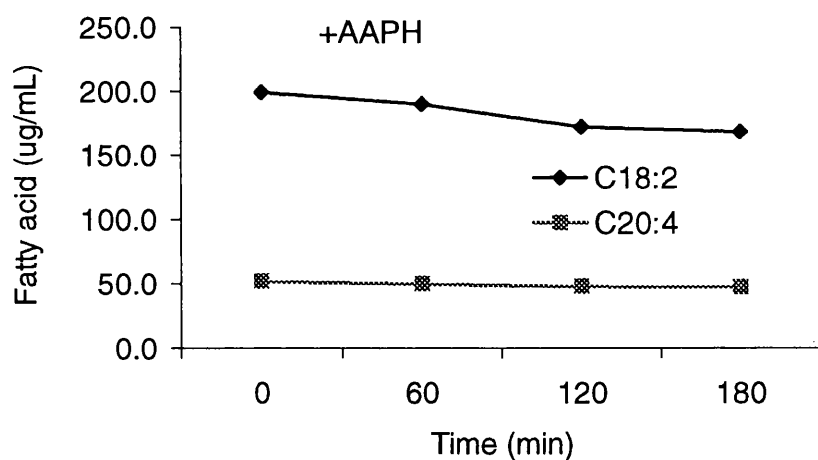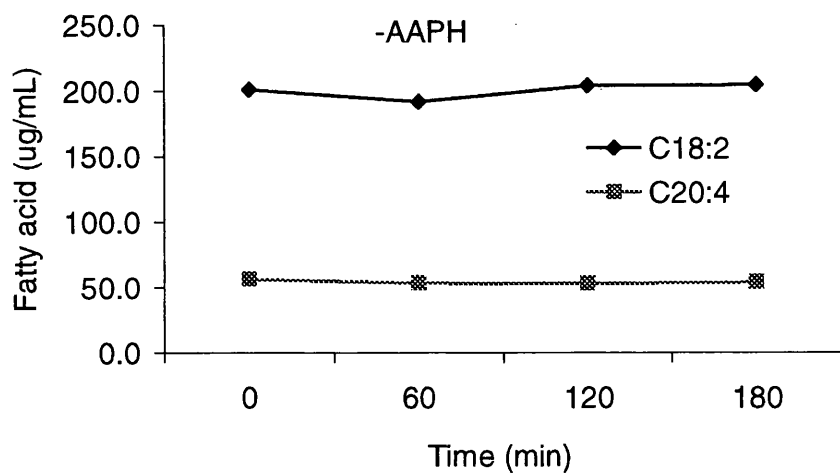
Figure 21: Changes in linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$) during incubation plasma in the presence or absence of 2'-Azobis—(2-amidinopropane) hydrochloride (AAPH). Plasma was mixed with AAPH and subsequently incubated at 60°C. Aliquots were removed at different time intervals and total lipids extracted with ethyl acetate. Fatty acids were converted to methyl esters and separated by gas chromatography using flame ionisation detector.

3.3.1.3. Quenching of peroxynitrite Pholasin® chemiluminescence
3.3.1.3.1. Optimisation of assay parameters

In initial experiments involving a total volume of 100 μl, different ratios of phosphate

buffer (50 mmol/L; pH 7.4) and Pholasin® (10 μg/ml) in the presence of 20 μl of SIN-1 (2

mg/ml in water) were tested to establish the optimal responses for the assay. Optimum

emission of light (signal to noise) was achieved with a combination of buffer to Pholasin®

in the ratio of 6/1 and was used for all subsequent experiments. For the assessment of

plasma antioxidant capacity, a sample volume of 5 μl was employed, sufficient to achieve a

reproducibly quenched light signal but avoiding excessive dilution of the plasma. The

assays were performed in microcuvettes in an LKB 1250 luminometer linked to a 121 LKB

recorder.

3.3.1.3.2. Assay protocol

One hundred microliters of Pholasin® (1.7 μg/ml) in phosphate buffer (50 mmol/L; pH

7.4) and plasma or buffer serving as a control (5 μl) were transferred to a microcuvette. The

reaction was initiated by adding SIN-1 (2 μl of 2 mg/ml $\equiv$ 2.5mmol l^{-1}) and the light signal

measured continuously until a maximal response was obtained.

3.3.1.3.3. Testing native and oxidised plasma

As displayed in Figure 22, significant changes in antioxidant capacity, expressed as the time

taken to reach maximum, were not observed in a control plasma sample incubated at 60°C

for 180 min. In the case of AAPH-oxidised plasma, a marked reduction in the antioxidant

capacity of the plasma sample was seen after 60 min incubation, and which was completely

depleted after 120 min incubation. In buffer controls incubated under the same condition as

the plasma, the presence or absence of AAPH had no effect on Pholasin® CL-profile

(4.50±0.35 min, 4.92±0.24 and 4.72±0.41 min, respectively). Thus, peroxyl radicals generated during thermal decomposition of AAPH had no effect on the signal in the presence or absence of SIN-1.
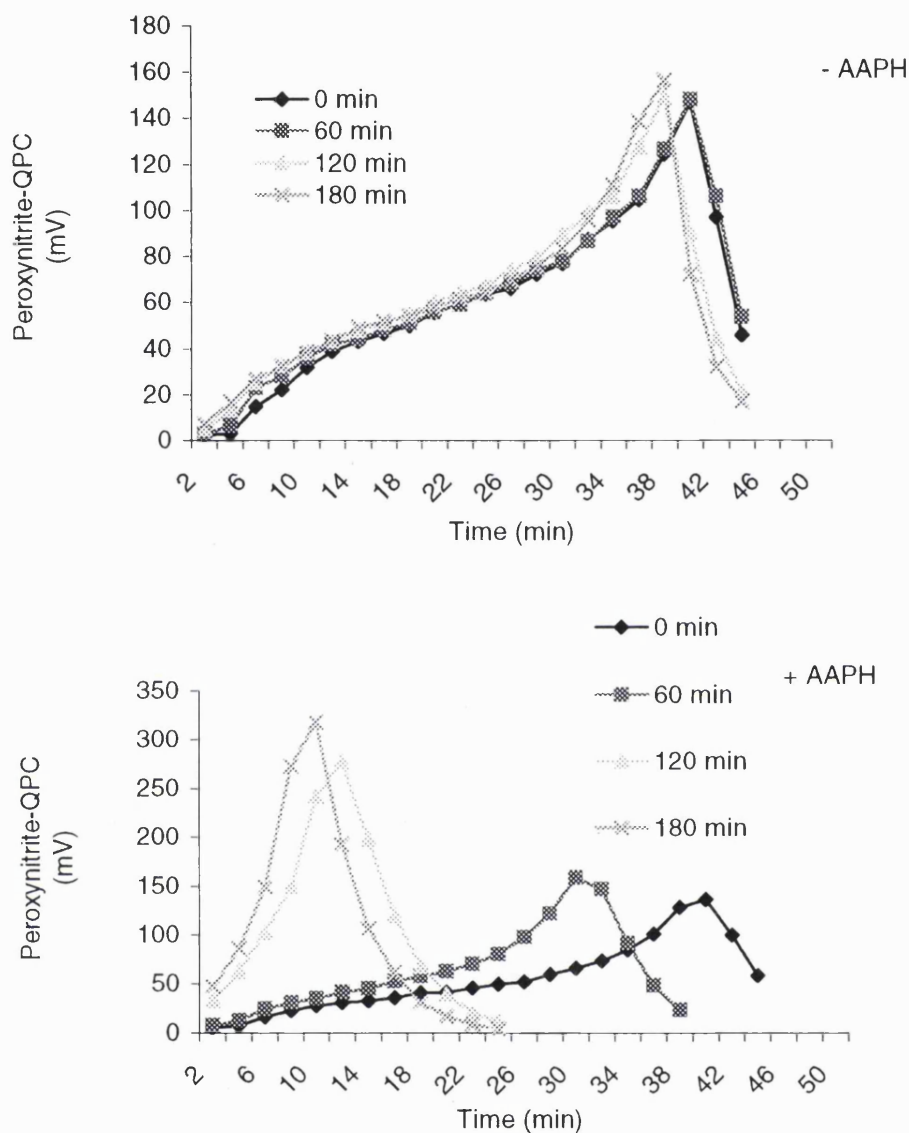
Figure 22: Changes in plasma antioxidant capacity as measured by quenching of peroxynitrite Pholasin® chemiluminescence (ONOO-QPC) during oxidation with AAPH at 60°C. After 0, 60, 120 and 180 min incubation time, Alquouts (5 µl) samples were transferred into microcuvettes at different time intervals (i.e. 0, 60, 120 and 180 min) and 100 µl Pholasin® (1.7 µg/ml) added. The reactions were initiated by adding 3-morpholino-sydnonimine HCl (2 µl of 2 mg/ml) and signal continuously monitored at 5 min intervals.

3.3.1.4. Quenching of superoxide anion Pholasin® chemiluminescence
3.3.1.4.1. Optimisation of assay parameters

Different ratios of phosphate buffer (50 mmol/L; pH 7.4), Pholasin® (10 μg/ml) in the presence of 20 μl of xanthine oxidase (0.5 U/ml in phosphate buffer) and xanthine (5 μl; 50 mmol/L) were tested to determine the optimal response for the assay. Optimum emission of light was achieved at a Pholasin® concentration of 0.5 μg/ml. At this level in the presence of plasma (5 μl), it was found that the emission of light by Pholasin® was weak to allow continuous and stable recording of the signal. The signal was not improved by increasing the amount of Pholasin® in the assay. By contrast, emission of light was substantially improved by the inclusion of Adjuvant K (50 μl/ml) as a light emission enhancer for Pholasin®.

3.3.1.4.2. Assay protocol

One hundred microliters of Pholasin® (0.5 μg/ml) and adjuvant K (50 μl/ml) in phosphate buffer (50 mmol/L; pH 7.4), plasma or buffer (5 μl) and xanthine (5 μl; 50 mmol/L) were pipetted into a microcuvette. The reaction was initiated by adding 20 μl of xanthine oxidase (0.5 U/ml) and the signal read over 5 min.

3.3.1.4.3. Testing native and oxidised plasma

Figure 23 shows the effect of AAPH on superoxide anion induced light emission by Pholasin® during oxidation of plasma at 60 °C for up to 180 min with and without AAPH. There were no changes in the antioxidant capacity measured in control plasma during incubation for 60 min. However, a slight in the intensity of emitted light was seen after 120 min but no further changes were detected when the samples were in incubation for up to 180 min. With AAPH-treated plasma, a modest increase in the intensity of light emitted

from Pholasin® was observed after 60 min. Only a 2-fold increase in the intensity of light emitted was noted after 180 min incubation. In buffer controls incubated under the same condition as the plasma samples, the presence of AAPH had no effect on in the intensity CL-signal (6.8± 0.2 mV vs 6.5±0.2 mV, 6.2±0.3 mV vs 6.3±0.4 mV and 6.2± 0.3 mV for incubation time 60, 120 and 180 min, respectively). These data suggest that the peroxyl radical generated during thermal decomposition of AAPH does not influence the antioxidant capacity test based on superoxide anion-induced chemiluminescence.
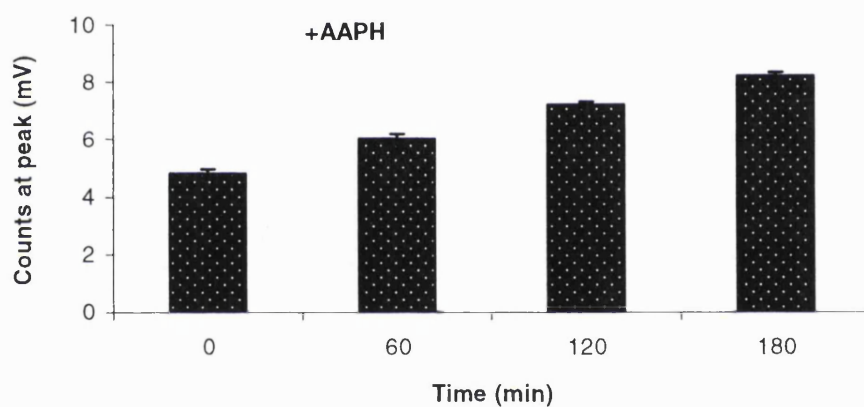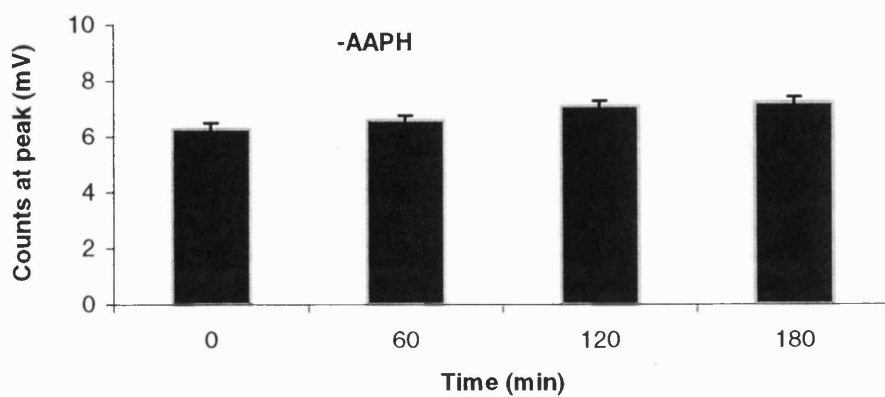
Figure 23: Changes in plasma antioxidant capacity as measured by quenching of superoxide anion Pholasin® chemiluminescence (O_2^--QPC) during incubation with AAPH at 60°C. Phosphate buffer (100 μl) containing Pholasin® (1.7 μg/ml) and adjuvant K (50 μl/ml) were transferred into microcuvettes and subsequently xanthine (5 μl; 50 mmol/L) and plasma samples (5 μl) were added. The reactions were initiated by adding 20 μl of xanthine oxidase (0.5 U/ml) and the signal read over 5 min. Data represent mean of duplicate analysis

3.3.1.5. Quenching of hypochlorous acid Pholasin® chemiluminescence

3.2.1.5.1. Optimisation of assay parameters

The antioxidant capacity test for HOCl was carried out using the same composition of phosphate buffer/ Pholasin® /Adjuvant K as that used for the O_2^--PCQ assay.

3.3.1.5.2. Assay protocol

One hundred microliters of Pholasin® (0.5 μg/ml) and adjuvant K (50 μl/ml) in phosphate buffer (50 mmol/L; pH 7.4) were pipetted into a microcuvette. Plasma or buffer (5 μl) was added and the reaction initiated by adding 20 μl of chloroamine-T (44 mmol/L in water) and the signal monitored over one min.

3.3.1.5.3. Native and oxidised plasma

As displayed in Figure 24, there were no changes in the antioxidant capacity measured during the incubation of control plasma for one hour at 60°C and further alteration in TAC occurred during the incubation of control plasma for 180 min. With AAPH-treated plasma, there was a 2-fold increase in the intensity of light emitted from Pholasin® after 60 min incubation. The corresponding increases for 120 and 180 min incubation were 11 and 17-fold, respectively. In buffer controls incubated under the same condition as the plasma samples, addition of AAPH had no effect on the CL-signal (221±5.9 mV vs 210±7.2 mV, 215±6.3 mV vs 208 ± 5.4 mV and 214±4.1 mV vs 216±6.7 mV for incubation times 60, 120 and 180 min, respectively). This suggests that AAPH per se was without effect on Pholasin®-CL.
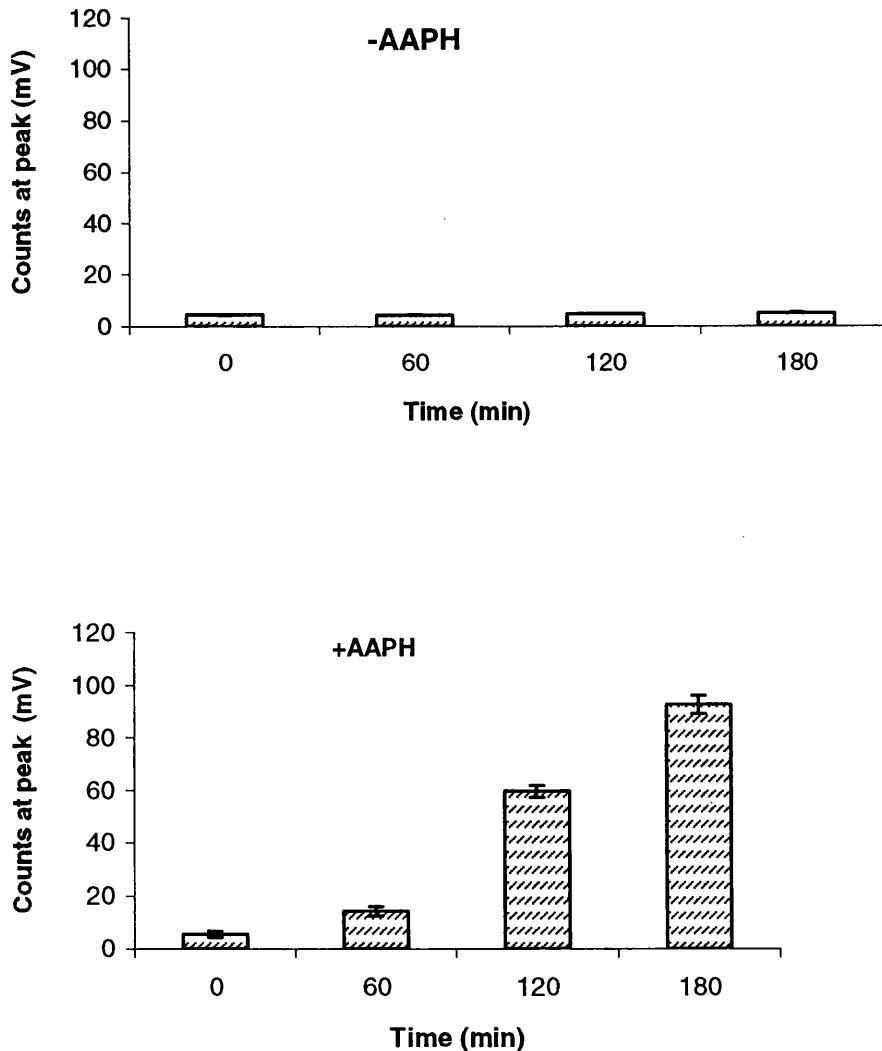
Figure 24: Changes in plasma antioxidant capacity measured by quenching of hypochlorous acid Pholasin® chemiluminescence (HOCl-QPC) during incubation with AAPH at 60°C. Phosphate buffer (100 μl) containing Pholasin® (1.7 μg/ml) and adjuvant K (50 μl/ml) were transferred into microcuvettes. The reactions were initiated by adding 20 μl of chloroamine-T (44 mmol/L in water) and the signal monitored over one min. Data represent mean of duplicate analysis.

In summary, it has been demonstrated that AAPH induce peroxyl mediated oxidation of plasma components produced an interesting profile of antioxidant change, whilst AAPH per se was without effect on Pholasin®-CL. At relatively short AAPH oxidation times when $C_{18:2}$ $_{(n-6)}$ and $C_{20:4\ (n-6)}$ concentrations were relatively stable large changes had already occurred to plasma TAC as measured by the ONOO-QPC and HOCL-QPC assay. In contrast, the superoxide quenching (O_2^--QPC) potential was less sensitive to plasma oxidation by AAPH. Taken together, these data suggest that plasma antioxidant tests based on the inhibition of light emission by Pholasin® in the presence of ONOO, O_2^- or HOCl should prove useful for the assessment of oxidative stress in biological fluids.

Chapter 4

Clinical application

4.1. Diabetic patients
4.1.1. Selection criteria

Patients for this study were recruited at the German Diabetic Research Institute, Dusseldorf. Inclusion criteria were: 1) Type 1 or type 2 diabetes according to the WHO/ADA criteria; 2) age>18 years. Informed consent was obtained from all subjects after the procedures that the study involved were fully explained. Subjects were interviewed to collect data on demographics, diabetes type, duration of diabetes, insulin treatment, medication, smoking habits and past history of neurological symptoms. Exclusion criteria were: 1) neuropathy other than that of diabetic origin; 2) smokers or ex-smokers <1 year; 3) use of antioxidants (vitamin C, vitamin E, lipoic acid, β-carotene, probucol) or iron supplementation within the last 3 months; 3) peripheral arterial disease (intermittent claudication or non-palpable foot pulse); 4) history of coronary heart disease, myocardial infarction and heart failure; 5) any medication that might adversely influence autonomic function; and 6) blood glucose levels > 22.4 mmol/L (400 mg/dl). It is of note that no information were avialable on socioeconomic background, diet or drug regime such as cholesterol lowering, antihypertensive or anti-inflammatory on these patients.

4.1.2. Polyneuropathy
4.1.2.1. Function tests

Electrophysiological tests, thermal discrimination and vibration perception thresholds were performed as previously described (Ziegler et al., 1991). Motor nerve conduction velocity (MNCV) was measured in the median and peroneal nerves, while sensory NCV (SNCV) was determined in the median and sural nerves at a skin temperature 33-34°C using surface electrodes (Sapphire, Medelec, Woking, UK). Quantitative sensory testing was evaluated by vibration perception threshold (VPT) at the second metacarpal bone and medial malleolus using the method of limits (Vibrameter, Somedic, Stockholm) and by

thermal perception thresholds (TPT) including warm and cold thresholds at the thenar eminence and dorsum of the foot using the method of limits (Path-Tester, Tönnies, Germany). Neurological examination was performed using the Neuropathy Impairment Score of the Lower Limbs (NIS-LL) (Dyck et al., 1993 & 1997) and the Neuropathy Symptom Score (NSS) (Young et al., 1993).

4.1.2.2. Criteria for diagnosis

Criteria for diagnosis of polyneuropathy (PNP) were based on: 1) slowing in motor nerve conduction velocity (MNCV) and/or sensory nerve conduction velocity (SNCV); 2) enhanced vibration perception threshold (VPT); 3) increased thermal perception threshold (TPT) warm and/or cold on the foot; 4) neuropathy impairment score (NIS) lower limb [LL] ≥ 2; and 5) neuropathy symptom scores (NSS) ≥ 3.

4.1.2.3. Criteria for staging

Criteria for staging of PNP were carried out according to the criteria published by Dyck et al. (1993 & 1997). These were: Stage 0 or no neuropathy) having <2 findings among criteria 1-3, NIS<2 and NSS<3); Stage 1 or asymptomatic neuropathy) having ≥ 2 findings among criteria 1-4 and NSS <3; Stage 2 or symptomatic neuropathy) having ≥ 2 findings among criteria 1-4 and NSS ≥ 3.

4.1.3. Cardiovascular autonomic neuropathy
4.1.3.1. Function tests

Autonomic reflex tests based on heart rate variability (HRV) were performed using a NeuroDiag II computer system (Dr. Vetter GmbH, Baden-Baden, Germany) as previously described (Ziegler et al., 1992ab). The systolic blood pressure response to standing was performed using a Dinamap 1846 SX monitoring system (Critikon, Norderstedt, Germany).

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4.1.3.2. Criteria for diagnosis

Criteria for diagnosis and definition of cardiovascular automonic neuropathy (CAN) were based on: 1) coefficient of variation (CV) at rest (5 min); 2) very low frequency (VLF) band power spectrum at rest (5 min); 3) low frequency (LF) band power spectrum at rest (5 min); 4) mean circulating resultant (MCR) during deep breathing; 5) maximum/minimum 30:15 ratio to standing up; 6) Valsalva ratio; and 7) change in systolic blood pressure on standing up.

4.1.3.3. Criteria for staging

CAN was defined as borderline if 2 out of 7 parameters were abnormal. Symptom was defined as CAN if 3 out of 7 parameters were abnormal.

4.2. Control subjects

Healthy volunteers (n=70) were recruited from the staff of the German Diabetic Research Institute, Dusseldorf and an ophthalmologist's practice in Langenfeld, a suburb of Dusseldorf. None of the controls were smokers, on any special diets, taking antioxidants, iron supplements or had taken medication related to diabetes for at least three months before taking part in this study. However, information is not avialable on socioeconomic or diet history on control subjects. The study was approved by the Ethical Committee of the German Diabetic Research Institute, Dusseldorf.

4.3. Biochemical information

The combined diabetic group showed a slightly higher male to female ratio and a higher mean body mass index (BMI) and elevated haemoglobin glycosylated (HbA1c) and triglyceride (TG) levels when compared to control subjects (Table 2). Low-density

lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol levels were slightly but significantly lower in diabetic patients than in control subjects.

When diabetic patients were separated according to the presence or absence of polyneuropathy (PNP) and/or cardiovascular autonomic neuropathy (CAN), the PNP-/CAN- group exhibited lower male to female ratio and a greater type 1 to type 2 ratio than patients with PNP+/CAN- and PNP+/CAN+, respectively. The number of patients on insulin therapy was similar in those with PNP-/CAN- or PNP+/CAN- but lower than in those with PNP+/CAN+. The mean of age of patients with PNP-/CAN- was lower and the duration of diabetes shorter than patients with PNP+/CAN- or PNP+/CAN+. In addition, triglyceride levels were lower in the PNP-/CAN- group. (Table 3). Patients with PNP-/CAN- also exhibited slightly lower glucose and HbA1c levels than in those with PNP+/CAN- or PNP+/CAN+. Diabetic patients with PNP-/CAN- exhibited a lower prevalence of albuminuria, retinopathy and hypertension than in PNP+/CAN- and PNP+/CAN+ respectively whilst the PNP+/CAN+ group exhibited fewer individuals with ketonuria than the PNP-/CAN- and PNP+/CAN+ groups.

Table 4 shows the demographic and biochemical data for the diabetic patients when separated into type 1 and type 2 diabetic patients. Type1 diabetic patients were younger, had a longer duration of diabetes and lower BMI than type 2 diabetic patients. Type1 diabetic patients exhibited high HDL-cholesterol concentrations and lower glucose, HbA1c, triglyceride and total cholesterol levels. The prevalence of retinopathy and hypertension were lower in patients with type 1 diabetes than in type 2 diabetes. Type 1 diabetic patients also demonstrated a higher degree of ketonuria but there were fewer individuals with albuminuria than type 2 patients.

Table 5 presents the demographic and biochemical characteristics for type1 diabetic subjects when divided according to the presence or absence of PNP and or CAN. The groups of patients with PN-/CAN- and PNP+/CAN+ had a similar ratios of males to females but higher than in the group with PNP+/CAN-. Patients with PN-/CAN- were younger and had shorter duration of diabetes than those with PNP+/CAN- or PNP+/CAN+. In addition, triglyceride, total cholesterol, LDL-cholesterol levels were lower. Type 1 diabetic patients had slightly lower glucose, HbA1c and HDL-cholesterol levels. Uinary creatinine levels were similar in patients with PNP-/CAN- or PNP+/CAN- but higher than those with PNP+/CAN+. Patients with PNP-/CAN- showed the lowest degrees of retinopathy and hypertensionthan in those with PNP+/CAN- or PNP+/CAN+. The prevalence of ketonuria and albuminuria was also the lowest in patients with PNP-/CAN-.

When type 2 diabetics were classified according to the presence or absence of PNP and CAN, the PNP-/CAN- group exhibited a lower male to female ratio, fewer individuals on insulin-treatment and shorter durations of diabetes than those in the PNP+/CAN- and PNP+/CAN+ groups, respectively (Table 6). The duration of diabetes was also shorter in patients with PNP-/CAN-. The prevalence of retinopathy and hypertension were lower in the PNP-/CAN- group. In addition, patients with PNP-/CAN- demonstrated lower degrees of albuminuria than the PNP+/CAN- or PNP+/CAN+ groups while the PNP+/CAN+ group exhibited the lowest degree of ketonuria.

In conclusion, reference to Table 1 indicates that the diabetic group as a whole and the control group were well matched with respect to mean age, body mass index (BMI).

Segregation of the patient into type 1 and type 2 diabetics or according to the presence of

PNP and/or CAN revealed that the respective subgroups were also optimally matched.

Table 2: Clinical characteristic of diabetic patients and control subjects.

Variables	Controls	Diabetics
N	70	185
Sex (m/f)	30/70	92/93
BMI (kg/m^2)	25.15±0.41	27.78±0.36 *
Age (Years)	50.50 ±2.06	51.78±1.08
Duration of diabetes (Years)	-	10.53±0.69
Glucose (mmol/L)	-	10.5±2.7
HbA1c (%)	5.71±0.06	9.55±0.14 *
Type 1/Type 2	-	61/124
Triglycrides (mmol/L)	1.3 2±0.10	1.94±0.11*
Cholesterol (mmol/L)	5.91±0.16	5.62±0.08
HDL-cholesterol (mmol/L)	1.61±0.05	1.28±0.03*
LDL-cholesterol (mmol/L)	3.61±0.13	3.47±0.08*
Plasma creatinine (μmol/L)	68.57±1.81	70.94±1.94
Urinary creatinine (mmol/L)	-	5.82±0.30
Uric acid (μmol/L)	-	307±6.74
Ketonuria (no/yes)	-	157/28
Albuminuria (no/yes)	-	119/66
Hypertension (no/yes)	-	95/86

Data are expressed as mean±SEM.
* Control subjects vs diabetic patients; $p < 0.05$

Table 3: Clinical characteristic of diabetic patients classified according to presence or absence of polyneuropathy (PNP) and/or cardiovascular autonomic neuropathy (CAN).

Variables	PNP-/CAN-	PNP+/CAN-	PNP+/CAN+
N	60	103	22
Sex (m/f)	21/39	58/44	12/10
BMI (kg/m^2)	27.68 ±0.66	28.29 ±0.51	26.70±0.98
Age (Years)	42.60±1.98	58.56±1.13*	54.36±2.86$
Duration of diabetes (Years)	7.30±0.88	12.04±1.00*	18.52±2.00$#
Glucose (mmol/L)	9.17±0.36	10.77±0.27	11.01±0.61
HbA1c (%)	9.09±0.25	9.67±0.18	9.77±0.33
Type 1/Type 2	30/30	23/80	8/13
Insulin treatment (no/yes)	15/45	28/75	2/20
Triglycrides (mmol/L)	1.85±0.33	1.99±0.11*	2.24±0.31$
Cholesterol (mmol/L)	5.25±0.15	5.80±0.11*	5.54±0.22
HDL-cholesterol (mmol/L)	1.33±0.4	1.28±0.05	1.28±0.12
LDL-cholesterol (mmol/L)	3.14±0.14	3.62±0.10*	3.23±0.27
Plasma creatinine (μmol/L)	65.12±1.79	71.84±2.26	74.74±10.05
Urinary creatinine (mmol/L)	6.42±0.61	5.57±0.39	5.11±0.92
Uric acid (μmol/L)	294±11.36	315±9.61	321±20.26
Ketonuria (no/yes)	46/13	79/23	20/2
Albuminuria (no/yes)	49/10	60/40	9/12
Hypertension (no/yes)	42/15	40/61	10/12

Values represent mean±SEM.
* PNP-/CAN- vs PNP+/CAN-; p<0.05
$ PNP-/CAN- vs PNP+/CAN+; p<0.05
# PNP+/CAN- vs PNP+/CAN+; p<0.05

Table 4: Characteristic of diabetic patients when segregated according to the type of diabetes.

Variables	Diabetic patients	
	type 1	type 2
N	61	124
Sex (m/f)	28/33	62/60
BMI (kg/m^2)	24.5±3.2	25.1±3.3*
Age (Years)	38.88±1.62	59.56±0.89*
Duration of diabetes (Years)	11.95±1.41	9.54±0.72
Glucose (mmol/L)	9.16±0.29	11.16±0.25*
HbA1c (%)	8.83±0.24	9.95±0.16*
Triglycrides (mmol/L)	1.38±0.09	2.27±0.18*
Cholesterol (mmol/L)	5.42±0.14	5.72±0.10
HDL-cholesterol (mmol/L)	1.46±0.06	1.18±0.04*
LDL-cholesterol (mmol/L)	3.35±0.13	3.53±0.10
Plasma creatinine (μmol/L)	76.69±3.99	69.56±1.77
Urinary creatinine (mmol/L)	6.35±0.57	5.55±0.35
Uric acid (μmol/L)	269±9.99	324±8.39*
Ketonuria (no/yes)	55/6	100/23
Albuminuria (no/yes)	49/12	75/48
Hypertension (no/yes)	47/12	44/78

Data are shown as mean±SEM
* Type 1 diabetics vs type 2 diabetics; $p < 0.05$

Table 5: Biochemical characteristics of type 1 diabetic patients when subdivided according to the presence or absence of polyneuropathy (PNP) and/or cardiovascular neuropathy (CAN).

Variables	PNP-/CAN-	PNP+/CAN-	PNP+/CAN+
N	30	23	8
Sex (m/f)	10/20	13/10	5/3
BMI (kg/m^2)	24.86±0.65	24.27 ±0.64	23.51±0.76
Age (Years)	31.17±1.64	48.23±2.81*	45.75±5.85$
Duration of diabetes (Years)	9.14±1.27	19.58±2.94*	21.87±3.64$
Glucose (mmol/L)	8.73±0.84	9.72±0.62	9.28±0.57
HbA1c (%)	8.17±0.30	8.72±0.40	9.14±0.57
Triglycrides (mmol/L)	1.10±0.08	1.62±0.18*	1.63±0.26$
Cholesterol (mmol/L)	4.76±0.15	5.85±0.20*	5.82±0.35$
HDL-cholesterol (mmol/L)	1.47±0.08	1.54±0.11	1.70±0.22
LDL-cholesterol (mmol/L)	2.77±0.08	3.61±0.18*	3.37±0.39
Plasma creatinine (μmol/L)	64.54±2.40	73.41±5.18	88.40±26.83
Urinary creatinine (mmol/L)	7.05±0.83	6.35±1.13	3.41±1.29$#
Uric acid (μmol/L)	254±10.93	269±17.84	289±44.48
Ketonuria (no/yes)	21/8	17/5	6/2
Albuminuria (no/yes)	27/2	17/5	3/5
Hypertension (no/yes)	27/1	15/6	5/3

Values are presented as mean±SEM.
* PNP-/CAN- vs PNP+/CAN- ; p<0.05
$ PNP-/CAN- vs PNP+/CAN+ ; p<0.05
# PNP+/CAN- vs PNP+/CAN+ ; p<0.05

Table 6: Characteristics of type 2 diabetic patients when subdivided according to the presence or absence of polyneuropathy (PNP) and/or cardiovascular neuropathy (CAN).

Variables	PNP-/CAN-	PNP+/CAN-	PNP+/CAN+
N	29	81	14
Sex (m/f)	11/28	45/36	7/7
BMI (kg/m^2)	30.53±0.91	29.45±0.55	28.52±1.26
Age (Years)	54.41±2.1	61.31±1.03*	59.29±2.25
Duration of diabetes (Years)	5.30±1.17	9.87±0.82*	16.61±2.29$#
Glucose (mmol/L)	11.12±0.53	11.02±0.30	12.00±0.80
HbA1c (%)	9.86±0.33	9.97±0.19	10.14±0.37
Insulin treatment (no/yes)	15/14	28/53	2/12
Triglycrides (mmol/L)	2.60±0.64	2.10±0.12	2.59±0.44
Cholesterol (mmol/L)	5.75±0.25	5.78±.12	5.37±0.28
HDL-cholesterol (mmol/L)	1.21±0.05	1.18±0.05	1.03±0.08
LDL-cholesterol (mmol/L)	3.49±0.21	3.63±0.11	3.15±0.37
Plasma creatinine (μmol/L)	66.45±2.69	71.05±2.40	66.93±4.70
Urinary creatinine (mmol/L)	5.67±0.91	5.32±0.37	6.16±1.24
Uric acid (μmol/L)	327±17.63	327±10.71	340±18.91
Ketonuria (no/yes)	25/4	62/19	14/0
Albuminuria (no/yes)	22/7	43/36	6/7
Hypertension (no/yes)	15/13	25/56	5/9

Data are shown as mean±SEM.
* PNP-/CAN- vs PNP+/CAN- ; p<0.05
$ PNP-/CAN- vs PNP+/CAN+ ; p<0.05
# PNP+/CAN- vs PNP+/CAN+ ; p<0.05

Chapter 5

Results

5.1. Plasma
5.1.1. Vitamin E
5.1.1.1. Background

The term vitamin E refers to a group of eight naturally occurring lipid-soluble molecules known as the tocopherols (Packer & Fuchs, 1993). α–Tocopherol accounts for over 80% of total vitamin E in human plasma. About one half of total plasma α–tocopherol is present in low-density lipoprotein (LDL) with the rest being equally distributed between very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). α-Tocopherol is an integral part of cellular membranes and protects against lipid peroxidation by donating a hydrogen atom to an acceptor molecule and converting it to a less reactive species (Burton et al., 1983). It quenches $^1O_2$ and protects against attack by O_2^- and OH˙ (Pryor, 1991 & 2000; van Acker et al., 1993; Brigelius-Flohe et al., 2002).

Vitamin E is essential for normal neurological structure and function in both humans and experimental animals (Romero, 1996). A deficiency of vitamin E results in the development of distal axonal neuropathy which predominantly involves the centrally directed fibres of sensory neurons, with large calibre myelinated fibres being particularly affected (Muller & Goss-Sampson, 1990). Why the nervous system should be particularly dependent on an adequate supply of vitamin E and the mechanisms involved in the neuronal deterioration, which occurs when it is deficient, are not completely understood.

There is some evidence indicating that pharmacological doses of vitamin E improve nerve conduction in humans. Tutuncu et al. (1998) have reported that defective nerve conduction in diabetic subjects with polyneuropathy is improved following treatment with vitamin E (900 mg/day) for 9 months. In addition, it has been shown that treatment with vitamin E

(600 mg/day) for 4 months improves the ratio of cardiac sympathetic to parasympathetic tone in subjects with type 2 diabetes (Manzella et al., 2001).

Dietary supplementation with vitamin E decreases the susceptibility of LDL to peroxidation in vitro (Esterbauer et al, 1991bc; Reaven et al., 1995) and has been shown to be associated with reduced protein glycation in diabetic patients (Paolisso et al., 1993). Vitamin E suppresses monocyte adhesion to cultured human endothelial cells and protects against impaired endothelium-mediated relaxation (Faruqi et al., 1994; Keegan et al., 1995). Moreover, a high intake of vitamin E has been shown to be inversely related to the incidence of heart disease in humans (Rimm et al., 1993; Stampfer et al., 1993; Salonen et al., 1995).

A number of previous studies have focused on measurements of plasma concentrations and/or lipid-standardised plasma contents of α-tocopherol from diabetic patients with or without complications compared to control subjects. While some studies have reported decreases in α-tocopherol levels, others have shown increases or no alterations. These discrepancies may be related to the fact that in some studies α-tocopherol was not standardised relative to lipid concentration which, in the case of hyperlipidaemic patients, can lead to misleading results.

The aim of the current study was to investigate the possible impact of type 1 or type 2 diabetes in patients with polyneuropathy (PNP) and/or cardiovascular neuropathy (CAN) on plasma α-tocopherol content compared to control subjects.

5.1.1.2. Findings

Figures 25 and 26 show data obtained for plasma α-tocopherol levels and α-tocopherol/cholesterol ratios in diabetic patients and control subjects. It was found that both measures of α-tocopherol were significantly lower in the diabetic patients as a whole than in control subjects [24.72±0.58 μmol/L vs 31.13±1.14 μmol/L; p<0.05 and 4.57±0.09 μgmol/mmol vs 5.38±0.17 μg/mg; p<0.05, respectively]. When the patients were classified according to the presence or absence of PNP and/or CAN, patients with PNP+/CAN- were found to have lower concentrations of α-tocopherol compared to controls followed by patients with PNP-/CAN- and PNP+/CAN+ [23.68±0.67 μmol/L, 25.93±1.00 μmol/L and 26.56±2.98 μmol/L vs 31.13±1.14 μmol/L; p<0.05]. However, the expression of plasma α-tocopherol concentration per unit cholesterol revealed a decrease in the α-tocopherol/cholesterol ratio in the patients with PNP+/CAN- compared to control subjects [4.12±0.18 μmol/mmol vs 5.38±0.17 μmol/mmol; p<0.05]. α-tocopherol/cholesterol ratio in patients with PNP-/CAN- and PNP+/CAN+ were similar but not different from control subjects [5.18±0.18 μmol/mmol and 5.11±0.36 μmol/mmol vs 5.38±0.17 μmol/mmol].

Plasma α-tocopherol concentrations in diabetic patients divided according to the presence of type 1 and type 2 diabetes were similar, but in both cases were significantly lower than in control subjects [24.75±1.00 μmol/L and 24.77±0.74 μmol/L vs 31.13±1.14 μmol/L; p<0.05]. The respective values for α-tocopherol/cholesterol ratios were 4.84±0.27 μmol/mmol and 4.48±0.18 μmol/mmol vs 5.38±0.17 μmol/mmol (p<0.05).

When type 1 diabetic patients were separated according to the presence of PNP and/or CAN, all three subgroups of patients demonstrated decreases in α-tocopherol

concentrations compared to control subjects. The largest differences in the plasma α-tocopherol concentrations were seen between diabetic subjects with PNP+/CAN+ and control subjects followed by patients with PNP+/CAN- and PNP-/CAN-, respectively [18.90±1.02 µmol/L, 25.45±1.74 µmol/L and 25.79±1.39 µmol/L vs 31.13±1.14 µmol/L; $p < 0.05$]. When α-tocopherol concentrations were expressed relative to cholesterol the largest differences were seen between patients with PNP+/CAN+ and control subjects followed by patients with PNP+/CAN- [3.31±0.35 µmol/mmol and 4.39±0.27 µmol/mmol vs 5.38±0.17 µmol/mmol; $p < 0.05$]. Patients with PNP-/CAN- produced a value for α-tocopherol/cholesterol ratios of 5.34±0.36 µmol/mmol.

In the case of type 2 diabetic patients, the greatest differences in α-tocopherol concentration were observed between patients with PNP+/CAN- and control subjects followed by those with PNP-/CAN- [23.17±0.67 µmol/L and 26.05±0.67 µmol/L vs 31.13±1.14 µmol/L; $p < 0.05$]. Patients with PNP+/CAN+ exhibited a value for α-tocopherol of 31.67±3.83 µmol/L. When plasma α-tocopherol levels were expressed relative to cholesterol concentration, the largest differences were observed between patients with PNP+/CAN- and control subjects followed by patients with PNP-/CAN- [4.03±0.18 µmol/mmol and 4.65±0.36 µmol/mmol vs 5.38±0.17 µmol/mmol; $p < 0.05$]. By contrast with the situation occurring with PNP+/CAN- and PNP-/CAN- increases were recorded for patients with PNP+/CAN+ versus control subjects [6.27±0.54 µmol/mmol vs 5.38±0.17 µmol/mmol].

There was a moderate but significant correlation between plasma α-tocopherol concentrations and triglyceride (TG) levels in diabetic patients [$r_{triglycerides} = 0.19$, $p = 0.05$]. No

relationships were seen between α-tocopherol levels and age, BMI, duration of diabetes, glucose, HbAlc, total-cholesterol, LDL-cholesterol, HDL-cholesterol or TG. In the case of control subjects, correlations were found between α-tocopherol levels and age, total-cholesterol, LDL-cholesterol and HDL-cholesterol [r_{age}=0.39, p<0.001; $r_{total-cholesterol}$ =0.34, p<0.01; $r_{LDL-cholesterol}$ =0.29, p<0.02 and $r_{HDL-cholesterol}$ =0.30, p<0.05]. In the case of α-tocopherol/cholesterol ratios, no correlations were seen in either diabetic patients or control subjects.
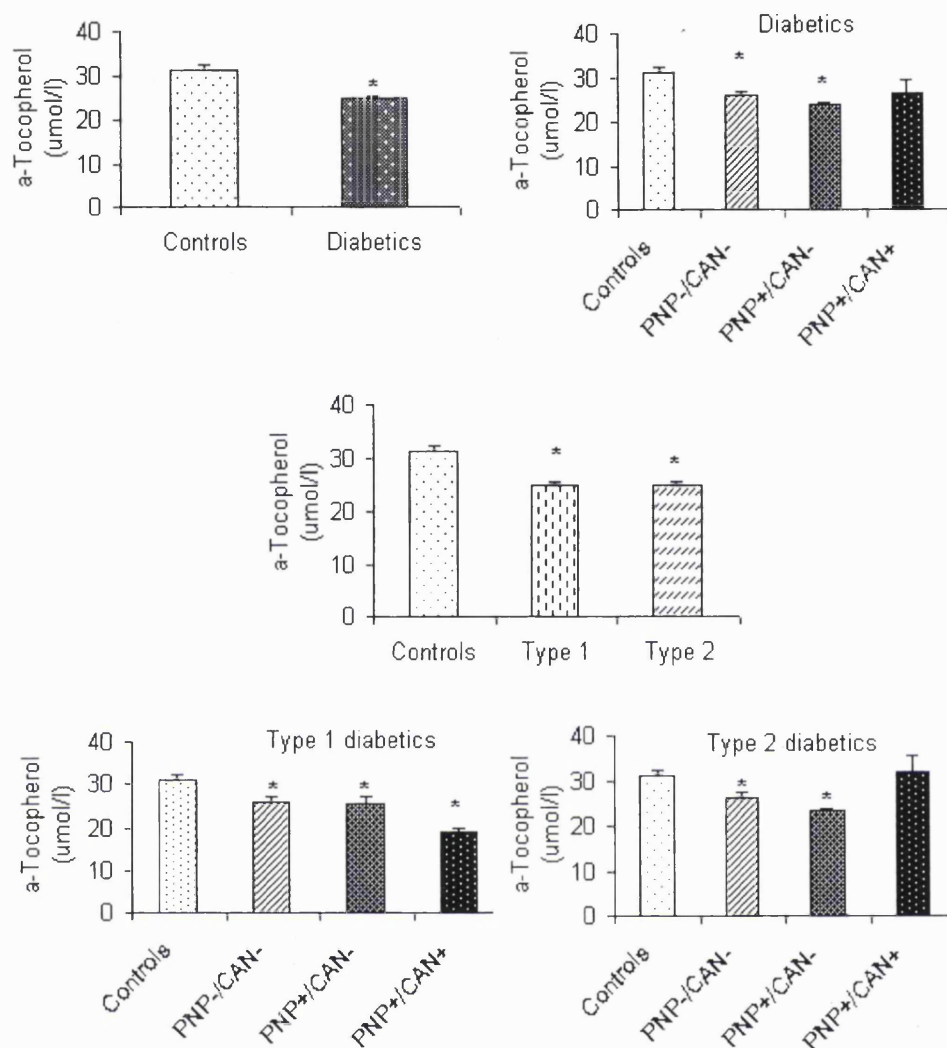
Figure 25: Plasma α–tocopherol levels in diabetic patients and control subjects. Plasma samples were spiked with γ–tocopherol as internal standard and total lipids partitioned with ethyl acetate. Tocopherols were separated by reversed-phase HPLC and signals read using fluorometric detection. Data are expressed as mean±SEM.

* Control subjects vs diabetic patients as a whole or diabetic subgroups; p<0.05. Data are presented as scatter plots (Appendix; Figures 58-62).
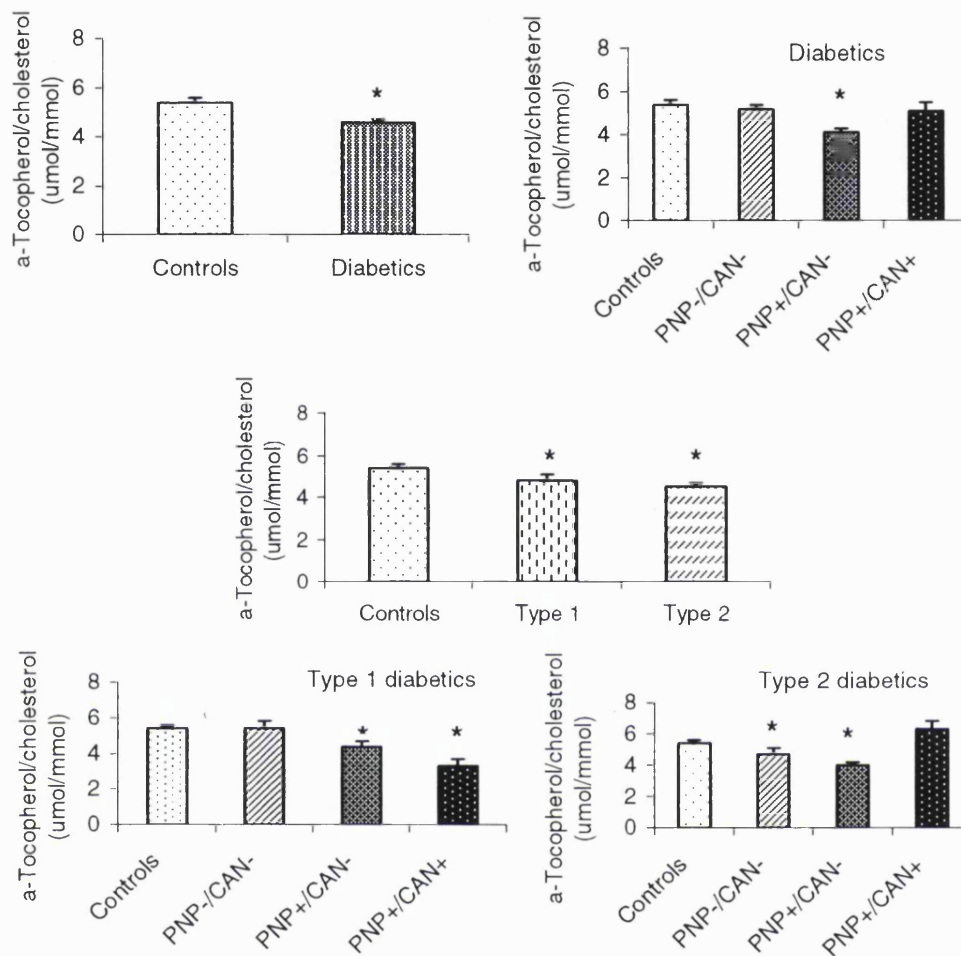
Figure 26: Plasma α–tocopherol/cholesterol ratios in diabetic patients and control subjects. Plasma samples were spiked with γ–tocopherol as internal standard and total lipids partitioned with ethyl acetate. Tocopherols were separated by reversed-phase HPLC and signals read using fluorometric detection. Values represent mean±SEM.

* Control subjects vs diabetic patients as a whole or diabetic subgroups; p<0.05. Data are illustrated as scatter plots (Appendix; Figures 62-67).

5.1.1.3. Discussion

Several analytical approaches exist for the determination of α-tocopherol in biological fluids including direct spectrophotometric/fluorometric, chromatographic i.e. HPLC linked to either photometric or fluorometric detectors and gas chromatographic methods (Kock et al., 1997; Prieto et al., 1999; Yap et al., 1999; Weinmann et al., 1999; Abidi, 2000; Gueguen et al., 2002; Roy et al., 2002). Of these, HPLC combined with photometric or fluorometric detection is the most frequently used. In this investigation, a simple HPLC/fluorometric method for the determination of plasma α-tocopherol was employed (Nourooz-Zadeh et al., 1995a). The analytical procedure involved the addition of γ-tocopherol as the internal standard, partitioning of total lipids with ethyl acetate and chromatographic separation on a C_{18}-column followed by fluorometic analysis. Fluorometric detection was chosen because it offers higher sensitivity and specificity and cleaner chromatograms compared to ultraviolet (UV) detection (Eitenmiller & Landen, 1999). The intra- and inter-assay coefficients of variations were <5%.

In the present study, plasma α-tocopherol concentration [31.13 μmo/L] in healthy control subjects were found to be comparable to previously reported values [29.67 μmol/L and 28.21 μmol/L] for healthy German individuals receiving an adequate amount of vitamin E in their diets (Schneider et al., 1995; Bergheim et al., 2003). The values observed for control subjects were also in good agreement with reference data for healthy subjects from studies conducted in France, Spain and Switzerland (Vuilleumier et al., 1983; Ascherio et al., 1992; Fernandez-Banares et al., 1993; Hercberg et al., 1994).

However, plasma α-tocopherol concentrations were significantly lower [20%] in diabetic patients than in control subjects, this decrease being similar to the 18% reduction previously

reported by Nourooz-Zadeh et al. (1997a). The finding concerning the reduction in plasma α–tocopherol levels in the diabetic patients in the present investigation is supported by previously reported studies (Asayama et al., 1993; Griesmacher et al., 1995; Sundaram et al., 1996; Osterode et al.1996; Olmedilla et al., 1997; Polidori et al., 2001). Taken together these data have suggested that low α-tocopherol concentrations seen in diabetes reflect increased consumption due to increased oxidative stress.

Contrasting with the above findings, there are reports that α-tocopherol levels are either unchanged (Galvan et al., 1996; Leonhardt et al., 1996; Dyer et al., 1997; Maxwell et al., 1997a; VanderJagt et al., 2001) or increased in diabetes (Murakami et al., 2000; Gopaul et al., 2001; Davison et al., 2002; Vessby et al., 2002). Reviewing the methods used for the measurement of α-tocopherol in these studies reveals that failure to show a decrease in plasma α–tocopherol levels in diabetic patients appeared to be linked to the use of UV-detection for its detection. This perhaps indicates that UV-detection lacks the specificity required for vitamin E determination when applied to hyperlipidaemic subjects. Further studies are, however, needed to establish if the observed differences could be linked to the detection method employed.

Assessment of absolute α-tocopherol, without an adjustment for a one or more of blood lipids, as index of antioxidant status has a major limitation in that the levels are influenced by lipid concentrations. The adjustment of plasma α-tocopherol concentrations relative to blood lipid content i.e. total cholesterol, triglyceride or total lipids (sum of triglycerides and cholesterol), phospholipid, lipoprotein and apolipoprotein has, therefore, been put forward to reduce the confounding of antioxidant with lipid concentrations (Horwitt et. al., 1972; Thurnham et al., 1986; Riemersma et al., 1991; Jordan et al., 1995; Gross et al., 2003). In

this investigation, plasma α-tocopherol concentrations were expressed relative to cholesterol because LDL is the major carrier of vitamin E in the plasma. The validity of this approach is supported by correlation between α-tocopherol levels and total cholesterol, LDL-cholesterol or HDL-cholesterol concentrations in control subjects. The second reason for expressing plasma vitamin E levels relative cholesterol concentration is that cholesterol levels were not significantly altered postprandially.

In this investigation, α-tocopherol/cholesterol ratios were 15% lower in diabetic patients than in control subjects. In a previous study, however, a reduction of 35% was recorded (Nourooz-Zadeh et al., 1997a). A possible explanation for the discrepancy between the two studies may be that diabetic patients and control subjects in the earlier study were not matched for plasma cholesterol content. Another observation made from the current study is that neither α–tocopherol concentrations nor α–tocopherol/cholesterol ratios seemed to be influenced by the type of diabetes. Differences were, however, observed in relation to the presence or absence of PNP and/or CAN.

In type 1 diabetic patients, α–tocopherol levels were similar in those with PNP-/CAN- or PNP+/CAN- but lower than in control subjects. The differences between patients with PNP-/CAN- or PNP+/CAN- were, however, increased when α–tocopherol levels were expressed per unit cholesterol. The increases in α–tocopherol/cholesterol ratios seen in patients with PNP-/CAN- reflect differences with respect to cholesterol content i.e. patients in this subgroup had significantly lower cholesterol levels than patients with PNP+/CAN- and control subjects respectively. In the case of patients with PNP+/CAN+ both α–tocopherol levels and α–tocopherol/cholesterol ratios were markedly lower than in control subjects. The reduced α–tocopherol concentrations seen in the patients with PNP+/CAN+ may be

due to increased oxidative stress since the differences could not be explained on the basis of variations in demographic, clinical or biochemical factors as the PNP+/CAN- and PNP+/CAN+ subgroups were similar with respect to these criteria.

As with type 1 diabetics subjects, α-tocopherol levels and α-tocopherol/cholesterol ratios were lower in type 2 diabetic patients with PNP-/CAN- when compared to control subjects. Both of these measures were 14% and 17%, respectively, lower in the patients with PNP+/CAN- than in those with PNP-/CAN-. This difference cannot be explained on the basis of variations in demographic, clinical and biochemical factors since both subgroups were adequately matched. By contrast, α-tocopherol concentrations and α-tocopherol/cholesterol ratios were slightly higher in patients with PNP+/CAN+ than those in control subjects. It is of note that α-tocopherol levels and α-tocopherol/cholesterol ratios in the patients with PNP+/CAN+ were also higher (17% and 25%, respectively) when compared to those in patients with PNP+/CAN-. These differences between patients with PNP+/CAN+ and PNP+/CAN- are difficult to explain since both subgroups were matched with respect to demographric and biochemical factors such as lipid levels. It is possible, however, that the observations made may reflect increased intake of vitamin E. Unfortunately, no information is available on drug history or diet history on these patients in order to establish the reason for the increased vitamin E levels in the patients with PNP+/CAN+ than in other diabetic subgroups or even control subjects.

In conclusion, this investigation has shown that plasma α-tocopherol concentrations and α-tocopherol/cholesterol ratios are significantly lower in diabetic patients and that both measures of plasma α-tocopherol do not appear to be influenced by the type of diabetes. It was, however, not possible to draw a firm conclusion on the impact of PNP and/or CAN on

circulating α–tocopherol levels because the results from both type 1 and type 2 diabetic

patients were inconclusive.

5.1.2. Vitamin C
5.1.2.1. Background

Vitamin C is an essential dietary component in humans. It cannot be synthesized from glucose due to the fact that humans lack the enzyme gulono-γ-lactone oxidase, which is required for the conversion of L-gulono-γ-lactone to ascorbic acid (Chatterjee et al., 1975; Smirnoff, 2001). Ascorbic acid is also required as a cofactor in many enzymatic reactions involved in the biosynthesis of collagen, neurotransmitters and carnitine, respectively (Friedman & Kaufman, 1965; Glembotski, 1984; Sauberlich, 1994; Bode, 1997; Jacob, 1999).

Ascorbic acid readily loses an electron forming semidehydroascorbic acid. This form of ascorbic acid is a free radical. The removal of a second electron from semidehydroascorbic acid yields dehydroascobic acid. Dehydroascorbic acid can be reduced back to ascorbic acid by dehydroascorbic acid reductase, which requires glutathione as a source of reducing power (Winkler, 1987; Bode, 1997; May et al., 2001). Upon further oxidation, dehydroascorbic acid is irreversibly degraded by hydrolytic opening of the lactone ring and the formation 2, 3-diketogulonic acid. Oxidation of ascorbic acid is accelerated on exposure to light, copper, heat and/or mildly alkaline conditions. Metabolic pathway of ascorbic acid is shown in Figure 27.

Higher intake of vitamin C is associated with the decreased risk of coronary heart disease (CHD) (Gokce et al., 1999; Carr & Frei, 2000; Yokoyama et al., 2000; Morel et al., 2003; Nam et al. 2003; Osganian et al., 2003). Increase intake of vitamin C is shown to improve both endothelial function and insulin sensitivity in patients with CHD (Hirai, 2000; Ellis et al., 2001; Hirashima 2001; Rossig et al., 2001; Erbs et al., 2003). It has also been shown that ascorbic acid protects isolated LDL and HDL during cell mediated as well as copper-mediated oxidation (Lehr et al., 1995; Martin & Frei, 1997; Carr & Frei, 2002; Alul et al.,

2003; Ashidate et al., 2003; Hillstrom et al., 2003). In addition, ascorbic acid exerts vasodilatory and anticlotting effects by altering the production of prostacyclin and other prostaglandins (Horrobin, 1996).
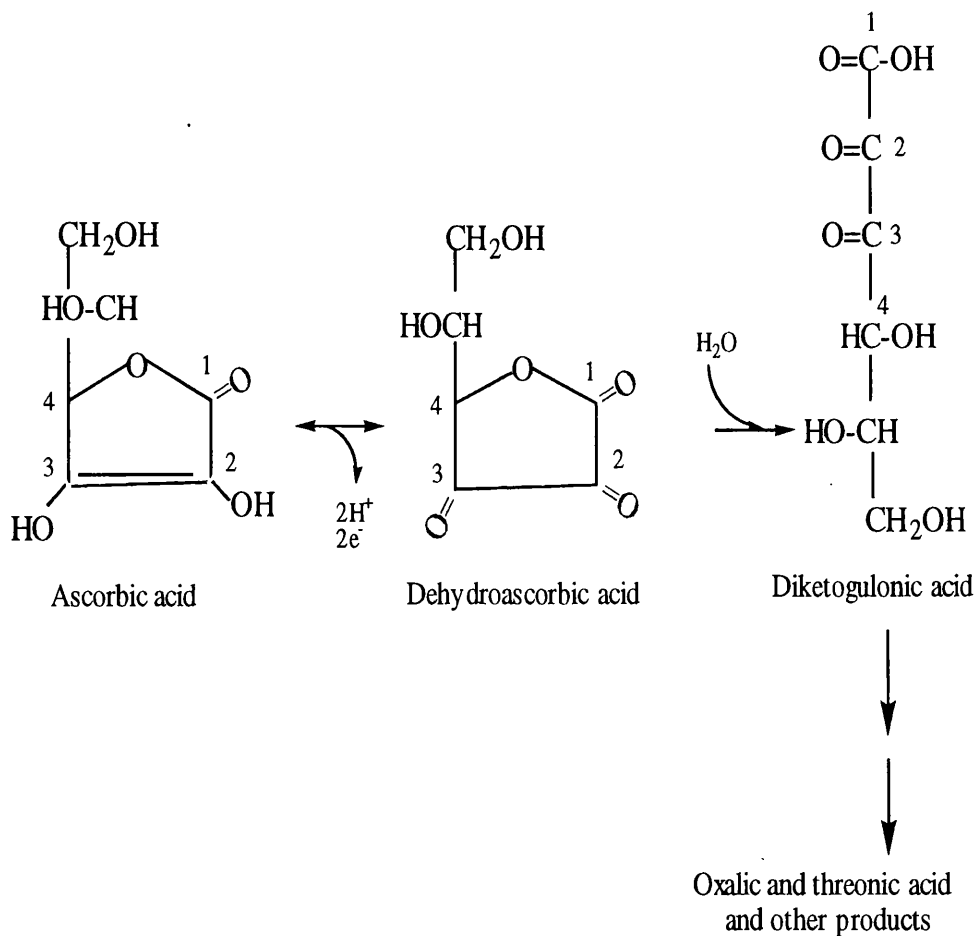
Figure 27: Metabolic pathway of ascorbic acid.

Plasma vitamin C concentrations in diabetic patients and control subjects have been examined by a number of previous studies. Some studies have reported decreases in ascorbic

acid levels in diabetic patients while no alterations seen others (For review see Wills & Byers, 1996). These discrepancies may be related to improper sample handling and/or heterogeneity of the studied populations.

The aim of the current study was to establish if type 1 and type 2 diabetics with varying degrees of neurological complications are associated with alteration in plasma ascorbic acid levels compared to healthy controls.

5.1.2.2. Findings

Diabetic patients had lower plasma ascorbic acid concentrations than control subjects [56.89 ± 1.82 μmol/L v 64.44 ± 2.33 μmol/L; $p < 0.05$]. When diabetic patients were subdivided according to the presence or absence of polyneuropathy (PNP) and autonomic cardiovascular neuropathy (CAN) ascorbic acid concentrations were found to be lower in all three patient subgroups relative to control subjects. The largest differences were seen between patients with PNP+/CAN- versus control subjects followed by patients with PNP+/CAN+ and PNP-/CAN-, respectively [54.64 ± 2.44 μmol/L, 55.83 ± 4.94 μmol/L and 61.34 ± 3.12 μmol/L vs 64.44 ± 2.33 μmol/l].

When patients were separated according to the type of diabetes the largest differences in ascorbic acid levels were noted between type 1 diabetic patients and control subjects [50.99 ± 2.44 μmol/L vs 64.44 ± 2.33 μmol/L; $p < 0.05$]. Type 2 diabetics yielded a value for ascorbic acid of 58.90 ± 2.39 μmol/L. When type 1 diabetics were subdivided according to the presence or absence of PNP and/or CAN all three subgroups had lower ascorbic acid levels than controls. The largest differences in ascorbic acid concentrations were observed between diabetics with PNP+/CAN- or PNP+/CAN+ versus controls followed by patients with PNP-/CAN- [52.08 ± 3.90 μmol/L, 52.08 ± 7.21 μmol/L and 55.38 ± 3.69 μmol/L vs 64.44 ± 2.33 μmol/L; $p < 0.05$].

In the case of type 2 diabetics, patients with PNP+/CAN- and PNP+/CAN+ had lower ascorbic acid levels versus control subjects [55.55 ± 2.95 μmol/L and 60.88 ± 6.70 μmol/L vs 64.44 ± 2.33 μmol/L]. By contrast, vitamin C levels were slightly higher in patients with PNP-

/CAN- than in controls [67.25±5.17 µmol/L v 64.44±2.33 µmol/L]. Figure 28 displays the data obtained for ascorbic acid levels in diabetic and control groups.

There was a moderate but significant correlation between ascorbic acid and LDL-cholesterol levels in diabetic patients [$r_{LDL-cholesteol}$=0.199, p<0.01]. Also, there was an inverse association between plasma ascorbic acid levels and total impairment score-lower limb (NIS-LL) in diabetic patients [r_{NIS-LL}=-0.262, p<0.001; Figure 29]. No relationships were seen between ascorbic acid levels and age, BMI, duration of diabetes, glucose, HbA1c, total-cholesterol, HDL-cholesterol or triglycerides. Correlations were absent in control subjects.
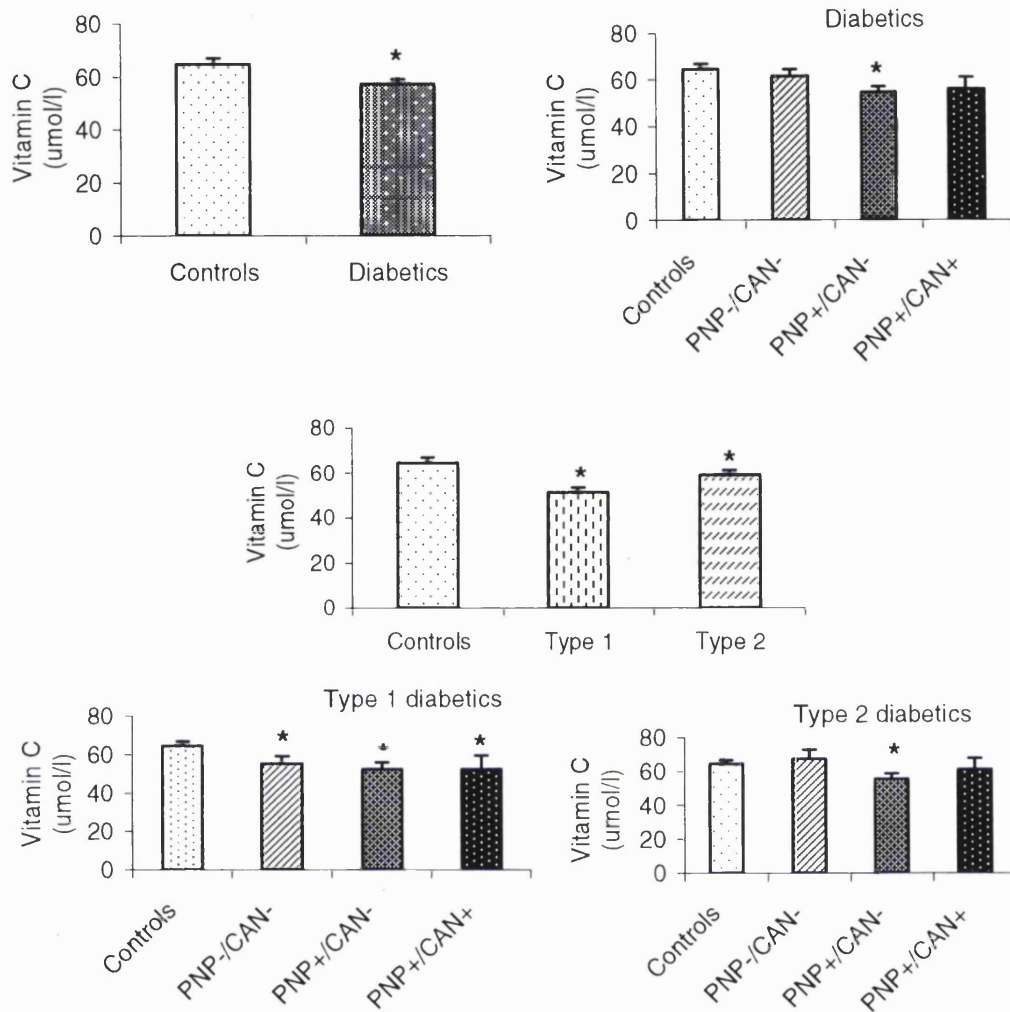
Figure 28: Plasma ascorbic acid concentrations in diabetic patients and control subjects. Plasma samples were deproteinisation with 20% metaphosphoric acid and aliquots of supernatant treated with ascorbate oxidase. Dehydroascorbic acid was reacted with o–pheylenediamine and signal was read fluorometrically. Data are expressed as mean±SEM.

* Control subjects versus diabetic patients as a whole or diabetic subgroups; $p < 0.05$.

Data are displayed as scatter plots (Appendix; Figures 68-72).
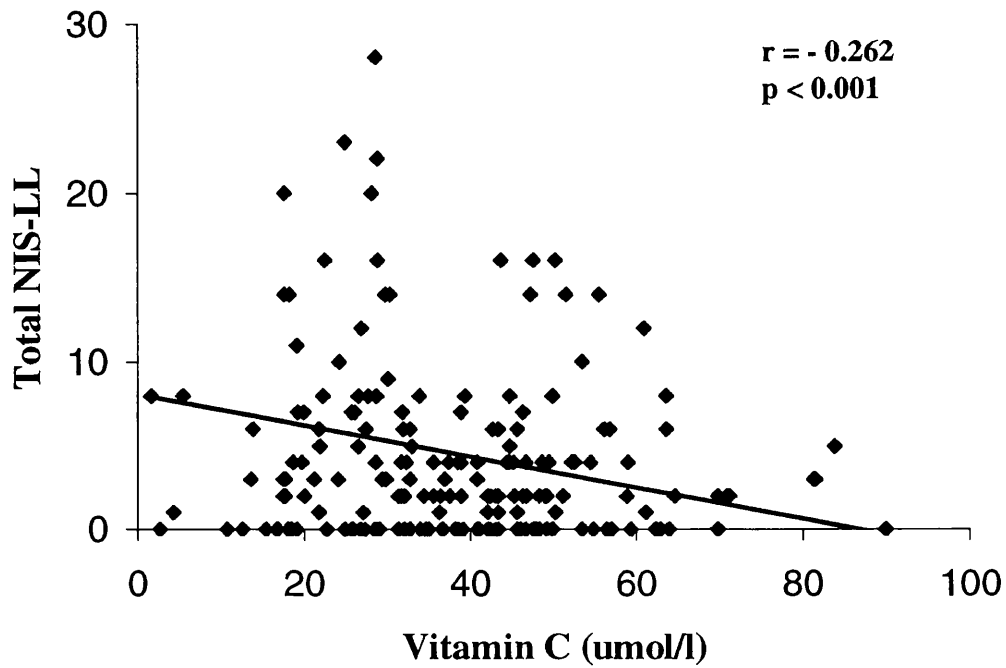
Figure 29: Association between plasma ascorbic acid levels and total neurological impairment score of the lower limb (NIS-LL). Vitamin C was determined fluorometrically as described under material and method chapter. Diagnosis and grading of diabetic neuropathy were carried out according to the criteria published by Dyck et al. (1993 & 1997).

5.1.2.3. Discussion

Several analytical approaches are available for the determination of ascorbic acid and dehydroascorbic acid in plasma (Eitenmiller & Landen, 1999). These include direct spectrophotometric and spectrofluorometric techniques (Moeslinger et al., 1995; Lee et al., 1997; Ihara et al., 2000), HPLC linked to electrochemical, fluorometric or ultraviolet detectors or a combination of fluorometric and ultraviolet detectors (Lopez-Anaya & Mayersohn, 1987; Dhariwal et al., 1991; Tessier et al., 1996; Esteve et al., 1997; Koshiishi & Imanari, 1997; Bode & Rose, 1999; Kall & Anderson, 1999; Hamad et al., 2002), and combined gas chromatographic/mass spectrometry (Deutsch, 1993 & 1997). Of these methods, HPLC combined with electrochemical detection is the method of choice and used by leading laboratories in the field. However, because the equipment employed is not standard tool in most laboratories, the method is not used generally.

The susceptibility of ascorbic acid to oxidation is a major concern for the accurate determination of its plasma levels (Bode et al., 1990; Dhariwal et al., 1991; Koshiishi & Imanari, 1997; Koshiishi et al., 1998). Plasma dehydroascorbic acid may be generated during sample deproteinisation in the presence of perchloric or trichloroacetic acids due to ferric ion release from haemoglobin and transferrin (Koshiishi et al., 1998). Ferric ion will transform ascorbic acid from a strong reducing agent to a pro-oxidant yielding a superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and subsequently hydroxyl radical (OH^-). By contrast, oxidation of ascorbic acid has not been observed with metaphosphoric acid (Margolis & Duewer, 1996; Koshiishi et al., 1998). Dehydroascorbic acid may also be generated during during plasma preparation at ambient temperature and/or improper storage. Plasma ascorbic acid is shown to stable during storage of sample for 2 hr at 4°C

(Boddy, 1999). By contrast, 30% of the ascorbic was lost when the plasma samples were stored at 24°C.

In this investigation, the previously established protocol of Obrosova et.al. (2001) was used for the determination of ascorbic acid. The procedure involved plasma preparation in the cold, deproteinisation with 20% metaphosphoric acid followed by conversion of ascorbic acid to dehydroascorbic acid. Dehydroacorbic acid condenses with o-pheylenediamine to form a quinoxaline derivative that emits light at 450 nm. This reaction can therefore be employed for the determination of vitamin C, as it is both specific and sensitive, and occurring at ambient temperature. The intra- and inter-assay coefficients of variations were <5%.

There have been two studies in which plasma ascorbic acid was measured in healthy German subjects. Schneider et al. (1995) reported a mean value for ascorbic acid of 75.2 μmol/L in a German population comprising 862 males and 1,144 females consuming 81 mg of vitamin C daily. A mean plasma value [63.3 μmol/L] was recorded for subjects taking 108 mg of vitamin C per day (Bergheim et al., 2003). The value for plasma ascorbic acid [i.e. 64.4 μmol/L] obtained for control subjects in the present investigation was therefore similar to that reported in previous studies (Schneider et al., 1995; Bergheim et al., 2003). The range of values [6.8-104.5 μmol/L] for plasma ascorbic acid obtained with control subjects in the current study were also comparable with the values reported previously [28.4-85.2 μmol/L; 39.7-140.3 μmol/L and 22.7-79.5 μmol/L] for healthy subjects with adequate vitamin C intakes (McCormick & Green 1994; Stahl & Seis, 1996; Benzie, 1999).

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Ascorbic acid levels were found to be 18% and 9% lower in type 1 and type 2 diabetic patients than in control subjects, respectively. Low ascorbic acid concentrations in diabetes probably reflect increased consumption due to enhanced free radical activity (Levine, 1986). For example, ascorbic acid donates electrons to the reactive metabolites of dietary flavonoids thereby sparing other cellular components from oxidation (Galati et al., 2002).

Another possible explanation is that hyperglycaemia may be responsible for the ascorbic acid deficit seen in diabetes. Exposure to high glucose may deactivate antioxidant enzymes and impair the intracellular regeneration of ascorbic acid by removing reducing equivalents in the form of NADPH for the polylol pathway (Stribling et al., 1989; Yue et. al. 1989; Feldman et al., 1992; Giugliano et al., 1996; Cameron & Cotter; 1999). As illustrated in Figure 30, the first step in the ascorbic acid regeneration from dehydroascorbic acid requires the formation of NADPH, which is provided by glucose-6-phosphate dehydrogenase (G-6-PD) or via isocitrate dehydrogenase action. NADPH then converts oxidised glutathione to reduced glutathione, which either reacts chemically with dehydroascorbate or via a reaction catalysed by a dehydroascorbate reductase enzyme, thioltransferease, or protein disulfide isomerase. During the course of the oxidation processes, ascorbic acid may pass through a highly unstable ascorbyl radical. The latter may spontaneously decay via the loss of an unpaired electron with the net product being dehydroascorbic acid and further degradation rather than regeneration to ascorbic acid through reduction of the ascorbyl radical by an ascorbyl free reductase (Bode, 1997).

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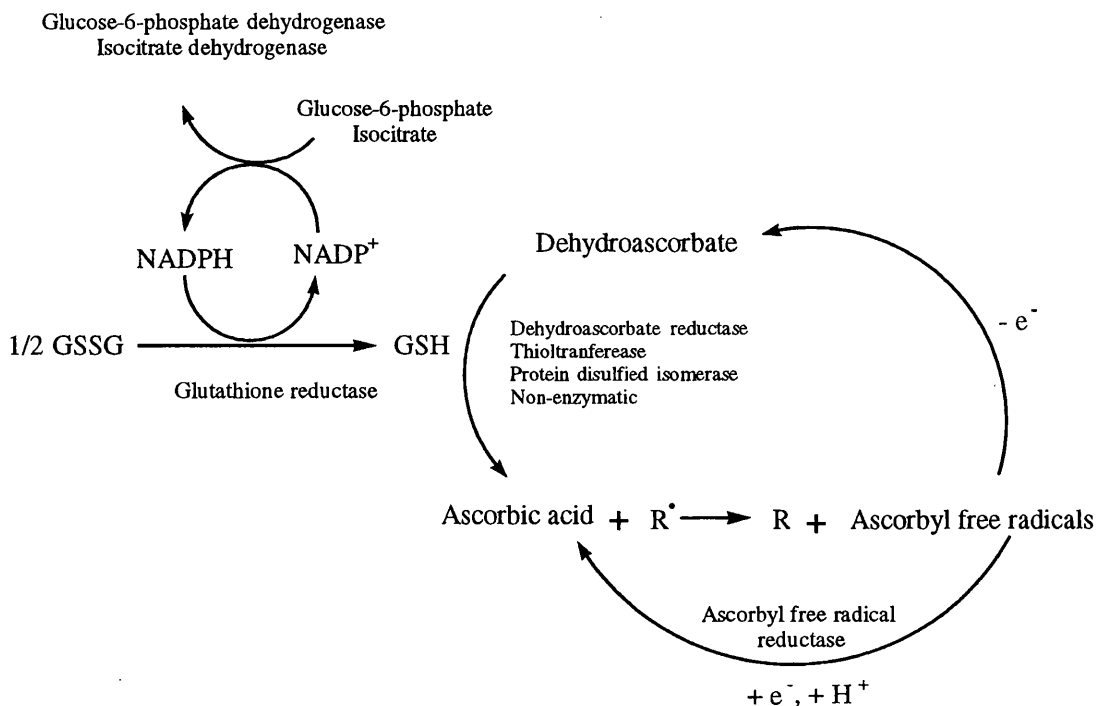
Figure 30: Proposed pathways for the regeneration of ascorbic acid.

A third explanation that cannot be rulled out, especially in type 1 diabetic patients, is an insulin-induced decrease in plasma vitamin C as has been reported by other works (Sherry & Ralli, 1942; Bigley & Stankova, 1974; Cunningham, 1988 & 1998; Winkler et al., 1994; Lindsay et al., 1998; Seghieri et al., 1998). The mechanism for this is uncertain but could involve acceletated tissue uptake or consumption. Further studies are needed to elucidate the relationship between insulin therapy and the metabolic factor(s) that regulates extracellular and intracellular vitamin C contents and the biological importance of these findings.

Reviewing published articles from 1990 to 2003 in which plasma ascorbic acid concentrations in diabetic and control subjects were compared. It was found that 4 articles focused on type 2 diabetes, one reported on type 1 diabetes and 2 articles on both type 1 and type 2 diabetes. In the case of type 2 diabetics, three studies showed an average reduction in ascorbic acid content of about 20% (Sundaram et al., 1996; Dyer et al., 1997; Skrha et al., 2003), two other studies reported 30-40% decreases (Sinclair et al., 1994; Maxwell et al., 1997a) while Srinivasan et al. (1997) observed a 64% reduction. Two principal studies have been conducted concerning the ascorbic acid in type 1 diabetic patients, reductions of 28% and 46%, respectively have been reported (Maxwell et al., 1997a; Seghieri et al., 1998). By contrast, no differences were seen by Skrha et al. (2003). Problems with previous studies have related principally to the numbers of patients studied and the heterogeneity of the populations concerned (for comprehensive review see Will & Byers, 1996).

The presence of PNP and/or CAN in type 1 diabetics was not found to be associated with any changes in ascorbic acid concentrations. This may indicate that the reduction seen in type 1 diabetic patients may occur before diabetic polyneuropathy develops. Regarding the data for type 2 diabetic subjects, it was not possible to draw any firm conclusions concerning the impact of neurological complications on ascorbic acid concentrations because patients with PNP-/CAN- had slightly higher vitamin C levels than control subjects. The observed differences in plasma ascorbic acid between patients with PNP-/CAN- and those with PNP+/CAN- or PNP+/CAN+ could not be explained on the basis of variations in demographic, clinical and biochemical factors. It is possible, however, that the observed increases in vitamin C levels in patients with PNP-/CAN- may reflect recent dietary vitamin C intake.

In conclusion, this study has shown that plasma ascorbic acid is lower in diabetic subjects and the levels appeared to be influenced by the type of diabetes. The presence of PNP and/or CAN did not appear to influence ascorbic acid levels in type 1 and type 2 diabetic patients. A potential observation from this study is the inverse association between plasma vitamin C concentration and total NIS-LL suggesting that this parameter may prove valuable tool in examining impact of therapeutic interventions on diabetic neuropathy.

5.1.3. Total antioxidant capacity
5.1.3.1. Background

A large body of evidence suggests that oxidative stress resulting from enhanced free-radical formation and/or reduced antioxidant defence are implicated in the pathogenesis of diabetic complications (Wolff et al., 1989; Baynes 1991; Kennedy & Lytons, 1997; Mercuri et al., 2000; Spector, 2000; Rosen et al., 2001). Attack by reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorinated species (RCS) on polyunsaturated fatty acids, proteins and DNA acids lead to the formation of various oxidatively modified species and the disruption of oxidant/antioxidant balance. Thus, oxidative stress can be assessed by the measurement of lipid peroxides, DNA-oxidation products, oxidatively modified proteins, antioxidant enzymes, nutrient antioxidants or total antioxidant capacity (TAC) (Halliwell, 1993; Jackson, 1999; Abuja & Albertini, 2001; Griffiths et al., 2002; Kohen & Nyska; 2002).

Various procedures have been employed for the assessment of plasma TAC (Lissi et al., 1995; Tubaro et al., 1998; Prior & Cao; 1999; Aldini et al., 2001; Gaboriau et al., 2002; Janaszewska & Bartosz, 2002; Schlesier et al., 2002). Of these techniques the total radical trapping parameter (TRAP) and total equivalent antioxidant capacity (TEAC) are the most frequently used. One major disadvantage with aforementioned methods for the assessment of plasma TAC is that these are end-point based and thus do not allow comparison of individual profiles. Another disadvantage is that they are not suited for routine screening of large numbers of samples.

In this study, three previously validated versions of the ABEL® (Analysis By Emitted Light) test, involving quenching of Pholasin chemiluminescence by peroxynitrite (ONOO),

superoxide anion (O_2^-) and hypochlorous acid (HOCl) , were utilised to assess the impact of type of diabetes with varying degrees of neurological complications on plasma total antioxidant capacity (TAC). Measurements were carried out in control subjects for comparison.

5.1.3.2. Findings
5.1.3.2.1. Quenching of peroxynitrite Pholasin® chemiluminescence (ONOO-QPC)

Figure 31 shows the data obtained for plasma TAC, measured by ONOO-QPC, in diabetic patients and control subjects. Diabetic patients had decreased plasma ONOO-QPC compared to control subjects [42.51 ± 0.80 min vs 49.86 ± 1.42 min; $P<0.05$]. Plasma ONOO-QPC was also found to be lower in diabetic patients with PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ than in control subjects [44.27 ± 1.33 min; 41.83 ± 1.14 min and 40.95 ± 2.81 min vs 49.86 ± 1.42 min; $p<0.05$].

ONOO-QPC values were similar in type 1 diabetic and type 2 diabetic patients whilst these were lower than in control subjects [43.17 ± 1.11 min and 42.16 ± 1.11 min and vs 49.86 ± 1.42 min; $p<0.05$]. Type 1 diabetics with PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ exhibited lower values for ONOO-QPC than in control subjects [43.79 ± 1.64 min, 44.35 ± 1.97 min, 37.50 ± 4.21 min vs 49.86 ± 1.42 min; $p<0.05$]. Decreases in plasma ONOO-QPC were also recorded in type 2 diabetic patients with PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ compared to control subjects [44.60 ± 2.24 min, 41.13 ± 1.34 min and 43.08 ± 3.71 min vs 49.86 ± 1.42; $P<0.05$].

5.1.3.2.2. Quenching of superoxide anion Pholasin® chemiluminescence ($O_2^{\cdot-}$-QPC)

Lower plasma $O_2^{\cdot-}$-QPC was observed in diabetic patients than in control subjects [4.05 ± 0.22 mV vs 2.59 ± 0.23 mV; $p<0.05$]. Plasma $O_2^{\cdot-}$-QPC values were diminished in patients with PNP-/CAN-, PNP+/CAN- or PNP+/CAN+ compared to control subjects [3.31 ± 0.34 mV, 4.28 ± 0.31 mV and 5.01 ± 0.68 mV vs 2.59 ± 0.23 mV; $p<0.05$].

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Type 1 diabetic patients had lower plasma O_2^--QPC when compared to type 2 diabetics and in both cases O_2^--QPC were lower than in control subjects [3.61±0.32 mV and 4.28±0.29 mV vs 2.9±0.23 mV; p<0.05]. Reduced plasma O_2^--QPC was recorded in type 1 diabetic patients with PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ compared to control subjects [3.15±0.43 mV, 3.62±0.50 mV and 5.40±0.94 mV vs 2.59±0.23 mV]. Moreover, diminished plasma O_2^--QPC was recorded in all three sub-groups of type 2 diabetic subjects i.e. PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ versus control subjects [3.56±0.56 mV, 4.45±0.37 mV and 4.78±0.97 mV vs 2.59±0.23 mV]. Figure 32 presents the data obtained for plasma TAC, measured by O_2^--QPC, in diabetic patients and control subjects.

5.1.3.2.3. Hypochlorous acid Pholasin® chemiluminescence (HOCl-QPC)

Figure 33 displays the data for plasma TAC assessed by HOCl-QPC in diabetic patients and control subjects. No differences were observed in plasma HOCl-QPC either between diabetic patients as a whole and control subjects [3.62±0.18 mV vs 3.52±0.30 mV] or patients subgroups with PNP-/CAN-, PNP+/CAN- or PNP+/CAN+ and control subjects [3.27±0.34 mV, 3.69±0.27 mV and 4.12±0.28 mV vs 3.52±0.30 mV, respectively]. When diabetic patients were divided according to type of diabetes no differences in plasma HOCl-QPC were seen between type 1 and type 2 diabetic patients and control subjects [3.35±0.30 mV and 3.77±0.24 mV vs 3.52±0.30 mV]. In addition, no differences were seen in HOCl-QPC between PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ in either type 1 or type 2 diabetics and control subjects [type 1 diabetics: 3.01±0.47 mV, 3.39±0.45 mV and 4.44±0.52 mV vs 3.52±0.30 mV; type 2 diabetics: 3.60±0.50 mV, 3.77±0.32 mV and 4.09±0.32 mV vs 3.52±0.30 mV].

5.1.3.2.4. Correlations

Strong correlations were found between TAC as assessed by ONOO-QPC and O_2^--QPC in diabetic patients and control subjects [$r_{diabetics}$=- 0.52; P<0.001 and $r_{controls}$=-0.67; P<0.001, Figures 34 and 35]. A positive correlation between ONOO-QPC and plasma uric acid content was seen in diabetic patients [$r_{uric\ acid}$ =0.27; P<0.005, Figure 36]. There were moderate associations between TAC as measured by ONOO-QPC or O_2^--QPC and total neurological impairment score (NIS-LL) [$r_{ONOO-QPC}$=-0.168; p<0.05 and r_{O2^-}-QPC=0.209; p<0.01, Figures 37 and 38].
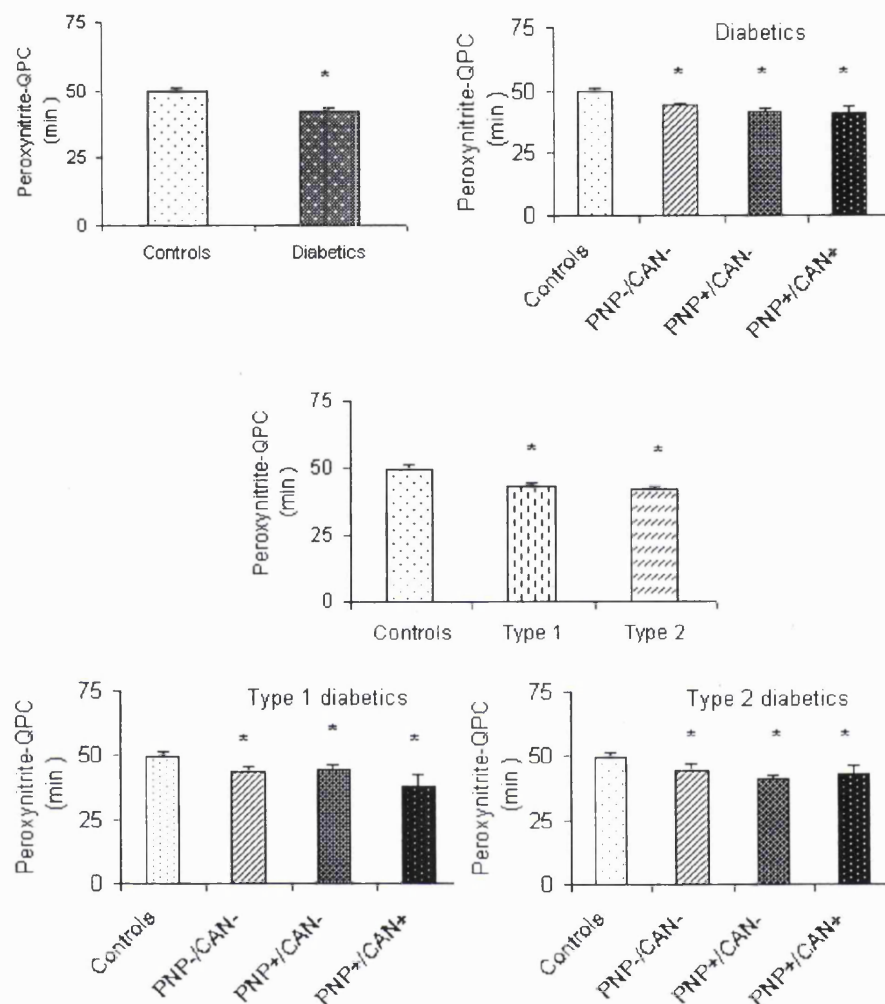
Figure 31: Total antioxidant capacity as measured by quenching of peroxynitrite Pholasin® chemiluminscence (ONOO-QPC) in diabetic patients and control subjects. Plasma samples (5 μl) were transferred into microcuvettes and 100 μl Pholasin® (1.7 μg/ml) added. The reactions were initiated by adding SIN-1 (2 μl of 2 mg/ml) and signal continuously monitored at 5 min intervals. Data represents mean±SEM.

* Control subjects versus diabetic as a whole or diabetic subgroups; $p < 0.05$.

Data are shown as scatter plots (Appendix; Figures 73-77).
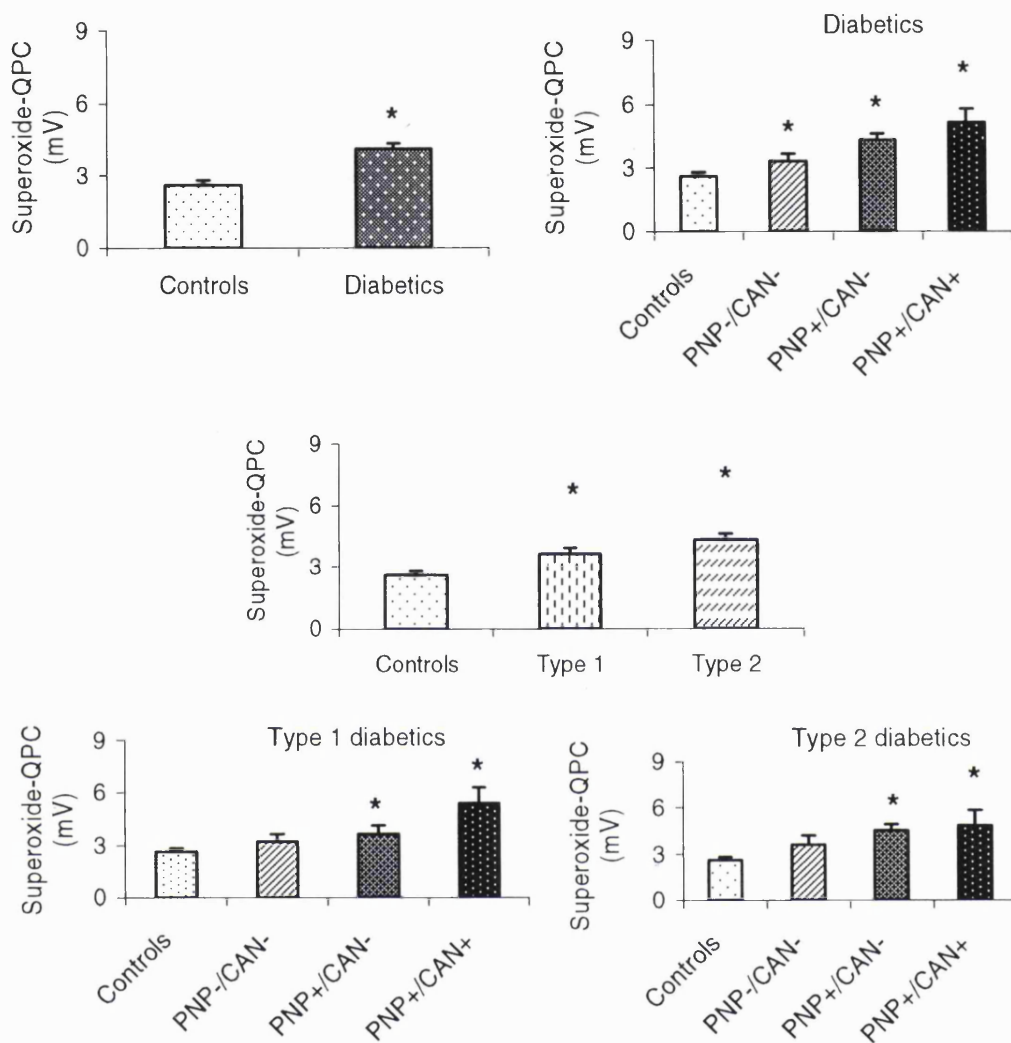
Figure 32: Total antioxidant capacity as measured by quenching of superoxide anion Pholasin® chemiluminscence (O_2^--QPC) in diabetic patients and control subjects. Phosphate buffer (100 µl) containing Pholasin® (1.7 µg/ml) and adjuvant K (50 µl/ml) were transferred into microcuvettes and subsequently xanthine (5 µl; 50 mM) and plasma samples (5 µl) were added. The reactions were initiated by adding 20 µl of xanthine oxidase (0.5 U/ml) and the signal read over 5 min. Results represents mean±SEM.

* Control subjects versus diabetic as a whole or diabetic subgroups; $p < 0.05$.

Data are presented as scatter plots (Appendix; Figures 78-82).

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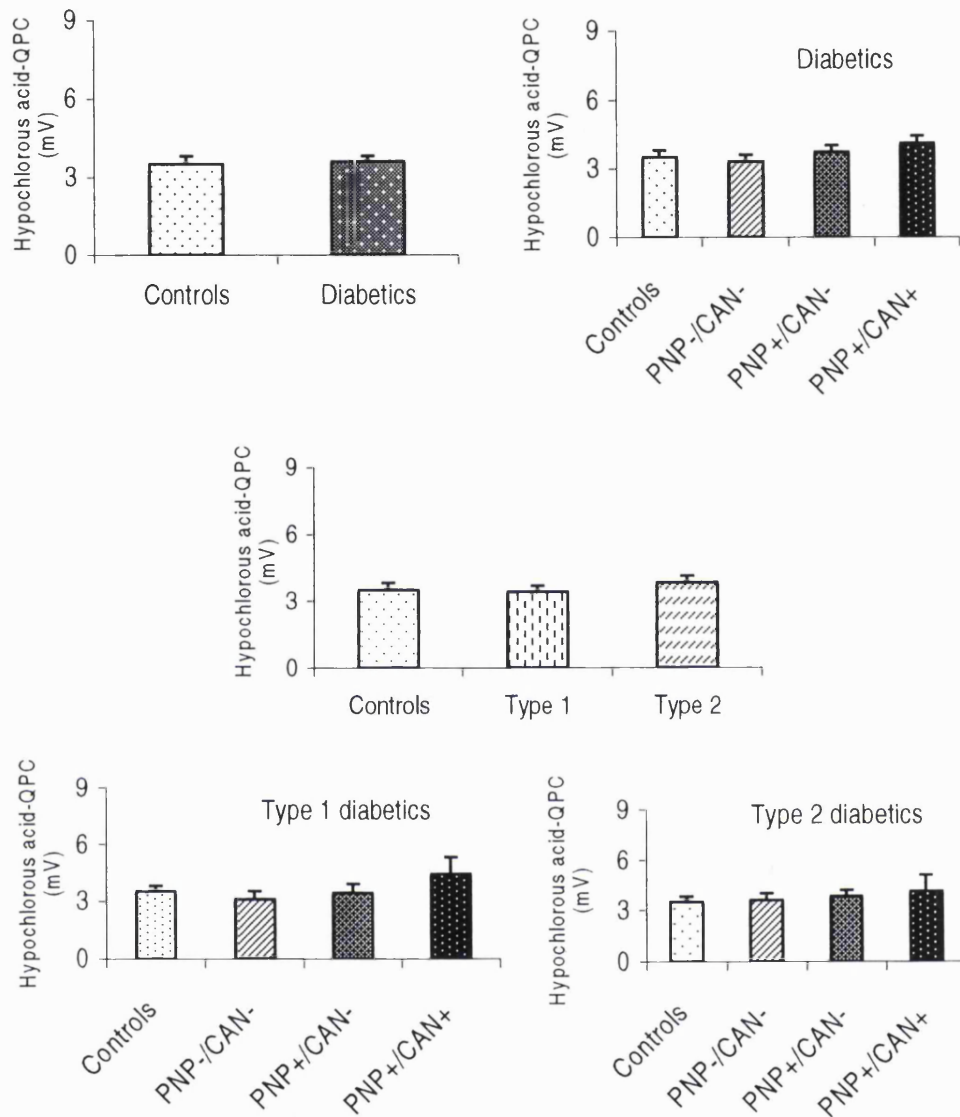
Figure 33: Plasma total antioxidant capacity as measured by quenching of hypochlorous acid Pholasin® chemiluminscence (HOCl-QPC) in diabetic patients and control subjects. Phosphate buffer (100 µl) containing Pholasin® (1.7 µg/ml) and adjuvant K (50 µl/ml) were transferred into microcuvettes. The reactions were initiated by adding 20 µl of chloroamine-T (44 mM in water) and the signal monitored over one min. Values are expressed as mean±SEM.

* Control subjects vesus diabetic as a whole or diabetic subgroups; p<0.05.

Data are shown as scatter plots (Appendix; Figures 83-87).
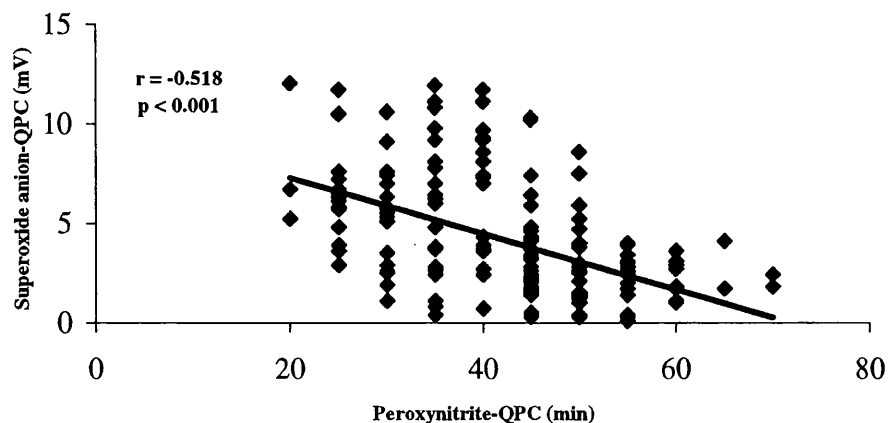
Figure 34: Correlation between plasma total antioxidant capacity assessed by quenching of peroxynitrite Pholasin® chemiluminscence (peroxynitrite-QPC) and by quenching of superoxide anion Pholasin® chemiluminscence (superoxide anion-QPC) in diabetic patients.
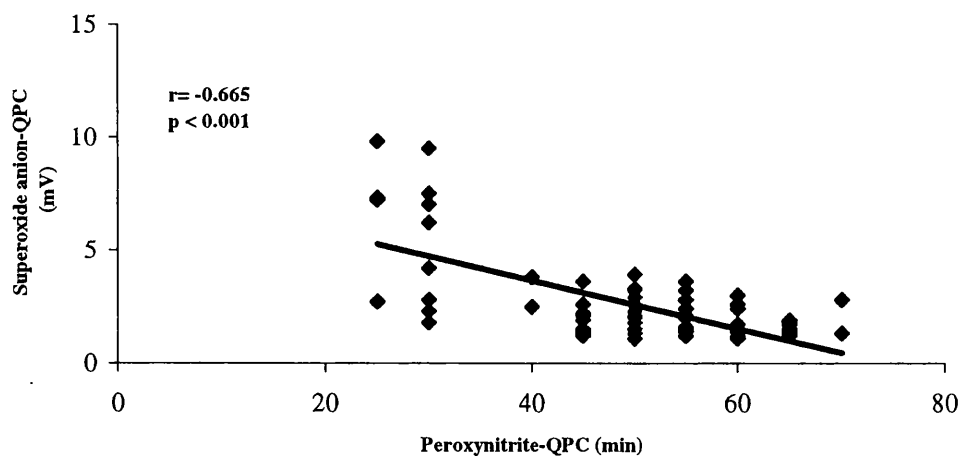
Figure 35: Association between plasma total antioxidant capacity assessed by quenching of peroxynitrite Pholasin® chemiluminscence (peroxynitrite-QPC) and by quenching of superoxide anion Pholasin® chemiluminscence (superoxide anion-QPC) in control subjects.
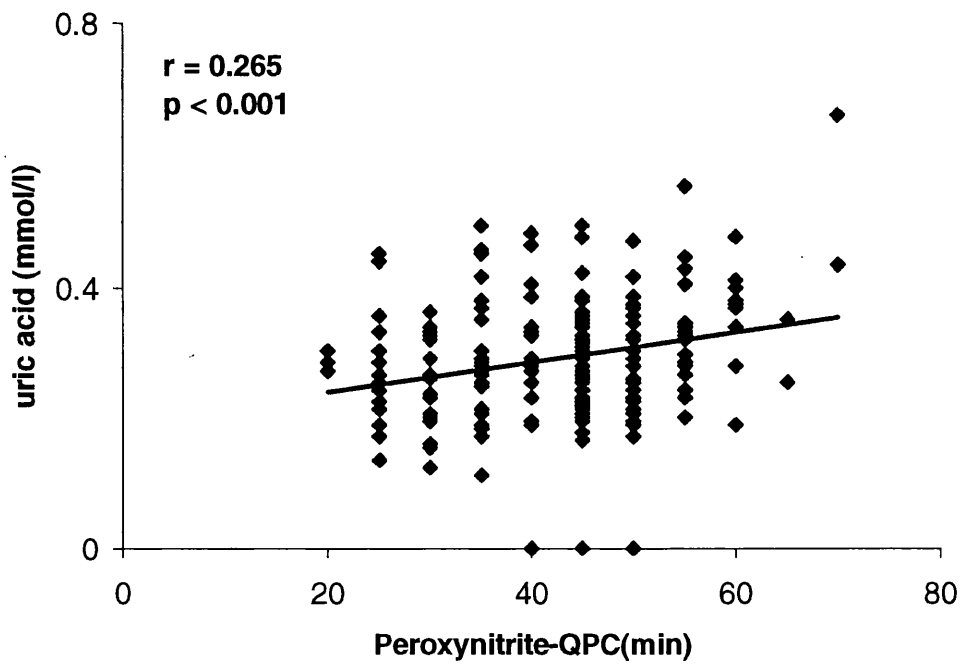
Figure 36: Relationship between plasma total antioxidant capacity assessed by quenching of peroxynitrite Pholasin® chemilumenescence (peroxynitrite-QPC) and plasma uric acid levels in diabetic patients.
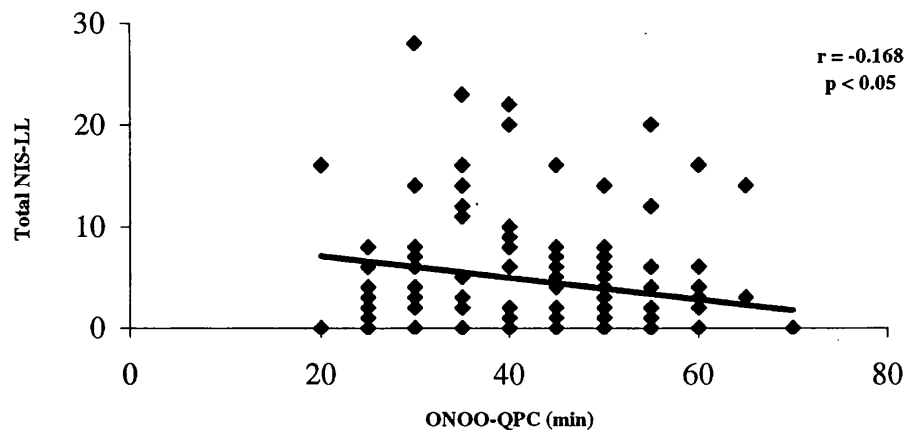
Figure 37: Relationship between plasma total antioxidant capacity assessed by quenching of peroxynitrite Pholasin® chemilumenescence (peroxynitrite-QPC) and total neurological impairment score-lower limb (NIS-LL). Diagnosis and grading of diabetic neuropathy were carried out according to the criteria published by Dyck et al. (1993 & 1997).
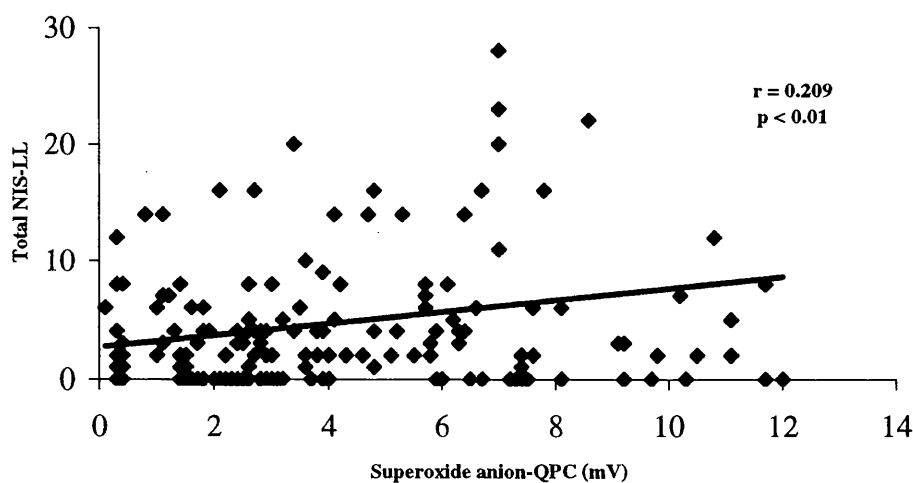
Figure 38: Association between plasma total antioxidant capacity assessed by quenching of superoxide anion Pholasin® chemilumenescence (superoxide anion-QPC) and total neurological impairment score-lower limb (NIS-LL). Diagnosis and grading of diabetic neuropathy were carried out according to the criteria published by Dyck et al. (1993 & 1997).

5.1.3.3. Discussion

The TAC is believed to reflect the combination of all the antioxidants present in a particular body fluid. It is, therefore, considered to be a more accurate representation of antioxidant status than that provided by the determination of individual antioxidant components. In this study, TAC was assessed using three previously validated versions of the ABEL® assay. The ABEL® assays are based on the capacity of a sample to scavenge free radicals and oxidants in the presence of the photoprotein Pholasin®, a luciferin type molecule found in the bioluminescent mollusc *Pholas dactylus*. Pholasin® emits light in the presence of different systems capable of generating free radicals (Knight et al., 2002).

Hence, if a sample has already been exposed to free radicals and/or prooxidants this will lead to a reduction in its complement of antioxidants and a reduced capacity to deal with the prooxidants generated in the assay. Evaluation of plasma TAC, measured by ONOO-QPC, is based on the measurement of the length of time Pholasin® chemiluminescence is inhibited. The higher, therefore, TAC the longer the lag-time until a maximum signal is reached. In the case of O_2^--QPC and HOCl-QPC, the assays are based on the measurement of the maximum amount of light emitted. Thus, the higher the free radical scavenging activity of a plasma sample the lower the signal is at maximum light emission.

An important observation from the study is that plasma total antioxidant capacity was found to be lower in diabetic patients than control subjects. The greatest differences in total antioxidant capacity between diabetics and control was achieved using ONOO-QPC or O_2^--QPC the decreases for ONOO-QPC and O_2^--QPC being 15% and 36%, respectively. There were significant relationships between plasma ONOO-QPC and O_2^--QPC in both diabetic patients and control subjects (Figures 34 and 35) suggesting that TAC, as measured by

ONOO-QPC, and O_2^--QPC are inter-related and could be used as surrogates. This is of interest from a practical point of view since the O_2^--QPC reaction is rapid, normally completed within 3 minutes whilst the ONOO-QPC assay is more time consuming, the analyses time being of the order of 30-70 min. Another potentially important observation was that there were moderate association between measures of TAC i.e. ONOO-QPC and O_2^--QPC and total NIS-LL suggesting that these markers are sensitive indicators of enhanced oxidative stress in diabetic patients with polyneuropathy. Also, these findings provide first evidence in support of the hypothesis that oxidative stress may be relevant in the pathogenesis of diabetic neuropathies.

In type 1 diabetic patients plasma O_2^--QPC were 16% lower than in type 2 diabetics, whilst the value for plasma ONOO-QPC was reduced by 10%. There were no significant differences in TAC, assessed by ONOO-QPC and O_2^--QPC, in type 1 diabetic patients with PNP-/CAN- and PNP+/CAN-. The additional presence of CAN was, however, associated with marked reductions in TAC, the values for ONOO-QPC and O_2^--QPC being reduced by 15% and 42%, respectively, compared with PNP-/CAN-. In the case of type 2 diabetic subjects, lower, TAC as assessed by ONOO-QPC and O_2^--QPC, were observed in patients with PNP+/CAN- compared to those with PNP-/CAN-. The additional presence of CAN was associated with a further reduction in O_2^- QPC only.

Previous studies using enhanced chemiluminescence enhanced assay have shown that uric acid accounts for up to 75% of plasma TAC (Wayner et al., 1987; Maxwell et al., 1997b; Ryan et al. 1997). Uric acid is a water-soluble antioxidant and in vitro acts as a potent scavenger of hydroxyl radicals, nitric oxide and peroxynitritie. In this investigation, a weak but significant correlation was seen between ONOO-QPC and plasma uric acid content in

diabetic subjects (Figure 36). The following observations, however, argue against endogenous uric acid influencing the assessment of TAC employing the ABEL® assays: 1) diminished TAC in diabetic patients with PNP-/CAN-, PNP+/CAN- or PNP+/CAN+ was not related to plasma uric acid concentration; and 2) the absence of major differences in TAC between type 1 and type 2 diabetic patients despite there being 20% less uric acid in the former.

In accordance with the findings of this investigation, reduced TRAP values have been reported in well-controlled type 1 diabetics without evident complications (Santini et al., 1997). Similarly, Vessby et al. (2002) have reported diminished plasma total antioxidant potential in type 1 diabetic patients compared to control subjects together with a direct correlation between TRAP values and plasma uric acid content. Valabhji et al. (2001) reported TEAC values in type 1 diabetic subjects with strong inverse correlations between TEAC and HbA1c or duration of diabetes. Moreover, a positive correlation was found between TEAC values and the presence of coronary artery calcification. Rocic et al. (1997) have reported that TRAP values are reduced in type 1 diabetic patients with positive islet cell antibodies when compared to both patients with negative islet cell antibodies and control subjects. TRAP values, however, were not influenced by gender, age and smoking-habits.

Using the TRAP assay, Ceriello et al. (1998) have shown that plasma antioxidant capacity is decreased in type 2 diabetic patients following acute hyperglycaemia. Opara et al. (1999) reported reduced TEAC in type 2 diabetic subjects with and without proteinuria although the reductions were greatest for the diabetic patients with proteinuria. Aguirre et al. (1998) have reported reduced plasma TRAP in type 2 diabetic patients together with a positive

association with uric acid and inverse correlations with fructosamine and glucose. Using the oxygen radical absorbance capacity (ORAC) assay, Pieri et al. (2001) reported that total antioxidant capacity is lower in type 2 diabetic patients and that an inverse association existed between ORAC and both HbA1c and age.

In conclusion, this study demonstrates that the ONOO-QPC and O_2^--QPC versions of the ABEL® assays are superior to the HOCl-QPC assay in assessing plasma TAC. This study also reveals that TAC is lower in diabetic patients and appears to be influenced by type of diabetes. The presence of PNP alone in type 1 diabetics did not have an impact on TAC while the additional presence of CAN was associated with a marked reduction in TAC. These markers may prove valuable tools in evaluating the impact of drugs or antioxidant interventions on TAC.

5.1.4. F$_2$-isoprostanes
5.1.4.1. Background

The accumulated data suggest that diabetes is associated with increased levels of free radicals and oxidative stress (Halliwell, 2002; Evans et al., 2003). One of the primary targets of free radicals in the human body is the polyunsaturated fatty acids e.g. arachidonic acid. Various methods have been employed for examination of lipid peroxidation products derived from arachidonic acid. These include measurement of conjugated-dienes (CD), fatty acid hydroperoxides, hydroxylated fatty acids, exhaled short chain alkanes and aldehydes. Of these methods, the assessment of CD and MDA are the most widely used due to their simplicity. These techniques, however, may be associated with problems relating to specificity and sensitivity (de Zwart et al., 1999).

A major step forward regarding the measurement of lipid peroxidation products came with the discovery of the isoprostanes (Morrow et al., 1990ab). The isoprostanes are a family of prostaglandin (PG)-like compounds produced by non-enzymatic peroxidation of polyunsaturated fatty acids (Roberts & Morrow; 2002; Nourooz-Zadeh et al., 1997b & 1998). Of these, 9α-11α-8-epi-PGF$_{2\alpha}$, (8-epi-PGF$_{2\alpha}$) has received the most attention because it is specifically derived from arachidonic acid (Marangon et al., 1999; Voutilainen et al., 1999; Wood et al., 2000; Dogra et al., 2001; Salahudeen et al., 2001; Weinberg et al. 2001; Klings et al., 2001; Cracowski et al., 2001abc; Dietrich et al., 2002; O'Byrne et al., 2002; Hodgson et al., 2002; Ikizler et al., 2002; Sinha et al., 2003). Circulating 8-epi-PGF$_{2\alpha}$ is mainly found bond to phospholipids and is released by the action of phospholipase A$_2$ (Morrow et al. 1992a; Kayganich-Harrison 1993). Figure 39 show structural differences between cyclooxygenase-derived PGF$_{2\alpha}$ and 8-epi-PGF$_{2\alpha}$.
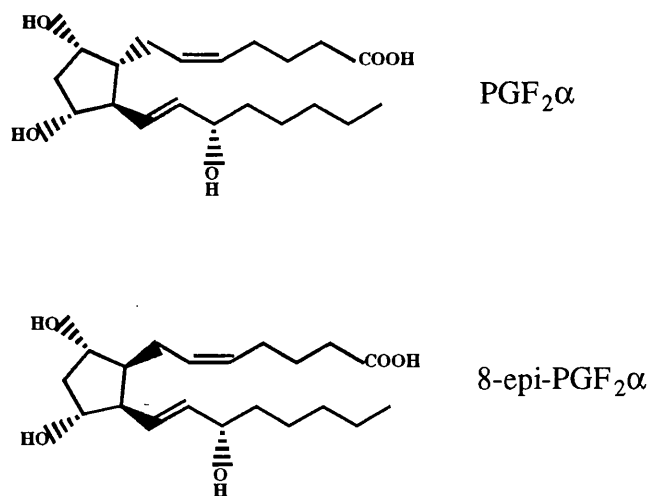
PGF$_2\alpha$

8-epi-PGF$_2\alpha$

Figure 39: Structural differences between enzymatic and non-enzymatic derived PGF$_2$-like compounds.

8-epi-PGF$_{2\alpha}$ is not only a specific marker of lipid peroxidation but also produces a wide range of biological effects. In rabbits, during the systemic infusion with 8-epi-PGF$_{2\alpha}$, renal blood flow and glomerular filtration were found to be reduced by 40-50% whilst systemic blood flow remained unchanged, indicating that the vasoactive actions of 8-epi-PGF$_{2\alpha}$ are selective for the renal vasculature. The renal effects of 8-epi-PGF$_{2\alpha}$ were abolished by the thromboxane receptor antagonist SQ29548, suggesting an action on the thromboxane receptor (Morrow et al., 1992; Takahashi et al., 1992; Fukunaga et al., 1993). In addition to its renal effects, 8-epi- PGF$_{2\alpha}$ has also been shown to be a potent constrictor of the pulmonary artery in rabbits and rats, and a bronchoconstrictor in the rat lung (Banerjee et al., 1992; Kang et al., 1993; Jourdan et al., 1997). It is also influences platelet function inducing shape change and reversible aggregation, which are inhibited by a thromboxane A$_2$/prostaglandin H$_2$ receptor blockade (Morrow et al., 1992b; Cranshaw et al., 2001).

Information on plasma 8-epi-PGF$_{2\alpha}$ in diabetic patients is rather limited. Gopaul et al. (1995 & 2001) reported a 3-fold increase in plasma total 8-epi-PGF$_{2\alpha}$ from poorly controlled type 2 diabetic patients compared to control subjects. Similarly, Handelman et al. (2001) reported 6-fold higher levels of plasma total 8-epi-PGF$_{2\alpha}$ in type 2 diabetic patients with end-stage renal failure.

The aim of the current study was to investigate the impact made by type of diabetes and its associated neurological complications on plasma total (sum of free and esterified) 8-epi-PGF$_{2\alpha}$ concentration in comparison with healthy controls.

5.1.4.2. Findings

Figure 40 displays the data obtained for plasma 8-epi-PGF$_{2\alpha}$ in diabetic patients and control subjects. These data reveal that 8-epi-PGF$_{2\alpha}$ levels were slightly but significantly higher in diabetic patients relative to control subjects [1.01±0.05 nmo/L vs 0.92±0.08 nmol/L]. When diabetic patients were grouped according to the presence or absence of polyneuropathy (PNP) and/or cardiovascular autonomic neuropathy (CAN), patients with PNP+/CAN- and control subjects exhibited values for 8-epi-PGF$_{2\alpha}$ of 1.04±0.08 nmol/L and 0.92±0.08 nmol/L, respectively. By contrast, 8-epi-PGF$_{2\alpha}$ concentrations were lower in diabetic patients with PNP-/CAN- and PNP+/CAN+ than in control subjects [0.81±0.08 nmol/L and 0.73±0.08 nmol/L vs 0.92±0.08 nmol/L].

Type 1 diabetic patients had lower 8-epi-PGF$_{2\alpha}$ levels compared to control subjects [0.78±0.06 nmol/L vs 0.92±0.08 nmol/L]. Type 2 diabetic patients, on the other hand, yielded a value for plasma 8-epi-PGF$_{2\alpha}$ of 1.01±0.08 nmol/L. When type 1 diabetics were subdivided according to the presence or absence of PNP and/or CAN-, the subgroups PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ were all found to have lower 8-epi-PGF$_{2\alpha}$ levels than control subjects. The largest decreases in 8-epi-PGF$_{2\alpha}$ levels were seen in patients with PNP-/CAN- relative to control subjects [0.71±0.08 nmol/L vs 0.92±0.08 nmol/L]. Type 1 diabetics with PNP+/CAN- and PNP+/CAN+ exhibited values for 8-epi-PGF$_{2\alpha}$ of 0.90±0.17 nmol/L and 0.87±0.11 nmol/L, respectively.

In type 2 diabetes, patients with PNP+/CAN- had higher 8-epi-PGF$_{2\alpha}$ concentrations compared to control subjects [1.09±0.11 nmol/L vs 0.92±0.08 nmol/L], whereas variations were not seen in patients with PNP-/CAN- [0.95±0.14 nmol/L vs 0.92±0.08 nmol/L].

Contrastingly, patients with PNP+/CAN+ had lower plasma 8-epi-$PGF_{2\alpha}$ levels than control

subjects [0.67±0.08 nmol/L vs 0.92±0.08 nmol/L].

There was a weak association between 8-epi-$PGF_{2\alpha}$ levels and plasma total cholesterol

[$r_{total\ cholesterol}$=0.177; $P<0.05$] whereas no correlations were seen between 8-epi-$PGF_{2\alpha}$

concentrations and age, BMI, duration of diabetes, glucose, HbA1c, LDL, HDL or TG.

Similar relationships were also absent in controls.
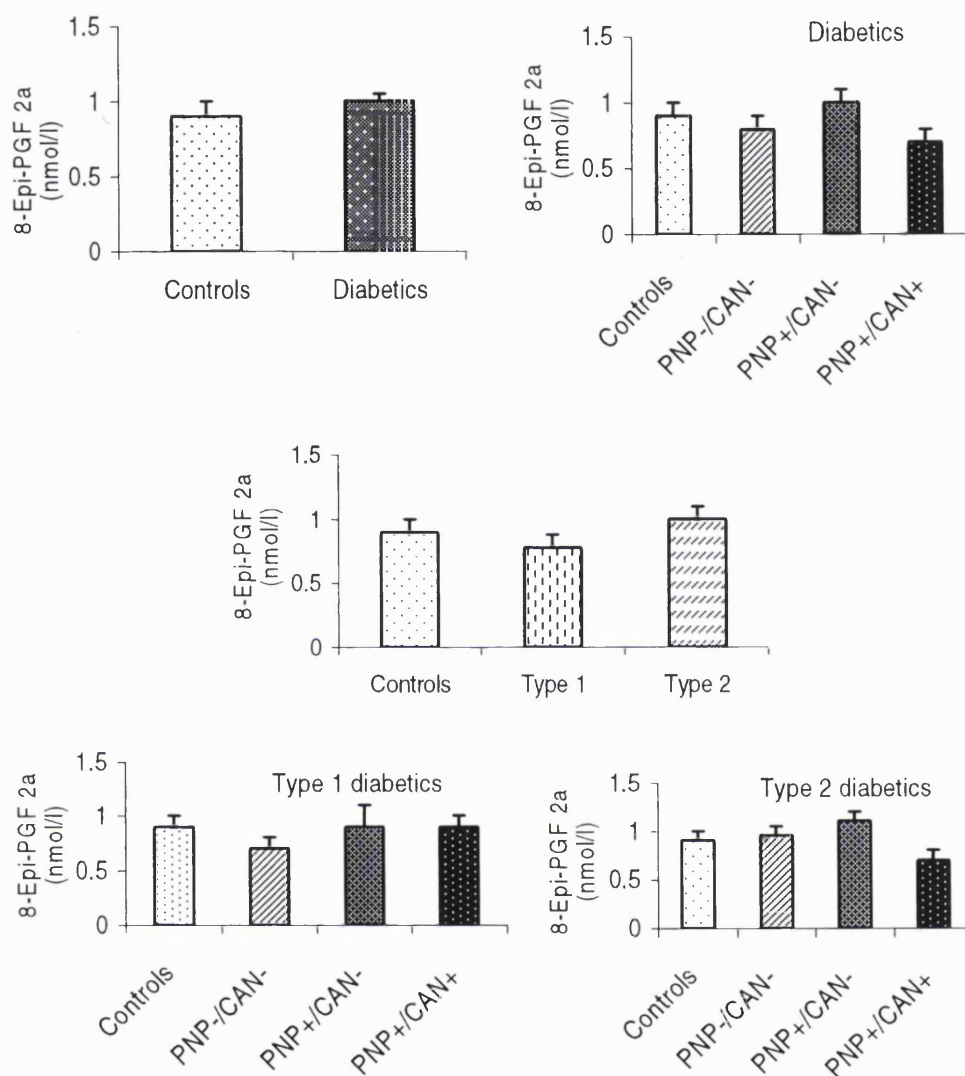
Figure 40: Plasma 8-epi-PGF$_{2\alpha}$ levels in diabetic patients and control subjects. Plasma total lipids were extracted with ethyl acetate and isoprostanes were isolated by chromatography on NH$_2$-cartridge. Isoprostanes were converted to TMS ether/PFB esters and analysed by GC-MS/NICI. Data represent mean±SEM. Data are illustrated as scatter plots (Appendix; Figures 88-92).

5.1.4.3. Discussion

A possible drawback with the measurement of plasma F_2-isoprostanes is that ex vivo generation of these species during sample handling and/or prolonged storage may occur. Thus, precautions must be taken to minimise artefactual F_2-isoprostane formation. Morrow et al. (1990a) was the first to draw attention to the fact that F_2-isoprostanes may be generated in plasma as a consequence of oxidative degradation occurring during prolonged and/or improper storage. It was reported that in freshly prepared plasma containing 14.5-115.8 pmol/L of free (non-esterifed) F_2-isoprostanes that concentrations rose to 2.89-11.57 nmol/l after storage at $-20°C$ for six months. The inclusion of the chain-breaking antioxidant butylated hydroxytoluene (BHT) at a final concentration of 25 µmol/L to the plasma samples, combined with storage at $-80°C$, was found to inhibit artefactual F_2-isoprostane formation without suppressing the F_2-isoprostane levels originally present.

Gopaul et al. (1995) established a protocol for maximising assay efficiency following an evaluation of the effects of sample handling and/or storage conditions on plasma total 8-epi-$PGF_{2\alpha}$ levels. The protocol involved the collection of blood samples into lithium heparin followed by immediate plasma separation at room temperature, the addition of BHT (25 µmol/L) and the storage of samples at $-70°C$ for a maximum of two months prior to the isolation of F_2-isoprostanes by solid-phase extraction and determination by GC-MS/NICI. This protocol was followed in the present study.

It was found in this study that the mean plasma total 8-epi-$PGF_{2\alpha}$ concentrations in healthy German control subjects were $0.96±0.72$ nmol/L. As illustrated in Figure 41, these values were in good agreement with previously reported data (Morrow et al, 1995; Mori et al., 1999; Gopaul et al., 2000 & 2001; Handelman, et al., 2001). Also, the range of values i.e.

0.58-2.31 nmol/L for plasma 8-epi-$PGF_{2\alpha}$ observed in this investigation were comparable with those reported previously; 0.78-1.07 nmol/L, 0.72-1.07 nmol/L and 0.29-1.74 nmol/L (Mori et al., 1999; Gopaul et al., 2000 & 2001; Handelman et al., 2001). These data taken together suggest that the large degree of variations in plasma 8-epi-$PGF_{2\alpha}$ in the present investigation may truly reflect variation in oxidative status in control subjects.
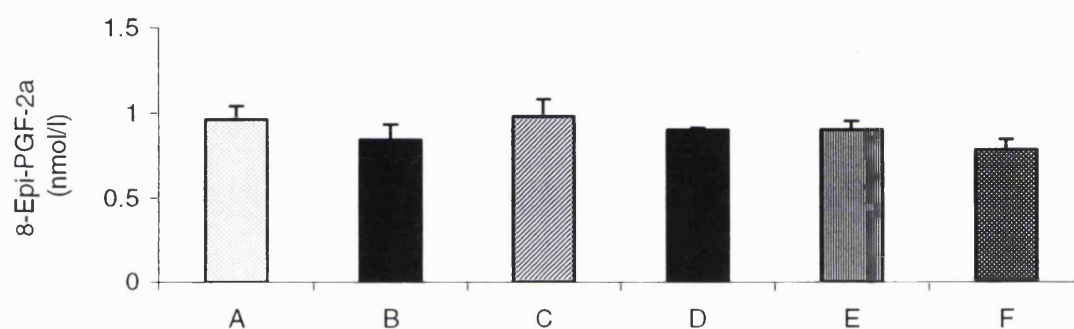
Figure 41 : Data obtained by different research groups on plasma 8-epi-$PGF_{2\alpha}$ concentrations in control subjects. A) Present study (n=71); B) Morrow et al., 1995 (n=8); C) Gopaul et al., 2000 (n=9); D) Gopaul et al., 2001 (n=39); E) Mori et al., 1999 (n=10) and F) Handelman et al., 2001 (n=23). Data represent mean±SEM.

Diabetic patients in the study exhibited 8-epi-$PGF_{2\alpha}$ levels that were 11% higher than control subjects. This value is considerably lower than the 60-70% reduction previously reported for type 2 diabetic patients (Gopaul et al., 1995 & 2001; Handelman et al., 2001). This investigation has involved both type 1 and type 2 diabetic patients and over 64% of the type 2 diabetic patients were receiving insulin. Hence, the differences regarding the patient groups examined and their treatments may account for the discrepancies in plasma 8-epi-$PGF_{2\alpha}$ levels observed between the present and previous studies

An interesting finding from this investigation is that plasma 8-epi-PGF$_{2\alpha}$ concentrations were 15% lower in type 1 diabetic patients than in type 2 patients. This may be related to the fact that hyperglycaemic control was better in type 1 diabetic patients than in type 2 diabetics (HbA1c 8.5% vs 9.9%). Hyperglycaemia may lead to increased free radical generation as a result of glucose auto-oxidation and subsequent formation of advanced glycation end products (AGEs), disruption of the polyol pathway, altered eicosanoid metabolism and reduction in antioxidant levels. The free radicals produced may then attack primary endogenous targets such as arachidonic acid leading to the formation of 8-epi PGF$_{2\alpha}$.

In accordance with the findings of this investigation, there is evidence that improvement in metabolic control is associated with a lowering in the amount of circulating lipid peroxidation products. Faure et al. (1993) studied the effect of insulin therapy on plasma lipid peroxidation. A significant reduction was found in the plasma concentrations of both TBARs and LOOHs (21% and 12%, respectively) after 14 days of insulin treatment. Berg et al. (1998) examined the effect of intensified-insulin treatment (CIIT) on plasma LOOHs in type 1 diabetic patients. Plasma LOOHs in the patients was found to fall by 31% over a 24-month period. The corresponding reduction in HbAc1 levels was 15%. Davi et al. (1999) have shown that improvement in metabolic control is associated with a marked reduction (32%) in urinary 8-epi-PGF$_{2\alpha}$ excretion. 8-epi-PGF$_{2\alpha}$ levels were found to be higher in type 1 diabetics with poor glycaemic control than in patients with good control (Hoeldtke et al., 2002 & 2003). Taken together these data lend support to the theory that hyperglycaemia is an important factor in the generation of endogenous lipid peroxides.

Another finding of the present study was that the presence of PNP in type 1 diabetic patients was associated with a 22% increased in 8-epi $PGF_{2\alpha}$ levels whilst the additional presence of CAN was without influence. The presence of PNP in type 2 diabetic subjects was also associated with increased 8-epi-$PGF_{2\alpha}$ formation. However, contrasting with the situation in type 1 patients, the additional presence of CAN was found to be linked with marked reductions in 8-epi $PGF_{2\alpha}$ levels. The decrease in plasma 8-epi $PGF_{2\alpha}$ concentrations observed in type 2 diabetic patients with PNP+/CAN+ may be related to insulin-treatment since this group had the highest degree of individuals on insulin-treatment i.e. 85% as compared with 48.3% and 65% for patients with PNP+/CAN- and PNP-/CAN-, respectively. It is of note that type 2 diabetic subjects with PNP+/CAN+ differed from those with PNP+/CAN- or PNP-/CAN- by exhibiting increases in vitamins E. Thus, reduced lipid peroxidation in these individuals may be related to improved antioxidant status and/or insulin treatment.

The current study has raised a number of concerns about the measurement of plasma 8-epi- $PGF_{2\alpha}$ as an accurate index of oxidative stress in clinical conditions associated with oxidative stress. First, there is a large degree of variance in plasma 8-epi-$PGF_{2\alpha}$ among both the diabetic and control groups. This appears not to be related to the method employed assay since inter and intra-assay co-efficient of variation for the assay were 5% and 8% and the mean values for diabetic patients and control subjects were in good agreement with previously reported data (Mori et al., 1999; Gopaul et al., 2000; Dogra et al., 2001; Handelman, et al., 2001). Second, the differences in plasma 8-epi-$PGF_{2\alpha}$ levels between diabetic patients and control subjects were borderline, contrasting with data reported elsewhere (Gopual et al., 1995 & 2001; Handelman et al., 2001). The matching of diabetic patients and control subjects and difference regarding inclusion criteria i.e. demographic,

clinical and biochemical factors may have also contributed to the differences observed between the current and previous studies.

In conclusion, the study demonstrates that plasma total (sum of free and esterified) 8-epi-$PGF_{2\alpha}$ concentrations are slightly higher in diabetic patients compared to control subjects, with insulin-treatment possibly influencing its levels. The presence of PNP in both type 1 and type 2 diabetics is associated with increased 8-epi-$PGF_{2\alpha}$ formation. The additional presence of CAN, however, did not appear to influence circulating plasma 8-epi-$PGF_{2\alpha}$ levels.

5.2. Urine
5.2.1. F_2-isoprostanes
5.2.1.1. Background

A body of evidence suggests that plasma 8-epi-$PGF_{2\alpha}$ concentrations are increased in conditions associated with oxidative stress and that treatment with antioxidants reduces its levels (Morrow et al., 1995; Collins et al., 1999; Voutilainen et al., 1999; Ames et al., 1998 & 1999; Basu et al., 2001; Klings et al., 2001; Dietrich et al., 2002; Sampson et al., 2002). A number of disadvantages, however, are associated with the measurement of plasma 8-epi-PGF_2. 8-Epi-$PGF_{2\alpha}$ is rapidly cleared from the circulation (about 16 min) indicating that its quantification in plasma will only provide information regarding a discrete point in time (Morrow et al., 1990a). As a result, if large changes occur in the production of lipid peroxides over a particular period the levels of 8-epi-$PGF_{2\alpha}$ measured in a single sample of blood will not provide an accurate integrated assessment of oxidative stress.

Artefactual generation of 8-epi-$PGF_{2\alpha}$ can occur with improper sample handling and/or prolonged storage leading to spurious results. The measurement of urinary 8-epi-$PGF_{2\alpha}$ has, therefore, been proposed as being superior to the measurement of circulating 8-epi-$PGF_{2\alpha}$ levels as it is believed to represent a more accurate systemic index of oxidative stress.

The aim of this study was to establish if type1 and type 2 diabetes with varying degrees of neurological dysfunction are associated with alterations in the urinary excretion of 8-epi-$PGF_{2\alpha}$.

5.2.1.2. Findings

Mean 8-epi-PGF$_{2\alpha}$ levels were found to be lower in diabetic patients than in control subjects [0.93±0.14 nmol/L vs 2.02±0.39 nmol/L]. When 8-epi- PGF$_{2\alpha}$ concentrations were expressed relative to creatinine the differences in 8-epi-PGF$_{2\alpha}$ content between diabetic patients and control subjects were less pronounced than those seen for absolute levels [22.3±0.32 pmol/mmol vs 31.8±15.9 pmol/mmol]. When diabetic subjects were divided according to the presence or absence of PNP and/or CAN, 8-epi-PGF$_{2\alpha}$ levels were lower in all three subgroups of diabetic patients than in control subjects. The largest decreases in 8-epi-PGF$_{2\alpha}$ levels relative to control subjects [2.02±0.39 nmol/L] were seen in patients with PNP-/CAN- followed by the PNP+/CAN+ and PNP+/CAN-, respectively [0.73±0.08 nmol/L, 0.93±0.22 nmol/L and 1.07±0.22 nmol/L]. The 8-epi-PGF$_{2\alpha}$/creatinine ratios for PNP-/CAN-, PNP+/CAN+ and PNP+/CAN- versus control subjects were 15.9±2.23 pmol/mmol, 25.44±6.36 pmol/mmol and 28.62±6.36 pmol/mmol vs 31.8±15.9 pmol/mmol.

8-Epi PGF$_{2\alpha}$ levels were similar in both type 1 and type 2 diabetic patients and were slightly lower than in control subjects [0.98±0.25 nmol/L and 0.89±0.14 nmol/L vs 2.02±0.39 nmol/L]. The respective values for 8-epi- PGF$_{2\alpha}$/creatinine ratios were 22.26±3.18 pmol/mmol and 12.72±3.18 pmol/mmol vs 31.80±15.94 pmol/mmol.

When type 1 diabetic patients were segregated according to the presence or absence of PNP and/or CAN, 8-epi-PGF$_{2\alpha}$ levels were found to be lower in all three diabetic groups compared to control subjects. 8-Epi-PGF$_{2\alpha}$ levels in patients with PNP-/CAN- and PNP+/CAN+ were similar but both lower than in control subjects [0.79±0.11 nmol/L and 0.78±0.53 nmol/L vs 2.02±0.39 nmol/L]. Patients with PNP+/CAN- produced a value for 8-

epi-PGF$_{2\alpha}$ of 1.26±0.64 nmol/L. When 8-epi-PGF$_{2\alpha}$ levels were expressed relative to creatinine content, the largest decreases were noted between patients with PNP-/CAN- and controls followed by patients with PNP+/CAN- [15.90±3.18 pmol/mmol and 25.44±6.36 pmol/mmol vs 31.8±15.9 pmol/mmol]. By Contrast, no differences were observed in 8-epi-PGF$_{2\alpha}$/creatinine ratios between PNP+/CAN+ patients and control subjects [34.98±19.02 pmol/mmol vs 31.8±15.9 pmol/mmol].

In the case of type 2 diabetic subjects, 8-epi-PGF$_{2\alpha}$ levels were lower in all three patient subgroups i.e. PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ compared to control subjects The largest differences in 8-epi-PGF$_{2\alpha}$ levels were seen between PNP-/CAN- patients versus control subjects [0.59±0.08 nmol/l vs 2.02±0.39 nmol/l]. 8-Epi-PGF$_{2\alpha}$ concentrations in patients with PNP+/CAN- and PNP+/CAN+ were similar but lower than in control subjects [1.01±0.22 nmol/L and 1.01±0.19 nmol/L vs 2.02±0.39 nmol/L]. The values for 8-epi-PGF$_{2\alpha}$/creatinine ratios for the three patient groups PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ compared to control subjects were 16.78±3.18 pmol/mmol, 26.32±6.36 pmol/mmol and 22.26±6.36 pmol/mmol vs 31.8±15.9 pmol/mmol. Figures 42 and 44 summarise the data obtained for the urinary 8-epi-PGF$_{2\alpha}$ concentrations and urinary 8-epi-PGF$_{2\alpha}$/creatinine ratios in diabetic patients and control subjects.

No correlations were seen between the urinary 8-epi PGF$_{2\alpha}$ levels in diabetic subjects and age, BMI, duration of diabetes, glucose, HbA1c, plasma total cholesterol, LDL, HDL or TG. Correlations were also absent in control subjects.
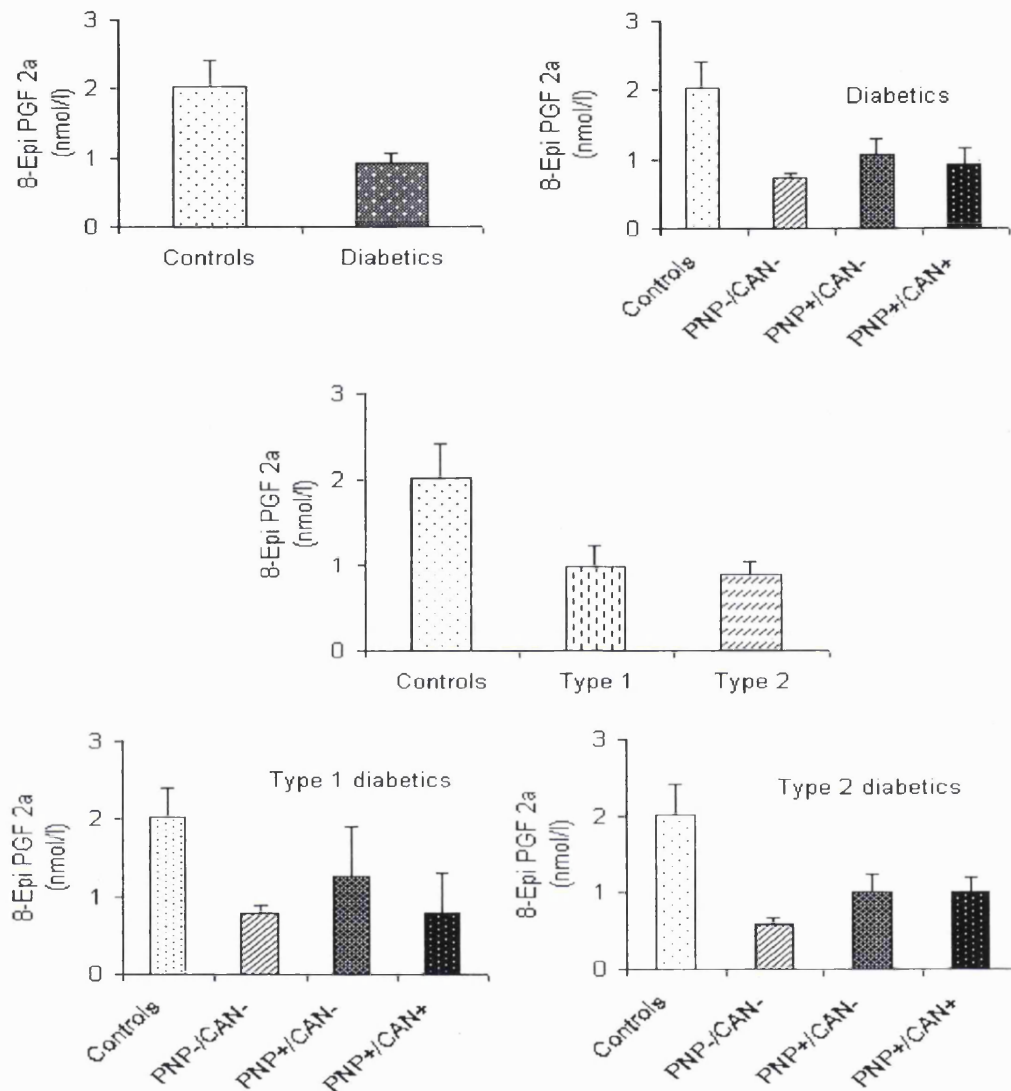
Figure 42: Urinary 8-epi-PGF$_{2\alpha}$ concentrations in diabetic patients and control subjects. Urine samples were acidified and isoprostanes were extracted with ethyl acetate. Isoprostanes were isolated by chromatography on NH$_2$-cartridge followed by Si-cartridge. Isoprostanes were analysed as TMS ether/PFB ester derivatives by GC-MS/NICI. Results are expressed as mean±SEM.

Data are shown as scatter plots (Appendix; Figures 93-97).

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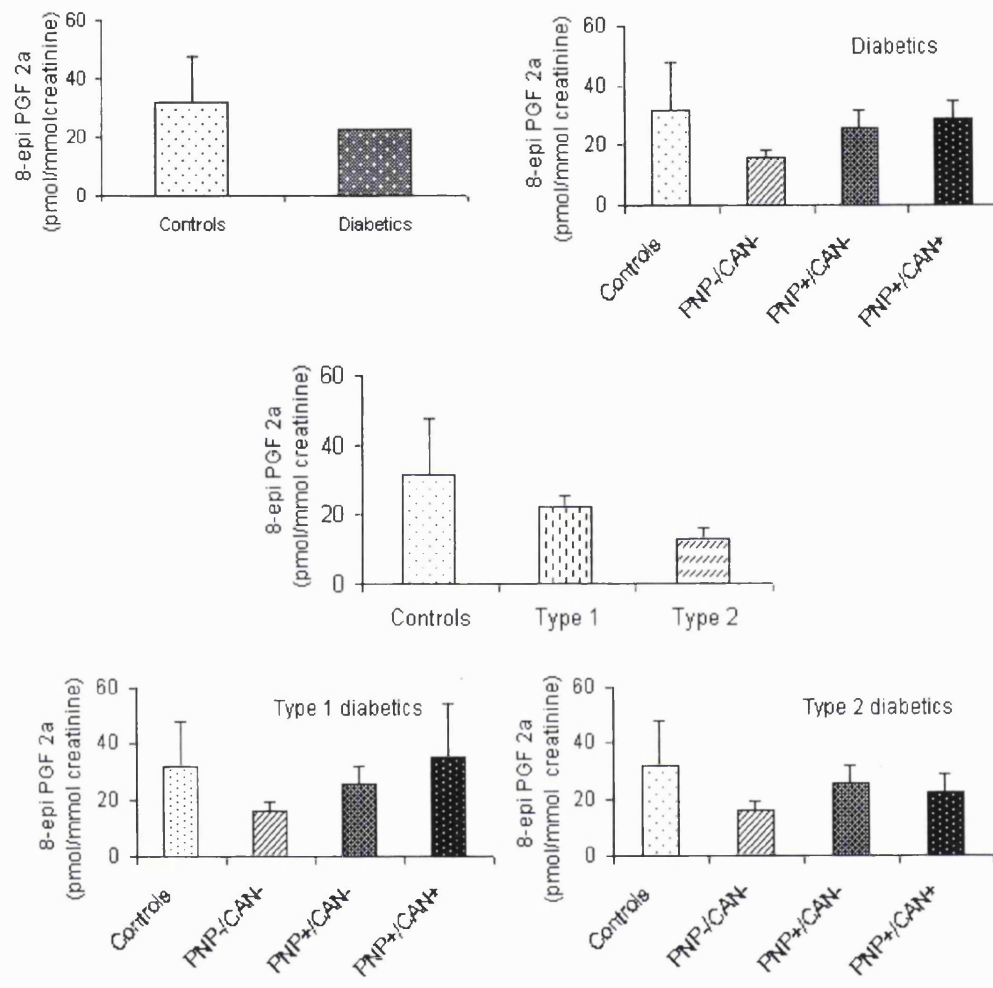
Figure 43: Urinary 8-epi-PGF$_{2\alpha}$/creatinine ratios in diabetic patients and control subjects. Urine samples were acidified and isoprostanes were extracted with ethyl acetate. Isoprostanes were isolated by chromatography on NH$_2$-cartridge followed by Si-cartridge. Isoprostanes were analysed as TMS ether/PFB ester derivatives by GC-MS/NICI.Results are expressed as mean±SEM.

Data are illustrated as scatter plots (Appendix; Figures 98-102).

5.2.1.3. Discussion

The development of accurate methods for the assessment of oxidative stress in vivo is a prerequisite for examining the relationship between oxidative stress and the progression of diabetic complications. In this study, a combined solid-phase extraction (SPE)/gas chromatography-mass spectrometry (GC-MS) procedure has been employed for the measurement of urinary 8-epi-$PGF_{2\alpha}$ as an index of oxidative stress in diabetic neuropathy. The assay involved total lipid extraction and chromatographic sample enrichment on NH_2- and Si-cartridges prior to final determination by GC-MS using negative ion chemical ionisation (NICI) mode. Inter- and intra assay coefficients of variation for urinary 8-epi- $PGF_{2\alpha}$ were acceptable being found to be 5% and 7%, respectively.

In the present investigation, 8-epi-$PGF_{2\alpha}$ levels and 8-epi-$PGF_{2\alpha}$/creatinine ratios in control subjects were 2.02±0.39 nmol/L and 31.8±15.9 pmol/mmol, respectively. As seen in Figure 44, these values were in the same range as those reported previously by other investigators employing GC-MS-NICI based assays (Richelle et al., 1999; Schwedhelm et al., 2000). Also, the values for 8-epi $PGF_{2\alpha}$ concentrations were comparable to those reported previously for male and female control subjects (1.49±0.36 nmol/L and 2.05±0.87 nmol/L, respectively) (Ferretti & Flanagan, 1997). These findings taken together indicate that both values for absolute 8-epi- $PGF_{2\alpha}$ and 8-epi-$PGF_{2\alpha}$/creatinine ratios obtained for control subjects in the current study fall well within the ranges reported by other investigators using GC-MS.
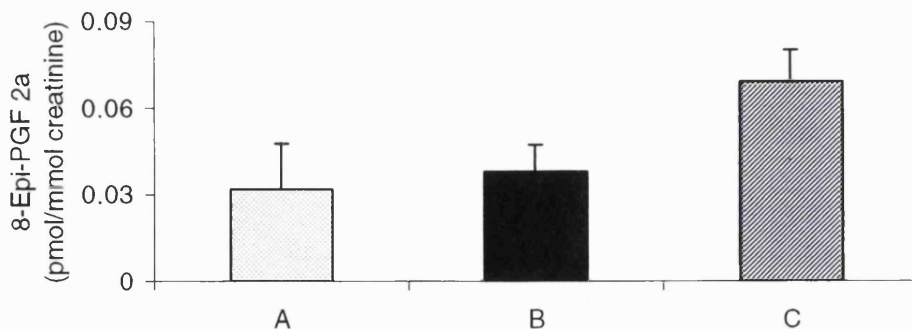
Figure 44: Data obtained by various laboratories on urinary 8-epi PGF$_{2\alpha}$/creatinine ratios in control subjects. A) Present study (n=14); B) Richelle et al., 1999 (n=4) and C) Schwedhelm et al., 2000 (n=14). Data represent mean±SEM.

It was found that 8-epi-PGF$_{2\alpha}$/creatinine ratios were 30% lower in diabetic patients than in control subjects. This decrease, however, did not achieve statistical significance possibly because of the considerable intra-individual variation in the values obtained for both diabetic and control subjects. Large degrees of variance (up to 15-fold) in urinary excretion rates for various prostaglandin-metabolites have been reported by several investigators (Falardeau et al., 1981; Fischer et al., 1983; FitzGerald et al., 1981 & 1983; Vesterqvist et al., 1984; Barrow et al., 1989; Ritter et al., 1989; Wennmalm, 1992; Riutta et al., 1994). Factors such as physical activity, smoking and daily sodium intake have all been suggested contributing to these large intra-individual variations.

Another finding from this study is that 8-epi-PGF$_{2\alpha}$/creatinine ratios were 43% higher in type 1 diabetics than in type 2 patients. The observed increase in the excretion of 8-epi-PGF$_{2\alpha}$ in type 1 diabetic patients may not be related to differences in renal function since type 1 diabetic patients exhibited lower degrees of albuminuria and hypertension than type 2 diabetics (33.0% vs 42.7% and 23% vs 63.7%, respectively).

The presence of PNP in type1 and type 2 diabetic patients was associated with a 38% increase in 8-epi-PGF$_{2\alpha}$/creatinine ratios compared to those patients with PNP-/CAN-. The observed increase in the excretion of 8-epi-PGF$_{2\alpha}$ in PNP+/CAN- compared to PNP-/CAN- may be related to differences in renal function since PNP-/CAN- patients exhibited lower degrees of albuminuria and hypertension than PNP-/CAN- patients (42.6% vs 63.8%; 42.6% vs 68.1% and 24.1% vs 46.9%; 48.3% vs 69.1%, respectively). The additional presence of CAN in type 1 diabetic patients was associated with a further 30% increase in 8-epi-PGF$_{2\alpha}$/creatinine ratios. By contrast, the additional presence of CAN in type 2 diabetic patients appeared to be without influence. The observed increases in 8-epi-PGF$_{2\alpha}$/creatinine ratios in type 1 diabetic patients with PNP+/CAN+ compared to those with PNP+/CAN- reflect differences in renal function as patients with PNP+/CAN+ had higher prevelance of individual with albuminuria and hypertension than PNP+/CAN- patients (63.8% vs 93% and 68.1% and 89.4%, respectively). These data indicate that the observed increase in excrtion of 8-epi-PGF$_{2\alpha}$ in type 1 and type 2 may reflect impaired renal function rather than the presence of PNP and/or CAN.

Several research groups have examined urinary excretion of 8-epi-PGF$_{2\alpha}$ in diabetic patients. Feillet-Coudray et al. (1999) studied urinary 8-epi-PGF$_{2\alpha}$ levels, as measured by enyzme immunoassay (EIA), in moderately controlled diabetics without renal disease. 8-epi-PGF$_{2\alpha}$ levels were found to be greater in diabetic patients than in control subjects with a positive relationship occurring between 8-epi PGF$_{2\alpha}$ and plasma glucose. Devaraj et al. (2001) employed an EIA assay to measure urinary 8-epi-PGF$_{2\alpha}$ in type 2 diabetics with and without macrovascular complications. In these patients, 8-epi PGF$_{2\alpha}$ levels were found to be raised, elevations being particularly marked for these patients with macrovascular complications. Moreover, it was shown that dietary supplementation with α–tocopherol

(1200 U/day) for 3 months led to a 50% reduction in 8-epi-PGF$_{2\alpha}$ concentrations in the macrovascular group. Davi et al. (1999) measured urinary 8-epi-PGF$_{2\alpha}$ by radioimmunoassay (RIA) and reported elevated levels of 8-epi-PGF$_{2\alpha}$ in type 1 and type 2 diabetic patients compared to control subjects. A 37% reduction in the urinary excretion of 8-epi-PGF$_{2\alpha}$ in type 2 diabetic subjects following vitamin E supplementation (600 mg daily for 2 weeks) was also observed. In contrast, Vessby et al., (2002) failed to detect any changes in urinary 8-epi-PGF$_{2\alpha}$ levels between type 1 diabetic patients and control subjects using RIA.

An explanation for the differences seen in urinary 8-epi-PGF$_{2\alpha}$ levels seen in diabetic patients in this study and in previous investigations may relate to the various analytical procedures used. It is well established that RIA and EIA are associated with shortcomings (Granstrom & Kindahl, 1978; Proudfoot et al., 1999; Bessard et al., 2001; Liang et al., 2003). These problems related primarily to substances present in biological fluids, which interfere with the assays. Immunoassays usually work well in buffer solutions or simple biological matrix. However, problems can arise when dealing with complex biological fluids and tissues. The data obtained in the present study would suggest that such problems do not arise with mass-spectrometry indicating that it may be a superior method.

Data from recent studies indicate that urinary excretion of 8-epi-PGF$_{2\alpha}$ is influenced by fasting-state, diet and exercise. Mori et al. (1999) examined the effects of dietary treatment with marine ω-3 fatty acids for 4 weeks and moderate exercise training on urinary 8-epi-PGF$_{2\alpha}$ excretion in type 2 diabetic subjects using a combined solid-phase extraction/GC-MS technique. They reported that each of these regimes led to significant reductions in urinary 8-epi-PGF$_{2\alpha}$ levels compared with the base-line concentrations. They also found

that combining dietary supplementation with ω-3 fatty acids with exercise training resulted in further reductions in urinary 8-epi-PGF$_{2\alpha}$ levels.

Richelle et al. (1999) studied the effects of fasting and various types of diet on urinary excretion of 8-epi-PGF$_{2\alpha}$ in healthy subjects using a gas chromatographic technique. A 15% increase in 8-epi-PGF$_{2\alpha}$ levels was observed after 24 hours of fasting compared to base-line values. A return to base-line levels occurred following food consumption suggesting that fasting induces oxidative stress and/or enhances the release of F$_2$-isoprostanes from adipose tissue. A low fat diet was found to lead to slight reductions in urinary 8-epi-PGF$_{2\alpha}$ levels as compared to the levels seen in subjects on a normal diet.

In conclusion, this study demonstrates that the urinary excretion of 8-epi PGF$_{2\alpha}$ exhibits considerable degrees of variability in both diabetic and control subjects and that 8-epi PGF$_{2\alpha}$/creatinine ratios are lower in diabetic patients than in controls. The excretion of 8-epi-PGF$_{2\alpha}$ appears to be influenced by type of diabetes and the presence of PNP in both type 1 and type 2 diabetics is associated with increased excretion of 8-epi-PGF$_{2\alpha}$. Further studies are needed in which the influence of factors such as diet, fasting state and renal function are examined to establish if urinary 8-epi-PGF$_{2\alpha}$ measurement constitutes a reliable index of oxidative stress. The possibility of diurnal variations in 8-epi-PGF$_{2\alpha}$ levels should also be considered.

5.2.2. F_2-isoprostane metabolites
5.2.2.1. Background

As described in the previous chapter, the F_2-isoprostanes are a series of prostaglandin F_2-(PGF_2)-like compounds produced by non-enzymatic peroxidation of arachidonic acid. Of these, 8-epi- $PGF_{2\alpha}$ has received the most attention due to its well-documented biological effects.

The measurement of 8-epi-$PGF_{2\alpha}$ in urine has been employed as a marker of oxidative stress by leading investigators in the field (Roberts & Morrow 2000 & 2002; Pratico et al. 2001). However, concern has been raised about the primary source of urinary unmetabolised 8-epi-$PGF_{2\alpha}$. This worry stems from observations that indicate that the kidney is a major source of urinary unmetabolised cyclooxyxgenase-derived $PGF_{2\alpha}$ in both man and animals (Frolich et al., 1975; Roberts & Morrow, 1997). Therefore, it has been suggested that the measurement of urinary 8-epi-$PGF_{2\alpha}$ metabolites including 2, 3-dinor-8-epi-$PGF_{2\alpha}$ (dinor-8-epi-$PGF_{2\alpha}$) and 2, 3-dinor-5, 6-dihydro-$PGF_{2\alpha}$ (dinor-dihydro-8-epi-$PGF_{2\alpha}$) may provide a better index of systemic non-enzymatic lipid peroxidation.

The aims of this study were to examine the impact of type 1 and type 2 diabetes in the presence or absence of polyneuropthay (PNP) and/or cardiovascular autonomic polyneuropathy (CAN) on the urinary excretion of dinor-8-epi-$PGF_{2\alpha}$ and dinor-dihydro-$PGF_{2\alpha}$. Measurements were made in healthy control subjects for comparison.

5.2.2.2. Findings
5.2.2.2.1. Dinor-8-epi-PGF$_{2\alpha}$

Dinor-8-epi-PGF$_{2\alpha}$ levels were lower in the diabetic patients as a group when compared to control subjects [24.33±4.13 nmol/L vs 34.61±5.54 nmol/L]. The difference in the values between the two groups was less pronounced when the levels were expressed relative to urinary creatinine [463.6±58.8 pmol/mmol vs 543.3±19.03 pmol/mmol]. The largest differences in dinor-8-epi- PGF$_{2\alpha}$ concentrations were seen between diabetic subjects with PNP+/CAN+ and controls followed by patients with PNP+/CAN- and PNP-/CAN- [12.36±2.95 nmol/L, 22.64±5.03 nmol/L and 31.39±31.39 nmol/L vs 34.611±5.54 nmol/L]. The values for dinor-8-epi- PGF$_{2\alpha}$/creatinine ratios for PNP+/CAN+, PNP+/CAN-, PNP-/CAN- and controls were 269.9±51.9 pmol/mmol, 494.8±100.34 pmol/mmol and 480.9±72.22 pmol/mmol vs 543.3±19.03 pmol/mmol.

Dinor-8-epi-PGF$_{2\alpha}$ concentrations were similar in type 1 and type 2 diabetic patients but both lower than in control subjects [23.41±7.55 nmol/L and 24.81±4.91 nmol/L vs 31±6.71 nmol/L]. The values for dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios for type 1 and type 2 diabetic patients versus control subjects were 482.4±83.04 pmol/mmol and 425.6±83.0 pmol/mmol vs 553.3±19.03 pmol/mmol]. Type 1 diabetic patients with PNP+/CAN+ had the lowest dinor-8-epi-PGF$_{2\alpha}$ levels compared to controls followed by those with PNP-/CAN- and PNP+/CAN-, respectively [5.08±1.53 nmol/L, 20.56±5.05 nmol/L and 33.17±18.70 nmol/L vs 34.61±5.54 nmol/L]. The values for dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios for the PNP+/CAN+, PNP-/CAN- and PNP+/CAN- groups versus control subjects were 228.0±103.38 pmol/mmol, 398.9±96.88 pmol/mmol and 529.4±183.3 pmol/mmol vs 543.3±19.03 pmol/mmol.

In the type 2 diabetic patients, dinor-8-epi-PGF$_{2\alpha}$ levels were found to be lower in patients with PNP+/CAN+ and PNP+/CAN- than in control subjects [16.88±4.25 nmol/L and 19.64±3.04 nmol/L vs 34.61±5.54 nmol/L]. Patients with PNP-/CAN- yielded a value for dinor-8-epi-PGF$_{2\alpha}$ of 34.61±5.54 nmol/L. The values for dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios for patients with PNP+/CAN+, PNP+/CAN- and PNP-/CAN- versus control subjects were 297.5±65.74 pmol/mmol, 484.4±117.6 pmol/mmol and 570.9±121.1 pmol/mmol vs 543.3±19.03 pmol/mmol. Figure 45 and 46 illustrates dinor-8-epi-PGF$_{2\alpha}$ levels and dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios in diabetic patients and controls subjects.

5.2.2.2.2. Dinor-dihydro-8-epi-PGF$_{2\alpha}$

Both dinor-dihydro-8-epi-PGF$_{2\alpha}$ levels and the dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios were lower in diabetic patients than in control subjects [8.02±1.73 nmol/L vs 18.12±5.45 nmol/L and 131.5±20.72 pmol/mmol vs 217.9±69.20 pmol/mmol, respectively]. The largest differences in dinor-dihydro-8-epi-PGF$_{2\alpha}$ concentrations were observed between patients with PNP+/CAN+ and control subjects followed by those with PNP+/CAN- and PNP-/CAN- [4.23±1.43 nmol/L, 7.87±2.68 nmol/L and 9.67±2.53 nmol/L vs 18.12±5.45 nmol/L]. The respective values for dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios for the three diabetic groups versus control subjects were 86.50±20.46 pmol/mmol, 138.5±30.69 pmol/mmol and 138.4±20.76 pmol/mmol vs 217.9±69.2 pmol/mmol.

When patients were segregated into type 1 and type 2 diabetic groups, both were found to yield lower values for dinor-dihydro-8-epi-PGF$_{2\alpha}$ than control subjects [8.75±3.87 nmol/L and 7.62±1.62 nmol/L vs 18.12±5.45 nmol/L]. The respective values for dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios were 124.6±38.06 pmol/mmol and 134.9±20.76 pmol/mmol vs

217.9±69.2 pmol/mmol. Type 1 diabetic patients with PNP+/CAN+ exhibited the lowest dinor-dihydro-8-epi-PGF$_{2\alpha}$ versus control subjects followed by patients with PNP-/CAN- and PNP+/CAN- [0.58±0.21 nmol/L, 6.68±1.31 nmol/L and 13.99±9.91 nmol/L vs 18.12±5.45 nmol/L]. The corresponding values for dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios all three patient groups versus controls were 24.22±10.38 pmol/mmol, 186.8±81.4 pmol/mmol and 100.3±20.7 pmol/mmol vs 217.9±69.2 pmol/mmol.

In type 2 diabetics, subjects with PNP-/CAN- and PNP+/CAN+ yielded similar values for dinor-dihydro-8-epi-PGF$_{2\alpha}$ but lower than control subjects [5.98±2.71 nmol/L, 6.47±2.10 nmol/L vs 18.12±5.45 nmol/L]. Patients with PNP+/CAN- produced a value of 12.35±3.07 nmol/L. The values for dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios for patients with PNP-/CAN-, PNP+/CAN- and PNP+/CAN+ versus control subjects were 124.6±46.47 pmol/mmol, 173.0±22.83 pmol/mmol and 124.5±34.60 pmol/mmol vs 217.9±69.2 pmol/mmol. Figure 47 and 48 illustrates dinor-8-epi-PGF$_{2\alpha}$ levels and dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios in diabetic patients and controls subjects.

5.2.2.2.3. Correlations

No correlations were seen in diabetic patients between dinor-8-epi PGF$_{2\alpha}$ or dinor-dihydro-8-epi PGF$_{2\alpha}$ in diabetics and age, BMI, duration of diabetes, glucose, HbA1c, plasma total cholesterol, LDL, HDL or TG. Relationships were also absent in control subjects. In diabetic patients, correlations were found between urinary creatinine and either dinor-8-epi PGF$_{2\alpha}$ or dinor-dihydro-8-epi PGF$_{2\alpha}$ [$r_{dinor-8-epi\ PGF2\alpha}$ = 0.367; p=<0.005 and $r_{dinor-dihydro-8-epi\ PGF2\alpha}$ =0.289; p<0.005] whilst such relationships were absent in control subjects. There were strong

associations between in both diabetic and control subjects [$r_{controls}$=0.704; p<0.01 and

$r_{diabetics}$=0.788; p<0.005] (Figures 49 and 50).
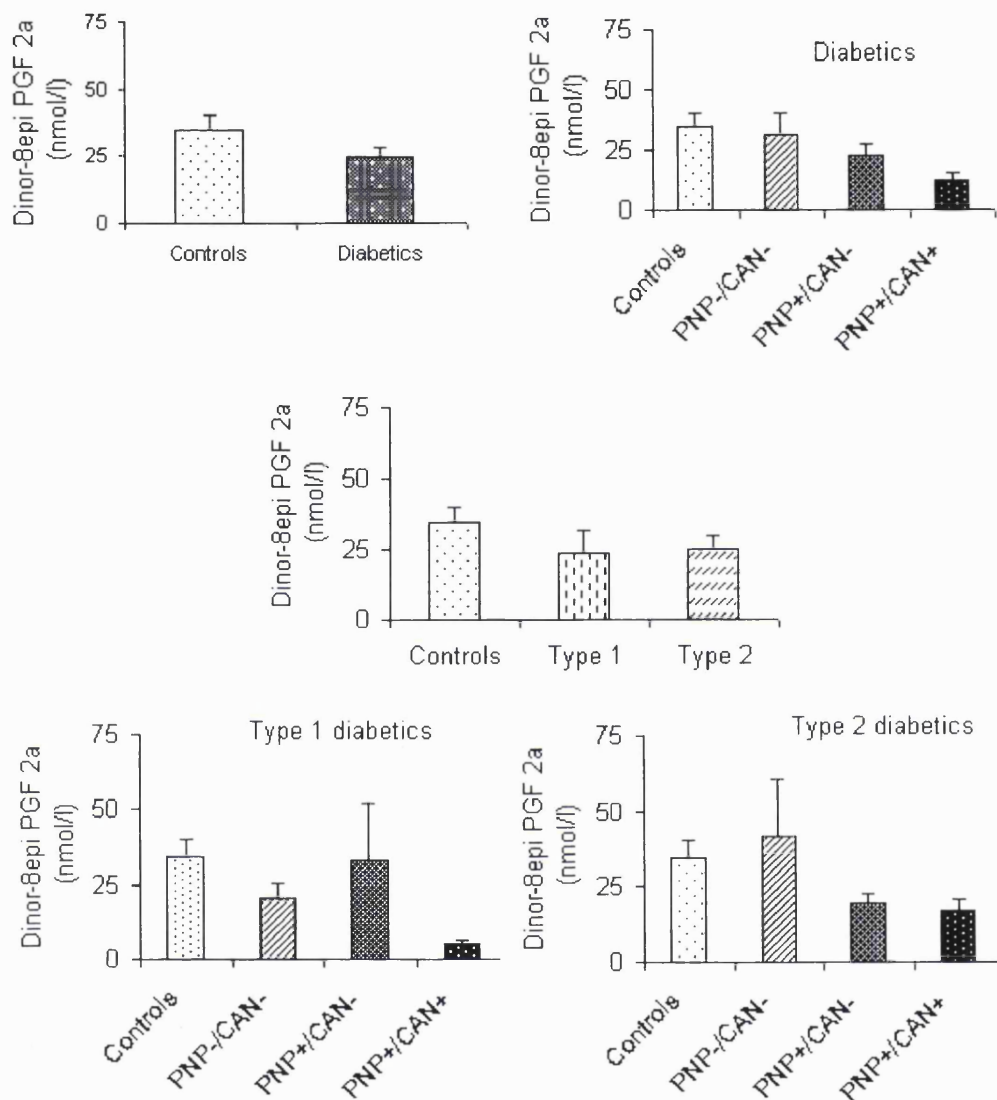
Figure 45: Urinary 2,3-dinor-8-epi-PGF$_{2\alpha}$ concentrations in diabetics and control subjects. Urine samples were acidified and isoprostanes were extracted with ethyl acetate. Isoprostanes were isolated by chromatography on NH$_2$-cartridge followed by Si-cartridge. Isoprostanes were analysed as TMS ether/PFB ester derivatives by GC-MS/NICI. Results are expressed as mean±SEM.

Data are displayed as scatter plots (Appendix; Figures 103-107).
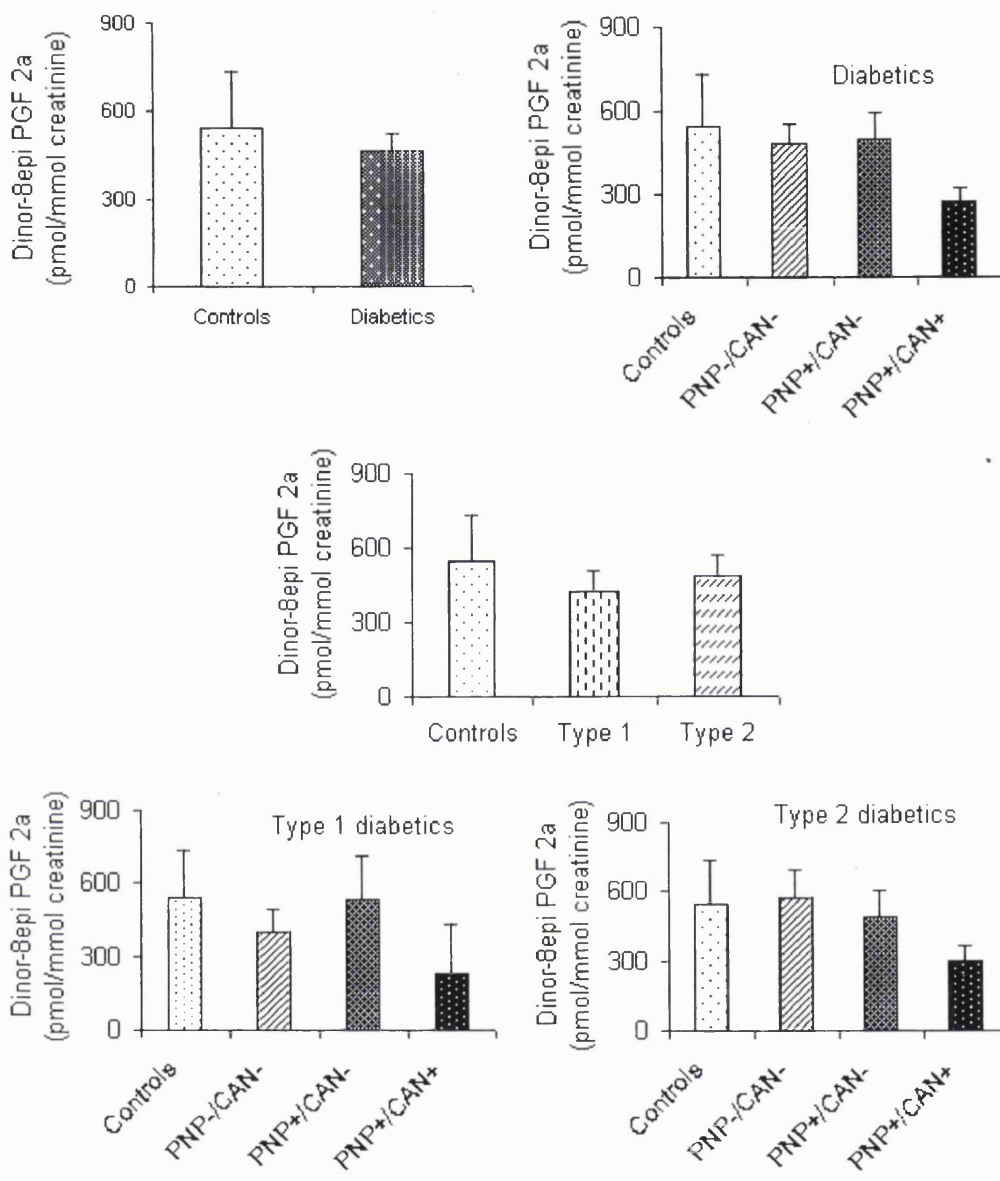
Figure 46: Urinary 2,3-dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios in diabetic patients and control subjects. Urine samples were acidified and isoprostanes were extracted with ethyl acetate. Isoprostanes were isolated by chromatography on NH$_2$-cartridge followed by Si-cartridge. Isoprostanes were analysed as TMS ether/PFB ester derivatives by GC-MS/NICI.

Values are presented as mean±SEM.

Data are illustrated as scatter plots (Appendix; Figures 108-112).
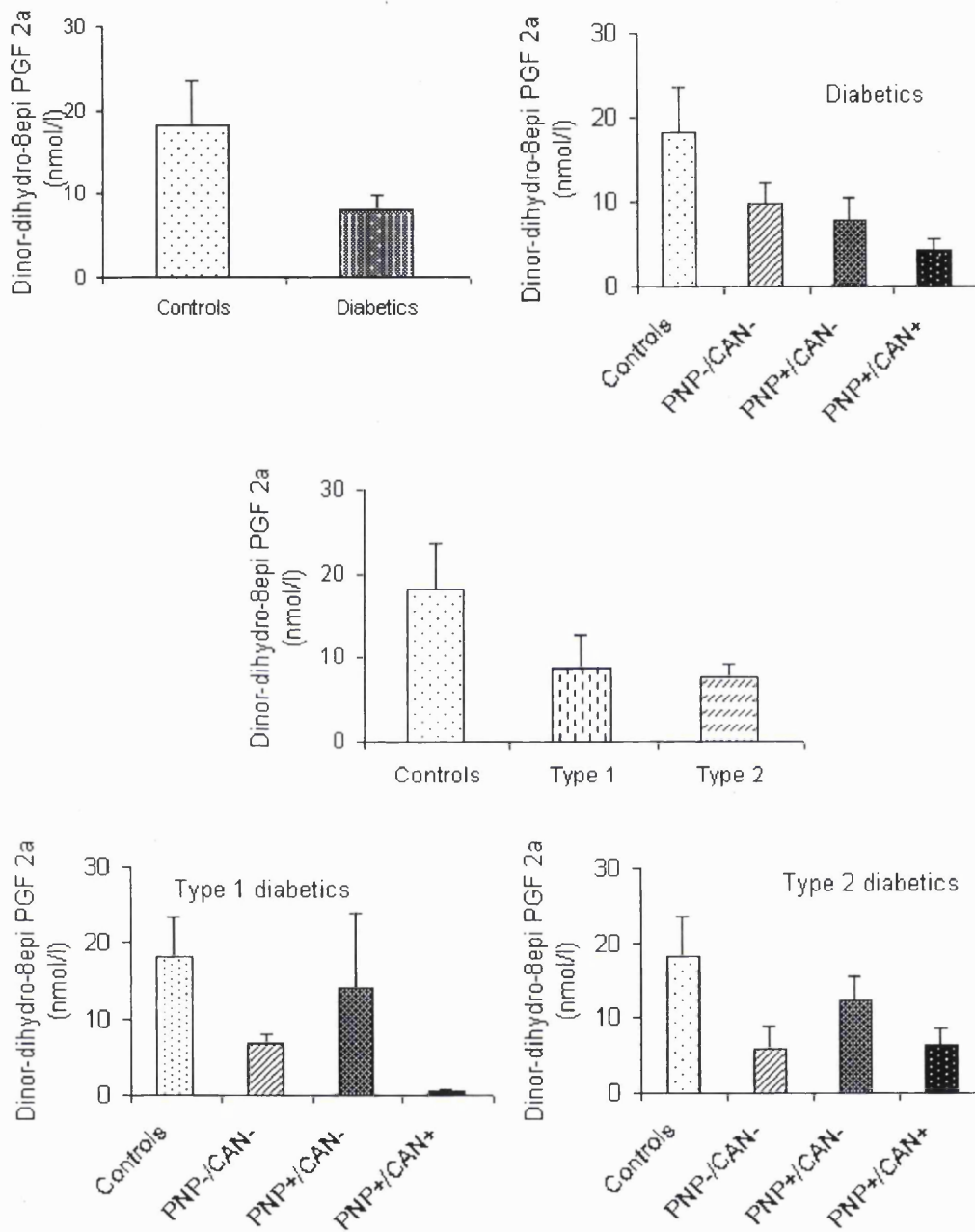
Figure 47: Urinary 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ levels in diabetic patients and control subjects. Urine samples were acidified and isoprostanes were extracted with ethyl acetate. Isoprostanes were isolated by chromatography on NH$_2$-cartridge followed by Si-cartridge. Isoprostanes were analysed as TMS ether/PFB ester derivatives by GC-MS/NICI. Data are shown as mean±SEM.

Data are presented as scatter plots (Appendix; Figures 113-117).

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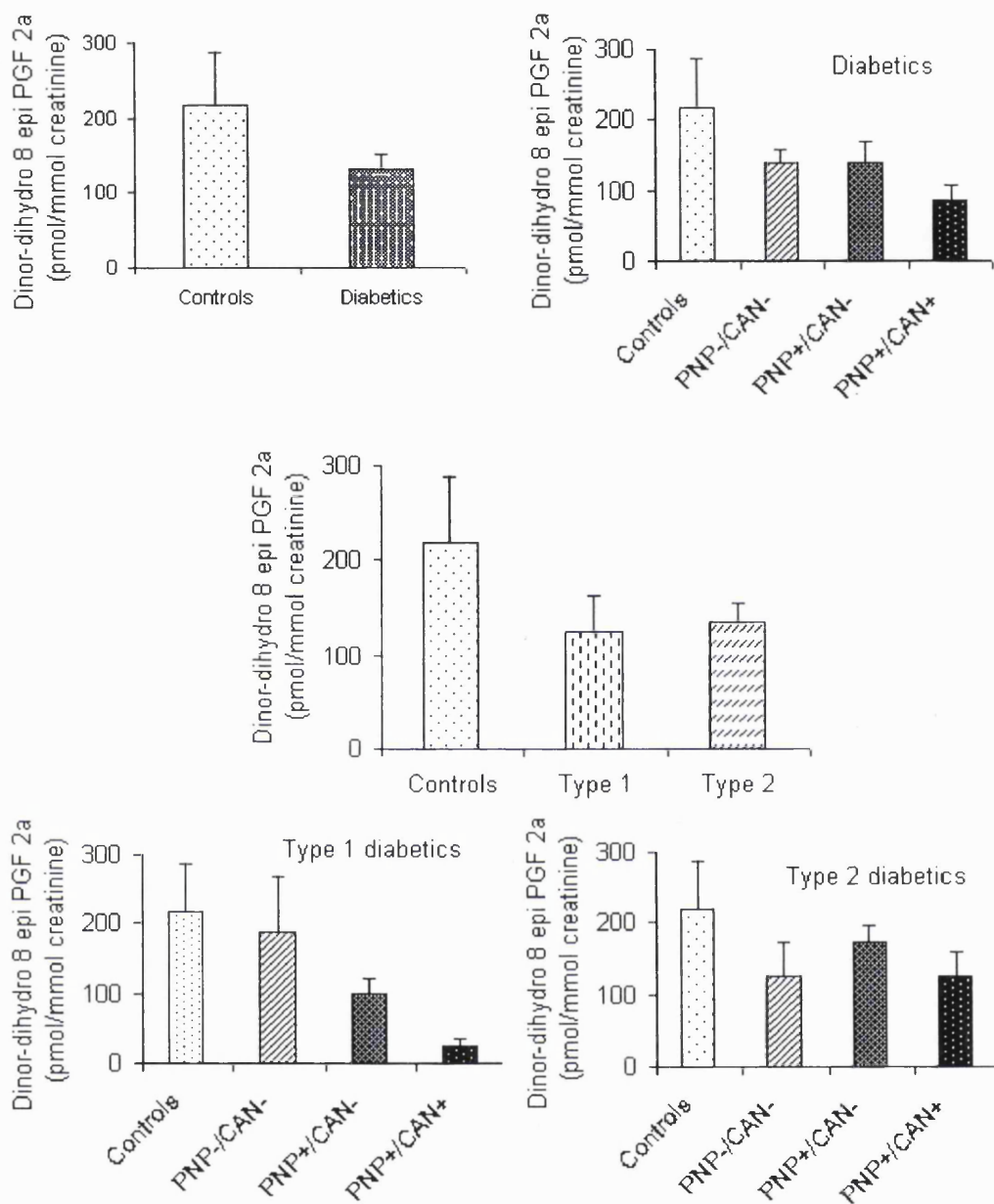
Figure 48: 2, 3-Dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ /creatinine ratios in diabetic patients and control subjects. Urine samples were acidified and isoprostanes were extracted with ethyl acetate. Isoprostanes were isolated by chromatography on NH$_2$-cartridge followed by Si-cartridge. Isoprostanes were analysed as TMS ether/PFB ester derivatives by GC-MS/NICI. Data are presented as the mean±SEM.

Data are illustrated as scatter plots (Appendix; Figures 118-122).
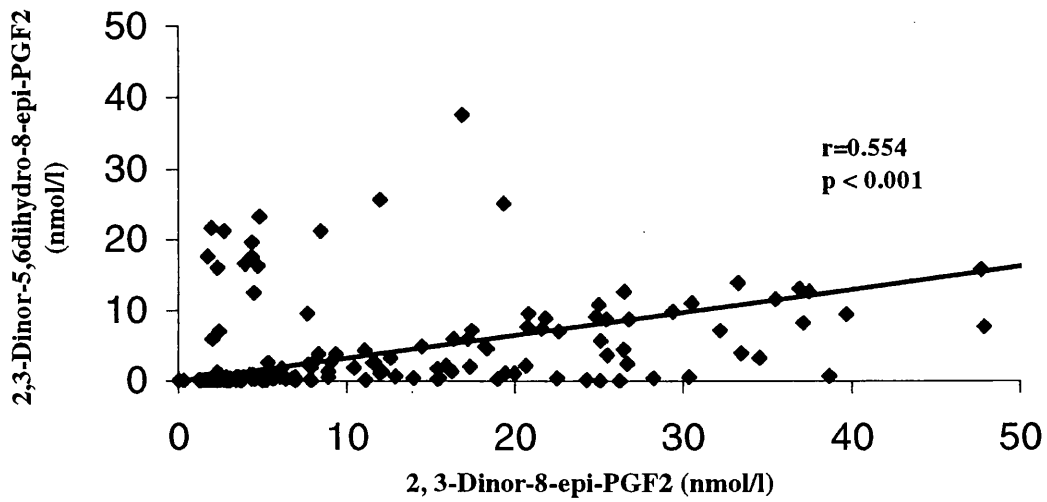
Figure 49: Correlation between urinary 2, 3-dinor-8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ in diabetic patients.
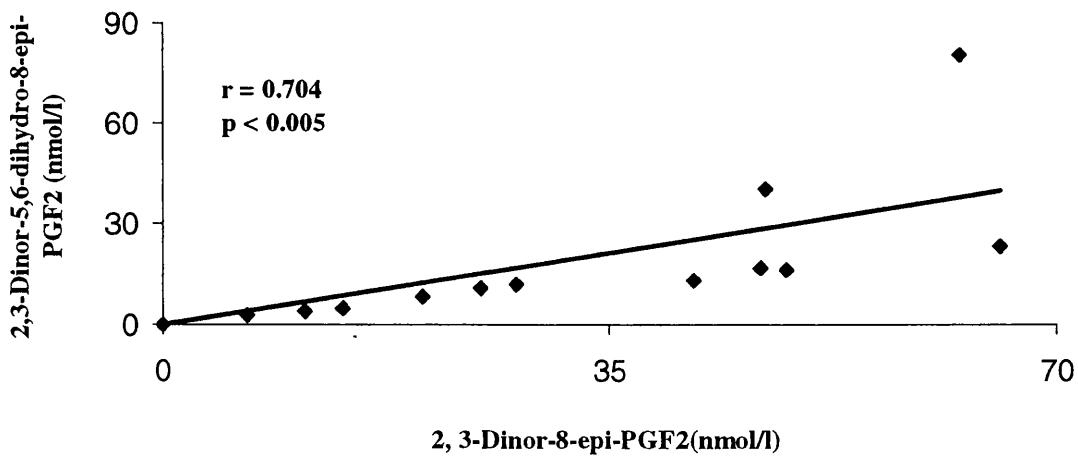
Figure 50: Association between urinary 2, 3-dinor-8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ in control subjects.

5.2.2.3. Discussion

The metabolic pathway for cyclooxygenase-derived $PGF_{2\alpha}$ was first established by Samuelsson et al. (1975). It was shown to occur via steps involving reduction of the hydroxyl group at the C_{15} position in the molecule to yield 15-keto-$PGF_{2\alpha}$, followed by reduction of the double bond at position C_{13} to form a 13, 14-dihydro-15-keto-$PGF_{2\alpha}$. The latter species subsequently undergoes both β- and ω-oxidation to form 5α, 7α-dihydroxy-11-oxo-tetranor-prosta-1, 16-dioic acid which is a major urinary metabolite (Figure 51).

Theoretically, it was expected that 8-epi-$PGF_{2\alpha}$ would undergo a similar metabolic degradation to that of cyclooxygenase-derived $PGF_{2\alpha}$. Roberts and co-workers (1996) reported in experiments involving the administration of radio-labelled 8-epi $PGF_{2\alpha}$ to primates and humans that 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ was the major urinary metabolite, accounting for about 20% of total urinary products. Chiabrando et al. (1999), on the other hand, demonstrated that 2, 3-dinor-8-epi-$PGF_{2\alpha}$ is the major urinary metabolite in rats and humans. Chiabrando et al. (2002) studied the metabolism of eight authentic diastereoisomers of 8-epi-$PGF_{2\alpha}$ by isolated rat hepatocytes and found that 8-epi-$PGF_{2\alpha}$ had the largest number of products in common with the cyclooxygenase-derived $PGF_{2\alpha}$. Figure 52 shows the proposed pathway for the metabolism of 8-epi- $PGF_{2\alpha}$.
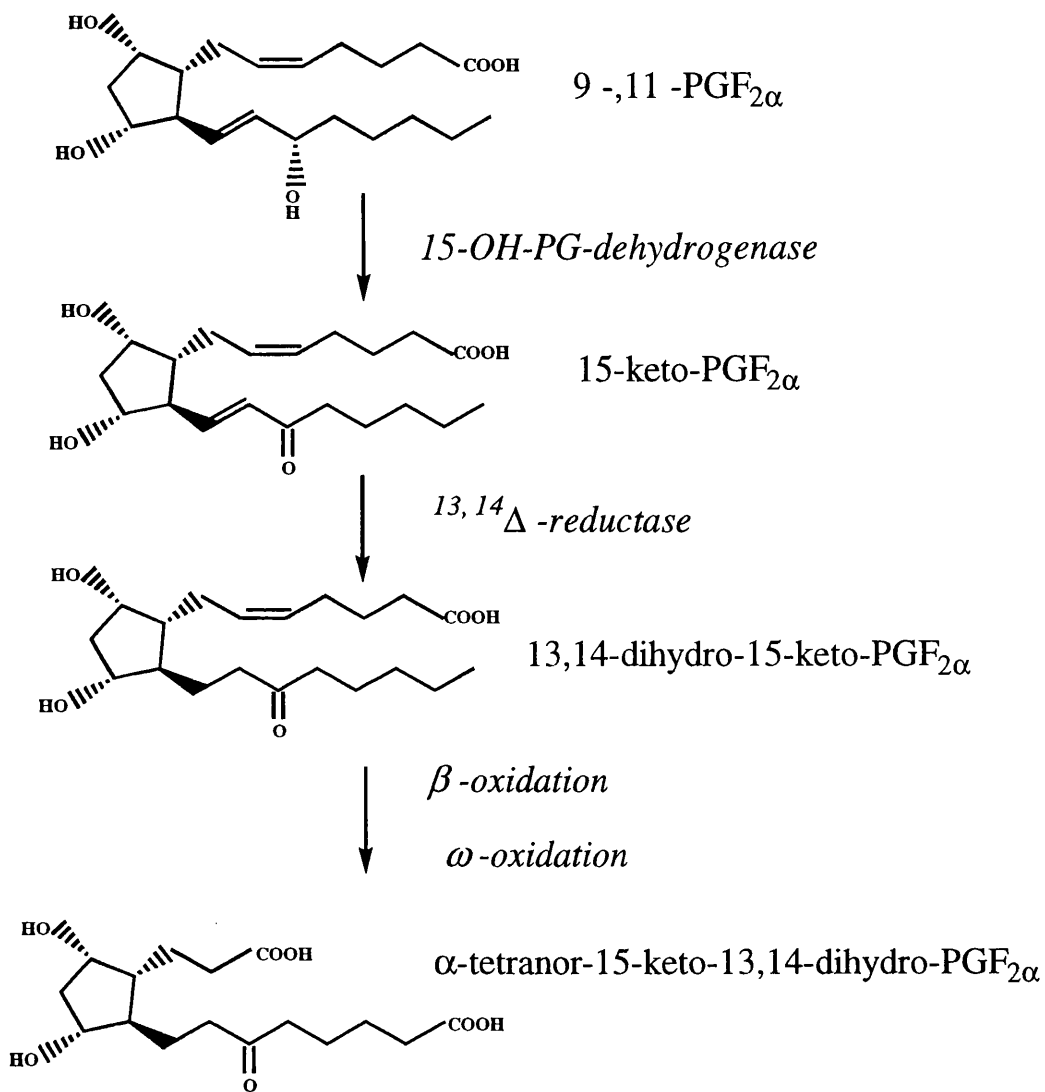
Figure 51: Pathway for the metabolism of cyclooxgenase derived $PGF_{2\alpha}$.

9-,11-$PGF_{2\alpha}$

15-OH-PG-dehydrogenase

15-keto-$PGF_{2\alpha}$

$^{13,\ 14}\Delta$ *-reductase*

13,14-dihydro-15-keto-$PGF_{2\alpha}$

β *-oxidation*

ω *-oxidation*

α-tetranor-15-keto-13,14-dihydro-$PGF_{2\alpha}$

HO////

HO\\''

O
H

COOH   8-epi-PGF$_{2\alpha}$

↓ *β-oxidation*

HO////

HO\\''

O
H

COOH   2, 3-dinor-8-epi-PGF$_{2\alpha}$

↓ *5,6Δ-reductase*

HO////

HO\\''

O
H

COOH   2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$
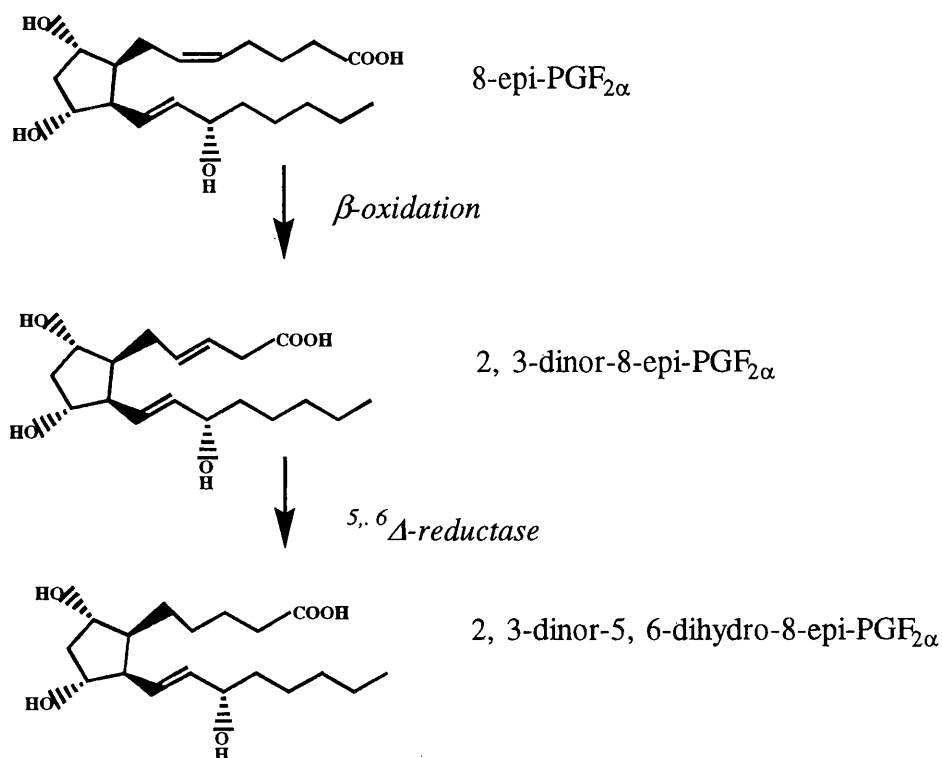
Figure 52: Proposed metabolic pathway for non-cyclooxygenase derived 8-epi-PGF$_{2\alpha}$.

In the present investigation, dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios for control subjects were 217.9±69.20 pmol/mmol. As illustrated in Figure 53, the value fall well within the ranges reported by other groups using GC-MS (Morrow et al., 1999; Schwedhelm et al., 2000; Morales et al., 2001). In addtion, the mean value for dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios [543.3±19.03 pmol/mmol] was similar to that reported by Liang et al. (2003). In addition, the ratios of dinor-8-epi-PGF$_{2\alpha}$ or dinor-dihydro-8-epi-PGF$_{2\alpha}$ to 8-epi-PGF$_{2\alpha}$ in the present investigation were in good agreement with values of 13.8 and 2.3 reported elsewhere (Schwedhelm et al., 2000; Liang et al., 2003). These data taken together confirm the reliability of the combined ethyl acetate/NH$_2$/Si procedure for the simultaneous isolation of urinary 8-epi PGF$_{2\alpha}$ and its metabolite.
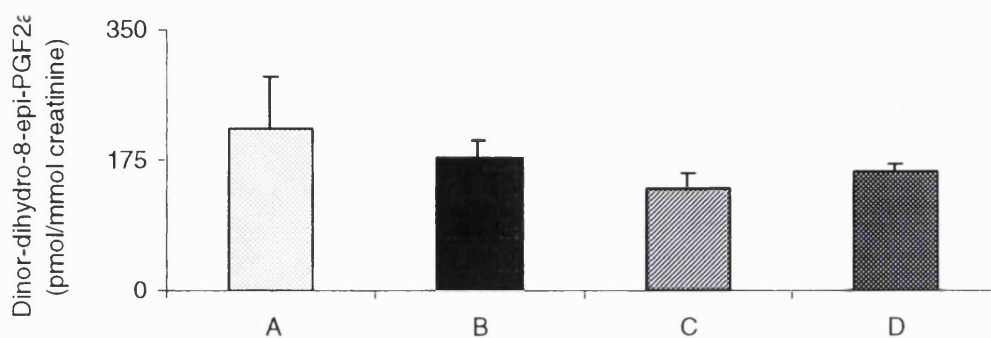
Figure 53: Values obtained by different laboratories on urinary 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios in control subjects. A) Present study (n=14); B) Schwedhelm et al., 2000 (n=14); C) Morrow et al., 1999 (n=10) and D) Morales et al., 2001. Data are expressed as mean±SEM.

In the present study, the excretion of both creatinine-standardised dinor-8-epi-PGF$_{2\alpha}$ and dinor-dihydro-8-epi-PGF$_{2\alpha}$ was found to be lower in diabetics than in controls. As is with the case with the measurement of unmetabolised urinary 8-epi PGF$_{2\alpha}$, however, these decreases did not achieve statistical significance due to the considerable intra-individual variation in the values obtained for both diabetic and control subjects. Differences in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios or dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios between type 1 and type 2 patients were not observed. Variations in the excretion of dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios and dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios were, however, seen in relation to the presence of PNP and/or CAN in type 1 and type 2 diabetics. The presence of PNP in type 1 diabetics was linked with substantial increases (25%) in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios. The increased excretion of dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios in patients with PNP+/CAN- is unlikely to be explained by impaired renal function

although patients with PNP+/CAN- exhibited higher degree of albuminuria and hypertension than the patients PNP-/CAN- group (63.8% vs 42.6% and 68.1% vs 42.6%, respectively). By contrast, the additional presence of CAN in type 1 diabetics was associated with a 43% reduction in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios when compared to patient group with PNP-/CAN-. The observed decline in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios in the patients with PNP+/CAN+ may be related to impaired renal function as these individuals had a higher degree of albuminuria and hypertension than PNP-/CAN- patients (93.6% vs 42.6% and 89.4% vs 42.6%, respectively).

By contrast, the presence of PNP in type 2 diabetic patients resulted in 15% reduction in the excretion of dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios. The observed reduction in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios is explained by difference in renal function since the PNP-/CAN- patient group exhibited a lesser degree of albuminuria and hypertension than PNP+/CAN- (24.1% vs 46.9 and 48.3% vs 69.1%, respectively). The additional presence of CAN was associated with 40% reductions in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios compared to PNP+/CAN- group. The observed decline in dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios in patients with PNP+/CAN+ is not explained by impaired renal function as both subgroups i.e PNP+/CAN- and PNP+/CAN+ were matched with respect to the prevalence of individuals with albuminuria and hypertension.

The presences of PNP in type 1 diabetic patients was linked with reductions in dinor-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios and were further reduced in the presence of additional CAN. These findings indicate the observed reduction in the excretion of dinor-dihydro-8-epi-PGF$_{2\alpha}$ may be related to impairment of renal function. By contrast, the presence of PNP in type 2 diabetic patients was found to be associated with increased dinor-dihydro-8-epi-

$PGF_{2\alpha}$/creatinine ratios whilst the additional presence of CAN appeared to be without influence. Taken together, these data suggest that measurement of dinor-8-epi-$PGF_{2\alpha}$/creatinine ratio is a better indicator impairment of renal dysfunction in type 2 diabetic patients in the presence of PNP and/or CAN whilst dinor-dihydro-8-epi-$PGF_{2\alpha}$/creatinine ratio is useful marker in type diabetic patients.

An important finding from this study is that dinor-8-epi-$PGF_{2\alpha}$ appears to be the major metabolite of 8-epi-$PGF_{2\alpha}$ in both diabetic and control subjects. This therefore provides confirmation of the report made by Chiabrandos et al. (1999) that dinor-8-epi-$PGF_{2\alpha}$ is the major urinary metabolite of 8-epi-$PGF_{2\alpha}$ in humans. The ratio of dinor-8-epi-$PGF_{2\alpha}$ to dihydro-8-epi-$PGF_{2\alpha}$ was slightly higher in diabetic patients than in control subjects (3.5 vs 2.5) but was not influenced either by type of diabetes. In type 1 diabetic patients, dinor-8-epi-$PGF_{2\alpha}$/dihydro-8-epi-$PGF_{2\alpha}$ were increased in the presence of PNP and were further enhanced in the presence of additional CAN. By contrast, the presence of PNP in type 2 diabetics was associated with marked reductions in the dinor-8-epi $PGF_{2\alpha}$/dihydro-8-epi-$PGF_{2\alpha}$ ratios. The presence of additional CAN was linked with further reductions in dinor-8-epi $PGF_{2\alpha}$/dihydro-8-epi-$PGF_{2\alpha}$ ratios. This suggests that simultaneous measurement of more than one 8-epi $PGF_{2\alpha}$ metabolite may be required in order to obtain an accurate picture of the systemic filtration of 8-epi-$PGF_{2\alpha}$ from the circulation.

In conclusion, this study indicates that the urinary excretion of dinor-8-epi-$PGF_{2\alpha}$ and dinor-dihydro-8-epi-$PGF_{2\alpha}$ is diminished in diabetic patients with dinor-8-epi-$PGF_{2\alpha}$ being the predominant urinary metabolite. Importantly, the urinary excretion of dinor-8-epi-$PGF_{2\alpha}$ and dinor-dihydro-8-epi-$PGF_{2\alpha}$ appears to be related to renal function. Further studies,

however, are needed to establish fully the impact of systemic renal filtration on the excretion of 8-epi-PGF$_{2\alpha}$ metabolites before their importance, as markers of oxidative stress can be determined.

5.3. Regression analysis
5.3.1. Background

A detailed assessment of potential biomarkers of oxidative stress may improve our understanding of the complex pathophysiology of late diabetic complications and lead to therapeutic regimes which delays the onset. Essentially, these biomarkers should be specific, reproducible and sensitive, and ideally easily determined and inexpensive.

Various biomarkers including lipid peroxidation products, DNA-oxidation products, protein oxidation products, thiols, antioxidant enzymes and nutrient antioxidants have been used to examine the association between oxidative stress and diabetic complications. However, none of these biomarkers have been validated simultaneously in cross-sectional investigations involving large numbers of subjects.

The objectives of this statistical survey are, therefore: 1) to examine the relationships between various measures of oxidative stress including, plasma total antioxidant capacity (TAC) as measured by quenching of peroxynitrite (ONOO-QPC), superoxide anion (O_2^--QPC) or hypochlorous acid (HOCl-QPC) Pholasin® chemiluminisce, vitamins C and E and 8-epi-$PGF_{2\alpha}$, and urinay 8-epi-$PGF_{2\alpha}$, 2, 3-dinor-8-epi-$PGF_{2\alpha}$ and 2, 3-dino-5, 6-dihydro-8-epi- $PGF_{2\alpha}$, and 2) to assess which of the aforementioned plasma measure(s) of oxidative stress is an independent determinant of diabetic polyneuropathy.

5.3.2. Findings
5.3.2.1. Single linear regression analyses

As seen in tables 7 & 8, there were strong correlations between the measures of plasma TAC i.e. ONOO-QPC, O_2^--QPC or HOCl-QPC in both diabetic patients and control

subjects. All three measures of TAC also correlated with vitamin C and vitamin E levels in diabetic patients whereas associations were only seen for ONOO-QPC and O_2^--QPC and vitamins C and E in control subjects. Correlations were seen between O_2^--QPC and plasma 8-epi-PGF$_{2\alpha}$ in both diabetic and control subjects.

Moderate associations were observed between all three measures of plasma TAC and urinary 8-epi-PGF$_{2\alpha}$ and its metabolites (i..e. 2, 3-dinor-8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$). By contrast, similar correlations were absent in control subjects.

Correlation was found between vitamin C and E in diabetic patients but was absent in control subjects. Associations were also seen between vitamin C levels and plasma 8-epi-PGF$_{2\alpha}$ in control subjects. By contrast, similar correlations were absent in diabetic patients. In the case of vitamin E, a relationship occurred with plasma 8-epi-PGF$_{2\alpha}$ in diabetic patients. On the other hand, correlations were absent between vitamin E and urinary 8-epi-PGF$_{2\alpha}$ and its metabolites. Interestingly, a strong correlation was seen between vitamin C and 2, 3-dinor-8-epi-PGF$_{2\alpha}$ in control subjects but was absent in diabetic patients.

There were strong associations between urinary 8-epi-PGF$_{2\alpha}$ and either 2, 3-dinor-8-epi-PGF$_{2\alpha}$ or 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ in both diabetic patients as well as control subjects (Figures 54-57).

5.3.2.2. Multiple linear regression analysis

To assess whether oxidative stress was an independent determinant of diabetic polyneuropathy, multiple linear regression analysis using forward stepping was performed with total neurological impairment score of the lower limb (NIS-LL) as the dependent marker of the severity of polyneuropathy. Independent variables entered into the model included age, sex, BMI, diabetes type, diabetes duration, height, HbA_{1c}, TG, HDL and LDL cholesterol, albuminuria, retinopathy, and hypertension. The only factors that were independently associated with total NIS-LL were diabetes duration and TG levels ($p < 0.05$) (Table 9).

When plasma O_2^--QPC values were added to the original model, O_2^- -QPC, diabetes duration and TG levels were independent factors found to be associated with total NIS-LL. Interestingly, plasma ONOO-QPC remained as the only factor associated with total NIS-LL when this variable was entered into the model. Adding vitamin C resulted in retinopathy being the only factor left in the model ($p < 0.05$). Duration of diabetes and type 2 diabetes constituted the factors associated with total NIS-LL ($p < 0.05$) when the vitamin E/cholesterol ratio was added to the original model (excluding TG, HDL, and LDL). When 8-epi-$PGF_{2\alpha}$ was added to the original model, diabetes duration and TG concentrations remained in the model as factors associated with total NIS-LL.

In conclusion, all three measures of plasma TAC i.e. ONOO-QPC, O_2^--QPC and HOCl-QPC were found to be inter-related. These assays also correlated with plasma vitamin C, vitamin E and 8-epi-$PGF_{2\alpha}$ as well as urinary 8-epi-$PGF_{2\alpha}$ and its metabolites in the diabetic group. Importantly, the measurement of plasma TAC was independently associated with the

severity of diabetic neuropathy. Taken together, these data indicate that the assessment of

TAC may prove to be the most useful tool in future studies for examining the role of

oxidative stress in the pathogenesis of diabetic complications.

Table 7: Correlations between plasma and urinary measures of oxidative stress in control subjects.

	Plasma						Urine		
	Vit C	Vit E	ONOO-QPC	O2⁻-QPC	HOCl-QPC	8-Epi	8-Epi	Dinor-8-epi	Dihydro-8-epi
Vit C		——	0.279*	-0.547***	——	-0.33**	-0.550***	-0.609*	——
Vit E	——		——	-0.423***	——	——	——	——	——
ONOO-QPC	0.279*	——		-665***	——	-0.310*	——	——	——
O2⁻-QPC	-0.547***	-0.423***	-0.665***		——	0.348***	——	——	——
HOCl-QPC	——	——	——	——		——	——	——	——
8-Epi	-0.33**	——	-0.310*	0.348***	——		——	——	——
8-Epi	——	——	——	——	——	——		0.884***	0.587*
Dinor-8-epi	-0.609*	——	——	——	——	——	0.884***		0.704***
Dihydro-8-epi	——	——	——	——	——	——	0.587*	0.704***	

8-Epi: 8-epi-PGF$_{2\alpha}$, ; Dinor-8-epi: 2, 3-dinor-8-epi-PGF$_{2\alpha}$; Dihydro-8-epi: 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$

*p<0.05; ** p<0.001; ***p<0.005.

Table 8: Relationships between plasma and urinary measures of oxidative stress in diabetic patients.

| | Plasma | | | | | | Urine | | |
	Vit C	Vit E	ONOO-QPC	O_2^--QPC	HOCl-QPC	8-Epi	8-Epi	Dinor-8-epi	Dihydro-8-epi
Vit C		0.190*	0.239**	-0.179*	-0.289***	――――	――――	――――	――――
Vit E	0.190*		0.273**	-0.451***	-0.225***	-0.158*	――――	――――	――――
ONOO-QPC	0.239**	0.273**		-0.518***	-0.469***	――――	――――	0.169*	0.197*
O_2^--QPC	-0.179*	-0.451***	-0.518***		0.500***	0.158*	――――	-0.234***	-0.227**
HOCl-QPC	-0.289**	-0.225**	-0.469***	0.500***		0.163*	――――	-0.256***	-0.181*
8-Epi	――――	-0.156*	――――	0.158*	0.163*		――――	――――	――――
8-Epi	――――	――――	――――	――――		――――		0.420***	0.526***
Dinor-8-epi	――――	――――	0.169*	-.0234**	-0.256**	――――	0.420***		0.788***
Dihydro-8-epi	――――	――――	0.197*	-0.227**	-0.181*	――――	0.526***	0.788***	

8-Epi:8-epi-$PGF_{2\alpha}$, ; Dinor-8-epi: 2, 3-dinor-8-epi-$PGF_{2\alpha}$; Dihydro-8-epi: 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$

*p<0.05; ** p<0.001; ***p<0.005

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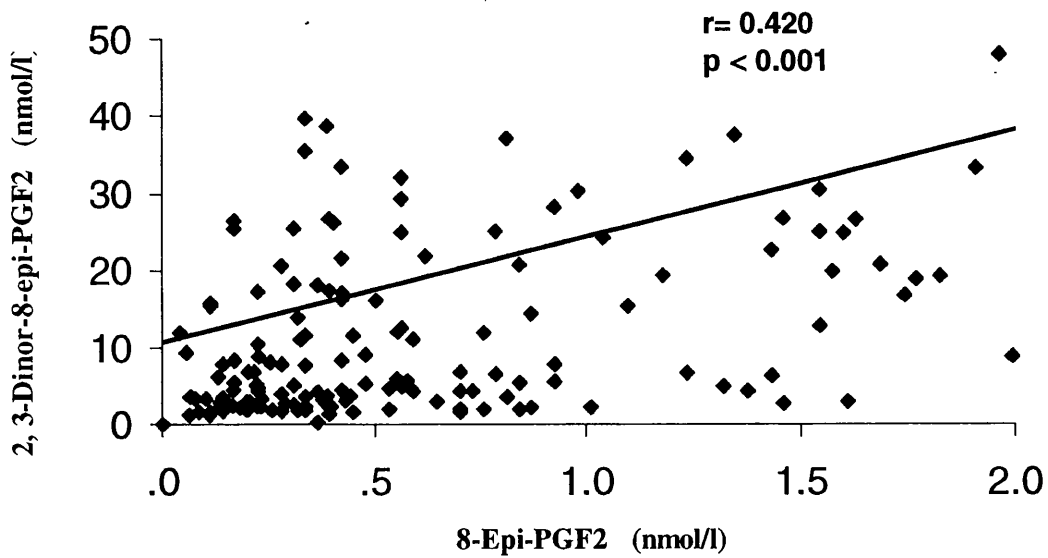
Figure 54: Correlation between 8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-8-epi-PGF$_{2\alpha}$ in diabetic patients.
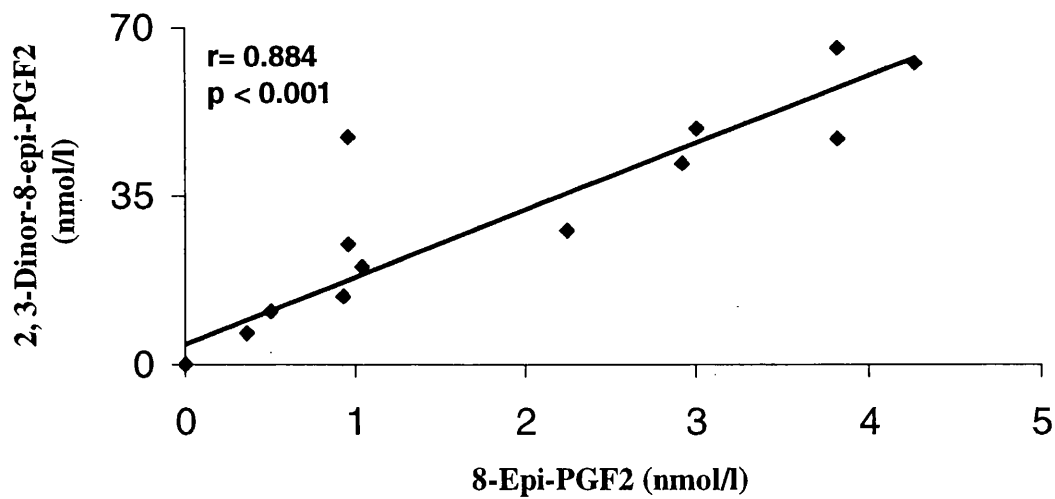
Figure 55: Correlation between urinary 8-epi-PGF$_{2\alpha}$ and 2, 3-dinor-8-epi-PGF$_{2\alpha}$ in control subjects.
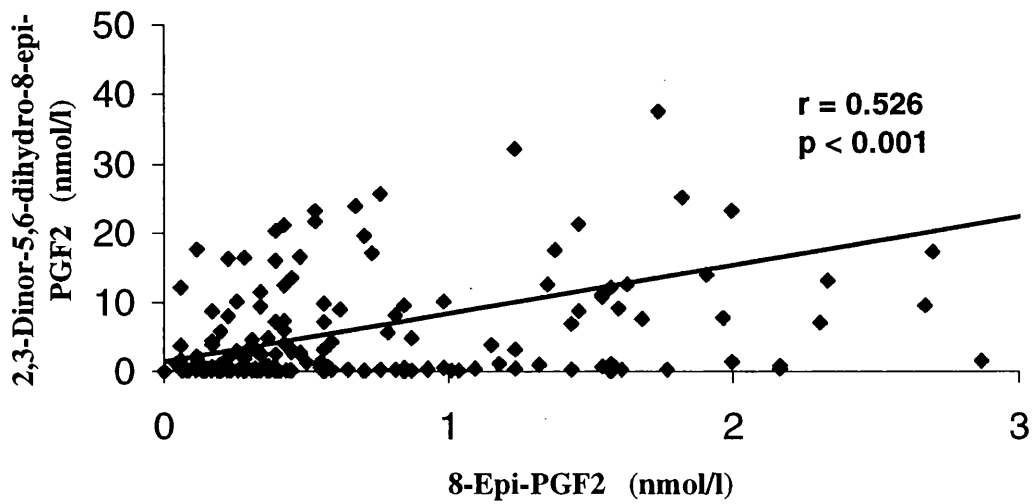
Figure 57: Correlation between urinary 8-epi-$PGF_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ in diabetic patients.
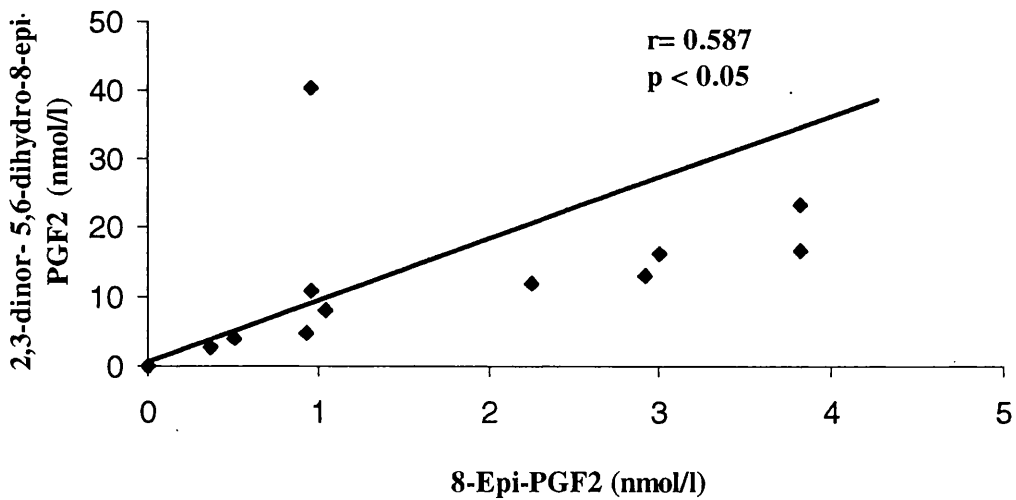
Figure 57: Association between urinary 8-epi-$PGF_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ in control subjects.

Table 9: Multivariate models identifying factors independently associated with total neurological

impairment score of lower limb (NIS-LL).

Variable added to the model	Independent variables associated with NIS-LL		
	Variable(s)	$\beta \pm SEM$	P
Original model	Duration of diabetes	0.137±0.053	0.010
	Triglycerides	0.014±0.006	
Original model + O_2^--QPC	Duration of diabetes	0.455±0.180	0.015
	Triglycerides	0.142±0.055	0.011
	O_2^--QPC	0.012±0.006	0.041
Original model + ONOO-QPC	ONOO-QPC	-0.124±0.052	0.019
Original model + vit C	Retinopathy	2.691±1.074	0.013
Original model + Vit E/Cholesterol	Duration of diabetes	0.157±0.057	0.007
	Type 2 diabetes	2.541±1.177	0.032
Original model + 8-epi- $PGF_{2\alpha}$	Duration of diabetes	0.144±0.057	0.013
	Triglycerides	0.015±0.006	0.019

Chapter 6

Concluding remarks

It has been proposed that oxidative stress may be associated with the development of diabetic complications. Therefore, potential biomarkers of oxidative stress may aid in the elucidation of disease-related pathophysiological mechanisms. In addition, once reliable determination of such biochemical marker(s) is established their use may prove valuable with respect to: 1) improved diagnostic specificity and sensitivity; 2) the monitoring of the progression of disease and, 3) the examination of the efficacy of therapeutic interventions.

Various methodological and/or mechanistic aspects of free radical generation in diabetes have examined previously (Nourooz-Zadeh et al., 1995a & 1997b; Rocic et al., 1997; Santini et al., 1997; Davi et al., 1997 & 1999; Opara et al., 1999; Gopaul et al., 2000; Matteucci & Giampietro, 2001; Ceriello et al., 1997 & 1998; Marra et al., 2002; Sampson et al., 2002; Vessby et al., 2002; Martin-Gallan et al., 2003). The principle problems with previous studies are that only single or at most two parameters were simultaneously measured as indices of oxidative stress providing only limited information. Second, the issue of the role of oxidative stress in diabetic neuropathy has not also been addressed specifically.

In this study, a battery of biomarkers including plasma total antioxidant capacity as measured by ONOO-QPC and O_2^--QPC, nutrient vitamins and 8-epi-PGF$_{2\alpha}$, and urinary 8-epi-PGF$_{2\alpha}$ and its metabolites (2,3-dinor-8-epi-PGF$_{2\alpha}$ and 2,3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$) were utilised to establish if oxidative stress is associated with the severity of diabetic PNP and CAN. Among the biomarkers determined in the present study, plasma 8-epi-PGF$_{2\alpha}$ and vitamin C have previously been reported to be associated with oxidative stress in human populations (Block et al., 2002).

Type 1 diabetic patients (PNP-/CAN-) had lower TAC as assessed by O_2^--QPC when compared to control subjects. Lower TAC (O_2^--QPC) were seen in the presence PNP and

that further reduction were observed in the presence of additional CAN. Type 2 diabetic

patients (PNP-/CAN-) also exhibited lower TAC (O_2^- -QPC) than in control subjects. The

presence of PNP resulted in further reduction in TAC (O_2^- -QPC) while the presence of

additional CAN was without influence. As the case with type O_2^--QPC , type 1 and type 2

diabetic patients (PNP-/CAN-) had lower TAC (ONOO-QPC) relative to control subjects.

The presence of PNP resulted in further reduction in TAC (ONOO-QPC) in type 2 diabetics

but not in type 1 patients. In the presence of additional CAN, type 1 diabetic patients

exhibited lower TAC (ONOO-QPC) than in patients with (PNP-/CAN-) whilst no

differences were seen in the case of type 2 diabetics.

Type 1 diabetics (PNP-/CAN-) exhibited lower vitamin E/cholesterol ratios relative to

control subjects and that no alterations were noted in the presence of PNP. However,

significant reductions were seen in vitamin E/cholesterol ratios in the additional presence of

CAN. In the case of type 2 diabetics, vitamin E/cholesterol ratios were lower in patients

(PNP-/CAN-) than in control subjects and that further reductions were noted in the presence

of PNP. On the contrary, the presence of additional CAN was associated with a marked

increased in vitamin E/cholesterol ratios. The reason for the observed increases in vitamin

E/cholesterol ratios in the presence of both PNP and CAN is unclear. It is possible,

however, that the observations made may reflect differences in dietary vitamin E intake.

Vitamin C levels were lower in type 1 diabetic patients (PNP-/CAN-) when compared to

control subjects. No further alterations were observed in vitamin C concentrations in the

presence of either PNP or the additional presence of CAN. In type 2 diabetes, no

differences were seen in vitamin C levels between patients (PNP-/CAN-) and control

subjects. Reductions were observed in vitamin C levels in the presence of PNP and that the presence of additional CAN was without influence.

Lipid peroxidation is a process whereby LDL and other lipid-containing molecules may become oxidised in the blood stream (Bruckdorfer, 1998). Products of lipid peroxidation exert adverse effects on a variety of processes, inhibiting antithrombin III activity, producing procoagulant activity, enhancing platelet aggregation, modulating vascular responses, and acting as mitogens. Increased formation of lipid peroxidation products is shown to be associated with neuronal damage in experimental diabetic neuropathy (Low & Nickander, 1991; Kihara et al., 1991; Lowitt et al., 1995; Nagamatsu et al., 1996; Nickander et al., 1996; Obrosova et al., 2000).

Type 1 diabetic patients (PNP-/CAN-) had markedly lower 8-epi-PGF$_{2\alpha}$ concentrations than control subjects. The presence of PNP was associated with increased 8-epi-PGF$_{2\alpha}$ formation and that the additional presence of CAN was without influence. No differences were seen in 8-epi-PGF$_{2\alpha}$ concentrations between type 2 diabetics (PNP-/CAN-) and control subjects. Elevated 8-epi-PGF$_{2\alpha}$ levels were observed in the presence of PNP. The additional presence of CAN, on the other hand, resulted in a marked reduction in 8-epi-PGF$_{2\alpha}$ concentrations. It is of note that type 2 diabetic patients (PNP+/CAN+) exhibited increases in plasma vitamin E compared to other subgroups. This group also had the highest degree of individuals on insulin-treatment i.e. 85% as compared with 48.3% for patients (PNP+/CAN-). Thus, reduced 8-epi PGF$_{2\alpha}$ concentrations in diabetics with (PNP+/CAN+) may be related to improved antioxidant status and/or insulin treatment.

Two principle investigations have shown that urinary excretion of 8-epi-$PGF_{2\alpha}$ is elevated in diabetic patients and that treatment with antioxidants reduces its levels its levels (Davi et al., 1999; Devaraj et al., 2001). In this study, it was found that urinary 8-epi-$PGF_{2\alpha}$ levels expressed relative to creatinine were reduced in diabetic patients than in control subjects. 8-Epi-$PGF_{2\alpha}$/creatinine ratios were lower in type 2 patients than type 1 diabetics. Type 1 and type 2 diabetic patients (PNP-/CAN-) exhibited lower 8-epi-$PGF_{2\alpha}$/creatinine ratios when compared to control subjects. The presence of PNP in type 1 diabetic patients was associated with increased excretion of 8-epi-$PGF_{2\alpha}$ and further increases were seen in the presence of additional presence of CAN. The presence of PNP in type 2 diabetics resulted in increased excretion of 8-epi-$PGF_{2\alpha}$ whilst the additional presence of CAN was without influence.

As is the case with 8-epi-$PGF_{2\alpha}$, excretion of creatinine-standardised 2, 3-dinor-8-epi-$PGF_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ were also lower in diabetic patients, with 2, 3-dinor-8-epi-$PGF_{2\alpha}$ being the predominant urinary metabolite. No differences in the excretion of 8-epi-$PGF_{2\alpha}$ metabolites between type 1 and type 2 diabetics. However, variations in the excretion of 2, 3-dinor-8-epi-$PGF_{2\alpha}$ and 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ were seen in relation to the presence of PNP and/or CAN in type 1 and type 2 diabetic patients.

Type 1 diabetic patients (PNP-/CAN-) exhibited lower 2, 3-dinor-8-epi-$PGF_{2\alpha}$/creatinine ratios than in control subjects. 2, 3-Dinor-8-epi-$PGF_{2\alpha}$/creatinine ratios were not altered in the presence of PNP. However, a reduction in 2, 3-dinor-8-epi-$PGF_{2\alpha}$/creatinine ratios were seen in the additional presence of CAN. No differences were seen in 2, 3-dinor-8-epi-

PGF$_{2\alpha}$/creatinine ratios between type 2 diabetic patients (PNP-/CAN-) and control subjects. 2, 3-Dinor-8-epi-PGF$_{2\alpha}$/creatinine ratios were slightly lower in the presence of PNP and that further reductions were observed in the additional presence of CAN.

In the case of 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ /creatinine ratios, no differences were noted between type 1 diabetic patients (PNP-/CAN-) and control subjects. Patients with (PNP+/CAN-) exhibited lower 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ /creatinine ratios and that further reductions were observed in the additional presence of CAN. Type 2 diabetic patients (PNP-/CAN-) had lower 2,3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ /creatinine ratios compared to control subjects. The presence of PNP was associated with a slight increase in 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$/creatinine ratios while reductions were observed in the presence of additional CAN. Taken together, there no a straightforward (yes or no) answer to question whether measurement of urinay excretion of 8-epi-PGF$_{2\alpha}$ and its metabolites represent a realiable intergrated marker(s) of non-enzymatic lipid peroxidation in the human body. Further studies are needed to examine the impact of renal contribution and/or deteroiation before their importance, as markers of oxidative stress can be determined.

Correlations occurred between plasma TAC, as assessed by ONOO-QPC and O$_2^-$ -QPC, vitamins C, vitamin E, 8-epi-PGF$_{2\alpha}$ in both diabetic and control subjects. This is of interest from a practical point of view since both ONOO-QPC and O$_2^-$-QPC are simple and inexpensive methods compared to the measurement of plasma and urinary 8-epi-PGF$_{2\alpha}$. Other advantages of the ONOO-QPC and O$_2^-$-QPC assays are that only small sample volumes (5μl) are required and the methods are easily adaptable to modern auto-analyser. In addition, the diagnostic value of the ONOO-QPC and O$_2^-$-QPC assays can be improved when used in conjugation with Trolox as an internal standard. Another potentially important

observation was that correlations occurred between TAC (ONOO-QPC and O_2^--QPC) and total NIS-LL. In addition, ONOO-QPC was found to be independently associated with the severity of neuropathy as assessed by NIS-LL.

In summary, these findings provide the first evidence in support of the hypothesis that oxidative stress is relevant to the pathogenesis of diabetic neuropathies and that assessment of total antioxidant capacity may prove to be a useful tool in the prevention or treatment of diabetic complications.

Chapter 7

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Chapter 8

Appendix
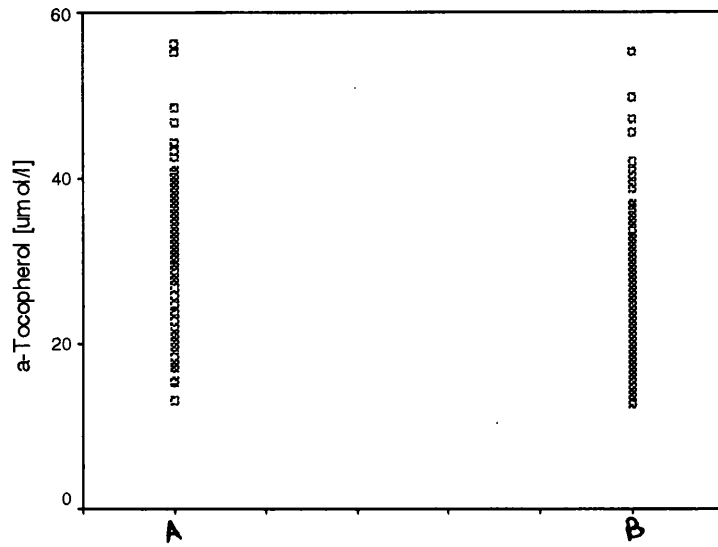
Figure 58: Plasma α–tocopherol levels in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
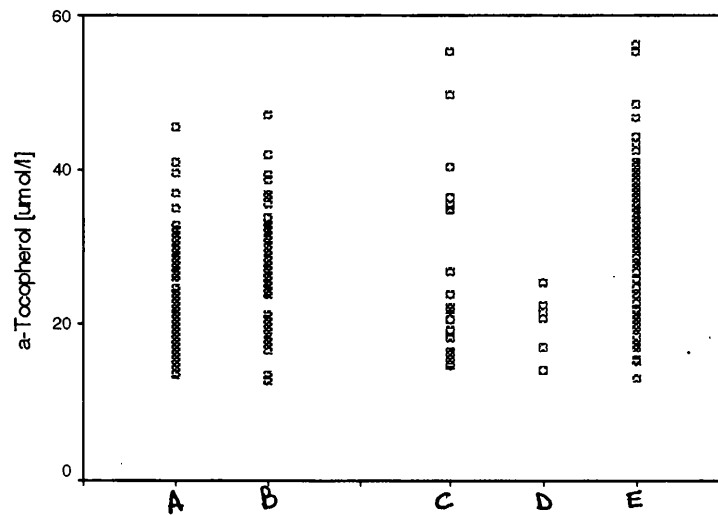
Figure 59: Plasma α–tocopherol levels in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetic patients; E) Control subjects.
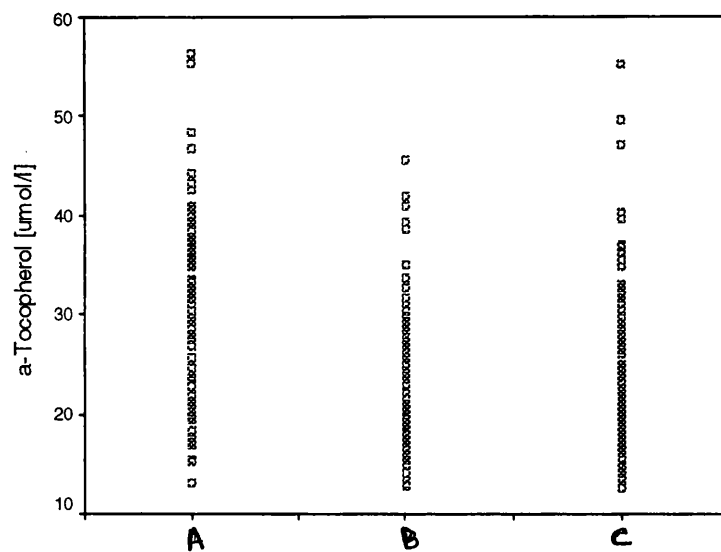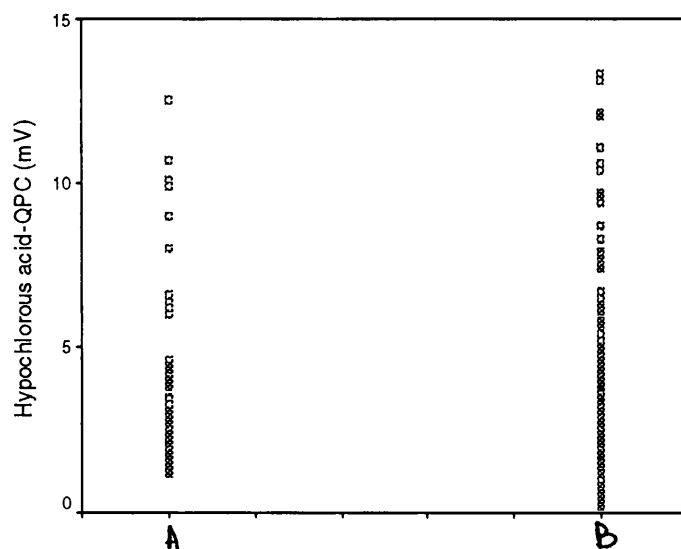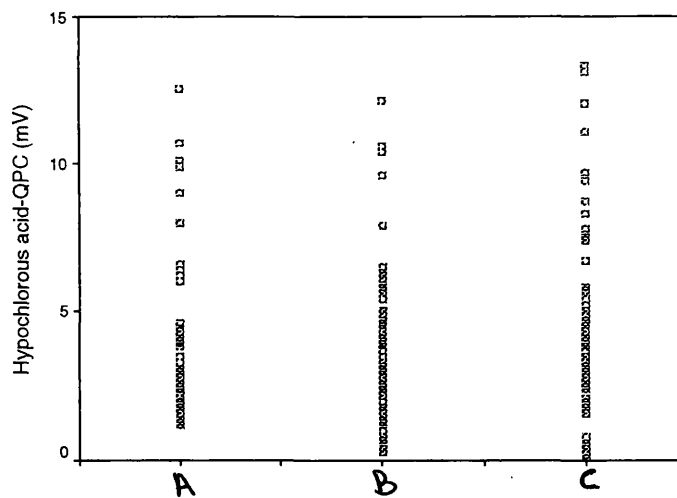
Figure 60: Plasma α–tocopherol levels in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.
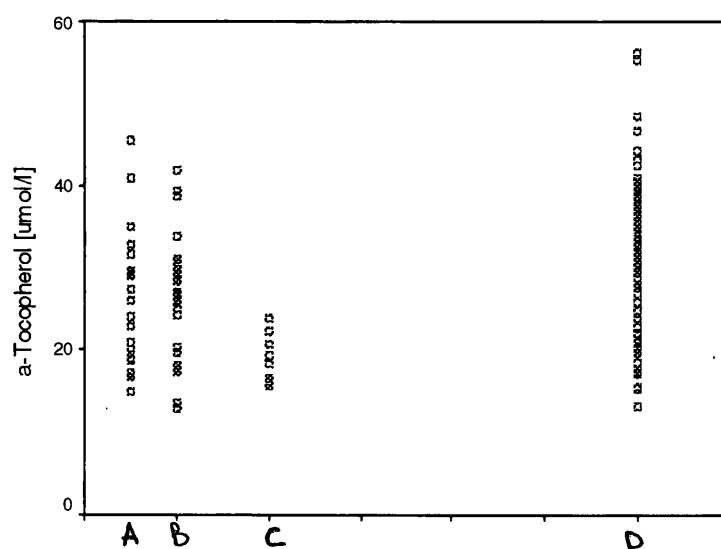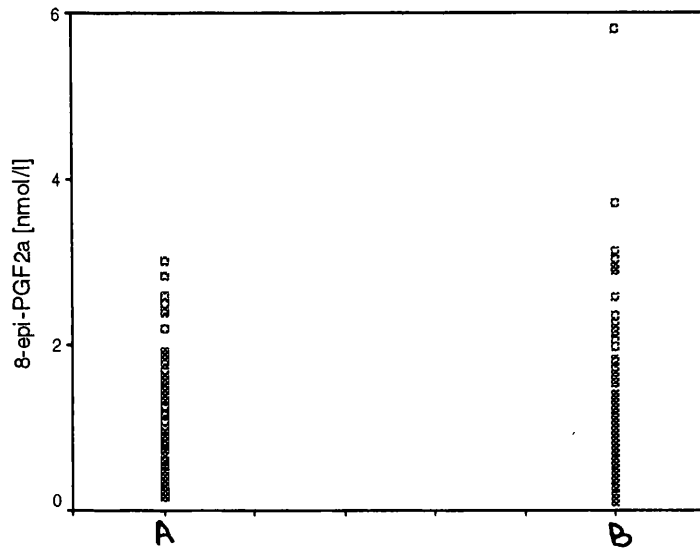

Figure 61: Plasma α–tocopherol levels in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
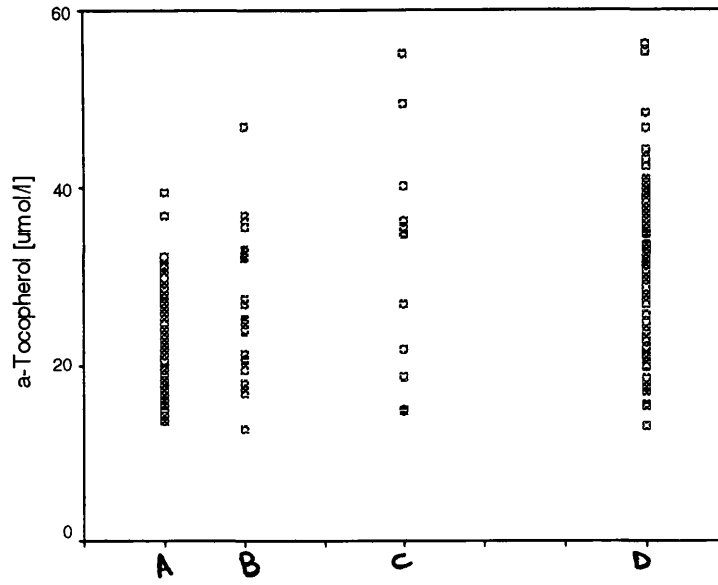
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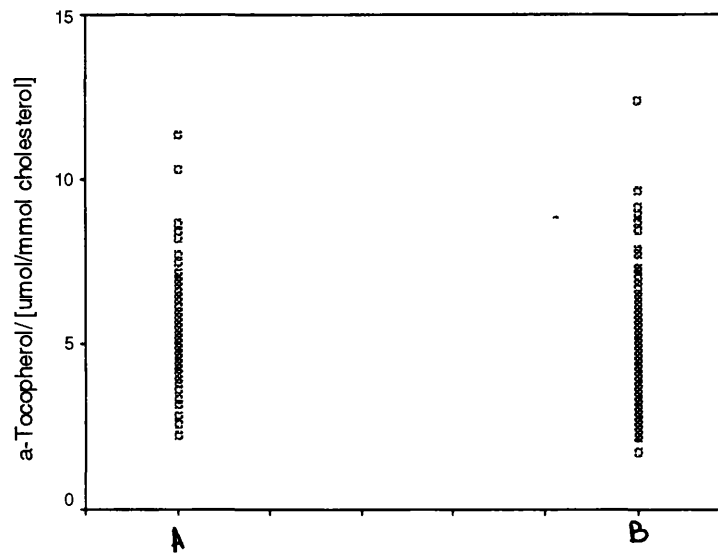

Figure 63: Plasma α–tocopherol/cholesterol ratios in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
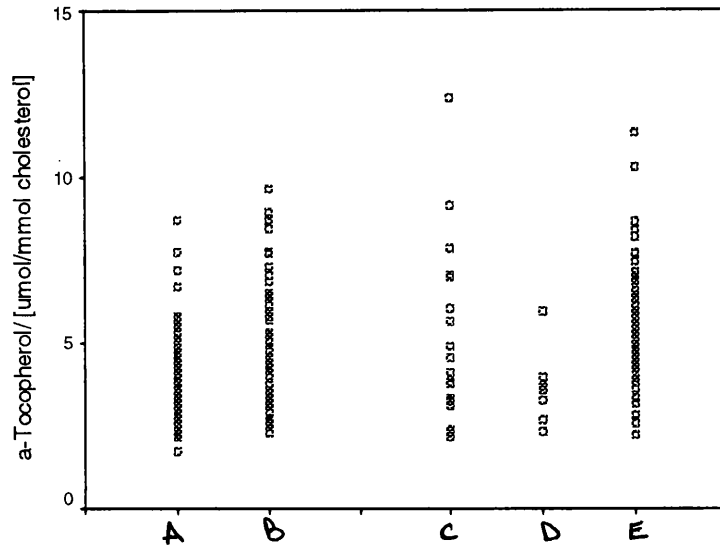
Figure 64: Plasma α–tocopherol/cholesterol ratios in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.
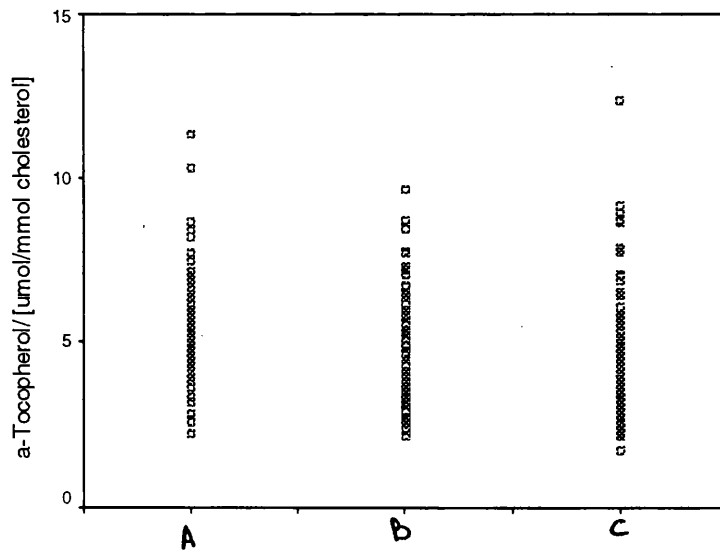
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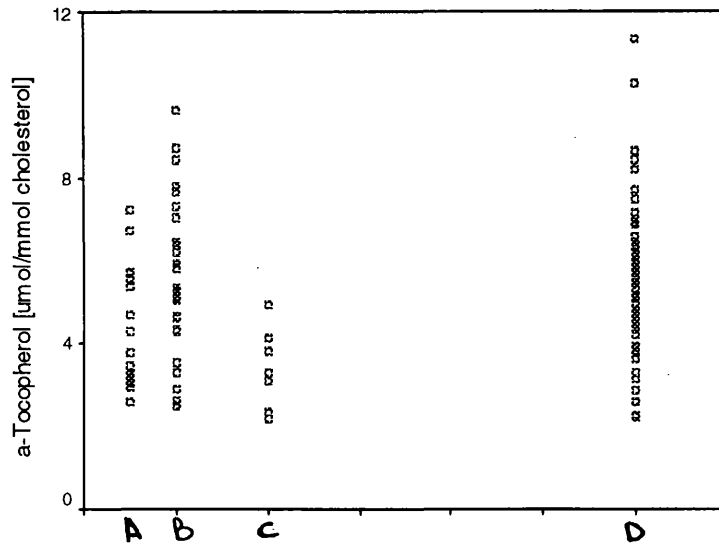
Figure 66: Plasma α-tocopherol/cholesterol ratios in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
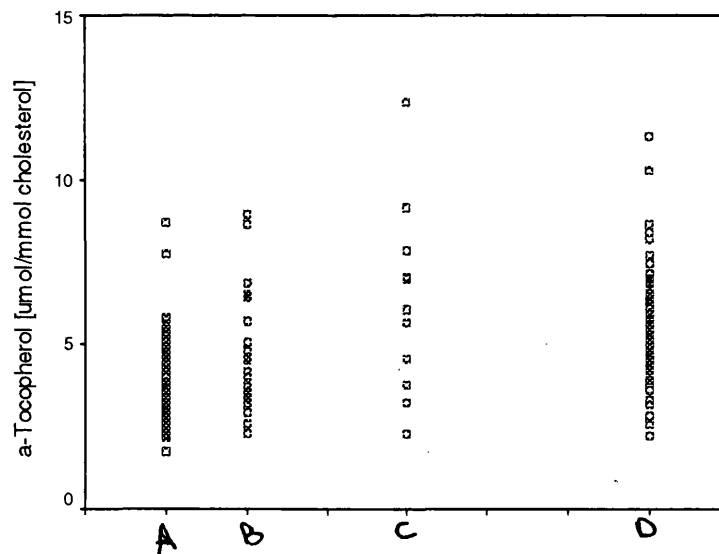
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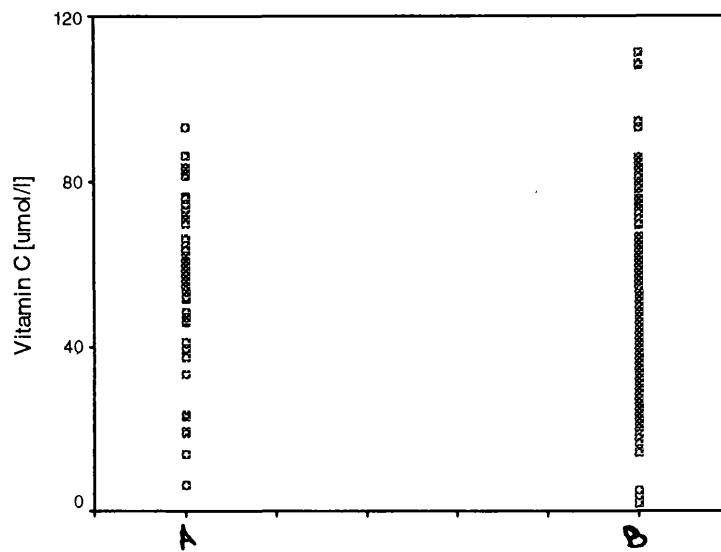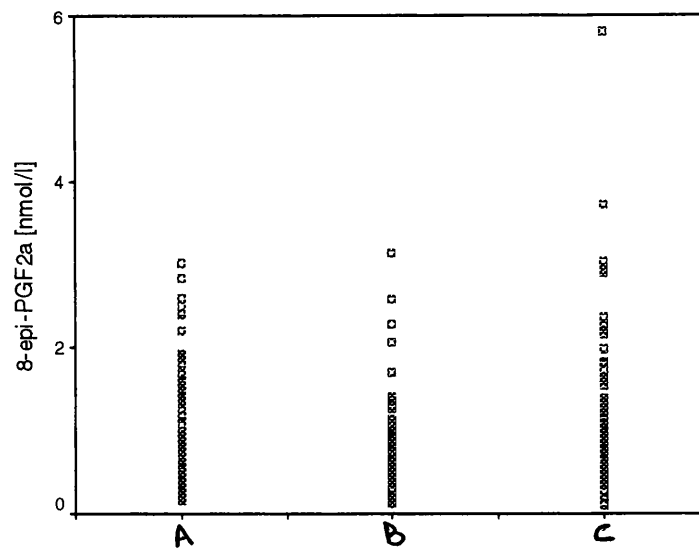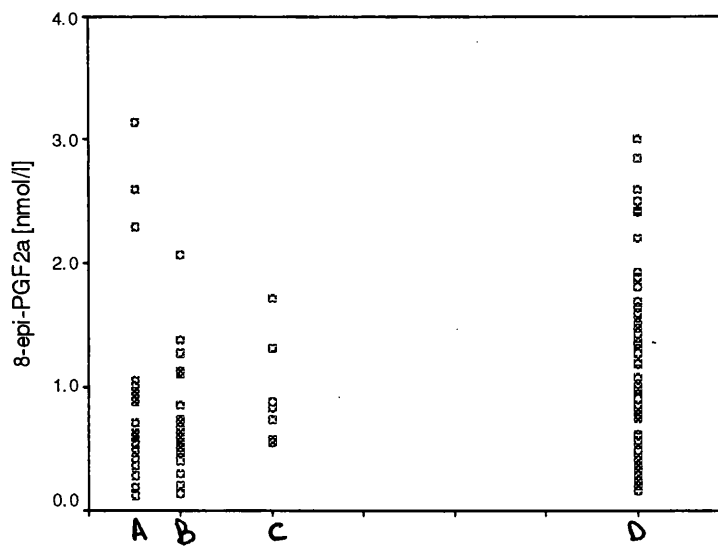
Figure 68: Plasma vitamin C levels in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
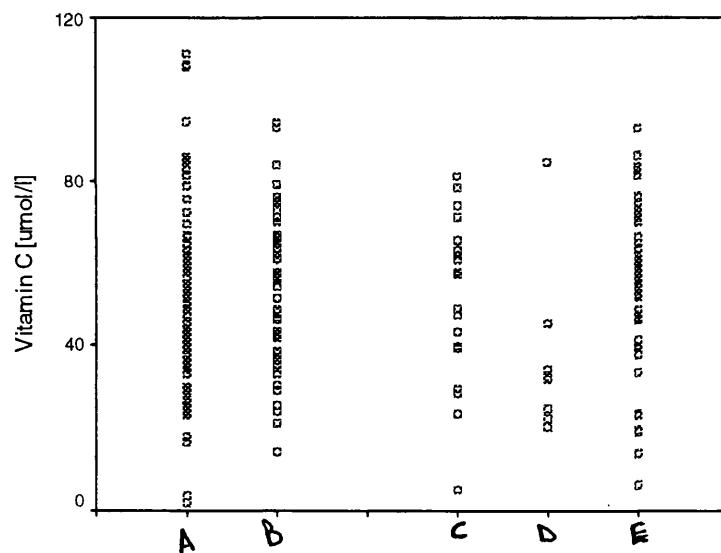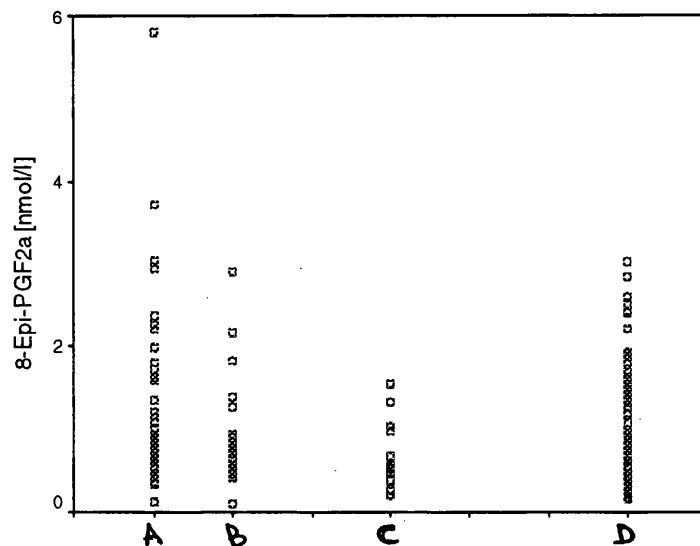

Figure 69: Plasma vitamin C levels in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; D) Control subjects.
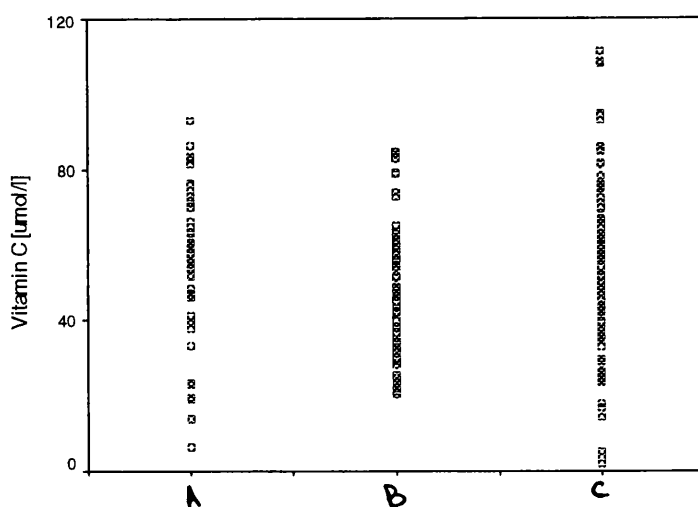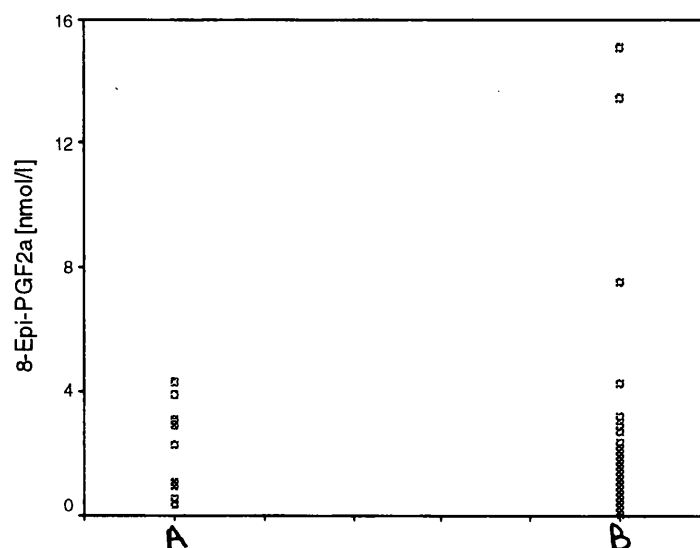
Figure 70: Plasma vitamin C levels in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; and C) type 2 diabetics.
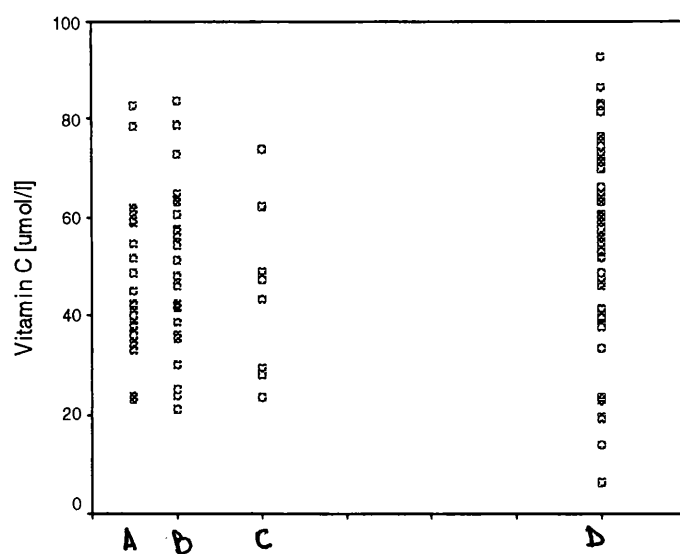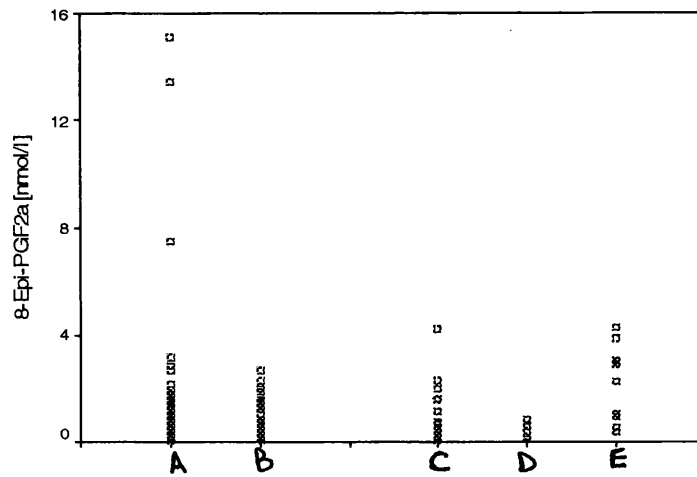

Figure 71: Plasma vitamin C levels in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
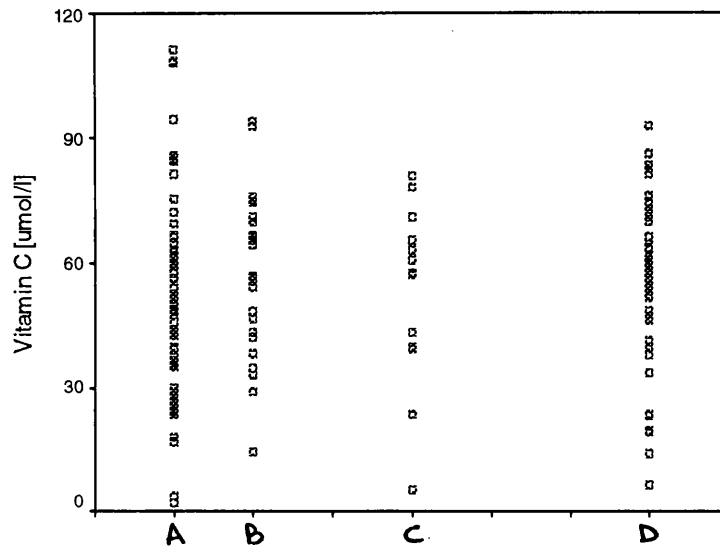
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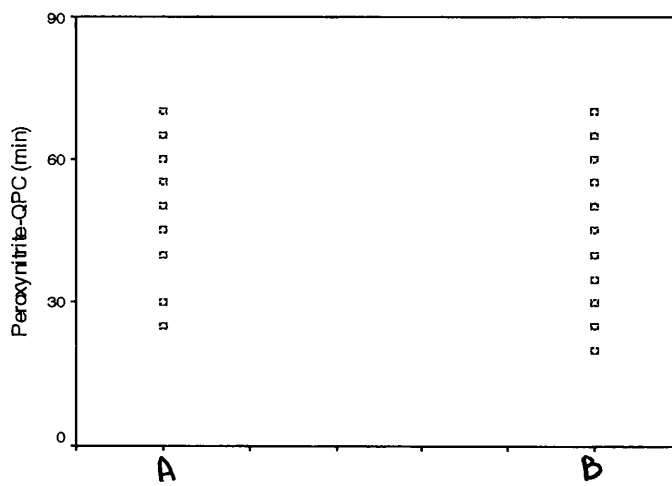

Figure 73: Plasma total antioxidant capacity (peroxynitrite-QPC) in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
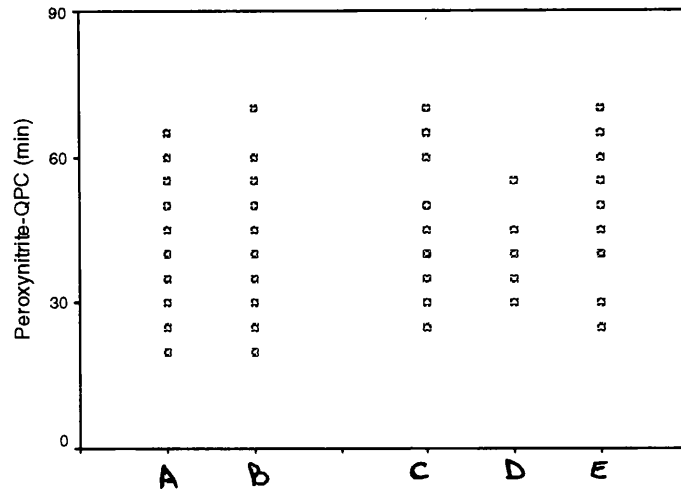
Figure 74: Plasma total antioxidant capacity (peroxynitrite-QPC) in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+ and D) Newly diagnosed type 1 diabetics; E) Control subjects.
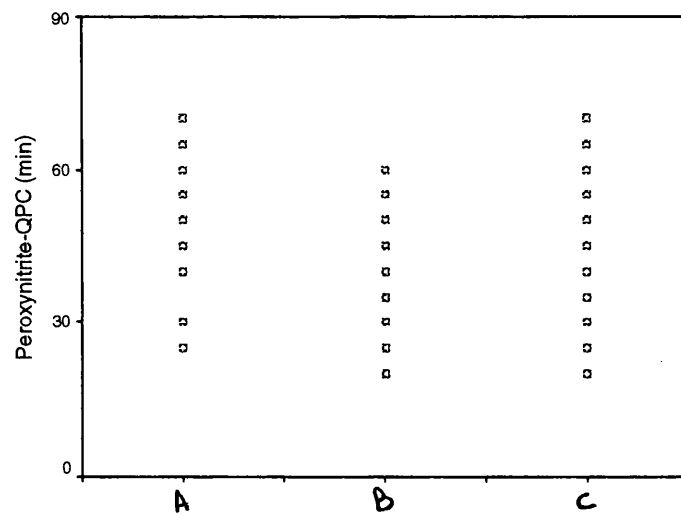
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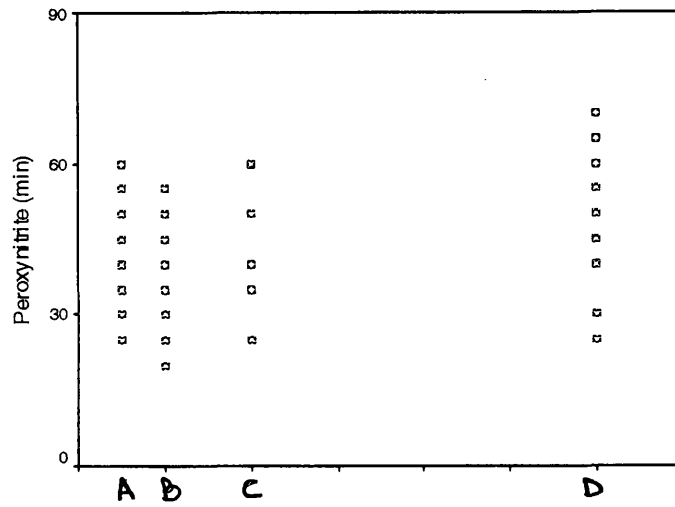
Figure 76: Plasma total antioxidant capacity (peroxynitrite-QPC) in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
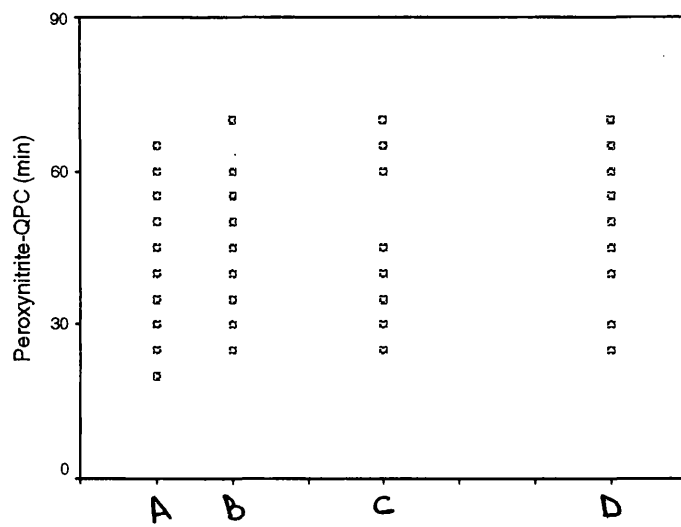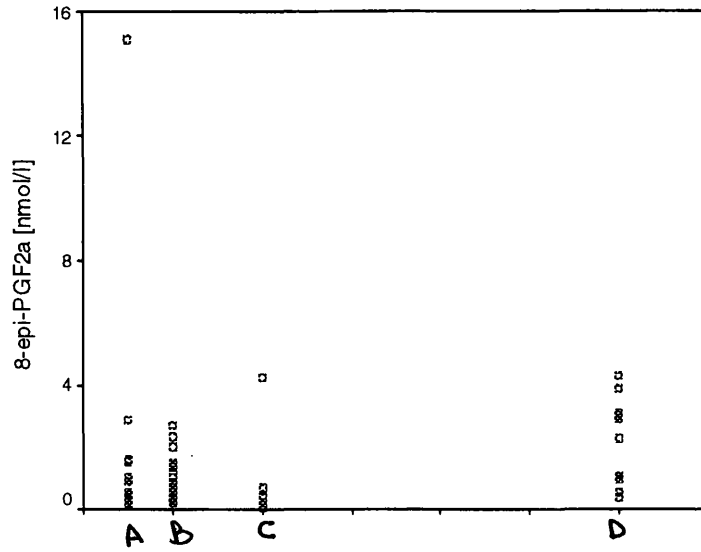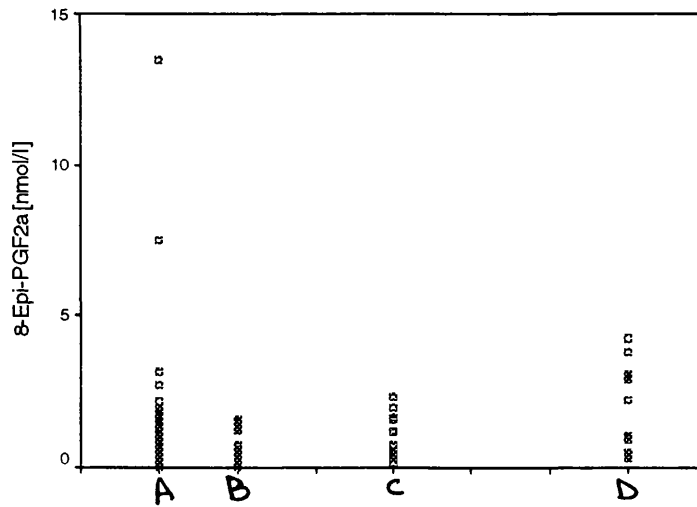

Figure 77: Plasma total antioxidant capacity (peroxynitrite-QPC) in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
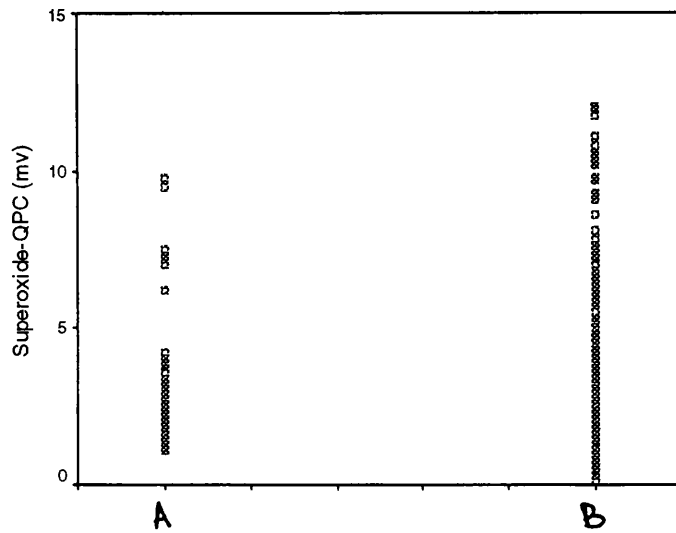
Figure 78: Plasma total antioxidant capacity (superoxide anion-QPC) in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
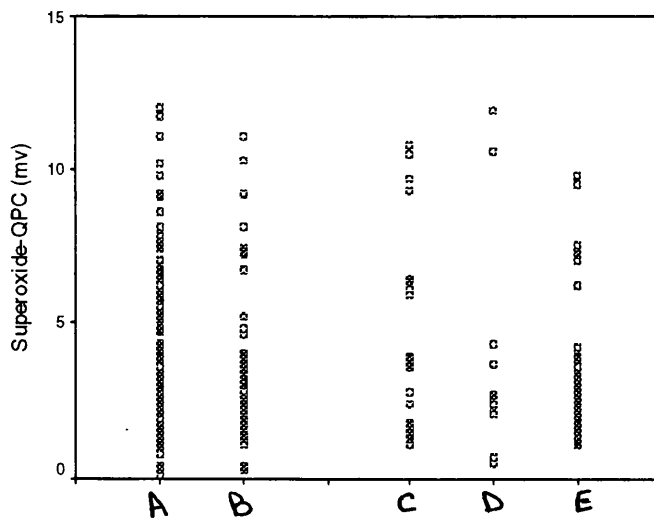
Figure 79: Plasma total antioxidant capacity (superoxide anion-QPC) in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.
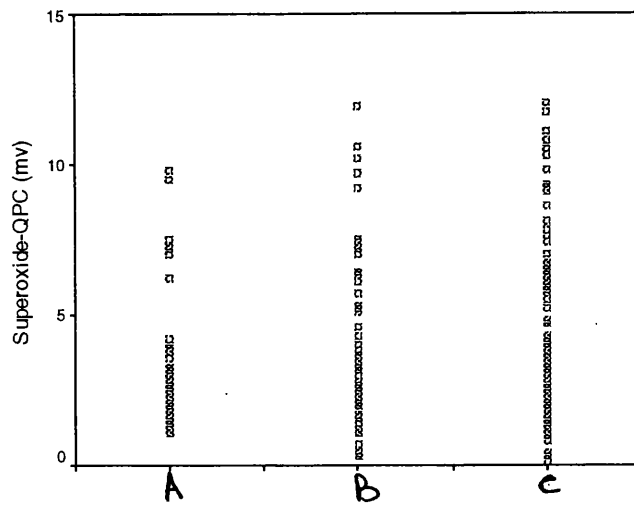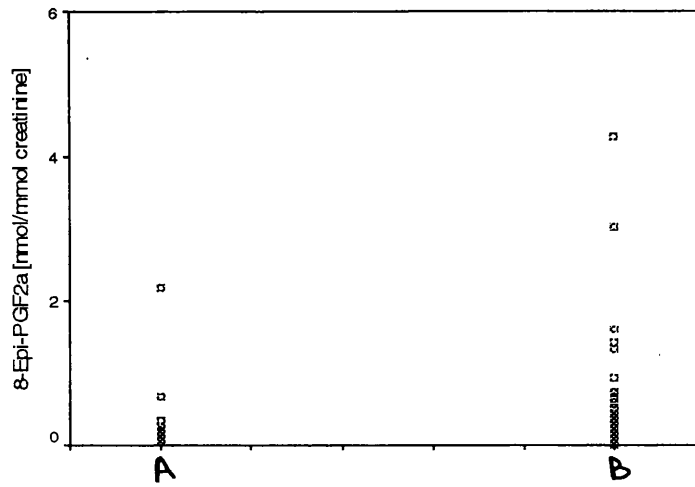
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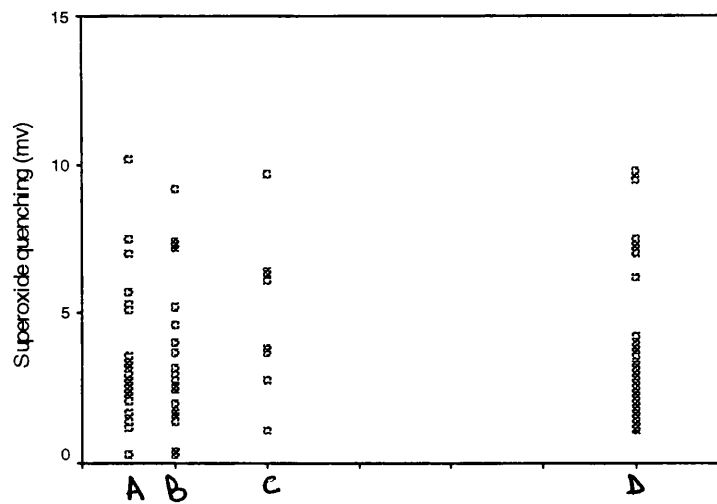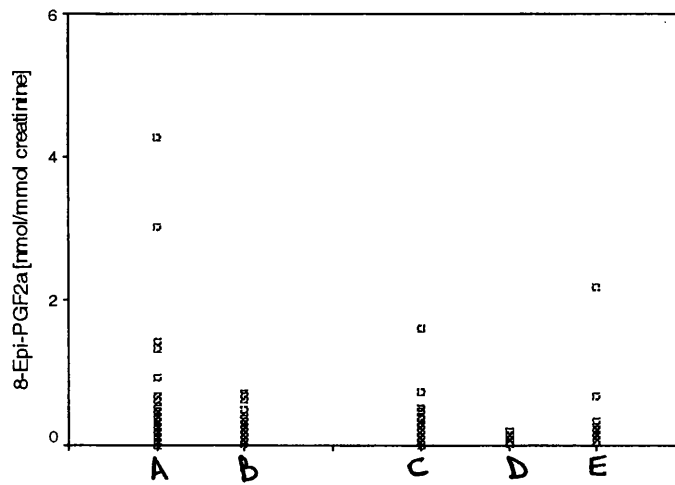

Figure 81: Plasma total antioxidant capacity (superoxide anion-QPC) in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) newly diagnosed type 1 diabetics; and E) Control subjects.
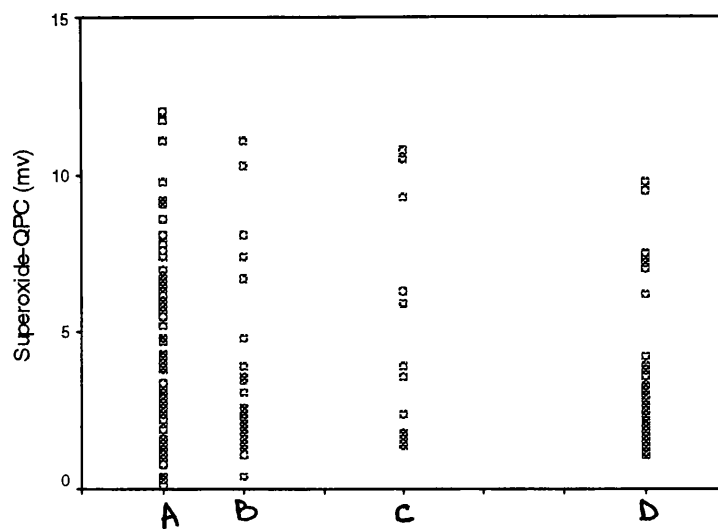
Figure 82: Plasma total antioxidant capacity (superoxide anion-QPC) in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.


Figure 83: Plasma total antioxidant capacity (hypochlorous acid-QPC) in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
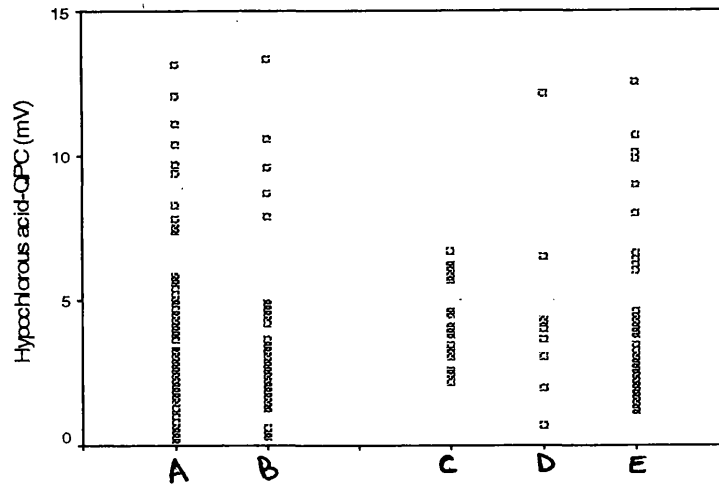
Figure 84: Plasma total antioxidant capacity (hypochlorous acid-QPC) in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.
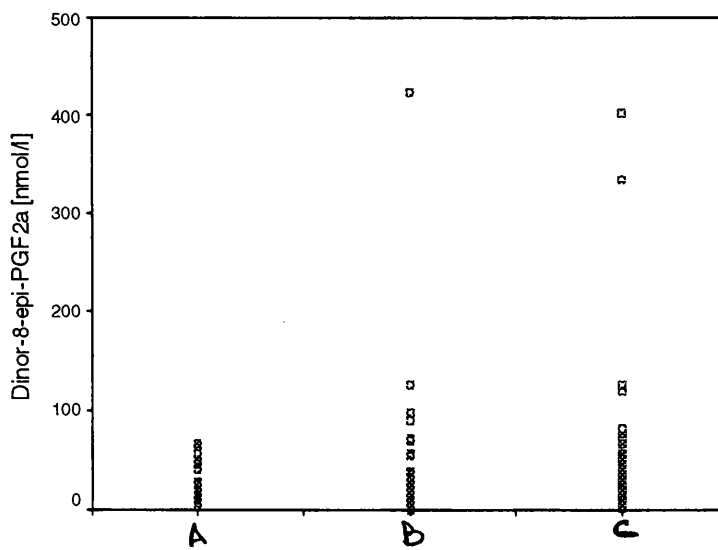

Figure 85: Plasma total antioxidant capacity (hypochlorous acid-QPC) in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.
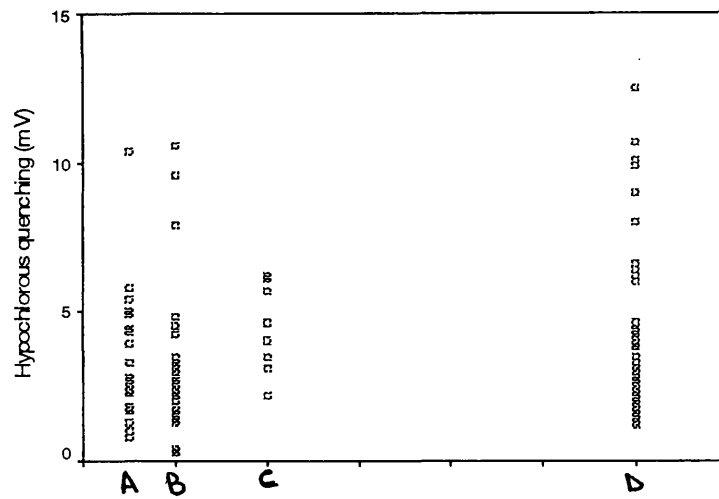
Figure 86: Plasma total antioxidant capacity (hypochlorous acid-QPC) in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
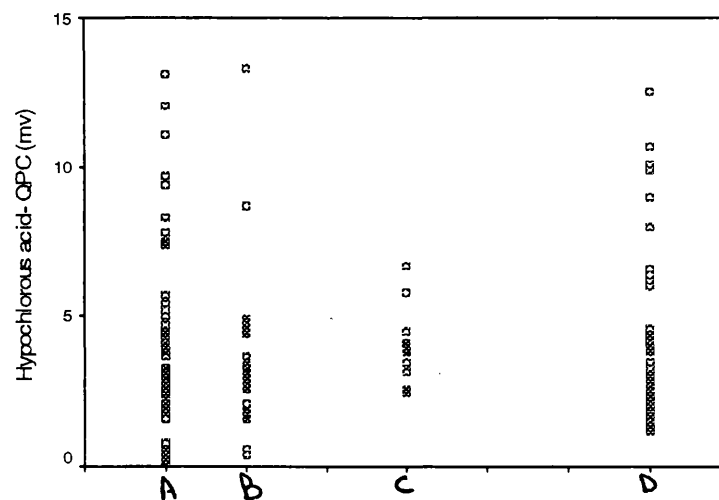
Figure 87: Plasma total antioxidant capacity (hypochlorous acid-QPC) in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
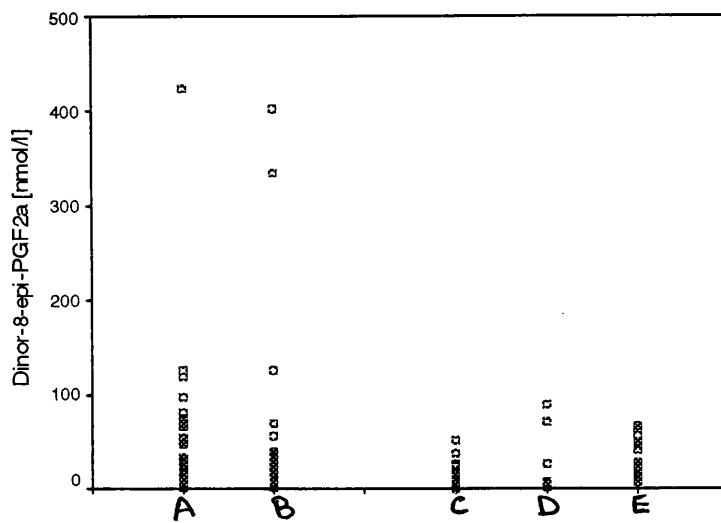
Figure 88: Plasma 8-epi PGF$_{2\alpha}$ in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
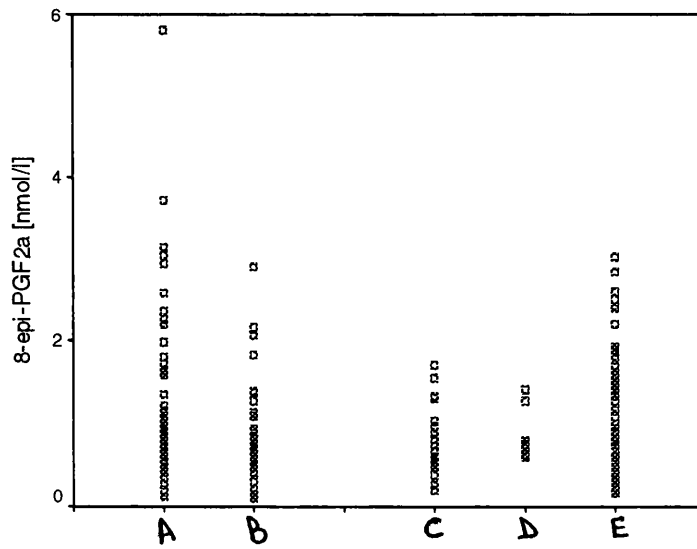
Figure 89: Plasma 8-epi PGF$_{2\alpha}$ in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.

Figure 90: Plasma 8-epi PGF$_{2\alpha}$ in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.

Figure 91: Plasma 8-epi PGF$_{2\alpha}$ in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
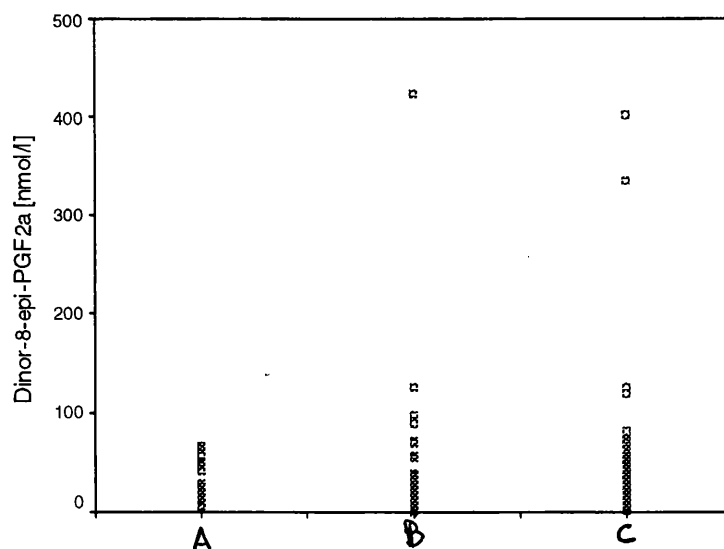
Figure 92: Plasma 8-epi $PGF_{2\alpha}$ in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

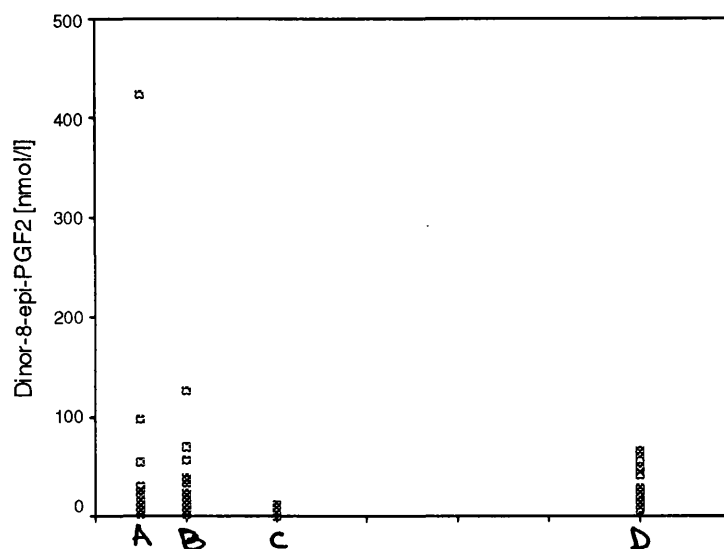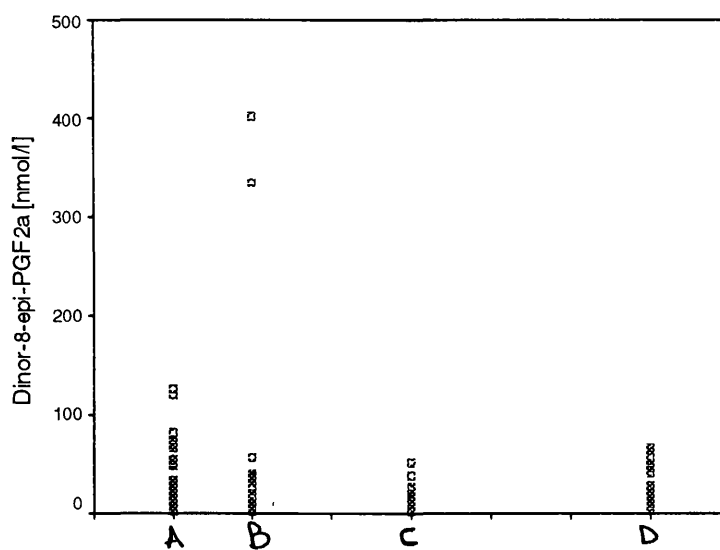**Figure 93**: Urinary 8-epi $PGF_{2\alpha}$ in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.

Figure 94: Urinary 8-epi $PGF_{2\alpha}$ in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.
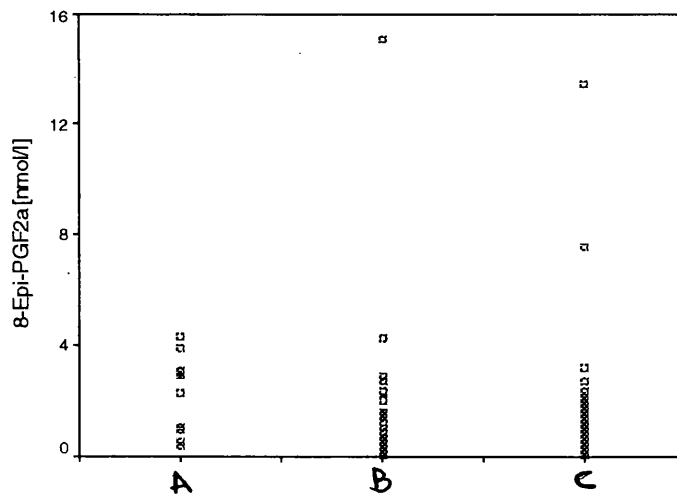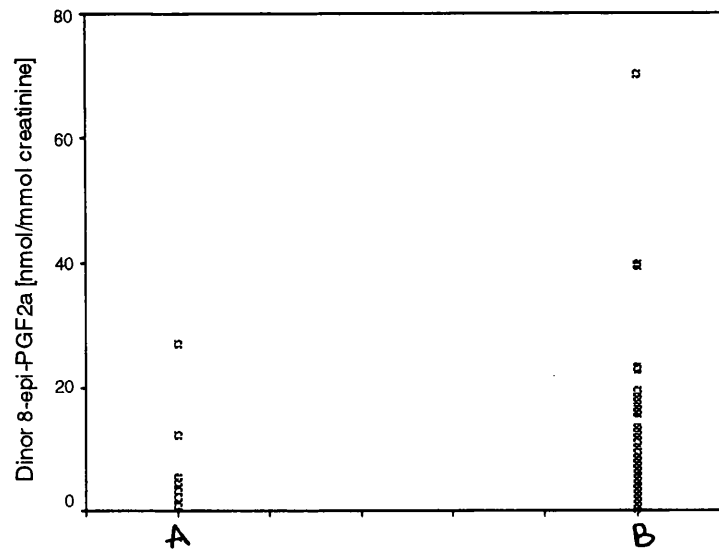

Figure 95: Urinary 8-epi $PGF_{2\alpha}$ in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; and C) type 2 diabetics.
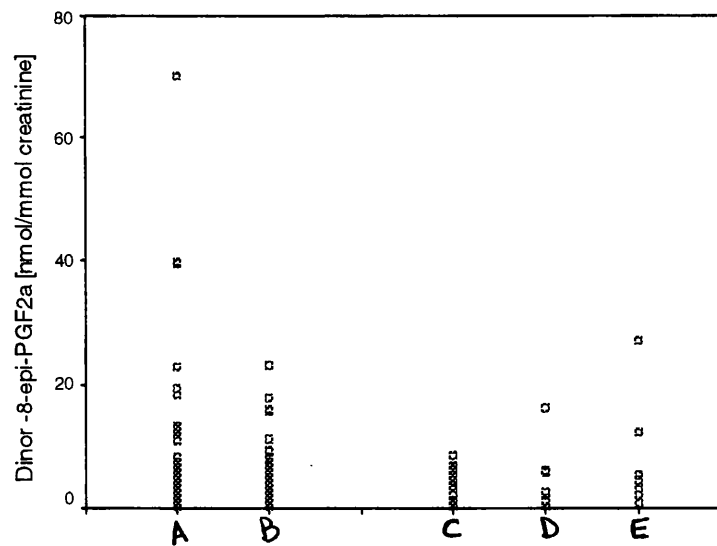
Figure 96: Urinary 8-epi $PGF_{2\alpha}$ in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

Figure 97: Urinary 8-epi $PGF_{2\alpha}$ in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
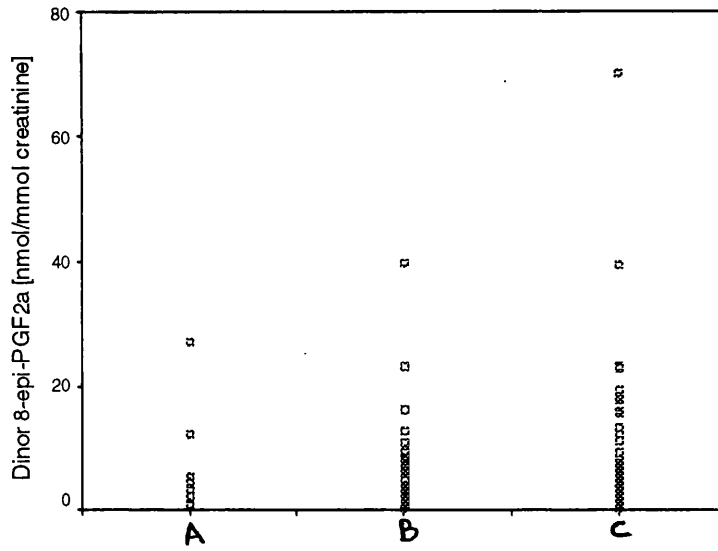
Figure 98: Urinary 8-epi PGF$_{2\alpha}$ /creatinine ratios in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.

Figure 99: Urinary 8-epi PGF$_{2\alpha}$ /creatinine ratios in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; D) Control subjects.
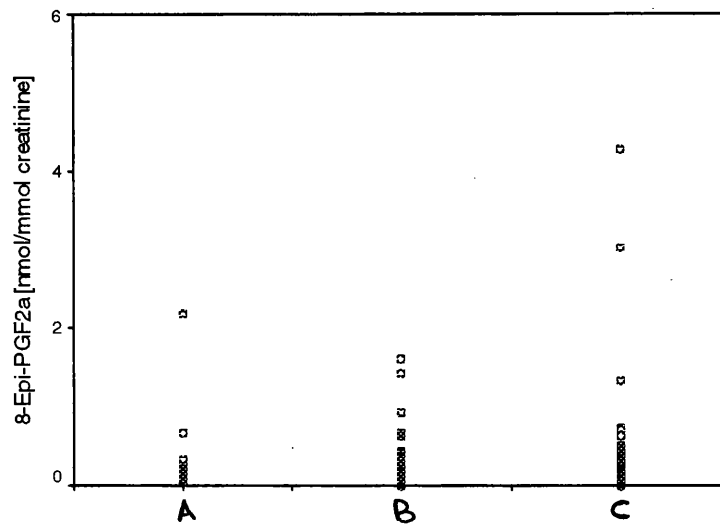
Figure 100: Urinary 8-epi $PGF_{2\alpha}$ /creatinine ratios in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.
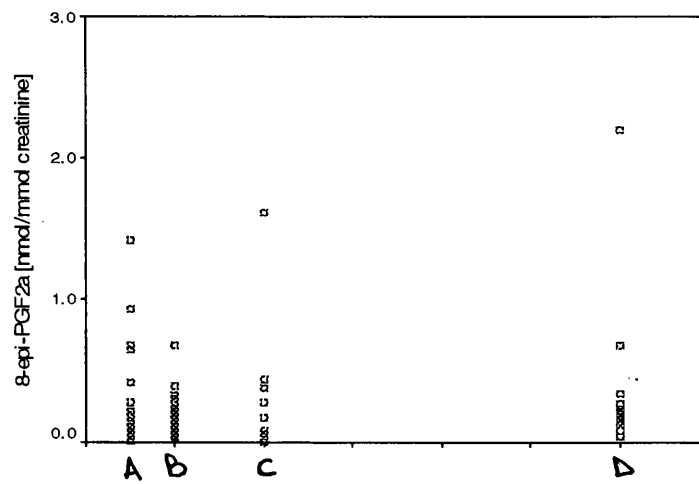
Figure 101: Urinary 8-epi $PGF_{2\alpha}$ /creatinine ratios in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
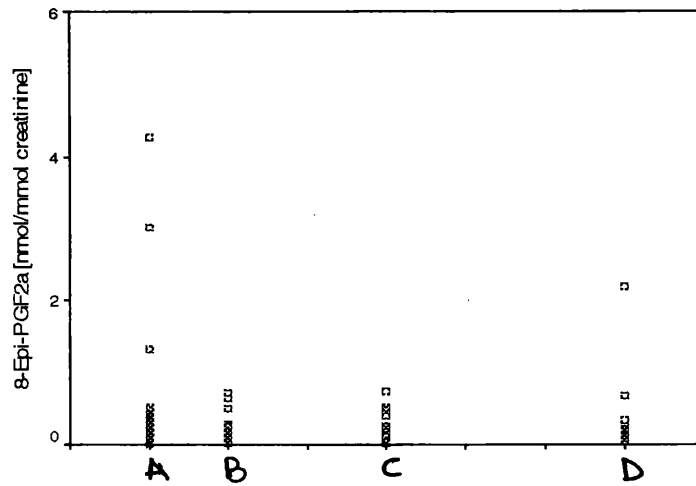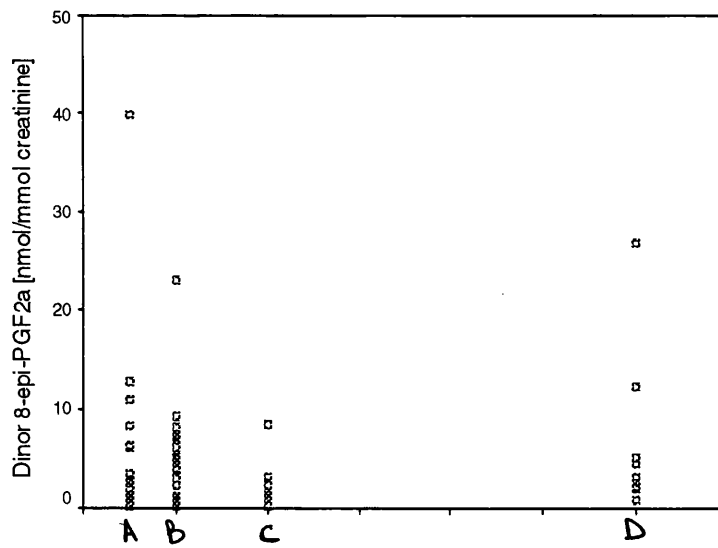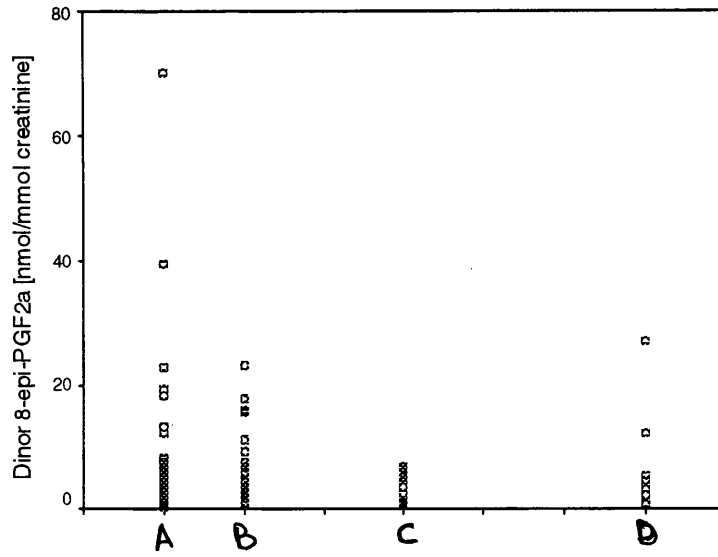
Figure 102: Urinary 8-epi $PGF_{2\alpha}$ /creatinine ratios in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

Figure 103: Urinary dinor-8-epi $PGF_{2\alpha}$ in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.

Figure 104: Urinary dinor-8-epi PGF$_{2\alpha}$ in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.
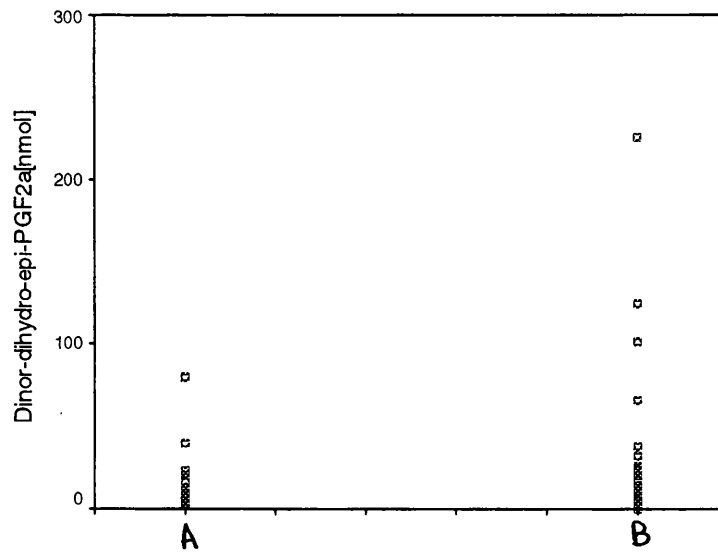

Figure 105: Urinary dinor-8-epi PGF$_{2\alpha}$ in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.
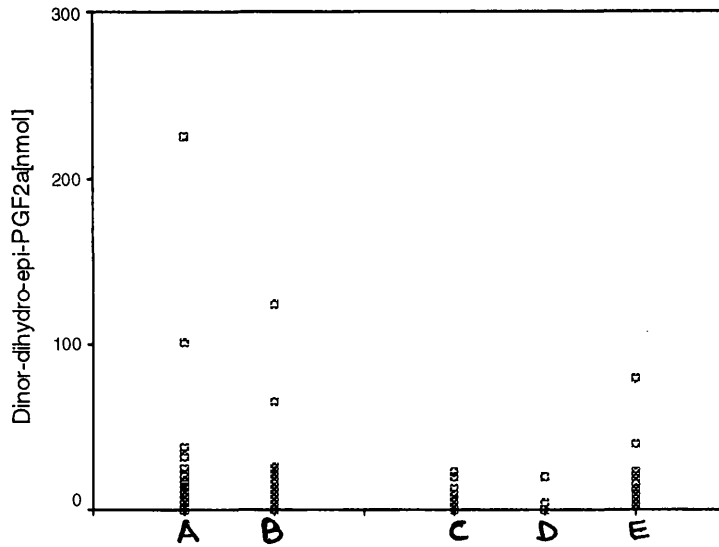
Figure 106: Urinary dinor-8-epi $PGF_{2\alpha}$ in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

Figure 107: Urinary dinor-8-epi $PGF_{2\alpha}$ in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
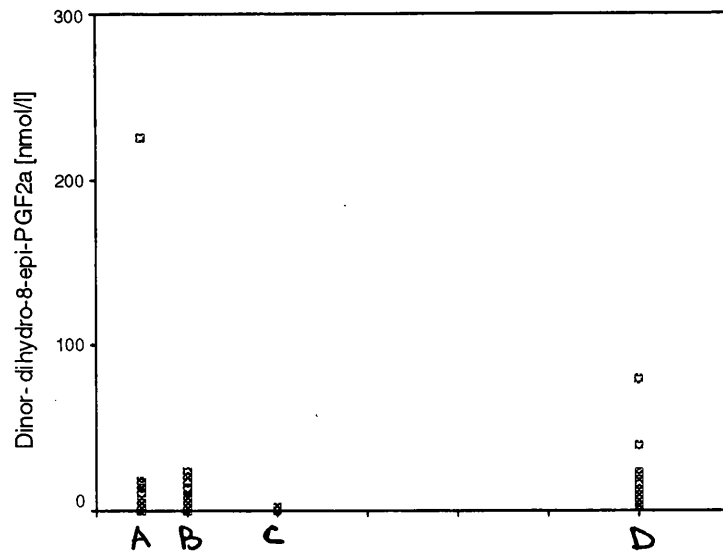
Figure 108: Urinary dinor-8-epi $PGF_{2\alpha}$/creatinine ratios in combined diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
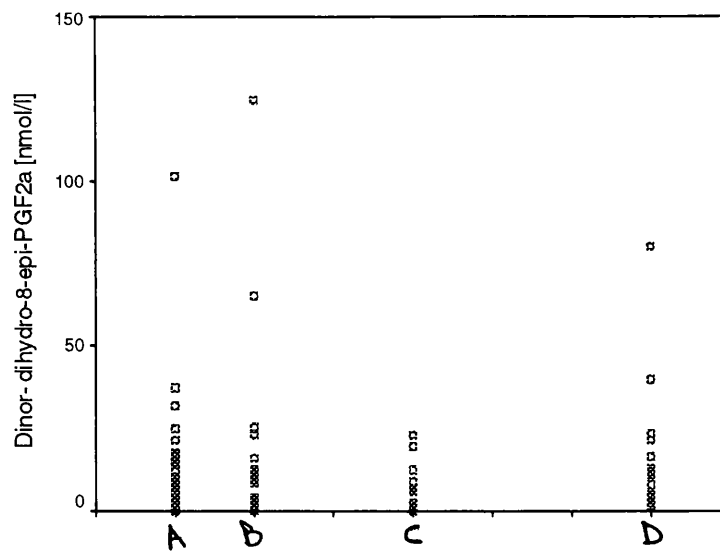

Figure 109: Urinary dinor-8-epi $PGF_{2\alpha}$/creatinine ratios in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed type 1 diabetic patients; E) Control subjects.
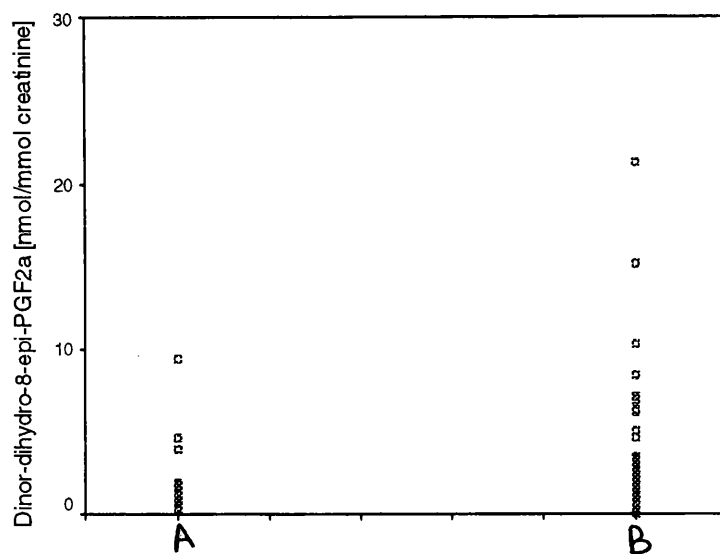
Figure 110: Urinary dinor-8-epi $PGF_{2\alpha}$ /creatinine ratios in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.

Figure 111: Urinary dinor-8-epi $PGF_{2\alpha}$/creatinine ratios in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

Figure 112: Urinary dinor-8-epi $PGF_{2\alpha}$ /creatinine ratios in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; and D) Control subjects.

Figure 113: Urinary dinor-dihydro-8-epi $PGF_{2\alpha}$ in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.

Figure 114: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-; C) PNP+/CAN+; D) Newly diagnosed type 1 diabetics; E) Control subjects.
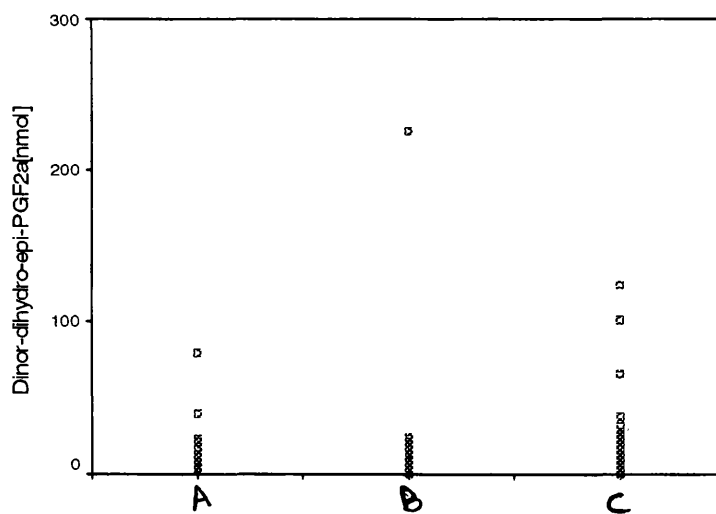
Figure 115: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.
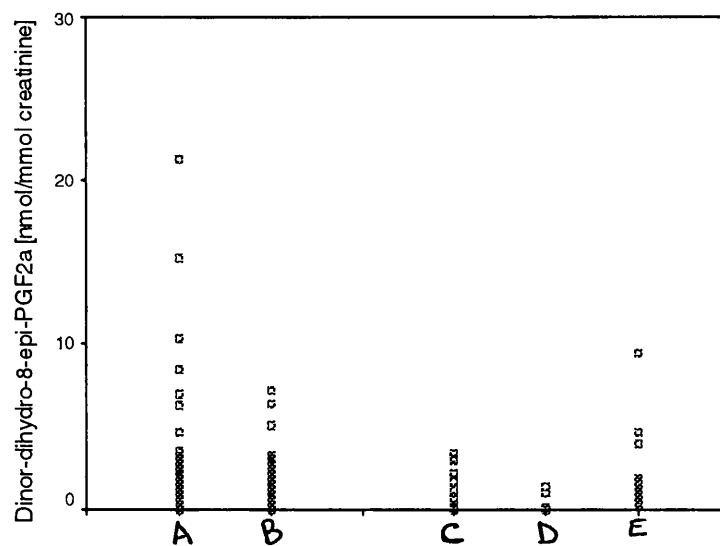
Figure 116: Urinary dinor-dihydro-8-epi $PGF_{2\alpha}$ in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.
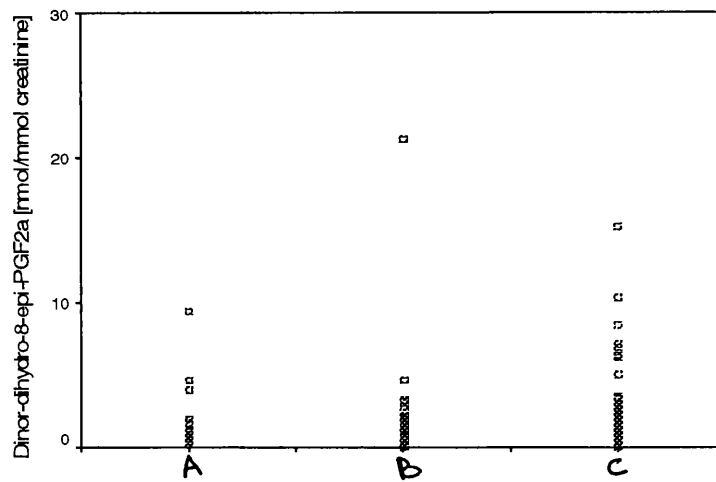

Figure 117: Urinary dinor-dihydro-8-epi $PGF_{2\alpha}$ in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

Figure 118: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ /creatinine ratios in diabetic patients and control subjects. A) Control subjects; B) Diabetic patients.
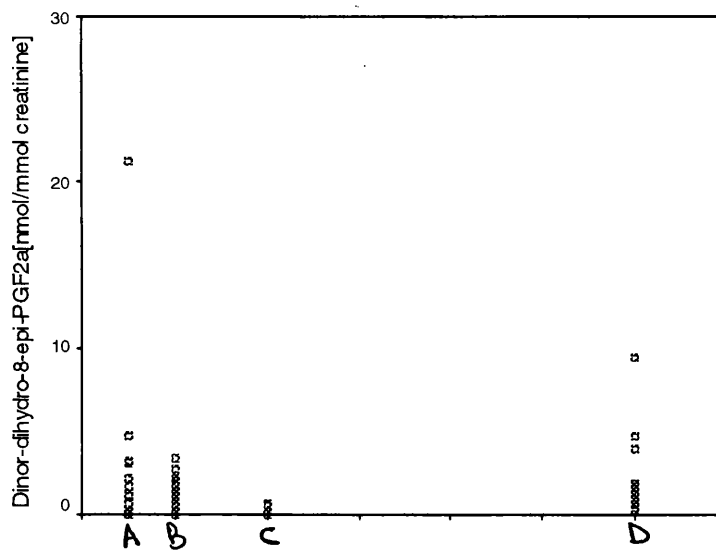

Figure 119: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ /creatinine ratios in diabetic subgroups divided according to the presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Newly diagnosed; E) Control subjects.
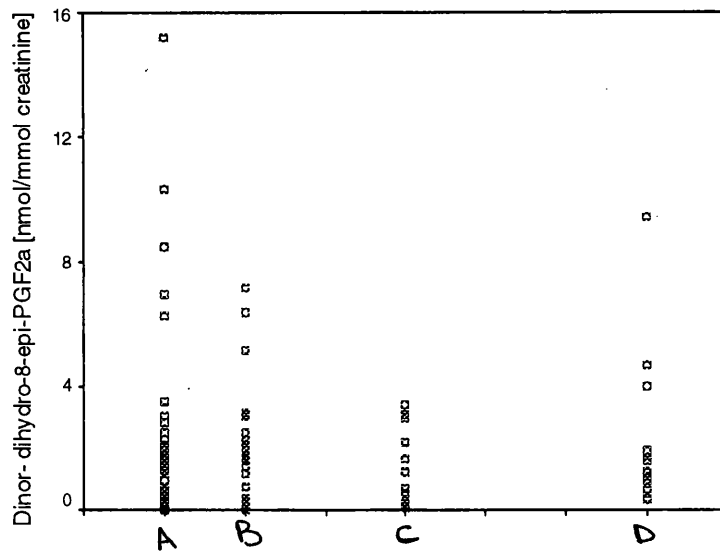
Figure 120: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ /creatinine ratios in diabetic subgroups segregated according to the type of diabetes. A) Control subjects; B) type 1 diabetics; C) type 2 diabetics.

Figure 121: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ /creatinine ratios in type 1 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.

Figure 122: Urinary dinor-dihydro-8-epi PGF$_{2\alpha}$ /creatinine ratios in type 2 diabetic patients split according to the type of presence of PNP and/or CAN. A) PNP+/CAN-; B) PNP+/CAN-); C) PNP+/CAN+; D) Control subjects.