NUTRITIONAL AND HORMONAL BIOMARKERS IN PROSTATE CANCER EPIDEMIOLOGY

Doctor of Philosophy Thesis

By

Alison J. Price

Oriel College

University of Oxford

Trinity 2012

Cancer Epidemiology Unit

Nuffield Department of Clinical Medicine

Oxford OX3 7LF

Nutritional and hormonal biomarkers in prostate cancer epidemiology

Alison J. Price, DPhil Student, Oriel College, University of Oxford

A thesis submitted for the degree of Doctor of Philosophy, Trinity term 2012

Evidence from international comparisons and migrant studies suggest that environmental factors, such as a Western diet, may be important in prostate cancer development, possibly through effects on hormone and growth factor secretion and metabolism. However, despite considerable research, convincing associations between diet and risk for prostate cancer have not been established. Random and systematic measurement error in dietary assessment using traditional survey methods may contribute to inconsistent findings, particularly as they may not capture adequately specific nutritional constituents of the diet that may be associated with risk, such as fatty acids or vitamins.

Validated biomarkers of nutritional factors and hormonal activity, as used in this thesis, provide more precise, objective and integrated measures of exposure, with the capacity to clarify potential mechanisms in the causal pathway of prostate cancer development. Nutritional and hormonal biomarkers investigated for their potential role in the development of prostate cancer include: folate and vitamin B_{12}, which are essential for DNA methylation, repair and synthesis; phytanic acid, obtained predominantly from ruminant fat intake and associated with an enzyme (α-Methylacyl-Coenzyme A Racemase (AMACR)) that is consistently over-expressed in prostate cancer tissue; and insulin-like growth factor (IGF-I), a growth factor influenced by diet and involved in the regulation of cell proliferation, differentiation, and apoptosis.

All work presented in this thesis is from the European Prospective Investigation into Cancer and Nutrition (EPIC) study of 500,000 European men and women, using prospectively collected diet and lifestyle data and biological samples. The large number of prostate cancer cases diagnosed during long-term follow-up of EPIC participants enabled investigation of heterogeneity in risk for prostate cancer by time from recruitment to diagnosis (of particular importance for a disease with a long pre-clinical phase) and cancer characteristics such as disease grade and stage.

Plasma phytanic acid concentration was highly correlated with dietary intake of fat from dairy products (r = 0.46) and beef (r = 0.30); capturing differences between countries in consumption of fat from these foods. Although phytanic acid is a useful biomarker of ruminant fat consumption, there was little evidence to support the hypothesis that the association between dairy products and prostate cancer risk (as suggested by previous work in EPIC and other studies) is mediated by phytanic acid (OR for doubling in concentration 1.05; 95%CI 0.91 – 1.21; P_{trend} = 0.53).

There was strong evidence for an association between higher circulating IGF-I concentration and risk for prostate cancer (OR for highest versus lowest fourth 1.69; 95% CI: 1.35, 2.13; P_{trend} = 0.0002). Furthermore, the positive association observed among men diagnosed with advanced stage disease and among men diagnosed more than seven years after blood

collection, supports the hypothesis that high IGF-I concentration is associated with clinically significant prostate cancer many years before diagnosis.

There was no evidence of an association between prostate cancer risk and dietary folate or vitamin B_{12} intake, or between circulating levels of folate (OR for doubling in concentration 1.05; 95%CI 0.95 – 1.15; $P_{trend} = 0.33$) or vitamin B_{12} (1.05; 95%CI 0.92 – 1.21; $P_{trend} = 0.47$) and only limited evidence for an increased risk associated with elevated vitamin B_{12} in a meta-analysis of six prospective studies, that included the present study.

All of these analyses were based on a blood sample taken at one point in time, with the assumption that this reflects the 'true' underlying concentration over the long-term. The poor to modest reliability estimates (intra-class correlation coefficients ranging from 0.18 to 0.48) for circulating concentrations of folate, IGF-I, phytanic acid and vitamin B_{12} taken in samples approximately six years apart in a sub-sample of participants from EPIC Oxford, show that estimates of usual concentrations based on a single blood measurement weaken the ability to detect associations with disease risk. Where small effect sizes are anticipated, this may bias associations toward the null.

In conclusion, there is convincing evidence that IGF-I is an important and potentially modifiable risk factor for prostate cancer many years before diagnosis. However, there is little evidence for an association between biomarkers of folate, vitamin B_{12} and phytanic acid concentrations and risk for prostate cancer. Future studies should, where possible, incorporate multiple blood samples taken several years apart to better characterise long term relationships between biomarkers of nutritional and hormonal exposure and disease risk and pool individual participant data from multiple prospective studies to strengthen the power to detect modest associations.

Table of contents

Nutritional and hormonal biomarkers in prostate cancer epidemiology i
Table of contents iii
Table of tables vii
Table of figures ix
Abbreviations x
Chapter 1: Thesis Introduction 1
1.1 Rationale 1
1.2 Research Aims 1
1.3 Outline of the thesis 2
1.4 The role of the author in this thesis 4
1.5 Publications relating to this thesis 5
Chapter 2 The European Prospective Investigation into Cancer and Nutrition (EPIC) 6
2.1 Introduction 6
2.2 Design and Methods 6
2.3 Recruitment 6
2.3.1 Data 6
2.3.2 Data entry and storage 7
2.3.3 Blood collection and storage 8
2.4 Ethics 8
2.5 Follow-up 8
2.6 Outcome of incident prostate cancer 8
Chapter 3 Prostate Cancer Epidemiology 10
3.1 Overview of prostate cancer epidemiology 10
3.1.1 Geographical Trends 10
3.1.2 Temporal trends 10
3.2 Risk Factors 11
3.2.1 Established Risk Factors for prostate cancer 11
3.2.2 Putative risk factors for prostate cancer 13
3.3 Latent prostate cancer 15
3.4 Prostate Specific Antigen (PSA) testing 15
3.5 Classification of prostate cancer 16
3.5.1 Implications for analyses included in this thesis 16
Chapter 4 Validation of Nutritional and Hormonal Biomarkers 22
4.1 Introduction 22

4.2 Biomarkers of interest 22
4.3 Aim of study 23
4.4 Study design 24
4.4.1 Study population 24
4.4.2 Laboratory analysis 24
4.4.3 Statistical analysis 25
4.5 Results 26
4.6 Discussion 28
4.6.1 Strengths of the study 30
4.6.2 Limitations of the study 30
4.7 Conclusions 31
Chapter 5 Phytanic acid and risk for prostate cancer 41
5.1 Introduction 41
5.1.1 History of phytanic acid 41
5.1.2 Metabolism of phytanic acid 42
5.1.3 Mechanism by which phytanic acid may increase prostate cancer risk 42
5.2 Aim of study 43
5.3 Methods 43
5.3.1 Study population 43
5.3.2 Follow-up for cancer incidence and vital status 44
5.3.3 Selection of case and control participants from the study cohort 44
5.3.4 Laboratory analysis 45
5.3.5 Statistical analysis 46
5.4 Results 48
5.5 Discussion 49
5.5.1 Strengths of the study 50
5.5.2 Limitations of the study 50
5.6 Conclusion 50
Chapter 6 Phytanic acid as a nutritional biomarker of fat from dairy, beef and other sources 56
6.1 Introduction 56
6.2 Aim of study 57
6.3 Methods 57
6.3.1 Study population 57
6.3.2 Laboratory methods 58
6.3.3 Statistical analysis 59

6.4 Results 60
6.5 Discussion 62
6.5.1 Strengths of the study 63
6.5.2 Limitations of the study 64
6.6 Conclusions 64
Chapter 7 IGF-I and risk of prostate cancer 72
7.1 Introduction 72
7.1.1 Physiology of IGF-I 72
7.1.2 Determinants of circulating IGF-I concentration 73
7.1.3 Mechanisms by which IGF-I may promote cancer 75
7.1.4 Evidence that circulating IGF-I is involved in prostate cancer progression 76
7.2 Aim of study 77
7.3 Methods 77
7.3.1 Study population 77
7.3.2 Follow-up for cancer incidence and vital status 78
7.3.3 Selection of case and control participants from the study cohort 78
7.3.4 Laboratory analysis 79
7.3.5 Statistical analysis 79
7.4 Results 80
7.5 Discussion 82
7.5.1 Strengths of the study 83
7.5.2 Limitations of the study 83
7.6 Conclusion 83
Chapter 8 Folate and vitamin B_{12} and risk for prostate cancer 90
8.1 Introduction 90
8.1.1 Sources of folate and vitamin B_{12} 90
8.1.2 Determinants of circulating folate and vitamin B_{12} concentrations 91
8.1.3 Physiology of folate and vitamin B_{12} 91
8.1.4 Pathophysiology of folate and vitamin B_{12} 92
8.1.5 Evidence that folate and vitamin B_{12} are involved in cancer development 92
8.2 Aim of study 94
8.3 Methods 94
8.3.1 Study cohort 94
8.3.2 Follow-up and case identification from the study cohort 95
8.3.3 Selection of case and control participants for the nested case control study 96
8.3.4 Laboratory analysis 96

8.3.5 Statistical analysis 97
8.4 Results 100
8.4.1 Cohort study 100
8.4.2 Nested case control study 101
8.5 Discussion 103
8.5.1 Strengths of the study 105
8.5.2 Limitations of the study 105
8.6 Conclusion 106
Chapter 9 Discussion 126
9.1 Overview 126
9.2 Aim of study 126
9.3 Main findings from this thesis 126
9.3.1 Phytanic acid and risk for prostate cancer 126
9.3.2 IGF-I and risk for prostate cancer 126
9.3.3 Folate and vitamin B12 and risk for prostate cancer 127
9.3.4 Folate, IGF-I, phytanic acid and vitamin B_{12}: reliability studies 127
9.4 Findings in context 127
9.5 Strengths of study 128
9.6 Limitations of study 129
9.7 Recommendations for future research 131
9.8 Conclusion 133
References 134
Appendix 161
9.9 EPIC baseline questionnaire 161

Table of tables

Table 3.1 Information relating to prostate cancer stage and grade classification in large prospective cohorts and randomised controled trials 19
Table 4.1 Insulin-like growth factor-I reliability studies 32
Table 4.2 Folate and vitamin B_{12} reliability studies 33
Table 4.3 Characteristics of 100 men and 100 women at baseline and follow-up 34
Table 4.4 Descriptive statistics, variance components and measures of reliability for folate, vitamin B_{12}, phytanic acid and IGF-I 35
Table 4.5.1 - 4.5.4: The proportion of participants changing tertile categories between baseline and follow-up with tertile cut-points based on the distribution of each analyte at the first blood collection 35
Table 4.6 Observed and true relative risks (RR) obtained when using a single blood measure given the ICCs observed in each study and assuming no additional error 39
Table 4.7 Sample Size[1] required for a width of confidence interval given a range of intra-class correlation coefficients obtained with two blood samples 40
Table 5.1 Baseline characteristics of prostate cancer cases and matched controls in EPIC 52
Table 5.2 Adjusted geometric mean phytanic acid concentrations and 95% CI among the 561[1] control participants 53
Table 5.3 Odds ratios (and 95% CI) for prostate cancer by thirds of plasma phytanic acid concentration in EPIC 54
Table 5.4 Odds ratios (and 95% CI) for prostate cancer associated with a doubling of phytanic acid concentration in EPIC 55
Table 6.1 Mean (standard deviation) food and nutrient intakes estimated using the food frequency questionnaire (FFQ) 65
Table 6.2 Geometric mean concentration of phytanic acid, pentadecanoic acid and heptadecanoic acid by country 66
Table 6.3 Associations between food and fat intake acquired from meat, dairy and fish and plasma concentrations of phytanic acid 68
Table 6.4 Associations between food and fat intake acquired from meat, dairy and fish and plasma concentrations of pentadecanoic acid (C15:0) 69
Table 6.5 Associations between food and fat intake acquired from meat, dairy and fish and plasma concentrations of heptadecanoic acid (C17:0) 70
Table 7.1 Prospective studies of circulating IGF-I concentration and risk of prostate cancer ... 84
Table 7.2 Characteristics of 1,542 prostate cancer cases and 1,542 control subjects in EPIC overall and subdivided into study phase 86
Table 7.3 Adjusted geometric mean IGF-I concentrations and 95% CI among 1,542 control participants according to baseline characteristics 87
Table 7.4 Odds ratios (95% CI) for prostate cancer associated with quartile concentrations of IGF-I or a doubling in IGF-I concentration in the European Prospective Investigation into Cancer and Nutrition 88
Table 8.1 Prospective observational studies of the association between circulating folate and vitamin B_{12} and risk for prostate cancer 108
Table 8.2 Characteristics of participants in the European Prospective Investigation into Cancer and Nutrition (EPIC) stratified by country 109

Table 8.3 Hazard ratios (HR) and 95% confidence intervals (CI) for prostate cancer among 142,252 men in the EPIC cohort by quintiles of dietary intake of folate and vitamin B_{12} 110
Table 8.4 Hazard ratios (HRs) and 95% confidence intervals (CI) for prostate cancer among 142,252 men in the EPIC cohort by dietary intake of folate and vitamin B_{12} 111
Table 8.5 Characteristics of 1,480 prostate cancer cases and 1,777 control participants in EPIC with measurement of circulating folate and vitamin B_{12} concentrations..... 112
Table 8.6 Adjusted geometric mean folate and vitamin B_{12} concentrations [95% CI] among control participants according to baseline characteristics 113
Table 8.7 Geometric mean [95% CI] folate and vitamin B_{12} concentrations by phase of study and country among control participants in the European Prospective Investigation into Cancer and Nutrition (EPIC) 114
Table 8.8 Odds ratios (ORs) and 95% confidence intervals [95% CI] for prostate cancer by quintiles of circulating folate and vitamin B_{12} concentration in the EPIC cohort 114
Table 8.9 Odds ratios (ORs) and 95% confidence intervals [95% CI] for prostate cancer by quintiles of circulating folate concentration in the EPIC cohort using country specific quintile cutpoints 116
Table 8.10 Odds ratios (ORs) and 95% confidence intervals [95% CI] for prostate cancer associated with a doubling of circulating folate and vitamin B_{12} concentration in the EPIC study 117

Table of figures

Figure 3:1 Age standardised prostate cancer incidence and mortality rates by world area, 2008. 18
Figure 3:2: Age-specific incidence rates per 100,000 population and the average number of new cases per year amongst men in the United Kingdom, 2008. 19
Figure 4:1.1-4.1.2 Geometric mean concentrations at time points 1 and 2 by third of concentration for each biomarker 37
Figure 5:1 Schematic diagram of phytanic acid metabolism from animal sources (ruminants and fish) to human metabolism within sub-cellular peroxisomes 51
Figure 6:1 Geometric mean dairy fat intake (g/day) by the geometric mean phytanic acid concentration[1,2] (µmol/L) 66
Figure 6:2 Geometric mean beef fat intake (g/day) by the geometric mean phytanic acid concentration[1,2] (µmol/L) 67
Figure 8:1 Folate and vitamin B_{12} metabolism 107
Figure 8:2 Fixed effects meta-analysis of the association of circulating folate and risk for prostate cancer by EPIC country 118
Figure 8:3 Fixed effects meta-analysis of the association of circulating folate and risk for prostate cancer 119
Figure 8:4 Fixed effects meta-analysis of the association of circulating folate and risk for prostate cancer in prospective studies, excluding Collin, 210 120
Figure 8:5 Fixed effects meta-analysis of the association of circulating vitamin B_{12} and risk for prostate cancer 121
Figure 8:6 Fixed effects meta-analysis of the association of circulating vitamin B_{12} and risk for prostate cancer, excluding Hultdin, 2005 122
Figure 8:7 Fixed effects meta-analysis of the association of circulating vitamin B_{12} and risk for prostate cancer in prospective studies excluding Hultdin, 2005 and Collin, 2010 123
Figure 8:8 Funnel plot of 8 studies of the association of circulating folate and risk for prostate cancer 124
Figure 8:9 Funnel plot of 6 studies of the association of circulating vitamin B_{12} and risk for prostate cancer 125

Abbreviations

24-HDR
: 24-hour dietary recall

AMACR
: α-methylacyl-coenzyme A racemase

BMI
: Body mass index

CRUK
: Cancer Research United Kingdom

CV
: Coefficient of variation

DHT
: Dihydrotestosterone

DNA
: Deoxyribonucleic acid

dTMP
: Deoxythymidylic acid*3*

dUMP
: Deoxyuridine monophosphate

ELISA
: Enzyme-linked immunosorbent assay

EPIC
: European Prospective Investigation into Cancer and Nutrition

FFQ
: Food-frequency questionnaire

GC/MS
: Gas chromoatography mass spectrometry

GH
: Growth hormone

GHRH
: Growth hormone releasing hormone

GWAS
: Genome wide association study

HR
: Hazard ratio

IARC
: International Agency for Research on Cancer

ICC
: Intra-class correlation coefficient

ICD-10
: International Statistical Classification of Injuries, Disease and Deaths

IGFBP
: Insulin-like growth factor binding protein

IGF-I
: insulin-like growth factor

mRNA
: Messenger ribonucleic acid

MTHFR
: Methylenetetrahydrofolate reductase

OR
Odds Ratio
PSA
Prostate specific antigen
QC
Quality control
RCT
Randomised controlled trial
RR
Relative risk
SAM
S-adenysl methionine
SIM
Selected ion monitoring
SNP
Single nucleotide polymorphisms
THF
Tetrahydrofolate
TNM
Tumour node metastasis
UK
United Kingdom
WHO
World Health Organisation

Chapter 1: Thesis Introduction

1.1 Rationale

Prostate cancer is one of the most common human cancers, particularly in economically developed countries where it is the third leading cause of cancer mortality among men (1), yet aside from age, ethnicity and family history of disease the aetiology is not understood. Ecological studies have shown a greater incidence of prostate cancer in countries with a Western diet (associated with a higher consumption of dietary fat and animal products (2)) and migrant studies (3-5) have shown cancer rates in successive migrant generations shift upwards in the direction of the prevailing rates in the host country. Systematic reviews of epidemiological studies suggest that diets high in meat and dairy products may be associated with an elevated risk for prostate cancer (6-8), however the evidence from prospective studies on diet and risk for prostate cancer are inconsistent (9). Furthermore, the specific mechanism for an association is not understood, although diet may mediate its affect on prostate cancer by moderating hormone and growth factor levels, as these are known to be essential for normal prostate development and suppression of androgen levels is a mainstay of prostate cancer treatment (10).

The assessment of dietary exposures by questionnaire methods may contribute to the inconsistent evidence for an association between diet and prostate cancer risk. Random error associated with the use of food tables, assessment of the frequency of food consumption, portion size, daily variation and failure to report usual diet or misreporting of food choice or amount (11) leads to misclassification of exposure status that may significantly attenuate diet-disease associations (even with correction for measurement error in the calculation of risk estimates). Furthermore, it is difficult to differentiate specific constituents of the diet that may be associated with risk with sufficient precision and accuracy using traditional assessment methods.

For studies with access to prospectively collected, and appropriately archived biological specimens, biomarker measurements offer opportunities to improve the precision of risk estimates by providing more accurate and objective measures of dietary constituents. Furthermore, biomarkers of nutritional status and endogenous hormone activity (which may be influenced by diet) can be related to known or suspected modifiable risk factors for cancer. However, prior to application of a biomarker in a large epidemiological study it is necessary to establish the determinants of biomarker levels and the extent to which a single measure represents an individual's usual or long-term exposure, as this measure is considered to be of most interest in the aetiological relationship with chronic disease.

1.2 Research Aims

The research documented in this thesis is concerned with investigating risk factors for prostate cancer, using validated biological markers as surrogate measures of nutritional exposures and endogenous hormone activity.

1.3 Outline of the thesis

Chapter 1 provides an introduction to the thesis, the rationale, general aim and an outline of the key research objectives that will be explored in Chapters 2 to 8.

Chapter 2 gives an introduction to the European Prospective Investigation into Cancer and Nutrition cohort study (EPIC) which provides the source of all data used throughout the thesis.

Chapter 3 reviews the background research relevant to prostate cancer epidemiology, including geographical and temporal trends in cancer incidence and mortality, established and putative risk factors. An overview of issues associated with prostate cancer disease characterisation and an explanation of the end-points used in the statistical analyses included in this thesis is also provided in this chapter.

Chapter 4 describes the design and results of reliability studies for circulating folate, IGF-I, phytanic acid, and vitamin B_{12}. These studies are performed on a small subset of EPIC Oxford participants (100 men and 100 women) who provided a baseline and follow-up blood sample (collected on average 6 years later). It is anticipated that biomarkers will reduce misclassification of exposure status and provide more precise identification of risk factors. However, the extent to which this end is achieved is determined largely by the ability of a single blood measure of a candidate risk factor to accurately and reliably inform relative risk estimates for prostate cancer in relation to long-term exposures. A manuscript relating to this work has been prepared for publication.

Chapter 5 investigates the association between plasma phytanic acid concentration, a biomarker of ruminant fat intake, and risk of prostate cancer using a nested case control design in which cases are matched with an equal number of controls on study centre, age at recruitment (+/- 6 months), time of blood collection (+/- 1 hour), length of follow-up and duration of fasting at blood collection (< 3, 3 - 6, > 6 hours). The effects of potential confounding variables (other than those accounted for in the matched design) are examined by including additional regression terms in the conditional logistic regression models and include body mass index (kg/m^2), height (cm), weight (kg), smoking (never, past, present, unknown), alcohol consumption (< 8, 8 - 15, 16 - 39, $\geq$ 40 g/day, unknown), physical activity (inactive, moderately inactive, active, unknown), marital status (married/cohabitating or not married/cohabitating, unknown) and educational attainment (primary or equivalent, secondary, degree level, unknown). A validated gas chromatography- mass spectrometry (GC/MS) assay is used to measure plasma phytanic acid concentrations. The work outlined in this chapter was published in the American journal of Clinical Nutrition (June 2010 vol. 91 no. 6 1769-1776) and is available at: http://www.ajcn.org

Chapter 6 evaluates the suitability of phytanic acid as a nutritional biomarker of ruminant fat consumption by quantifying the major dietary sources among European men. The study comprises a cross-sectional analysis of 560 control men nested within the EPIC cohort and utilizes dietary data from an existing EPIC database. The correlation between various foods of ruminant origin, including dairy products and beef, and plasma concentrations of phytanic acid and the saturated fatty acids, pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), are investigated.

Chapter 7 explores the association between circulating concentrations of insulin-like growth factor (IGF-I) and risk for prostate cancer. High circulating IGF-I concentrations have been associated with increased risk for prostate cancer in several prospective epidemiological studies however, prostate cancer is a disease with a long pre-clinical phase, and few studies have had sufficient follow-up time to adequately investigate heterogeneity in risk by duration of follow-up, leaving open the possibility of reverse causality. EPIC previously reported a marginally significant increased prostate cancer risk for highest versus lowest category of IGF-I concentration (OR 1.35; 95%CI 0.99 - 1.82; P_{trend} = 0.08) using data from 630 men with an incident prostate cancer diagnosis (matched with 630 control men) and a median time from blood collection to diagnosis of 3.4 years (range, 0.1 – 8.5 yrs (12). The study described in this chapter is an expansion of this study with extended follow-up of the cohort. The study uses a nested case-control design with 1,542 prostate cancer cases (matched with 1,542 control men) who have a median time from blood collection to diagnosis of 5.8 years (range 0.1 – 12.8 yrs) and is sufficiently powered to explore associations by stage of disease and amongst men diagnosed after prolonged follow-up. Matching is performed on study centre, age at recruitment (+/- 6 months), time of blood collection (+/- 1 hour), length of follow-up and duration of fasting at blood collection (< 3, 3 - 6, > 6 hours). The work described in this chapter has been published in Cancer Epidemiology Biomarkers and Prevention and is available at: http://cebp.aacrjournals.org

Chapter 8 investigates the association of circulating concentrations of folate and vitamin B_{12} and risk for prostate cancer using a nested case control design in which cases are matched on study centre, age at recruitment (+/- 6 months), time of blood collection (+/- 1 hour), length of follow-up and time spent fasting at blood collection (< 3, 3 - 6, > 6 hours). The effects of potential confounding variables (other than those accounted for in the matched design) are examined by including additional regression terms in the conditional logistic regression models. The results from this study using blood measures of folate and vitamin B_{12} are compared with that of the association of dietary intake of folate and vitamin B_{12} and risk of prostate cancer using a cohort analysis. An aggregate meta-analysis of prospective studies of the association of circulating folate and vitamin B12 is also included. A manuscript relating to this work has been prepared for publication.

Chapter 9 reviews the main findings from the thesis and provides recommendations for future work. Appendix A contains a copy of the baseline questionnaire used in the EPIC UK cohort (Cambridge and Oxford), which includes a standardised food-frequency questionnaire and a detailed lifestyle questionnaire.

It is anticipated that this work furthers our understanding of the risk factors for prostate cancer through application of validated biomarkers of nutritional and hormonal exposures. All analyses will utilise data from the European Prospective Investigation into Cancer and Nutrition (EPIC).

1.4 The role of the author in this thesis

All work presented in this thesis is my own, except where otherwise stated, and conducted under the supervision of Dr Naomi Allen and Professor Timothy Key. The literature reviews, the statistical analyses and authorship of all chapters comprising this thesis are my own. The initial concept of studying the association of phytanic acid[1] and risk for prostate cancer was developed by Professor Tim Key. Tim secured funding from Cancer Research UK (CRUK) for a DPhil student to design a study to test the hypothesis that high plasma levels of phytanic acid increase the risk for prostate cancer. It was anticipated that a second phase of this work would involve immunohistochemistry to detect α-methylacyl coenzyme A racemase (AMACR)[2] expression in tissue microarrays constructed from tumour samples, and to examine the relation between plasma phytanic acid and AMACR expression. However, collection of tissue samples from National Health Service trusts proved difficult to achieve within the resource and time constraints of my DPhil studies and was not pursued further.

The idea to develop my research experience in the design and application of biomarkers of nutritional and hormonal exposures was conceived in consultation between me and my superviors, Dr Naomi Allen and Professor Tim Key. The biomarkers, IGF-I, folate and vitamin B_{12} were chosen in addition to phytanic acid as earlier work had suggested an association with risk for prostate cancer but to provide the statistical power to investigate associations by cancer characteristics such as grade, stage and time to diagnosis, more information was required using data with a larger number of cases and prolonged follow-up time.

All of the laboratory assays for phytanic acid, IGF-I, folate and vitamin B_{12} were conducted by laboratory technicians, although I participated in the retrieval and preparation of plasma samples for transport to the HFL Sports Ltd, United Kingdom, where the phytanic acid assays were conducted. I also visited HFL Sports Ltd to understand better the laboratory methods.

[1] Phytanic acid is metabolized by α-methylacyl-coenzyme A racemase (AMACR), an enzyme which plays a key role in peroxisomal β-oxidation of branched-chain fatty acids. Earlier research has shown that AMACR is consistently over-expressed in prostate cancer tissue compared with normal tissue (83-86, 172).

[2] AMACR is considered a reliable diagnostic marker of prostate cancer in the clinical setting, although its role in the aetiology of prostate cancer has not yet been established (173). The experimental finding of increased AMACR protein expression in cultured prostate cancer cells exposed to a high (25 μM) concentration of phytanic acid (174) suggests that this fatty acid may have a regulatory role in the over-expression of AMACR protein in prostate cancer.

1.5 Publications relating to this thesis

At the time of writing, two manuscripts have been published from work described in this thesis. These include "Phytanic acid and risk of prostate cancer: results from the European Prospective Investigation into Cancer and Nutrition" and "Insulin-like growth factor and risk of prostate cancer: results from the European Prospective Investigation into Cancer and Nutrition".

My co-authors for the papers read and provided advice on the content and methodology and to this extent, each paper may not be considered my own work. Two manuscripts have also been prepared for publication related to the work outlined in Chapter 4 (on page 22) and Chapter 8 (on page 90).

Chapter 2 The European Prospective Investigation into Cancer and Nutrition (EPIC)

2.1 Introduction

The principal aim of the EPIC study is to investigate the association between nutrition and lifestyle factors and risk of cancer within the European population.

2.2 Design and Methods

The EPIC study is a prospective observation study with ongoing follow-up that is designed to collect data in relation to diet and lifestyle from a large number of people across countries with heterogeneous diet and lifestyle patterns so that the relationship between these factors and cancers (and other diseases) can be investigated. The study design takes advantage of the wide variation in dietary intake across countries, and in doing so reduces the proportion of variability attributable to random error in the dietary measures.

The prospective collection of blood samples and a broad range of nutritional, lifestyle and anthropometric data from approximately 350,000 women and 150,000 men, across 10 European counties, consisting of 23 region centres, facilitates comparisons in cancer rates, dietary and lifestyle habits between countries and exploration of interactions between nutritional, genetic, hormonal and lifestyle factors.

2.3 Recruitment

2.3.1 Data

Men and women aged mostly between 35 to 70 years were recruited between 1992 and 2000. The majority of participants were recruited from the general population living in defined geographical areas. However, exceptions to this recruitment approach included: France, where participants were members of a health insurance scheme for state school employees; Florence (Italy) and Utrecht (the Netherlands), where women were attending breast cancer screening; Italy and Spain, where participants in some centres were blood donors and; Oxford (United Kingdom), where participants were recruited from throughout the United Kingdom via General Practices and via the post through the Vegetarian Society in order to include as many non-meat eaters as possible. In France, Norway, Utrecht (The Netherlands) and Naples (Italy) the regional cohorts only recruited women (13).

Individuals were invited to participate by mail or in person and those who agreed also provided written consent. At enrolment, participants completed a lifestyle questionnaire and dietary questionnaire using country-specific, validated dietary assessment instruments (13, 14). Participants who completed the questionnaires at home were invited to a study centre for venepuncture, anthropometric measurements and blood pressure assessment.

The dietary assessment methods used included either a self-administered quantitative dietary questionnaire (Italy, the Netherlands, Germany, Greece, Spain and France), a semi-quantitative food-frequency questionnaire (Denmark, Norway, Naples (Italy), and Umeå (Sweden), a combination of a semi-quantitative food-frequency questionnaire and a 7 day record (United Kingdom) or a non-quantitative food-frequency questionnaire and a 14-day record on hot meals (Malmo, Sweden) (13). Individual portion sizes were estimated by using a common picture book throughout all centres and other tools, such as depictions of household measurements, bread shapes, standard units, and portion sizes were also used. For dietary questionnaires, the conversion of food quantities into nutrient intakes was obtained by means of country-specific nutrient databases.

To facilitate comparison of dietary measurements between cohorts, to adjust for possible systematic over- or under-estimation in dietary intake measurements, and to correct for attenuation bias in relative risk estimates, a calibration study was performed that involved an additional dietary assessment, using 24 hour dietary recall method in an age and gender stratified random sample of 36,900 participants (8%), that was common across all study population (15). This calibration procedure meant that individual dietary intakes could be expressed according to the same reference scale (16). The sample size requirement was weighted by the expected numbers of cases of cancer in age-gender categories over 10 years of follow-up (15). A software programme, called EPIC-SOFT was specifically designed to standardise the measurements across study populations (15) and a linear regression calibration model was used to adjust for possible systematic over- or under-estimation in dietary intake measurements and to correct for attenuation bias in relative risk estimates(17, 18). Overall the response rate to the calibration study ranged between 46.5% in the Oxford cohort to 91.6% in Germany and with the exception of education and physical activity, in most cohorts there were no significant differences in lifestyle factors and anthropometric measures between the calibration sample and the rest of the cohort of men and women (15).

Non-dietary information was collected for lifestyle factors associated with risk for cancer (such as smoking), related to nutritional status or considered to be potential risk factors for cancer. These co-factors included marital status, educational attainment, current and past employment, lifetime history of smoking and alcohol consumption, medical history (including previous illnesses and disorders, although not family history of prostate cancer), physical activity at work and leisure. For women information was also collected on reproductive history and use of contraceptives and hormone replacement therapy. A copy of the EPIC lifestyle and food frequency questionnaire administered in the UK is provided in Appendix A (on page 161).

2.3.2 Data entry and storage

EPIC lifestyle and dietary data including centre-specific and EPIC wide standardised variables and formats are stored centrally at IARC, Lyon (France) in the EPIC ORACLE database and personal identifying information are stored at local centres in participating countries. Following receipt and transformation of data to standard EPIC variables quality control checks are applied in order to identify and manage irregular or implausible values (13).

2.3.3 Blood collection and storage

Blood samples were collected from 386,000 participants and separated into plasma, serum, erythrocyte and buffy coat fractions. Centrally purchased syringes and 0.5ml straws were distributed to centres to ensure standardised procedures for collection and storage and in most centres half of the aliquots (n=28) were transported to IARC, France, to be stored in liquid nitrogen at -196 while the remaining samples were stored locally at the centre. In all countries, except Denmark and Sweden, blood was separated into 0.5 mL fractions (serum, plasma, red blood cells, and buffy coat for DNA extraction) and placed into straws, which were heat-sealed and stored under liquid nitrogen. In Denmark, 1.0 mL blood fraction aliquots were stored locally in Nunc tubes at −150°C under nitrogen vapour and in Sweden samples were stored in freezers at −80°C.

2.4 Ethics

All participants provided written informed consent and local ethics committees in the participating countries and the Internal Review Board of the International Agency for Research on Cancer (IARC) approved the study.

2.5 Follow-up

Follow-up for cancer diagnosis is based on population cancer registries in Denmark, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom. In France, Germany and Greece follow-up is achieved through a combination of health insurance records, cancer and pathology registries and active-follow-up through participants and their next of kin. A standardised protocol is used for the collection of clinical and pathological data however clinical and pathology records are not reviewed and standardised centrally. Follow-up for vital statistics is collected in all centres from death registries, insurance records and death certificates. Details pertaining to the methods employed in specific countries are discussed in the methods sections of each analysis included in this thesis.

2.6 Outcome of incident prostate cancer

Prostate cancer cases were defined as code C61 according to the 10th revision of the International Statistical Classification of Injuries, Disease and Deaths (ICD-10). Cases were defined as men who developed prostate cancer after the date of recruitment and before the end of the study period, as determined by the latest date of follow-up in each study centre.

At the time of analysis, closure dates for the study period were defined as the latest date of complete follow-up of both cancer incidence and vital status and corresponded to December 2007 in Denmark, June 2010 in Germany, December 2009 in Greece, May 2009 in Italy, December 2007 in the Netherlands, May 2008 in Spain, December 2008 in Sweden and December 2009 in the United Kingdom (UK).

Overall, 142,252 men, including 4,728 prostate cancer cases were available for analysis at the end of follow-up for the study period, after exclusion of men with; a previous diagnosis of cancer, missing information on the date of cancer diagnosis or end of follow-up, missing dietary or other lifestyle data or an improbable total energy intake (in the top or bottom 1% of the ratio of estimated energy intake to energy requirement).

Tumour stage information (TNM staging score) was derived from pathology reports and cancer registry data was used to define localized and advanced stage categories and information on Gleason score or the World Health Organization (WHO) grading system is used to define low and high grade disease.

Chapter 3 Prostate Cancer Epidemiology

3.1 Overview of prostate cancer epidemiology

3.1.1 Geographical Trends

Prostate cancer is a leading cause of cancer incidence and mortality worldwide (accounting for 14% (n = 903,500) of total incident cancers and 6% (n = 258,400) of total cancer deaths in men in 2008 (19)) but there are significant differences in geographic trends between countries, as illustrated in **Figure 1** (on page 18). The highest age standardised incidence of prostate cancer are reported in Western countries, with rates per 100,000 population ranging from 85 to 104 in North America, Western Europe and Australia, while the lowest rates, between 8 and 4 per 100,000 population, are reported in Northern Africa, China and Eastern Asia (South-Central Asia)(19).

In contrast to the wide variation in incidence of prostate cancer between economically developed and developing countries, there is considerably less variation in mortality rates. Age standardised mortality rates for prostate cancer are high in Western countries (between 9 and 15 per 100,000 population), especially Northern Europe, Australia, Western Europe and the USA, although the highest rates are experienced in the Caribbean, with 26 prostate cancer deaths per 100,000 population (20). The lowest age standardised mortality rates are experienced in parts of Asia, especially China, with less than 3 prostate cancer deaths per 100,000 population (19-21).

3.1.2 Temporal trends

Much of the observed recent geographic variation in prostate cancer incidence may be due to differences in screening practices and the prevalence of prostate-specific antigen (PSA) testing, which increases early detection of latent disease (22). Following the introduction of widespread PSA testing in the United States in the mid 1980s, prostate cancer incidence increased by 100% over a 7 year period, reaching a peak incidence in 1992 (in excess of 100 cases per 100,000 population (23)) and subsequently declined to 85 cases per 100,000 population in 2008, as the proportion of prevalent cases diminished (19). Similar incidence trends have been observed in countries, such as Australia and Canada, with higher uptake of PSA testing. In the United Kingdom incidence rates continue to increase, albeit gradually, which may reflect the lower prevalence of PSA testing, with only 6% of men aged 45-84 years tested in 2002 (24) compared with 57% in the United States (25), and 34% in Canada (26) in 2001.

Prostate cancer mortality in developed countries has been decreasing over the past 10 years and this may, in part, be attributable to the reduced incidence of advanced stage disease following the introduction of PSA testing. However, the role of PSA testing in mortality reduction is contentious as a large randomized controlled trial in the United States (27) reported no reduction in mortality with PSA testing after 10 years of follow-up while a European trial (28) reported a 20% reduced rate of death from prostate cancer. In contrast to

the downward trend observed in Northern America, Western and Northern Europe and Australia, mortality appears to be increasing in Eastern Europe and several Asian countries, including Japan (29).

While some of the variation in incidence and mortality rates between countries and over time is attributable to differences in screening and diagnostic practices (30, 31), access to health care, treatment practices and death certificate reporting (32, 33), the magnitude of the difference suggests that lifestyle, environmental and genetic factors do play a role in the aetiology of this disease.

3.2 Risk Factors

3.2.1 Established Risk Factors for prostate cancer

Advanced age (20), a family history of disease (34-41) and African origin (21, 42), are the only established risk factors for prostate cancer.

3.2.1.1 Age

Worldwide very few cases of prostate cancer occur in men aged less than 50 years but incidence and mortality rates increase exponentially with age. In 2008, worldwide age-specific rates per 100,000 person years were 1.2 in men aged 40-44 but rose exponentially to 4.9, 19.1, 51.8, 114.6, 204.6, 293.2 for each successive 5 year age group, with the highest rate of 392.1 in men 75 years or older (43). In most countries, incidence in men over the age of 75 years is between 20 and 80 times higher than in men aged between 50-54 years (43). **Figure 2** (on page 19) shows age-specific incidence rates per 100,000 population and the average number of new cases per year amongst men in the United Kingdom (UK), 2008.

3.2.1.2 Family History

There is considerable evidence from studies of familial aggregation of cancer that inherited genes, a shared environment and potentially gene-environment interactions are important in the development of prostate cancer (38-41).

Studies have shown that men with a first degree relative (father, brother) with prostate cancer have incident rates at least 2.5 times greater than men without a first degree relative with the disease and risk appears to increase with an increasing number of affected family members (38, 39). While differences in detection rates may bias results (as men with a family history of prostate cancer have increased diagnostic activity (44)) there is some evidence to suggest that having a brother with prostate cancer infers a higher risk than a father with disease (38), and among men with a brother or father with prostate cancer, over 50% of risk is considered attributable to familial clustering of the disease (38). Furthermore, family history of prostate cancer is associated with a higher risk in men aged less than 65 years than in men aged 65 years or older at diagnosis (38-41).

Evidence from twin studies suggest the heritability of prostate cancer is approximately 40% (37) and genome wide association studies (GWAS)[1] of prostate cancer have identified more than 30 genetic variants which together are reported to contribute 20% of the familial risk of prostate cancer (45-52) and suggest that heritability is not due to single high penetrance genes. As most common variants individually or in combination confer only small increments in risk there appears to be considerable missing heritability. Postulated explanations for this missing heritability include; rarer variants with greater penetrance that are not adequately captured using existing genotyping arrays, gene-gene interactions and shared environmental determinants among relatives that are not adequately measured(53).

3.2.1.3 Ethnicity

The incidence of prostate cancer in American black men is estimated to be 50-60% greater than that for American white men (54). Between 2002 and 2006, the prostate cancer incidence was 146.3 and 231.9 cases per 100,000 population in whites and blacks, respectively (1), although this may be an under estimate as PSA testing uptake is lower in black men (55). Incidence rates of prostate cancer in black men living in Africa and the Caribbean are lower than those observed in American black men but the extent to which this difference reflects a true lower risk or less case ascertainment via PSA testing is difficult to determine. In a study conducted in the United Kingdom, higher rates of prostate cancer were observed in black (166 per 100,000 population; 95% CI 151-180 per 100,000 population) than in white men (56.4 per 100,000 population; 95%CI 53.3-59.5 per 100,000 population) and the higher risk in black men compared with white men was more apparent in younger age groups (P heterogeneity <0.0001) (42).

Prostate cancer mortality rates are generally high in predominantly black populations, with age standardised mortality rates of 26 and 18 per 100,000 population in the Caribbean and sub-Saharan Africa, respectively, while rates less than 4 per 100,000 are observed in Eastern Asia (54). Furthermore, the CONCORD study found that amongst 628,000 men in 31 countries diagnosed with incident prostate cancer during 1990-94 and followed until 1999, the age standardised 5 year survival rate for prostate cancer was lower in blacks (85.8%) than in whites (92.4%) (56). In the United States this disparity may reflect to some extent inequality in access to and uptake of health care, as black men are less likely to be diagnosed with localised prostate cancer than white men (1).

The mechanisms for racial differences are unclear, although increased genetic susceptibility and different levels of androgen hormone activity within the prostate may contribute to the increased risk in black men (42). A recent GWAS detected a marker of risk for prostate cancer on chromosome 17q21 that appears specific to men of African ancestry (45) and a GWAS of prostate cancer in a Japanese population (52) identified five new susceptibility loci (not identified in White populations) on chromosomes 2p24, 5p15, 6p21, 6q22 and 13q22. While these findings highlight the potential heterogeneity of prostate cancer susceptibility among

[1] GWAS investigate the association between common genetic variation and disease and enable extensive searches for cancer loci without specifying particular candidate genes.

ethnic populations, the biological contribution of these alleles to prostate cancer risk is unknown.

3.2.2 Putative risk factors for prostate cancer

Investigations of life-style factors such as obesity, tobacco smoking, alcohol consumption, diabetes, sexual activity and sexually-transmitted infections, vasectomy and physical activity have provided inconsistent evidence for an association with prostate cancer risk, although many studies have been too small or with limited follow-up to stratify by cancer characteristics such as grade and stage of disease or time to diagnosis from recruitment. Other putative risk factors for prostate cancer include genetic polymorphisms, endogenous hormone activity and other lifestyle factors such as diet.

As discussed earlier in this text (section 3.2.1.2), GWAS have identified multiple single nucleotide polymorphisms (SNPs) that are each associated with a modest increase in prostate cancer risk that may, for men who carry large numbers of these variants, result in substantially higher risk. At least three distinct loci on chromosome 8 (8q24) contain variants that are associated with risk for sporadic prostate cancer (47-51, 57) and a recent meta-analysis of genome-wide and replication case-control association studies identified an additional 13 independent risk foci (58) but neither the specific genes nor the functional significance of these loci have been established.

Variation in lifetime exposure to endogenous androgen sex hormone may contribute to inter-individual and ethnic differences in prostate cancer risk. Sex hormones are known to play an essential role in normal prostate development and growth (59) and use of finasteride, a treatment which blocks the conversion of testosterone to its more active form, dihydrotestosterone (DHT), leading to lower levels DHT in the prostate, reduces prostate cancer incidence by up to 25% (10). These factors strongly suggest a role for androgens in prostate cancer development however results from a pooled analysis of prospective data of the association of circulating sex hormones and prostate cancer risk do not support an association with mid-adulthood circulating androgen concentrations (60). Furthermore, evidence from investigations of polymorphisms of the candidate gene CYP17 that encodes proteins involved in androgen synthesis and metabolism do not support an association (61), although a recent meta-analysis suggests that the rs743572 polymorphism in this gene is associated with risk in Black populations but not in White or Asian populations (62).

3.2.2.1 Evidence for IGF-I and risk for prostate cancer

Insulin-like growth factors (IGFs) and associated binding proteins have been the subject of considerable investigation in relation to cancer because of their role in the regulation of cell proliferation, differentiation, and apoptosis (63) and a recent pooled analysis of individual participant data found that high IGF-I concentrations (within the normal physiological range) are associated with increased risk of prostate cancer (OR 1.38; CI 95% 1.19 -1.60; p trend < 0.001; in the highest vs. lowest quintile) (64). It has also been shown that animal protein may increase levels of IGF-I(12, 65) and this may be a mechanism through which a Western-style

diet may influence risk. To date circulating IGF-I is the strongest candidate risk factor identified for prostate cancer.

3.2.2.2 Evidence for diet and risk for prostate cancer

The hypothesis that a Western-style diet may be a risk factor for cancer emerged in the 1970s as it was evident that the incidence of colo-rectal, breast, prostate, endometrial and lung cancer was greater in Western countries than in Asian countries where diets were much lower in animal products, fat and sugar (2). Furthermore, evidence from ecological comparisons (20) and migrant studies (3-5) suggested that a Western lifestyle and diet may play an important role in the aetiology of prostate cancer, as migrates were shown to acquire the cancer incidence rate of their new host country within one generation (66). These observations prompted research into the associations between different lifestyle factors and diet and risk of developing prostate cancer.

Early case control studies may have contributed to a number of misleading associations between diet and prostate cancer due to small numbers and the effects of recall and selection bias. Subsequent research conducted in large prospective cohort studies has not established convincing protective or causal associations between constituents of a Western diet and prostate cancer risk (8, 9). Although the contribution nutrition plays in the development of prostate cancer remains unclear, there is some evidence to suggest an increased risk with high intake of dairy products (67-78). A possible mechanism underlying an association between dairy products and risk of prostate cancer is an increase in the production of insulin like growth factor-I (IGF-I) (65). An alternative hypothesis is that the phytanic acid content of dairy products increases the risk of prostate cancer (79) by up-regulating the expression of α-Methylacyl-Coenzyme A Racemase (AMACR)[1] (80-82), an enzyme consistently over-expressed in prostate cancer tissue compared with normal tissue (83-86).

Folate and vitamin B_{12} have been investigated in relation to several cancers, including colo-rectal, pancreas, breast and prostate cancer, for their essential role in deoxyribonucleic acid (DNA) methylation, repair and synthesis (87). Folate (obtained from green leafy vegetables, fruit and grains in the diet) interacts with vitamin B_{12} (largely acquired from intake of red meat and offal) in the synthesis of nucleotides and amino acids, including methionine, and deficiency results in reduced S-adenosylmethionine that leads to DNA instability and the development of several conditions including neural tube defects (in developing embryos) and megaloblastic anaemia. It is disruption to DNA methylation, repair and synthesis that is considered important in cancer development however, there are concerns over a dual, time and dose dependent effect on cancer risk (88). Experimental studies in colo-rectal cancer suggest that folate administration prior to the presence of pre-neoplastic lesions reduces cancer growth while folate given in the presence of early cancers increases the rate of tumour progression (89, 90). Moreover, results from a meta-analysis of six randomised controlled trials of folic acid supplementation (a synthetic and more bio-available form of folate) show an increased risk of prostate cancer with supplementation (91). Although individual observational studies of the association between circulating concentrations of folate and vitamin B_{12} and risk

[1] AMACR is essential for ββ-oxidation of phytanic acid inside sub-cellular peroxisomes.

for prostate cancer show less consistent results, a recent meta-analysis of prospective studies has shown an increased risk associated with higher circulating concentrations of folate (OR per 10nmol/L increment increase in folate 1.19; 95%CI 1.03-1.37) and vitamin B12 (OR per 100pmol/L increment increase in vitamin B12 1.10; 95%CI 1.03-1.18) (92); discussed further in Chapter 8 section 8.1.5.2.

In summary, the inconsistent evidence for an association between diet and risk for prostate cancer may reflect methodological difficulties associated with accurately measuring long term dietary habits using traditional survey methods (93). It is possible that dietary assessment methods do not capture adequately specific nutritional constituents of the diet that may be associated with risk and interactions between dietary factors and other environmental or genetic risk factors that may alter overall risk of disease (13). However, it is anticipated that validated biomarkers of nutritional exposure (and endogenous hormonal activity), as used in this thesis, may provide more accurate measures of exposures and intermediate phenotypes in order to clarify potential mechanisms in the causal pathway of prostate cancer development.

3.3 Latent prostate cancer

As discussed in section 3.1.1, prostate cancer is a very common disease, particularly in elderly men (>65 years) (20), and although many cancers remain small and undetected (94), some men will develop an aggressive, lethal form of the disease. Clearly differentiating non-aggressive from aggressive disease is crucial for determining the best treatment approach (and preventing men undergoing invasive and unnecessary treatments for cancers that many never become life threatening) and it may also be important for determining possible aetiological differences. However, prostate tumour progression is difficult to predict (as the duration between onset of asymptomatic disease and clinical symptoms has been estimated to be up to 12 years (95)) and some men will develop a form of disease that has little impact on health even in the absence of treatment (96). Autopsy studies have shown microscopic, well-differentiated tumours of the prostate gland in 30-40% of men aged over 50 years (94) and in 10-14% of men between the age of 40 and 50 years (97). By 80 years of age it is estimated that 80% of all men have asymptomatic prostatic carcinoma (98).

The latent nature of prostate cancer has implications for the design and analysis of prospective observational studies. Because study participants may have sub-clinical disease at enrolment there is the possibility of reverse-causation, whereby before diagnosis, a developing prostate cancer alters the availability of a factor of interest in the association with risk. There is also the possibility of outcome misclassification, with participants harbouring subclinical disease during follow-up assigned to the control group of the study.

3.4 Prostate Specific Antigen (PSA) testing

Increased use of prostate-specific antigen (PSA) testing has led to much earlier detection of asymptomatic disease (estimated to be as high as 12 years in men aged 55 years (99)), decreased numbers of advanced cancers and a concomitant increase in the proportion of early localised cancers (of which many may never progress to clinical disease (100)).

Although formal prostate cancer screening programmes are not operational in the European countries participating in the EPIC study, more wide spread use of PSA testing has increased the rate of diagnosis of latent cancers, advancing a prostate cancer diagnosis by as much as 10 years in some men and potentially ascribing a prostate cancer diagnosis to a proportion of men who may not have developed clinical symptoms during their lifetime (101). Unfortunately it is not possible to determine the extent of over-detection of prostate cancer within the EPIC cohort as general practice medical records and information on PSA testing history are not available. As a result, in the work presented here it is not possible to investigate heterogeneity in the associations with prostate cancer risk by PSA testing history.

While it is not possible to determine definitively the impact of increased uptake of PSA testing on changes to prostate cancer diagnosis during the follow-up of EPIC participants (as PSA testing history information was not collected), the relatively long duration of the study means that sufficient follow-up time exists to adequately investigate heterogeneity in risk for prostate cancer by duration from time of recruitment to cancer diagnosis, thereby examining the impact of latent disease on associations between biomarkers of diet and hormones and prostate cancer risk (i.e. the extent of reverse causation).

3.5 Classification of prostate cancer

The prostate cancer TNM tumour grading system (TNM) (102) and the Gleason score (103), devised over 40 years ago, are the standard benchmarks for determining the clinical stage and pathological grade of prostate cancer. However diagnostic practices and criteria have evolved during this time with implications for the interpretation of stage and grade information. In recent years 18 gauge ultrasound guided needle biopsy tissue (collected at diagnosis) has become available for grading disease while previously this information was taken from radical prostatectomy tissue. The implication of this change in diagnostic practice is a trend toward higher grade allocation to cancers that would have previously been classified as less-aggressive forms of the disease (102, 104, 105). To accommodate heterogeneous patterns of early disease (detected by PSA testing) that were not apparent when the Gleason criteria were first devised, scoring recommendations have also been updated (106). In the original system the two predominant Gleason scores contributed to the overall grade however the current recommendation is to use the most predominant score and the tertiary score (105). This change has also contributed to the upward trend in grade allocation overtime (105).

3.5.1 Implications for analyses included in this thesis

For prospective studies, changes in diagnostic practices that alter disease classification during the follow-up period have the potential to introduce error and possibly obscure relations between exposures and risk. To address this issue, some prospective studies and clinical trials have standardised prostate cancer grade (75) and stage (107) data and collected detailed information on a participants PSA screening history (108). However, for large, international and/or multi-centre studies, this may not be feasible and to date, standardisation of histopathology across centres participating in the EPIC study has not been performed. Furthermore, for studies without information on screening it becomes difficult to ascertain the

likely lead time associated with an individual's diagnosis and account for this in the analytical methodology.

A review of the classifications used in other large prospective cohort studies and randomised controlled trials (RCT) to characterise advanced stage and high grade cancer revealed considerable variation, shown in **Table 1**(on page 19). It was also notable that very few studies combined stage and grade data to generate an 'aggressive' disease category. Several studies include Gleason score 7 in their definition of high grade disease, although some experts consider this insufficient to classify aggressive disease as there is some evidence to suggest that amongst men diagnosed with localised prostate cancer, those with a Gleason score of 8 - 10 have the greatest risk of death (60-80%) within 15 years of diagnosis compared with 18-30% for those men diagnosed with a lower Gleason score (109).

In the EPIC study tumour stage information (TNM staging score) is derived from pathology reports and cancer registry data and used to define the following categories:

- localised (T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised);
- advanced (T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic and;
- unknown stage.

Information on Gleason score or the World Health Organization (WHO) grading system is used to generate categories of:

- low grade (Gleason score ≤7, or cases coded as well differentiated or moderately differentiated);
- high grade (Gleason score >7, or cases coded as poorly differentiated or undifferentiated), and;
- unknown grade

All of the analyses included in this thesis will use these definitions to evaluate the relation between population characteristics and putative risk factors and risk of non-aggressive and aggressive prostate cancer. In the EPIC study information on PSA measurements, prostate cancer treatment and cause of death were not available. It is anticipated that future research will identify diagnostic and prognostic biomarkers of prostate cancer that provide a clear differentiation between aggressive and non- aggressive disease but there are currently no such validated biomarkers.

Figure 3:1 Age standardised prostate cancer incidence and mortality rates by world area, 2008.

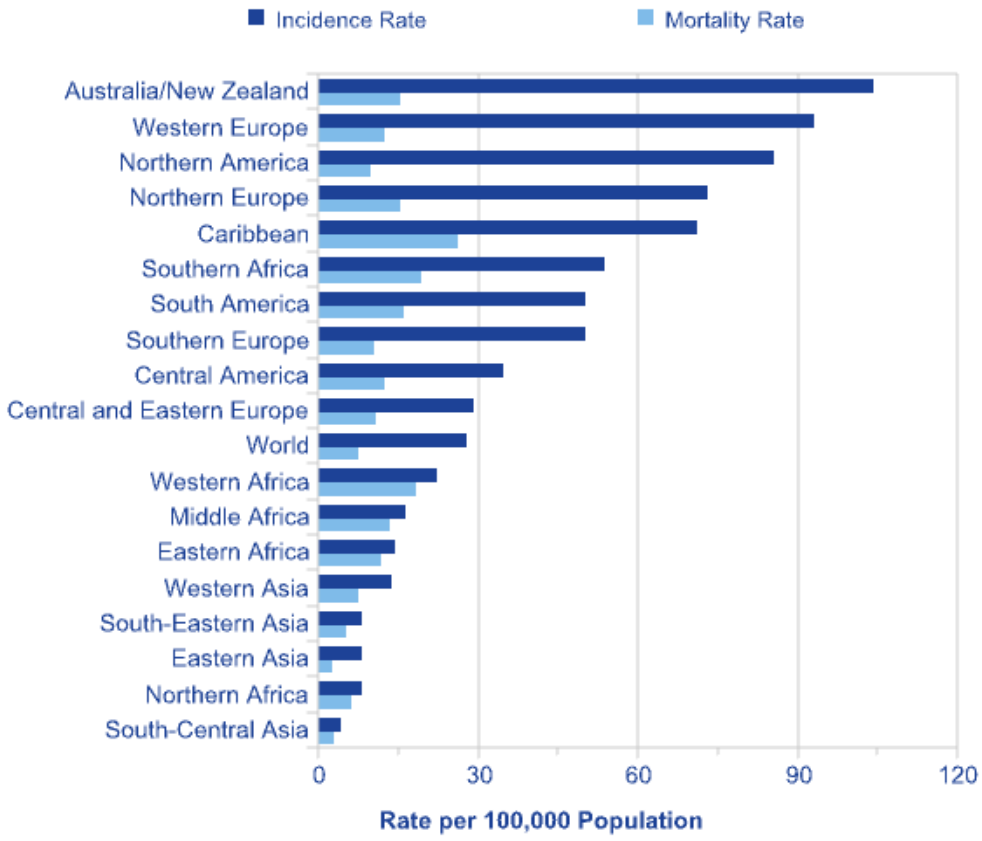


Figure 3:2: Age-specific incidence rates per 100,000 population and the average number of new cases per year amongst men in the United Kingdom, 2008.

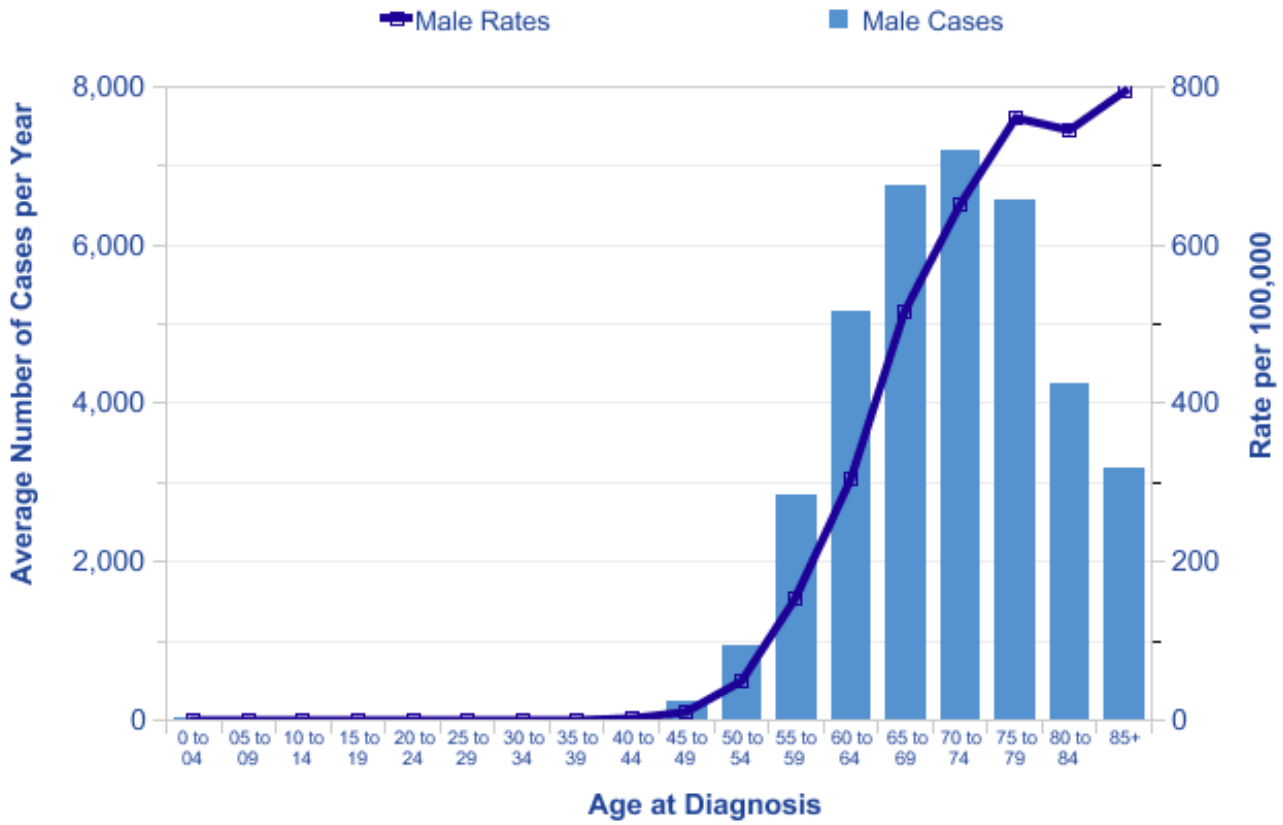


Table 3.1 **Information relating to prostate cancer stage and grade classification in large prospective cohorts and controled trials**

Study Name	Study Design	Data	Biological	Country	Recruitment	Sex	Number	Age	Clinical Staging and Grading	Other information
Health Professionals Follow-Up Study	Prospective Cohort	Lifestyle questions every 2 years Diet questions every 4 years	No	USA	1986	male	51,529	40-75	Advanced= N0M0 with seminal vesicle invasion through capsule (stage T3b or T4 or C2) Non-advanced= T1 or T2 and N0M0 but excluding stage T1a + includes tumors with capsular invasion (stage C1 or T3a) Stage A = occult or incidental finding (A1 = focal and A2= diffuse) Stage B = confined to prostate Stage C = localized to periprostatic area Stage D = metastatic disease (D1 metastatic disease involving only regional LN and D2 metastases to other organs) Advanced = Stage C, D and fatal Metastatic = Stage D and fatal High Grade= ≥ 7 Low Grade= < 7	Self reported cancer and verified from cancer registry information and medical records A study physician staged the prostate cancers Any info from initial work up included- staging prostatectomy, bone scan TNM staging info updated in 1992 (see Schroder, 1992) Cause of death based on death certificate & primary medical records Death due to prostate cancer assigned if extensive metastatic disease Advanced disease used as indicator of aggressive disease 2007 paper Giovannucci, In J Cancer, 121,1571-1578 A1 cases dropped because innocuous/more prone to detection bias
Cancer Prevention Study II Nutrition Cohort (CPS)	Prospective cohort	1992 Lifestyle question 1997 Nutrition question	1998-2001 blood from 17,411 men with nutrition data	USA	1992	male	86,404	50-74	Localized stage= intracapsular involvement or invasion not beyond capsule Regional= extracapsular extension and invasion into ureter,rectum regional LN Distant= distant LN, mets Grade- well, mod and poorly differentiated Aggressive Prca = Gleason Score ≥ 8, grades 3-4, or stage D at diagnosis	Self reported cancer and verifed by med records and cancer register PSA info collected in 1997 on Questionnaire [didn't distinguish if screen detected]
Alpha-Tocopherol, Beta-Carotene Cancer (ATBC) Prevention Study	RCT-blind	1985-1993 Lifestyle and dietary question	No	Finland	1985-1988	male	29,133	50-69	Non-advanced= Stage 0-II Advanced= stage III or IV Low grade = with grades 1 or 2 High Grade= with grades 3 or 4	Medical records reviewed by 2 study oncologists to confirm diagnosis and stage Hitopath and cytology specimens reviewed by 1-2 pathologists to confirm cancer and histologic type/grade TNM classification for stage

Table 3.1 (cont) Information relating to prostate cancer stage and grade classification in large prospective cohorts and randomised controled trials

Study Name	Study Design	Data	Biological	Country	Recruitment	Gender	Number	Age	Clinical Staging and Grading	Other information
Physicians Health Study	RCT-blind 1982-1995	Lifestyle and short diet question	Yes	USA	1982		22.071		Advanced = stage C or D (on Whit Jew scheme) or poor histologoical differentiation at diagnosis or Gleason score ≥ 7 at diagnosis	Whitmore-Jewett classification scheme
Multi-Ethnic Cohort 5 ethnic groups	Prospective cohort	Lifestyle and diet question	Yes collected 2001-2006	California and Hawaii	1993-1996	male	82,483	45-75	Advanced= regional or metastatic (not in situ or localized) High Grade= Gleason Score ≥ 7 or poorly differentiated	PSA info collected in 2000-2002
The Netherlands Cohort	Prospective cohort	Lifestyle and diet question	No	Netherlands	1986	male	58279	55-69	Localized= T0-2, M0 Advanced= T3-4, M0	TNM classificatiom
Prostate Cancer Prevention Trial	RCT	Screened yearly for PSA and DRE	Annual PSA	USA	1994-1997	male	18,882	55+	low grade (Gleason score< 7) high grade (Gleason ≥ 7) or < 8 and ≥ 8 Localized stage = organ confined disease (≤ stage T2 and N0M0) and organ confined low- and high-grade disease Advanced stage = > T2 or N1 or M1	All prostate biopsies reveiewed centrall interpretations arbitrated be a referee p At recruitment non-cancer if PSA (< 3n negative + noclinical history of benign
EPIC (European Investigation into Cancer and Nutrition)	Prospective cohort	Lifestyle and diet question	Yes	Europe	1992-2000	male	150,000	20+	localised = T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised advanced = T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic low grade = combined Gleason score < 7 or well differentiated or moderately differentiated high grade = combined Gleason score ≥7 or equivalent [cases	No information on PSA testing Grade and stage not standardised acros

Chapter 4 Validation of Nutritional and Hormonal Biomarkers

4.1 Introduction

Prospectively collected and archived biological specimens from epidemiological studies provide opportunities to investigate associations between biomarkers of diet and hormones and subsequent risk for disease. Important design elements of these studies include standardised procedures for collection, transport and storage of biological specimens, reliable and valid laboratory techniques (110) and measurement of co-factors that may influence biomarker levels, which may include season of blood collection, fasting status, genetic polymorphisms, medications and other lifestyle factors (111).

Although biological measures are not subject to the errors associated with human memory or opinions and perceptions that may influence recall for dietary assessment methods, the extent to which a single biomarker measure represents an individual's usual or long-term exposure level is critical, as this measure is of most interest in the aetiological relationship with chronic disease. Day to day, seasonal and longer-term intra-individual variation in exposure levels reduces the strength of the correlation between single estimates and underlying long term exposure. The larger the ratio of intra-individual variance to between individual variance the greater the number of repeat measures required to obtain a reliable estimate of an individual's usual exposure level. Furthermore, measurement error and misclassification caused by a single estimate reduces the statistical power of a study and can lead to systematic underestimation of the strength of the association with disease risk toward the null in risk analyses; known as 'regression dilution bias' (18, 112). However, in a multivariate analysis, inclusion of correlated exposure variables measured with imprecision can alter the strength and/or the direction of the association with risk (113-116). Repeat blood measures from an individual taken several years apart may improve the reliability of the measure and correct for this effect or if only single measures are available in the whole study population (as is the case for most observational studies), repeat measures in a subset of representative individuals (either within or external to the study population) can be used to calculate 'correction factors' to inflate the regression coefficient relating the exposure to risk (113). Nonetheless, use of replicate measures in the entire study population is advantageous as it avoids analytical assumptions that must be made when applying a correction factor from a sub-group to un-validated subjects (117).

In summary, without knowledge of the reliability of the biomarker it may be difficult to interpret if a null result between a biomarker and a health outcome reflects the absence of a true association with disease risk or rather, attenuation of a modest risk estimate toward the null because the biomarker is an inadequate measure of usual or long-term hormonal or nutrient status.

4.2 Biomarkers of interest

The biomarkers of interest in this thesis are circulating concentrations of folate, vitamin B_{12} and insulin-like growth factor-I (IGF-I) because of their potential role in the development of

several cancers including breast, colo-rectal, pancreatic and prostate cancer (92, 118-120), and phytanic acid for its possible role in prostate cancer development (121).

In brief, folate is a water soluble B vitamin that is found predominantly in green leafy vegetables, although it also acquired from supplements and fortified foods in the form of folic acid. In contrast, vitamin B_{12}, also known as cyanocobalamin, is mostly acquired from consumption of red meat. Folate and vitamin B_{12} are essential for DNA methylation, repair and synthesis and deficiency in these nutrients is associated with anaemia, cardiovascular disease, neural tube defects and neuropsychiatric disorders (122, 123). Results of a recent meta-analysis of folic acid supplementation in randomised controlled trials (91) suggests a modest increased risk of prostate cancer with supplementation and a meta-analysis of circulating folate concentrations in prospective observational studies(92) suggests an increased risk of prostate cancer with higher levels of both B vitamins (this is discussed in greater detail in Chapter 8 (section 8.1.5.2).

Phytanic acid is a branched chain fatty acid that is not synthesized in the human body and is acquired mostly from consumption of ruminant fat, and to a lesser extent from pelagic fish. It has been hypothesized that the phytanic acid content of dairy products may increase the risk of prostate cancer (79) by up-regulating the expression of αα-Methylacyl-Coenzyme A Racemase (AMACR)[1] (80-82), an enzyme consistently over-expressed in prostate cancer tissue compared with normal tissue(83-86)(more details are provided in Chapter 5; section 5.1.3).

Insulin like growth factor-I (IGF-I) is a polypeptide hormone secreted mainly from the liver, although produced in many body tissues throughout life, and involved in cell differentiation and proliferation and prevention of cell death and has been shown to be associated with prostate cancer risk (see Chapter 7; section 7.1.3).

There have been numerous investigations of the reliability of a single IGF-I measure over the short- to medium-term and although most studies have been small in size, reliability estimates suggest moderate to good reliability, as shown in **Table 4.1**[2](124-136). However, there are limited published data on the reliability of circulating folate and vitamin B_{12}, shown in **Table 4.2**[1](137-140), and no previous studies for phytanic acid.

4.3 Aim of study

The aim of this study was to use repeat blood samples, collected on average 6.4 (standard deviation 0.54) years apart, to investigate the effect of variability in blood concentrations on

[1] AMACR is essential for ββ-oxidation of phytanic acid inside sub-cellular peroxisomes.

[2] Reliability studies were identified by using medline and scopus databases and the search terms “reliability”, “repeatability” and “intra-class correlation coefficient” in combination with biomarker names(“folic acid” or “folate”; “vitamin B_{12}” or “cobalamin”; “phytanic acid” or “3,7,11,15-tetra methylhexadecanoic acid”)

estimates of reliability for circulating levels of folate, IGF-I, phytanic acid and vitamin B_{12} in participants in the European Prospective Investigation into Cancer and Nutrition (EPIC).

4.4 Study design

4.4.1 Study population

Between 1993 and 1999, approximately 65,000 British men and women, aged between 35 to 70 years, were recruited into the Oxford arm of the EPIC cohort. General practice surgeries were used to recruit members of the general population from Greater Manchester, Oxfordshire and Buckinghamshire and postal recruitment was used to target non-meat eaters via vegetarian and vegan societies, health magazines and health food shops. At recruitment all participants completed a lifestyle questionnaire that included a semi-quantitative food-frequency questionnaire (FFQ) relating to usual diet over the past year and 19,241 participants provided a non-fasting blood sample at their general practice surgery. Approximately 5 years after recruitment, 38,027 participants completed a follow-up questionnaire and 1,039 randomly selected individuals also gave a repeat non-fasting blood sample. Information collected at both time points includes weight, height, smoking status, alcohol intake and energy intake. Blood samples were sent in the post at ambient temperature to an EPIC laboratory, in Norfolk (UK) at recruitment and in Oxford (UK) at follow-up, where samples were processed and separated into serum, plasma, red cells and buffy coat fractions prior to storage at -196°C in liquid nitrogen tanks. Sodium citrate anticoagulant was used for all of the plasma samples included in this study. All participants provided written informed consent and local ethics committee in Oxford, UK approved the study.

To be eligible for inclusion in this study participants must have provided a baseline and follow-up blood sample and not have had a registered diagnosis of cancer at the time of recruitment. Fasting restrictions were not applied prior to either blood collection and approximately 80% of participants had consumed either food or drink within 3 hours of blood collection at both time points.

The reliability study for folate, vitamin B_{12} and phytanic acid included 100 male participants (because of their potential role in prostate cancer development) and the IGF-I study included a further 100 female participants (as IGF-I has been associated with both prostate and breast cancers), randomly selected within age strata (40-49, 50-59, ≥60 yrs) and diet group (meat eater, vegetarian and vegan) and matched by time since last meal (<6, ≥≥6hours since last meal).

4.4.2 Laboratory analysis

Serum folate and vitamin B_{12} concentrations were measured in singleton at BEVITAL (Bergen, Norway). A *Lactobaillus leichmannii* and a *Lactobacillus casei* microbiological assay, adapted to a microtiter plate format and performed by a robotic workstation (Microlab AT plus2; Hamilton Bondaduz) was used for folate and vitamin B_{12}, respectively. The lowest point of detection for folate (2 nmol/L) and vitamin B_{12} (30 pmol/L) provided adequate sensitivity to

detect the lowest concentration in the study samples. Further assay details are provided in **Chapter 8** (section 8.3.4)**.**

Plasma phytanic acid concentrations were measured in singleton using a gas chromatographic/mass spectrometric (GC/MS) assay at the HFL Sport Science Ltd (Fordham, Cambs, UK). Using quality control samples, phytanic acid demonstrated good stability at room temperature, with less than 6% variation between samples left at room temperature for 4 hours compared to samples that were extracted immediately. Furthermore, phytanic acid was stable following several freeze-thaw cycles (at -80˚C), with less than 4% variation between samples that were subjected to the freeze-thaw process and those that were not (141). Further details of the laboratory methods are described **Chapter 5** (section 5.3.4).

Serum IGF-I concentrations were measured in duplicate in the Cancer Epidemiology Unit (Oxford, UK) using enzyme-linked immunosorbent assays (ELISA) from Diagnostic Systems Laboratories (DSL). Baseline and follow-up samples from each individual were assayed in the same batch, with inclusion of duplicate, blinded quality control samples of low, medium and high concentrations. A more in-depth discussion of the laboratory methods is presented in **Chapter 7** (section 7.3.4).

The intra-assay and inter-assay co-efficient of variation (CV) was 10.3% and 12.5% for folate, 4.0% and 11.7% for vitamin B_{12}, 3.9% and 6.9% for phytanic acid and 4.4% and 12.4% for IGF-I, respectively. Folate, vitamin B_{12} and phytanic acid measures for one participant were excluded due to a missing baseline serum sample.

4.4.3 Statistical analysis

Concentrations of folate, vitamin B_{12}, phytanic acid and IGF-I were log-transformed to approximate normal frequency distributions from the observed positively skewed data. One way analysis of variance tests were conducted to estimate components of variance associated with variability between- ($\sigma^2_{s)}$ and within-individuals (σ^2_e). The reliability of measurements was assessed by computing intra-class correlation coefficients (ICC) from the following model: $Y_{ijk} = \mu + \alpha_i + \epsilon_{j(i),}$ where y denotes the mean log values obtained from assay measurements for individuals i (i= $1,2\ldots,100$) at time point j(i) (j=$1,2$), with μ as the true mean of the log values of the biomarker, and α_i and $\epsilon_{j(i)}$ as independent zero mean variables with variance σ^2_s and σ^2_e, respectively. Using the variance estimates, the ICC was calculated as: $p = \sigma^2_s / (\sigma^2_s + \sigma^2_e)$, where σ^2_s and σ^2_e are the between-individual and within-individual components of variance, respectively, and p is the proportion of between-individual variation relative to the total variation.

The number of biomarker measurements required to limit the attenuation of the correlation coefficient to 5%, 10% and 20% between a biomarker and another variable was calculated by using the formula: $n_p = [P^2/(1-P^2)][S^2_w/S^2_b]$, where $P = 1$ minus the maximum attenuation desired, S^2_w is the within-individual variance and S^2_b is the between-individual variance (142).

To account for within-individual variability of both the analyte and another factor, the number of measurements required to limit attenuation of a regression coefficient was estimated using the formula: $n_\beta = [P/(1-P)][S^2_w/S^2_b]$. All estimates were rounded up to the nearest integer if more than 0.2 higher than the nearest lower integer.

Attenuation of the expected value of the observed coefficient that results from use of *n* measurements was estimated using the formulas: $\text{Attenuation}_p = \sqrt{(n / ((S^2_w/S^2_b) + n))}$ and $\text{Attenuation}_B = n / ((S^2_w/S^2_b) + n)$, where $n = 1$ for correlation based on a single measurement (143). The attenuation coefficient (denoted by λ_p for correlation and λ_β for regression), measures the degree to which the estimated parameter differs from the value that would be obtained in the absence of intra-individual variation, and ranges between 0 and 1, with values close to 0 indicative of severe attenuation.

The utility of a single measurement to correctly classify longer term concentrations into tertile categories was assessed by comparing tertiles of concentration as measured by the first blood sample with tertiles of concentration in the second sample with cut-points defined by the distribution of values in the first blood sample. Cut-points were also defined by the distribution of values at each blood sample in order to assess the adequacy of tertile categories as a measure of usual concentration.

The ICC generated from each reliability study was used to calculate observed relative risk estimates that would result if a single biomarker measurement was used, assuming a range of hypothetical true relative risks (RR), with the formula: Observed $RR = \exp^{[\log \text{true RR x ICC}]}$. Estimation of sample sizes required to achieve a range of 95% confidence interval widths around the observed ICC estimates were calculated using methods described by Shoukri et al (144). All statistical analyses included in this chapter were performed using Stata Statistical Software: Release 12 (College Station, TX, USA: StataCorp LP).

4.5 Results

The descriptive statistics for each analyte are shown in **Table 4.4** (on page 34). At the first blood sample collection the mean age of men and women was 55.2 (SD 8.6) and 55.4 (SD 8.0) years, and the mean body mass index (BMI) was 23.6 (SD 2.7) and 23.4 (SD 4.0) kg/m^2 for men and women, respectively. Recruitment and follow-up blood samples were stored on average for 14 and 7 yrs, respectively. The majority (>50%) of participants consumed food or drink within 3 hours of blood collection and had blood samples collected before 12 midday at both time points. At recruitment 48% of men and women consumed meat and 6% (n=3) of meat eating men and approximately 23% (n=11) of meat eating women reported to have changed to a vegetarian diet at the second blood sample. 32% of men and women were vegetarians at recruitment and 0% of vegetarian women and 12.5% (n=4) of vegetarian men had changed from a vegetarian diet to a diet that included meat at the second blood sample. 20% of men and women in the data set maintained a vegan diet throughout the duration of follow-up. The geometric mean concentration of each analyte varied by no more than 25% between each

sample collection and the distribution of values for each analyte were within the normal physiological range and consistent with those observed in earlier EPIC studies (12, 121, 141, 145, 146).

Table 4.4 (on page 35) shows the ICCs and measures of reliability for folate, IGF-I, phytanic acid and vitamin B_{12} for two blood samples taken, on average, 6 years apart. The ICCs for folate, IGF-I, phytanic acid and vitamin B_{12} were 0.18, 0.43, 0.48 and 0.34 respectively, reflecting the higher ratio of within- to between-individual variation in concentration for folate, and vitamin B_{12} compared to the lower ratios observed for IGF-I and phytanic acid. A single blood measure is associated with severe attenuation of the expected correlation coefficient for folate (137.0%) and vitamin B_{12} (72.6%) and moderate attenuation of the correlation coefficient for phytanic acid (44.5%) and IGF-I (52.4%). To limit attenuation of the correlation coefficient to 20%, two measures of phytanic acid, at least three measures of IGF-I, four measures of vitamin B12 and eight measures of folate are required. At least a doubling in the number of measurements for each analyte would be required to limit attenuation of the correlation coefficient to 10% (results not shown) or to limit attenuation of the regression coefficients to 20% (see Table 4.4) and 10 times as many to limit the attenuation of the regression coefficients to 5% (results not shown). Overall, the attenuation from a single measure for each analyte suggests that the true correlations, calculated by multiplying the observed correlations by the inverse of the attenuation factor, could be at least 40% larger than those observed.

The proportion of participants changing tertile categories between baseline and follow-up, using tertile cut-points for each analyte defined by the distribution of values at the initial blood collection are shown in **Table 4.5.1 - 4.5.4.** These tables illustrate that for each analyte between 48.5 and 63.6% of participants who were categorised in the top tertile at baseline remained in the highest tertile at the second blood collection, except for phytanic acid with approximately 40% remaining in the top tertile. Amongst those in the bottom tertile at baseline approximately 50% remained in this category, except for phytanic acid with near to 70% remaining in the lower tertile. Overall, approximately 50% of participants were classified in the same tertile category at each time point (47.5%, 47.5%, 52.5% and 46.5% for folate, IGF-I, phytanic acid and vitamin B_{12}, respectively).

Figure 4:1.1-4.1.2 Geometric mean concentrations at time points 1 and 2 by third of concentration for each biomarker (starting on page 37) shows the geometric mean concentrations (95% CI) at time point 1 and 2 by third of concentration for each biomarker. The graphs show evidence of regression to the mean whereby measures at a second time point are closer to the overall mean than the initial measures. Regression to the mean is evident in each figure, with the lowest and highest tertile categories at baseline including people whose usual concentration (represented by the follow-up sample) is higher or lower, respectively. The difference in geometric mean concentration between the top and bottom tertile is markedly greater for each analyte at baseline than at follow-up; with a 2.1- and 2.7-fold higher difference (between the upper and lower tertile) observed at baseline than follow-up for folate and phytanic acid, respectively, and a 2.8-fold higher difference observed for IGF-I and vitamin B_{12}.

Table 4.6 (on page 39) shows the extent of attenuation of the true relative risk (RR) when a single measure of each biomarker is used, assuming an ICC of 0.18, 0.43, 0.48 and 0.34 for folate, IGF-I, phytanic acid and vitamin B_{12}, respectively, and no additional sources of error. A single blood measure of folate, with a very low ICC (of 0.18), attenuates greatly a true RR of 2 to an observed RR of 1.13. Stated another way, an observed RR of 1.13 may represent a true RR of 2, given the ICC estimated here. For IFG-I an observed RR of 1.38 for the association with prostate cancer risk (as described in Chapter 7) would represent a true RR of 2.1, given the ICC of 0.43. For phytanic acid the larger ICC of 0.48 would result in slightly less attenuation of RR estimates, such that an observed RR of 1.11 may represent a true RR of 1.25. The sample size required to obtain a range of intra-class correlation coefficients with a specified width of confidence interval using two blood samples (144), is shown in **Table 4.7**. The calculations indicate that even with two blood samples a very large study sample is required to improve the precision of the reliability estimate when the ICC is very low. A very large sample size of nearly 1,420 individuals is required in order to achieve a 95% confidence interval width of 0.1 around the ICC estimate, given an ICC of 0.18 (as was observed for folate).

4.6 Discussion

In epidemiological research the long-term average intake of a nutrient or circulating concentration of a hormone is considered to be the important exposure in the evaluation of risk for chronic disease such as cancer. However, the extent to which a reliable estimate of usual concentration can be obtained from a single blood sample collected at one time point, is dependent on a number of factors. These include appropriate handling and storage of archived biological samples, use of reliable laboratory methods and the degree of within-individual variation in biomarker concentration over the period of investigation; affected by phenomena such as varying patterns of dietary intake, hormonal activity, gastrointestinal absorption, interactions with drugs and alcohol, tissue metabolism and excretion. Variation attributable to specimen handling, instrument inaccuracy (which includes laboratory assay intra-and inter-batch variation) and within-individual change introduce measurement error that, when unrelated to risk for disease (random error), result in attenuation of relative risk estimates and reduce statistical power for detecting exposure-disease relationships.

Our findings suggest that a single measurement of circulating folate, IGF-I, phytanic acid or vitamin B_{12} is not sufficient to characterise adequately an individual's blood concentration within 20% of their true usual concentration. At least two phytanic acid, three IGF-I, four vitamin B_{12} and eight folate measurements are required to limit attenuation in correlation coefficients to 20% and twice this number of measurements is required to adequately characterise an individual for regression coefficients (where the analyte is an independent factor).

The large number of samples needed in order to improve estimate precision for usual folate and vitamin B_{12} concentration is impracticable for large scale observational studies for reasons of expense and limited resources. However, for prospective observational studies with blood collected on two occasions several years apart, calculation of the mean concentration of these

measures will provide a more reliable estimate of usual concentrations for IGF-I and phytanic acid.

The poor ICC observed for plasma folate in the current study contrasts with excellent reliability measures reported in earlier studies(137-140) of circulating folate over a shorter time interval (weeks-3 months; refer to **Table 4.2**). These different findings suggest that while a single circulating folate measure is a very good estimate of short term levels it does not provide an informative estimate of long-term usual concentrations. Differences between baseline and follow-up in the duration of time whole blood samples spent in the post may have contributed to the large within-person variation obtained for folate in this study, as reductions as large as 30% have been observed for folate after a 24 hour delay in processing and 10% within 2 hours (147, 148). The poor reliability for folate demonstrated by the low ICC may result in attenuation of risk estimates toward the null, making a small association difficult to detect (as observed in the nested case control analysis of folate on risk for prostate cancer; discussed in Chapter 8).

While acceptable stability has been reported for vitamin B_{12}, delayed processing may increase blood concentrations due to movement out of red blood cells (147). Moreover, negligible intake of vitamin B_{12} associated with a meat-free diet (leading to low circulating levels), may have contributed to the large within-person variation observed in the vegetarian and vegan diet groups (previously described in this population (149)), as blood concentrations decline with duration of dietary restriction and depletion of red blood cell reserves (123).

The higher ICC observed for phytanic acid, corresponds to greater between-individual to intra-individual variation, which may reflect greater stability of phytanic acid with storage and transport (compared with the B vitamins), the range of dietary patterns including meat-eater, vegetarian and vegan (that captures a broad range of phytanic acid concentrations) and limited change in these patterns, with less than 10% of men changing diet group during follow-up. In a Western diet it is likely that meat eaters and vegetarians maintain a regular intake of phytanic acid from ruminant meat and/or dairy products such as butter and milk, while vegans experience an almost negligible intake due to their plant based diet. The good reliability observed for phytanic acid, the absence of endogenous synthesis of this branched chain fatty acid in the human body and the strong correlation previously observed with dietary dairy fat intake (121, 141) suggest that it may be a useful surrogate measure of long term exposure to ruminant fat, particularly fat from beef and dairy products.

The modest ICC observed for IGF-I in the current study is consistent with the findings of several earlier studies (133, 135), although ICCs of higher magnitude (0.81- 0.90) have also been observed in studies of small sample size and with shorter time intervals between blood collection (128, 132, 134). Attenuation of the expected correlation coefficient results in underestimation of the association between IGF-I and cancer risk. For example, after correction for attenuation the observed relative risk estimate for prostate cancer of 1.38 in a collaborative analysis of 12 studies (64) would correspond to a larger true relative risk of 2.13

assuming an ICC of 0.43. However, correction factors are sensitive to the magnitude of measurement error (and associated size of the reliability estimate) and where confounding exists application of a correction factor may over inflate risk estimates (115, 116). Furthermore, altering risk estimates using correction factors from reliability studies assumes that "usual" exposure levels are the risk factor of interest and not the peaks (or troughs) of exposure (which may be more relevant for nutritional biomarkers with distinct seasonal variation).

Classification of folate, IGF-I, phytanic acid and vitamin B_{12} by tertiles characterised approximately 50% of individuals correctly after approximately a six year period which suggests that categories of exposure may be an informative means of investigating the dose-response association with disease, even if random fluctuations in absolute concentration over time (as illustrated in Figure 4:1.1-4.1.4) result in a degree of misclassification of usual concentration and systematic underestimation of the slope of the association between a biomarker and disease.

4.6.1 Strengths of the study

Strengths of our study include the use of validated laboratory methods, the long follow-up (mean 6.4 years) and the wide age range of participants, with heterogeneous dietary habits (meat-eaters, vegetarians, vegans). Small intra-and inter-batch coefficients of variation were calculated for each assay method (using technician blinded quality control samples), both samples from an individual were assayed in the same batch and all assays were analysed in the same time period.

4.6.2 Limitations of the study

Although the study presented here is large in terms of other reliability studies (see **Table 4.1 and Table 4.2**), the small sample size (n=100 men and n =100 women (IGF-I only)) meant that the study was underpowered to investigate reliability estimates within age, fasting status or diet strata. Although standardised protocols for collection and storage of blood samples were used, it is possible that differences in processing time of samples (largely attributable to time spent in post) may have contributed to some of the variation observed, especially for folate. Sets were not matched on season of blood collection, weight, energy intake or vitamin supplement status and these factors are likely to contribute to biological variation (although greater matching restricts the population and underestimates the variation that exists in the full cohort). It is also conceivable that stronger correlation coefficients for phytanic acid would have been observed if sets were matched on fasting status, as significant differences have been observed amongst fasted and non-fasted men (see Chapter 5, section5.4) (121). A further limitation of this study is that participants are of white European origin and this may limit the extent to which the findings are generalisable to populations of different ethnicity and dietary patterns. Participants did not have a history of cancer diagnosis at the time of the baseline blood collection but a breast cancer diagnosis was registered in two women, a malignant skin cancer diagnosis in one woman and a colon cancer diagnosis was registered in one man prior to the second blood sample collection. It is possible that cancer development

may result in changes to circulating concentrations of biomarkers (as discussed further in Chapter 7).

4.7 Conclusions

Our folate, vitamin B_{12}, phytanic acid and IGF-I reliability studies show that estimates of usual concentrations based on single blood measurements weaken the ability to detect associations with disease risk and, where small effect sizes are anticipated, may bias associations toward the null. These findings highlight the need for studies to incorporate multiple blood samples taken several years apart to better characterise relationships between biomarkers of longer - term nutritional and hormonal exposure and disease risk.

Table 4.1 Insulin-like growth factor-I reliability studies

Study	Author; year	Number, age and sex[1]	Sample number	Assay method[3]	Sample	Analyses[4]	Time between samples	Findings[5]
IGF-I								
Rancho Bernado Heart and Chronic Disease Study	Goodman-Gruen, D; 1997	24	2	IRMA	Serum	Pearson correlation coefficients	8-54 days	r = 0.94; p=0.001
Health Professional Follow-up Study	Platz, E; 1999	149 men	2	ELISA (DSL)	EDTA plasma	Spearman correlation coefficients	3.0 ± 0.5 years	r = 0.70
New York University Women's Health Study (NYUWHS)	Kaaks, R; 2000	46 case-control sets (1case: 2 controls)	2	IRMA (DSL)	Serum	Spearman correlation coefficients	(0.75 – 4.75 years)	r = 0.87 r = 0.82 for IGF-I adjusted for IGFBP-3
Physicians' Health study	Chan, JM; 2002	49 men	2	ELISA (DSL)	EDTA plasma	Pearson correlation coefficients	5 years	r = 0.70
ORDET study	Muti, P; 2002	59 women	2	IRMA (DSL)	Serum	ICC	1 year	r = 0.81
Healthy volunteers	Borofsky, N; 2002	13 men (24-77 years) 13 women (23-56 years)	2	IRMA (DSL)	Serum	ICC	1.2-6 years (mean 4.5 years)	r = 0.74 (95% CI 0.55-0.93)
NYUWHS, Northern Sweden Health and Disease and ORDET	Lukanova, A; 2004	76 women	2	IRMA (DSL)	Serum	ICC	14 months	r= 0.66 (95% CI 0.51– 0.77)
CARDIA Male Hormone Study	Gapstur, SM; 2004	622 black 796 white	2	IRMA (DSL)	Serum	Spearman correlation coefficients	5 years 8 years	r = 0.39 for 5 years between repeat samples r= 0.47 for 8 years between repeat samples
Nurses' Health Study II	Missmer, S; 2006	113 women	3	ELISA (DSL)	Plasma	ICC	1 blood sample yearly for 3 years	Follicular sample r = 0.69 (95% CI: 0.56 – 0.79) Luteal sample r = 0.83 (95% CI: 0.77 – 0.87)
Melbourne Collaborative Cohort Study	Severi, G; 2006	44 men	2	ELISA (DSL)	Plasma	ICC	1 year	r = 0.39 (95% CI: 0.27, 0.51)
PLCO Screening Trial	Weiss, JM; 2007	20 men	2	ELISA (DSL)	Serum Plasma	ICC ICC	4 weeks 4 weeks	r = 0.90 r = 0.97
Melbourne Collaborative Cohort Study	Baglietto, L; 2007	71 women	2	ELISA (DSL)	Plasma	ICC	1 year	r = 0.42 (95% CI 0.27-0.57)
Seattle Metropolitan Population	Chia, V M; 2008	22 men 16 women	2	ELISA (DSL)	Serum	ICC	4 months	r = 0.88 (95% CI 0.8 -0.95)

[1] Sex and age not specified for all studies

[2] IMRA refers to an immunoradiometric assay and ELISA refers to an enzyme-linked immunosorbent assay

[3] DSL refers to assays supplied by Diagnostics Systems Laboratry (Webster, Texas, USA)

[4] ICC refers to the intra-class correlation coefficient; calculated as the proportion of between-person variation relative to the total variation

[5] 95% Confidence intervals not provided for all studies

Table 4.2 Folate and vitamin B_{12} reliability studies

Study	Author; year	Number, age and sex[1]	Sample number	Assay method	Sample	Analyses[2]	Time between samples	Findings[3]
Folate								
University of Ulster (staff and students)	McKinley, M; 2001	22	4 one at the end of each season	Microbiological assay	Serum	ICC	3 months	r = 0.94
NHANES III	Lacher, D; 2005	2426 Approx. half male, half 20–39 years and half 40+ years	2	Microbiological assay	Serum	ICC	17 days	r = 0.35
NHANES III	Kant, A; 2008	934 men and women	2	Radioassay	Serum	ICC ICC	17 days	r = 0.09 (0.08, 010) r = 0.13[4]
Oregon Health & Science University	Bowman, G; 2011	19 men 19 women	2	Radioimmunoassay (ALPCO, Salem, NH)	Plasma	ICC	1 month	r = 0.92[3]
Vitamin B_{12}								
University of Ulster (staff and students)	McKinley, M; 2001	22 sex not specified	4	Microbiological assay	Serum	ICC	3 months one at the end of each season	r = 0.97
NHANES III	Lacher, D; 2005	2426 Approx. half male, half 20–39 years and half 40+ years	2	Microbiological assay	Serum	ICC	17 days	r = 0.32
Oregon Health & Science University	Bowman, G L; 2011	19 men 19 women	2	Radioimmunoassay (ALPCO, Salem, NH)	Plasma	ICC	1 month	r = 0.97

[1] Sex and age not specified for all studies

[2] ICC refers to the intra-class correlation coefficient; calculated as the proportion of between-person variation relative to the total variation

[3] 95% Confidence intervals not provided for all studies

[4] muli-variant adjustment including age, education, area of residence, alcohol, fasting, season, time between blood samples and socio-economic status

Table 4.3 Characteristics of 100 men and 100 women at baseline and follow-up

	Men			Women		
	Baseline	Follow-up	P value[4]	Baseline	Follow-up	P value[4]
Number	100	100		100	100	
Mean Age; yrs (SD)	55.2 (8.6)	61.6 (8.8)		55.4 (8.0)	61.8 (8.0)	
Mean BMI; kg/m^2 (SD)	23.6 (2.7)	24.1 (2.9)		23.4 (4.0)	23.9 (4.5)	
Mean blood storage time; yrs (SD)	14.0 (0.5)	7.2 (0.3)		13.7 (0.6)	7.16 (0.3)	
Geometric mean concentration[3] (95% CI):						
Folate; nmol/L	21.4 (18.8, 24.5)	27.6 (24.2, 31.5)	0.01	N/A	N/A	
Vitamin B_{12}; pmol/L	302.7 (273.7, 334.9)	288.5 (260.8, 319.1)	0.51	N/A	N/A	
IGF-I; nmol/L	24.9 (23.4, 26.5)	23.8 (22.4, 25.4)	0.32	19.6 (18.3, 21.1)	21.0 (19.6, 22.6)	0.17
Phytanic acid; μmol/L	2.95 (2.5, 3.5)	2.3 (2.0, 2.8)	0.06	N/A	N/A	
	Remaining within the same category[2] at both time points					
Time since last meal[2]; n (%)	65 (65.0)			61 (61.0)		
Time of day of blood collection[2]; n (%)	51 (54.3)			51 (54.3)		
Diet Group[2]; n (%)	93 (93.9)			89 (89.0)		
BMI Group[2]; n (%)	80 (85.1)			85 (91.4)		
Smoking[2]; n (%)	88 (88.9)			99 (93.9)		
Alcohol intake[2]; n (%)	63 (63.0)			41 (41.0)		

[1] Repeat samples were measured in 100 men and 100 women for IGF-I, and 99 men for folate, phytanic acid and vitamin B_{12}

[2] Unknown for some participants

[2] Categories of time since last meal (< 3, ≥ 3 hours), time of day of blood collection (< 12:00, 12 -17:59, 18:00 - 24:00), diet group (Omnivore, Vegetarian, Vegan), BMI (< 25, ≥25kg/ m^2), smoking (never, former, current) and alcohol intake (none, < 10, ≥10gm/ day).

[3] Adjusted for batch: folate (1-3), vitamin B_{12} (1-2), phytanic acid (1-6) and IGF-I (1-6)

[4] P values relate to the likelihood ratio chi square tests of difference for categorical variables

Table 4.4 Descriptive statistics, variance components and measures of reliability for folate, vitamin B_{12}, phytanic acid and IGF-I

			Variance[1]				Correlation Coefficients			Regression Coefficients		
			Variance				Attenuation[3] (%) from n measurement/s:		No. of measurements[4] to reduce attenuation to:	Attenuation[3] (%) from n measurement/s:		No. of measurements[4] to reduce attenuation to:
	Timepoint	Number	Between	Within	ICC[2]	95% CI	1	2	20%	1	2	20%
Folate	1	99										
	2	99	0.09	0.44	0.18	[0.00, 0.37]	137.0	82.0	8	460.0	230.0	19
Vitamin B_{12}	1	99										
	2	99	0.09	0.17	0.34	[0.16, 0.51]	72.6	41.0	4	198.0	98.9	8
Phytanic acid	1	99										
	2	99	0.54	0.58	0.48	[0.33, 0.63]	44.5	24.3	2	109.0	54.5	5
IGF-I	1	200										
	2	200	0.06	0.08	0.43	[0.32, 0.54]	52.4	28.9	3	132.3	66.2	6

[1]Variance for the log transformed values of folate, vitamin B_{12}, phytanic acid and IGF-I

[2]Unadjusted ICC calculated as the percentage of total variability in folate, vitamin B_{12}, phytanic acid and IGF-I explained by differences between women

[3]Attenution of the correlation and regression coefficients for n measurements using the formulas: $\lambda p = \sqrt{(n/((S2w/S2b) + n))}$ and $\lambda_B = n/((S2w/S2b) + n)$

[4]The number of measurements is rounded up if more than 0.2 higher than the nearest lower integer; otherwise rounded down

Table 4.5.1 - 4.5.4: The proportion of participants changing tertile categories between baseline and follow-up with tertile cut-points based on the distribution of each analyte at the first blood collection

Table 4.5.1: Proportion of participants changing tertile categories between baseline and follow-up: tertile cutpoint based on the distribution of folate at time point 1

	Tertiles at time 2			
Tertiles at time 1	1 3.3 – 16.27 nmol/L	2 16.28 – 27.14 nmol/L	3 27.15 – 147.20 nmol/L	Total
1 0.83 – 16.75 nmol/L	16 48.5%	7 21.2%	10 30.3%	33 100.0%
2 16.76 – 28.49 nmol/L	6 18.2%	10 30.3%	17 51.5%	33 100.0%
3 28.50 – 83.62 nmol/L	6 18.2%	6 18.2%	21 63.6%	33 100.0%
Total	28 28.3%	23 23.2%	48 48.5%	99 100.0%

Table 4.5.2: Proportion of participants changing tertile categories between baseline and follow-up: tertile cutpoint based on the distribution of vitamin B_{12} at time point 1

	Tertiles at time 2			
Tertiles at time 1	1 46 – 253.65 pmol/L	2 253.66 – 390.48 pmol/L	3 390.49 – 2367.59 pmol/L	Total
1 92.95 – 255.11 pmol/L	16 48.5%	10 30.3%	7 21.2%	33 100.0%
2 255.12 – 387.20 pmol/L	15 45.5%	14 42.4%	4 12.1%	33 100.0%
3 387.21 – 721.80 pmol/L	6 18.2%	11 33.3%	16 48.5%	33 100.0%
Total	37 37.4%	35 35.4%	27 27.3%	99 100.0%

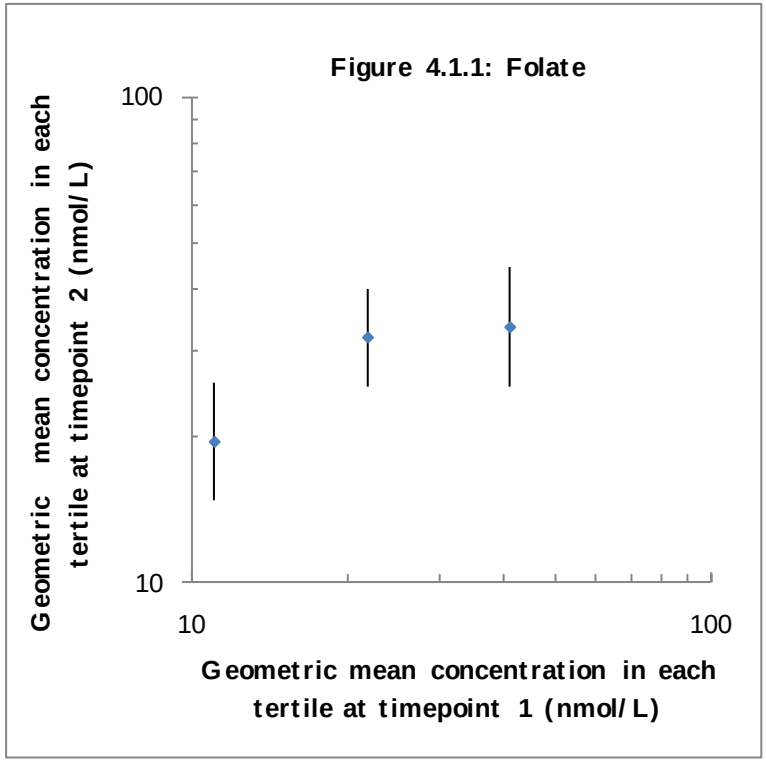
Table 5.5.3: Proportion of participants changing tertile categories between baseline and follow-up: tertile cutpoint based on the distribution of phytanic acid at time point 1

	Tertiles at time 2			
Tertiles at time 1	1 0.28 – 2.34 umol/L	2 2.35 – 4.87 umol/L	3 4.88 – 17.10 umol/L	Total
1 0.26 – 2.29 umol/L	23 69.7%	6 18.2%	4 12.1%	33 100.0%
2 2.30 – 5.08 umol/L	11 33.3%	16 48.5%	6 18.2%	33 100.0%
3 5.09 – 35.88 umol/L	7 21.2%	13 39.4%	13 39.4%	33 100.0%
Total	41 41.4%	35 35.4%	23 23.2%	99 100.0%

Table 4.5.4: Proportion of participants changing tertile categories between baseline and follow-up: tertile cutpoint based on the distribution of IGF-I at time point 1

	Tertiles at time 2			
Tertiles at time 1	1 6.95 – 18.92 nmol/L	2 18.93 – 26.64 nmol/L	3 26.65 – 50.73 nmol/L	Total
1 6.96 – 18.93 nmol/L	33 49.3%	22 32.8%	12 17.9%	67 100.0%
2 18.94 – 26.66 nmol/L	18 26.9%	28 41.8%	21 31.3%	67 100.0%
3 26.67 – 49.86 nmol/L	10 15.2%	22 33.3%	34 51.5%	66 100.0%
Total	61 30.5%	72 36.0%	67 33.5%	200 100.0%

Figure 4:1.1-4.1.2 Geometric mean concentrations at time points 1 and 2 by third of concentration for each biomarker



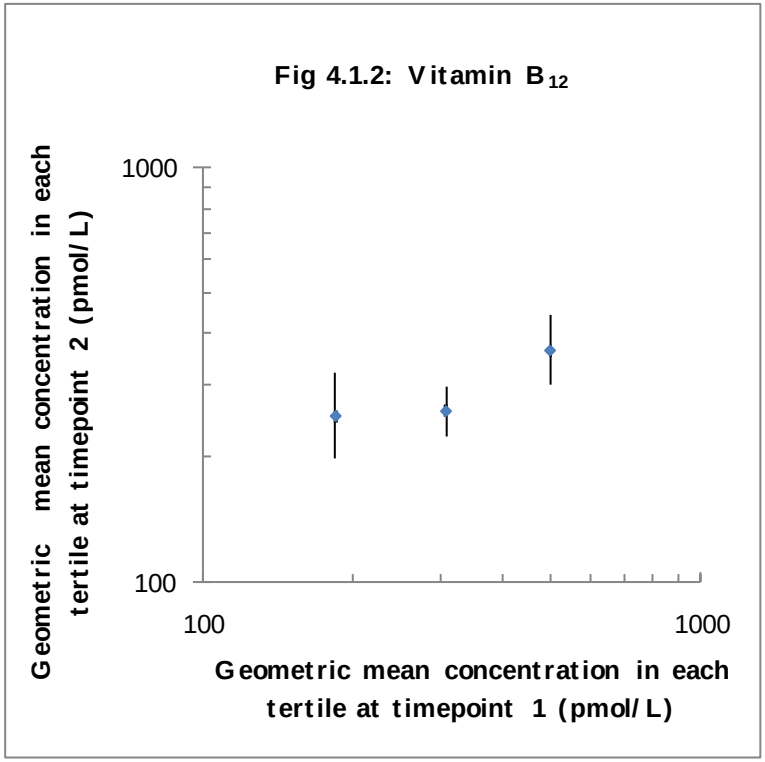
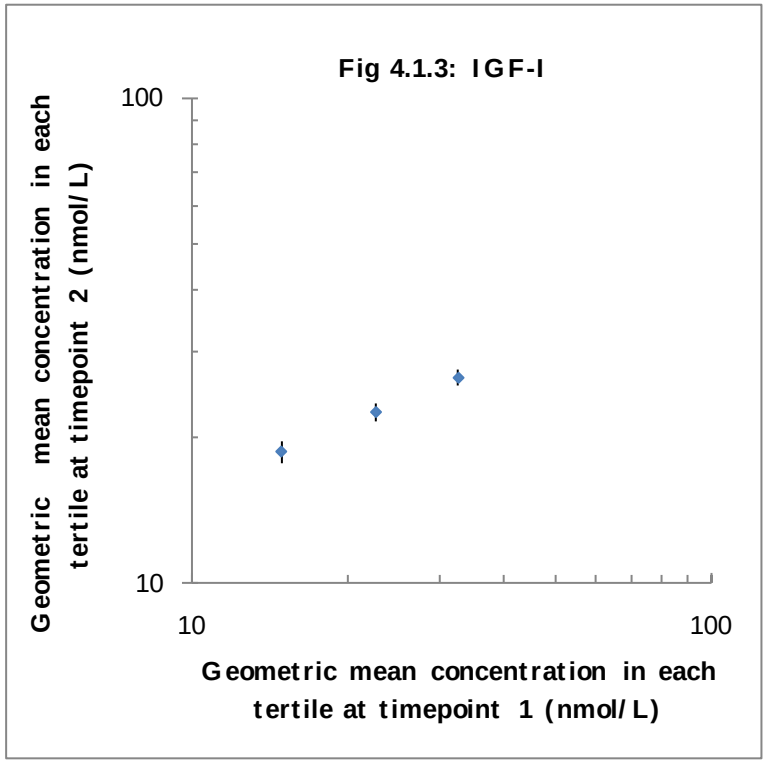


Figure 4.1.3-4.1.4(continued): Geometric mean concentrations at time points 1 and 2 by third of concentration for each biomarker


Fig 4.1.3: IGF-I
Geometric mean concentration in each tertile at timepoint 2 (nmol/L)
100
10
10
100
Geometric mean concentration in each tertile at timepoint 1 (nmol/L)

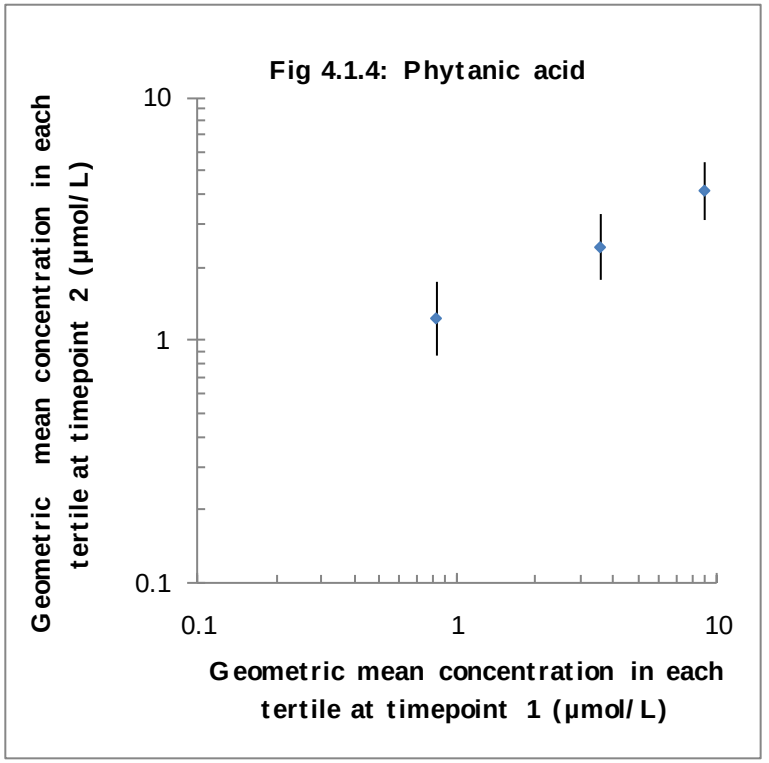

Fig 4.1.4: Phytanic acid
Geometric mean concentration in each tertile at timepoint 2 (µmol/L)
10
1
0.1
0.1
1
10
Geometric mean concentration in each tertile at timepoint 1 (µmol/L)

Table 4.6 Observed and true relative risks (RR) obtained when using a single blood measure given the ICCs observed in each study and assuming no additional error

True RR[2]	1.25	1.5	1.75	2.00	2.15	2.25	2.5	2.75	3	5.00
Folate (ICC = 0.18)										
Observed RR	1.04	1.08	1.11	1.13	1.15	1.16	1.18	1.20	1.22	1.34
Attenuation of RR (%)	16.72	28.29	36.80	43.36	46.62	48.57	52.83	56.37	59.38	73.28
IGF-I (ICC = 0.43)										
Observed RR	1.10	1.19	1.27	1.35	1.39	1.42	1.48	1.54	1.60	2.00
Attenuation of RR (%)	11.94	20.64	27.31	32.64	35.36	37.01	40.68	43.82	46.54	60.04
Phytanic acid (ICC = 0.48)										
Observed RR	1.11	1.21	1.31	1.39	1.44	1.48	1.55	1.63	1.69	2.17
Attenuation of RR (%)	10.96	19.01	25.25	30.26	32.84	34.41	37.90	40.91	43.52	56.70
Vitamin B12 (ICC = 0.34)										
Observed RR	1.08	1.15	1.21	1.27	1.30	1.32	1.37	1.41	1.45	1.73
Attenuation of RR (%)	13.69	23.48	30.88	36.71	39.66	41.45	45.38	48.71	51.57	65.43

[1] The ICC observed for each analyte was used to calculate observed relative risk estimates that would result if a single biomarker measurement was used, assuming a range of hypothetical true relative risks, with the formula: Observed RR = $\exp^{[\log \text{true RR} \times \text{ICC}]}$.

Table 4.7 Sample Size[1] required for a width of confidence interval given a range of intra-class correlation coefficients obtained with two blood samples

ICC	Width of the 95% Confidence Interval						
	0.01	0.05	0.1	0.15	0.2	0.25	0.3
0.2	141,613	5,665	1,417	630	355	228	158
0.3	127,245	5,091	1,273	567	319	205	142
0.4	108,422	4,338	1,085	483	272	174	121
0.5	86,434	3,458	865	385	217	139	97
0.6	62,939	2,519	630	281	158	102	71
0.7	39,968	1,600	401	179	101	65	45
0.8	19,915	798	200	89	51	33	23
0.9	5,548	223	56	26	15	10	7

[1] Estimation of sample sizes required to achieve a range of 95% confidence interval widths around the observed ICC estimates calculated using methods described by Shoukri et al, 2004.

Chapter 5 Phytanic acid and risk for prostate cancer

5.1 Introduction

Evidence from ecological comparisons (20) and migrant studies (3-5, 150) suggest that some aspect of a Western lifestyle, such as a diet rich in animal products, may play an important role in the aetiology of prostate cancer. Whilst there is little evidence for an association with meat intake (7), results from many prospective studies (67-78, 108) including a meta-analysis of 12 cohort studies (151), have shown an increased risk of prostate cancer in men with a high consumption of dairy products, although the results are not entirely consistent (152-156). It has been suggested that the association between dairy foods and prostate cancer risk may be due to its high calcium or protein content (78), although an alternative hypothesis is that the phytanic acid content of dairy products increases the risk of this disease.

5.1.1 History of phytanic acid

During the early 1950s Shorland and Hansen isolated an incompletely characterised C20 saturated fatty acid in butterfat (157, 158), later known as phytanic acid or 3,7,11,15-tetra methylhexadecanoic acid. Over the next 10 years, phytanic acid was also identified in ox-plasma (159), ox perinephric fat (160) and fish oils (161).

In the 1960s experimental work showed that phytanic acid biosynthesis did not occur *in vivo* following administration of a precursor substance (mevalonic acid). However, free phytol (obtained from green vegetables) appeared to be a significant precursor of phytanic acid (162). The quantitative importance of phytol as a source of phytanic acid was investigated by Baxter in 1968 using two separate experiments. In the first, subjects were given oral 14C-labeled pheophytin (Chlorophyll), and in the second, subjects were fed 250gm of cooked spinach. Of the total 14C-labeled pheophytin consumed, 95% passed into the subject's faeces without being absorbed and of the 155mg of phytol contained in 250gm of cooked spinach, 152mg (98%) passed unabsorbed in the subject's faeces over a period of 9 days (163). The results of both studies indicated that little phytol (<5%) is absorbed from chlorophyll in the diet and as such could not be viewed as a quantitatively important source of phytanic acid.

An investigation of dietary exclusion of exogenous sources of phytanic acid showed that participants with a diet restricted in phytanic acid had significantly reduced serum and adipose tissue concentrations compared to unrestricted counterparts (164); a finding which suggested that accumulation of phytanic acid depends upon the intake of precursor substances.

In summary, phytanic acid (3,7,11,15-tetra methylhexadecanoic acid) is a C20 branched chain fatty acid with negligible endogenous *de novo* synthesis (163). It is acquired in a Western diet mostly from the consumption of meat and dairy products high in ruminant (i.e. cow, sheep, goat) fat (160, 165-167) and to a lesser extent, from the consumption of plankton eating pelagic fish, such as mackerel, (161, 167)because these animals are able to digest chlorophyll in the their diet.

5.1.2 Metabolism of phytanic acid

After consumption, chlorophyll (a green pigment in plant material and plankton) is esterified by bacteria within the gastrointestinal system of ruminants and fish to a phytol moiety which is then available for metabolism to phytanic acid. This process (of phytol release from chlorophyll) is known only to occur in ruminants and pelagic fish. Once phytanic acid is absorbed into the blood stream, it is distributed to the liver where it is further metabolised to produce energy for normal cellular functions or it is stored in adipose tissue as fat. Distribution of phytanic acid to the adipose tissue of the mammary gland and subcutaneous fat stores surrounding muscle in ruminants and fish means that humans who consume fat containing milk and milk products such as cheese, butter and yoghurt and fatty meats or fish, also consume concentrations of phytanic acid.

The estimated average daily intake of phytanic acid in Western societies is 50-100mg, of which 50% is absorbed and metabolized (80, 81). The half-life of phytanic acid in circulation is unknown and although there have been studies in people with Refsum's disease, there are no published studies in healthy people.

Refsums disease, first diagnosed in 1945, is a hereditary disease of the nervous system associated with phytanic acid accumulation. The disease typically manifests in late childhood with the onset of progressive neurological dysfunction that includes demyelinating neuropathy, pes cavus, cerebellar ataxia, sensorineural deafness, anosmia and cranial nerve dysfunction (168). This condition indicates that phytanic acid can have deleterious effects at very high concentrations.

Following ingestion phytanic acid is rapidly incorporated into the triglyceride portion of the lipid fraction in plasma (169) and preferentially taken up by the liver where it may account for up to 50% of free fatty acid pool in hepatocytes. This pool is labile and can be acutely mobilized by stress, infection and starvation (170). From plasma, phytanic acid enters the peroxisome (a sub-cellular organelle), in association with the sterol carrier protein-2(SCP-2). Here it is metabolized by a series of oxidation steps including an essential β-oxidation step that requires the enzyme α-methylacyl-coenzyme A racemase (AMACR)(171). **Figure 5:1** provides a simple illustration of the acquisition and metabolism of phytanic acid.

5.1.3 Mechanism by which phytanic acid may increase prostate cancer risk

Integral to phytanic acid degradation, via β-oxidation in sub-cellular peroxisomes, is the enzyme AMACR. This enzyme has been shown to be consistently over-expressed in prostate cancer compared with normal tissue (83-86, 172) and, although its role in the aetiology of prostate cancer has not yet been established, AMACR is considered a reliable diagnostic marker of prostate cancer in the clinical setting (173). The observation that AMACR protein expression is increased in cultured prostate cancer cells exposed to a high (25 μM)

concentration of phytanic acid (174) suggests that this fatty acid may have a regulatory role in the over-expression of AMACR protein in prostate cancer.

Phytanic acid is also known to bind and activate at least two types of nuclear hormone receptors- retinoid acid receptors (RARs) and peroxisome proliferator-activated receptors (PPAR-α), that have been implicated in carcinogenesis via their role in cell proliferation, differentiation, inflammatory signalling and apoptosis (175).

5.2 Aim of study

The aim of this work is to test the hypothesis that high plasma levels of phytanic acid increase the risk for prostate cancer, which may be due to up-regulated expression of α-methylacyl coenzyme A racemase. This work will improve our understanding of the aetiology of prostate cancer and may lead to the development of dietary recommendations for reducing prostate cancer risk.

Reported in this chapter are findings, using a nested case-control design, on the relation between plasma phytanic acid concentration and risk of prostate cancer among 566 men with prostate cancer and 566 controls who are participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The association between phytanic acid and risk of prostate cancer according to clinical stage, histological grade, and other factors are also investigated.

5.3 Methods

5.3.1 Study population

The methods of recruitment used in the EPIC cohort study have been outlined in detail in **Chapter 2,** section 2.3(13). In brief, during recruitment between 1992 and 2000, approximately 360,500 women and 153,400 men, mostly aged 35-70 years, from 23 centres in ten European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom), completed either a standardised food frequency questionnaire or an interview based dietary questionnaire, and a detailed lifestyle questionnaire. Approximately 385,700 participants (139,600 men) also provided a blood sample. All participants provided written informed consent and local ethics committees in the participating countries and the Internal Review Board of the International Agency for Cancer Research (IARC) approved the study.

The present study includes prostate cancer cases and their matched controls from Germany, Greece, Italy, the Netherlands, Spain and the United Kingdom. The sub-cohorts from Denmark and Sweden were excluded because blood samples were not available for this analysis and Norway and France were not eligible as these cohorts only included women. With the exception of the Oxford centre in the United Kingdom (UK), whole blood was transported in the collection syringe to a local laboratory where samples were processed and separated into

serum, plasma, red cells and buffy coat fractions within 24-hours of collection, prior to storage at -196°C in liquid nitrogen tanks. Sodium citrate anticoagulant was used for all of the plasma samples included in this study. Blood samples from the Oxford cohort participants were sent in the post at ambient temperature to a laboratory in Norfolk (UK), with an average transit time of 1.5 days.

5.3.2 Follow-up for cancer incidence and vital status

Follow-up was provided through record linkage with population-based cancer registries in Italy, the Netherlands, Spain and the United Kingdom. In Germany and Greece active follow-up was achieved through study subjects and their next of kin and verified through health insurance or medical records and cancer and pathology registries or reports. Prostate cancer cases included in this study were defined as code C61 according to the 10th revision of the International Statistical Classification of Injuries, Disease and Deaths (ICD-10). For each EPIC centre closure dates of the study period were defined as the latest date of complete follow-up for both cancer incidence and vital status (March 2004, May 2002, December 2001 and June 2000, for Germany, Greece, Italy and the Netherlands, respectively, and December 2001 for both Spain and the United Kingdom), with a mean follow-up time of 4.8 years.

5.3.3 Selection of case and control participants from the study cohort

Cases were defined as men who developed prostate cancer after the date of blood collection and before the end of the study period, as determined by the latest date of follow-up in each study centre. Tumour stage information (TNM staging score), derived from pathology reports and cancer registry data, was used to define the following categories: localised (T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised; n = 255), advanced (T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic; n = 116), and unknown stage (n = 195). Information on Gleason score or the World Health Organization (WHO) grading system was used to generate categories of low grade (Gleason score $\leq$7, or cases coded as well differentiated or moderately differentiated; n = 381), high grade (Gleason score >7, or cases coded as poorly differentiated or undifferentiated; n = 69), and unknown grade (n = 116).

An incidence density sampling protocol was used to select control participants at random from the cohort of men who were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case and who were matched on study centre, length of follow-up, age at blood collection (+/- 6 months), time of blood collection (+/- 1 hour) and duration of fasting at blood collection (< 3, 3 - 6, > 6 hours).

Of the 666 prostate cancer cases identified during the follow-up period, 594 had provided a baseline blood sample. After excluding 9 cases who had a previous diagnosis of another cancer and 19 cases for whom there was insufficient or unavailable plasma, 566 prostate cancer cases and 566 matched controls were included in this analysis. With this sample size,

there will be 80% power to detect a risk of 1.5 for men in the top third of phytanic acid relative to men in the lowest third.

5.3.4 Laboratory analysis

5.3.4.1 Determination of plasma phytanic acid

Extraction and quantification of total plasma phytanic acid, which includes free and conjugated forms, was performed in singleton using a gas chromatographic /mass spectrometric (GC/MS) assay established at HFL Sport Science Ltd (Fordham, Cambs, UK), and validated over a concentration range of 0.032 – 9.6 µmol/L (141).

Plasma (50 µl) was thawed at room temperature before adding [3-methyl-$^2H_3$]phytanic acid (Dr H. J. ten Brink, VU Medical Center, Amsterdam, the Netherlands) in toluene (50 µl; 1·3 µg/ml). To release free phytanic acid in the greatest concentration plasma samples were subjected to acidic hydrolysis or saponification using ethanolic potassium hydroxide (5 % (w/v), 1 ml) for 1 h at 60°C. After this step, water (1 ml) and hexane (4 ml) were added and then the samples were shaken for 20 minutes before discarding the organic layer. In a subsequent step, glacial acetic acid (100 µl) and hexane (4 ml) were added and the samples shaken for a further 20 min before the hexane layer was removed and dried in a centrifugal vacuum evaporator. Prior to gas chromatographic-mass spectrometric analysis, the dried samples were derivatised to tert-butyldimethylsilyl ethers by adding pyridine (50 µl) and *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (50 µl) and heated at 80°C for 30 minutes.

5.3.4.2 Gas–liquid chromatography–mass spectrometry analysis

A Trace 2000 Series gas chromatograph (ThermoQuest, Waltham, MA, USA), with a BPX5 column (25 m x 0.25 mm, 0.22 µm film thickness; SGE, Ringwood, Victoria, Australia) using helium as the carrier gas, was used for all analyses. Data acquisition and processing were carried out using Xcalibur 1.1 and LCQuan 1.1 software (ThermoQuest).

Phytanic acid was ionised using electron ionisation and detected via selected ion monitoring (SIM) on a MD800 mass spectrometer (Fisons Instruments, later ThermoQuest). The two ions monitored were *m/z* 369 for phytanic acid and *m/z* 372 for the internal standard [3-methyl-$^2H_3$]-phytanic acid. The operating conditions were as follows: injector port temperature 300°C; injection volume 1 µl in splitless mode; carrier gas helium at a constant flow of 1.1 ml/min; oven temperature program 100°C held for 0.5 minutes then increased at 30°C/minute to 210°C and held for 1 minute, then at 10°C/minute to 300°C and held for 1 minute; source temperature 220°C; ionisation by electron ionisation (70 eV); emission current 150 µA. Analysis was performed in the selected ion monitoring mode. The two ions monitored were *m/z* 369 for phytanic acid and *m/z* 372 for the internal standard [3-methyl-$^2H_3$]-phytanic acid. The lower limit of quantification (0.032µmol/L) provided adequate sensitivity to detect the lowest concentration in the study samples, although 42 (3.5%) samples exceeded the validated upper limit (9.6µmol/L). These samples were diluted prior to re-extraction and analysis.

Assays were conducted by a laboratory technician, blind to both the case-control and quality control status of the plasma samples. Case-control sets were analysed in the same batch and blinded duplicate quality control samples were included in each, with a mean intra- and inter-batch coefficient of variation (CV) of 1.6% and 6.5%, respectively.

5.3.4.3 Quality control and stability

Three drug-free plasma samples, well characterised for their phytanic acid concentration, were used for quality control (QC) purposes (Richmond Pharmacology Ltd, Tooting, London, UK). The CVs were based on the analysis of QC samples across six batches, with at least two replicates in each batch. The mean intra- and inter-assay CV were 1·8 and 3·2 %, respectively.

In order to assess the stability of phytanic acid when left at room temperature, concentrations were compared in five replicates of each of two QC samples; one which had been left at room temperature for 4 hours before extraction, and one which was extracted immediately after thawing in the normal way. The phytanic acid concentration for the samples left for 4 hours varied from those that were extracted immediately by an average of 5.0 and 5.8 %, for each of the two sets of samples. This is much less than the 15 % variation generally considered acceptable for 4 h stability and suggests that phytanic acid concentrations are stable at room temperature over the timescale used for the extraction process.

To evaluate the stability of phytanic acid concentrations in samples that may have undergone several freeze–thaw cycles, five replicates of each of two QC samples were subjected to zero, one, two, or three freeze–thaw cycles (at − 80°C), with at least 24 hours between each thawing period. The range of deviations of phytanic acid concentration in the samples subjected to freeze–thaw cycles from those not subjected to freeze–thaw cycles was 0.4 to 3.3 % across both samples, suggesting that repeated freezing and thawing of samples does not substantially affect phytanic acid concentrations. However, all samples in the present analysis had undergone no previous freeze–thaw cycle before the phytanic acid assay.

Samples with phytanic acid concentrations above the upper detection threshold of 9.6μmol/l (4% of all samples) were diluted 1:1 with PBS (25μl of each) before re-extraction and analysis. When tested with three well-characterised QC samples, this procedure was found to produce deviations from the target values of 0.2, 0.8 and 3.6%, and was therefore considered appropriate.

5.3.5 Statistical analysis

Differences in baseline characteristics between case and control participants were compared using paired-sample t-tests for continuous variables and conditional logistic regression for categorical variables. Logarithmic transformation of plasma phytanic acid values was performed to approximate a normal distribution. To identify possible outliers, the distribution

of phytanic acid values was examined using box and whiskers plots and letter-value displays. No observations were identified as severe outliers on the logarithmic scale (> 3 times the inter-quartile range), although four observations were identified as moderate outliers (> 1.5 times the inter-quartile range). Sensitivity analyses excluding these observations made no material difference to the risk estimates and all observations were included in the final models.

Analysis of variance was used to examine whether the geometric mean concentration of plasma phytanic acid varied according to participant characteristics (country, centre, fasting status, month of blood collection, age at blood collection, body mass index (BMI), smoking status, alcohol consumption, physical activity, marital status and educational attainment). Where appropriate, adjustment was made for centre, age at blood collection, fasting status, month of blood collection and batch. Geometric mean concentrations were calculated after excluding five duplicate control observations (due to the use of incidence density sampling).

Conditional logistic regression models were used to calculate the odds ratios (ORs) for overall prostate cancer risk by thirds of phytanic acid concentration, with cut points defined by the distribution amongst control participants for all centres combined. Owing to the use of incidence density sampling for the selection of controls, the odds ratios presented estimate the incidence rate ratios for phytanic acid in relation to prostate cancer in the study cohort (176). Tests for linear trend were calculated using the natural logarithm of phytanic acid as a continuous variable.

The effects of potential confounding variables (other than those accounted for in the matched design) were examined by including additional regression terms in the conditional logistic regression model. Additional adjustment for BMI (continuous), smoking (never, past, present), alcohol consumption (< 8, 8 - 15, 16 - 39, ≥ 40 g/day), physical activity (inactive, moderately inactive, moderately active, active), marital status (married/cohabitating or not married/cohabitating), educational attainment (primary or equivalent, secondary, degree level) and calibrated intake of protein from dairy foods (quintiles) and calcium from dairy foods (quintiles) made little difference to the risk estimates or their 95% confidence intervals (<10% change). However, all models included adjustment for month of blood collection owing to seasonal variation in dietary patterns and to ensure comparability within matched sets.

Likelihood ratio χ^2 tests were used to examine heterogeneity of trends in prostate cancer risk associated with a doubling (log 2) of phytanic acid concentration by cancer stage (localised, advanced), cancer grade (low, high), age at blood collection (< 60, ≥ 60 years), age at diagnosis (< 60, ≥ 60 years), time between blood collection and diagnosis (< 48, ≥ 48 months), fasting status (< 3, ≥ 3 hours), country and BMI (< 25, ≥ 25 kg/m²).

All statistical analyses were performed using STATA 11 statistical software (StataCorp LP, College Station, TX, USA) and two-tailed tests of statistical significance with P values below 0.05 were considered significant.

5.4 Results

Plasma phytanic acid measurements were available for 566 matched sets; 199 from Germany, 9 from Greece, 62 from Italy, 25 from the Netherlands, 94 from Spain and 177 from the United Kingdom. As shown in **Table 5.1**, there were no significant differences between cases and controls according to baseline age, BMI, height, smoking status, alcohol consumption, physical activity, marital status and educational attainment. However, controls were on average heavier than cases (80.2 [SD 11.6] versus 78.9 [SD 10.9] kg; $P = 0.04$). The mean age at diagnosis was 64.8 years (SD 6.3) and the median time between blood collection and diagnosis was 3.7 years (range 1 month – 9.1 years; data not shown).

The geometric mean phytanic acid concentrations according to baseline characteristics amongst the controls are shown in **Table 5.2**. Phytanic acid concentrations were lower in older men compared with their younger counterparts ($P_{trend} = 0.02$) and in men with a longer duration of fasting at blood collection ($P_{trend} = 0.002$). There was significant variation by country ($P_{heterogeneity} < 0.0001$), with concentrations lowest in Spain, Greece and Italy (1.28 – 1.56 µmol/L), and highest in Germany, the United Kingdom and the Netherlands (2.91 – 3.97 µmol/L) ($P_{heterogeneity} < 0.0001$). This pattern closely reflected differences in dairy fat intake, with the lowest mean intake in Spain (4.5% of total energy intake) and the highest intake in Germany (11.5% of total energy intake). Plasma phytanic acid concentration also varied significantly by fasting status, with a 27% higher concentration in men who fasted less than 3 hours compared with those who fasted for more than 6 hours ($P_{heterogeneity} = 0.01$). Phytanic acid concentration did not vary significantly according to BMI, smoking status, alcohol consumption, physical activity, marital status, educational attainment or month of blood collection, following adjustment for centre, age at blood collection, fasting status, month of blood collection and batch, where appropriate.

The risk of prostate cancer by thirds of phytanic acid concentration is shown in **Table 5.3**. Phytanic acid concentration was not significantly associated with the risk of prostate cancer overall. In comparison to the lowest third, the risk of prostate cancer in the highest third of plasma phytanic acid concentration was 1.13 (95% CI: 0.76, 1.68: $P_{trend} = 0.53$) adjusted for month of blood collection and 1.22 (95% CI: 0.82, 1.84: $P_{trend} = 0.34$) in the fully adjusted model.

The odds ratios for prostate cancer associated with a doubling of plasma phytanic acid concentration, subdivided by case participant characteristics, are shown in **Table 5.4**. The association of phytanic acid with risk did not differ between localized and advanced stage disease ($P_{heterogeneity} = 0.22$) or between low- and high-grade disease ($P_{heterogeneity} = 0.64$). Similarly, there was no evidence of significant heterogeneity in the trend for prostate cancer risk by time from recruitment to diagnosis ($P_{heterogeneity} = 0.07$) or by age at blood collection ($P_{heterogeneity} = 0.73$). There was some evidence of heterogeneity in the association of phytanic acid with prostate cancer risk by age at diagnosis ($P_{heterogeneity} = 0.02$) but the risk estimates were not significant for either age group (≥ 60 and < 60 years). There was also some evidence

of heterogeneity in the risk estimates by fasting status, with a positive association in men who had fasted for 3 hours or more at blood collection (OR 1.27; 95% CI: 1.01, 1.60; P_{trend} = 0.04) and no association in men who had fasted for less than 3 hours (OR 0.89; 95% CI: 0.73, 1.09; P_{trend} = 0.25; $P_{heterogeneity}$ = 0.04). There was no significant heterogeneity in trends for prostate cancer risk by country ($P_{heterogeneity}$ = 0.48) or by BMI ($P_{heterogeneity}$ = 0.13; data not shown). Sensitivity analyses excluding men who had a diagnosis of prostate cancer within 12 months of blood collection (n = 67) made no material difference to these associations (data not shown).

5.5 Discussion

This study, nested within the EPIC cohort, is the first prospective investigation of the relation between plasma phytanic acid concentration (derived largely from consumption of dairy and other ruminant fat) and subsequent risk of prostate cancer. Overall, circulating phytanic acid concentration was not significantly associated with prostate cancer risk. Our finding of no association based on 566 cases and 566 controls contrasts with one case-control study that found phytanic acid concentration to be significantly higher in 49 cases compared with 55 controls (0.10 versus 0.08 mg/100mL; *P* = 0.04) (177). However, due to the small size and retrospective study design, its findings should be interpreted with some caution.

There was no evidence that the association of phytanic acid concentration with risk differed by stage or grade of disease, and although there was some evidence that the association differed according to age at diagnosis, the associations were not statistically significant in either group of men. While it is possible that the observed heterogeneity reflects some real differences between early-onset and late-onset disease, this finding may be due to chance given the number of statistical tests performed.

The range of phytanic acid in this European population was comparable with findings from previous studies in healthy individuals (141, 170). The higher mean plasma concentration of phytanic acid observed in non-fasted men compared to those who had fasted may be due to the presence of phytanic acid in triacylglycerol rich lipoproteins (178, 179) which are known to increase in the circulation following consumption of food containing fat (180, 181), although two smaller studies have found no significant association with fasting status (141, 170). It is possible that measurement of phytanic acid in fasting blood samples represents better the estimate of risk associated with 'usual' plasma phytanic acid concentration. However, further work is needed to examine how circulating phytanic acid concentration is modified by recent intake of food. The extent to which plasma phytanic acid concentration varies with seasonal change in dietary patterns and whether phytanic acid concentration interacts with variants of the AMACR gene to affect prostate cancer risk (182, 183) also warrant further investigation.

The finding that plasma phytanic acid concentration differed between countries corresponded to differences in dairy fat consumption (as described in two earlier studies (141, 177)). The relation between phytanic acid and dairy products is discussed in more detail in **Chapter 6** (on page 56). Overall, there is little evidence to support the hypothesis that the association between dairy products and prostate cancer risk is mediated by phytanic acid. Nonetheless

the findings should be interpreted with caution as this study was underpowered to detect a modest effect size of the magnitude observed (OR highest vs. lowest tertile 1.22 (95%CI 0.82 - 1.84). Other components of dairy foods, such as protein or calcium, and their association with insulin-like growth factor-I (IGF-I)(65) may be more important, as outlined in more detail in **Chapter 7** (on page 72).

5.5.1 Strengths of the study

The prospective nature of the study design together with the relatively large size, the considerable heterogeneity in ruminant product consumption and use of a validated technique for phytanic acid measurement are notable strengths of this EPIC study. Phytanic acid has shown moderate reliability (ICC = 0.48) over a 6 year period (as discussed in Chapter 4), is not synthesized in the human body and demonstrates high levels of stability under laboratory conditions (see section 6.3.2 of this chapter). It is also strongly correlated with dietary intake of fat from dairy and beef products (results presented in Chapter 6; section 6.4) thereby providing a useful biological measure of habitual consumption of ruminant fat with potential for application in studies investigating the relation between animal fat intake and risk for disease.

5.5.2 Limitations of the study

Weaknesses include the relatively short follow-up time and possible inclusion of men with sub-clinical cancer at recruitment. It is also possible that a measurement of phytanic acid concentration taken during middle to late adulthood may not reflect the aetiologically important time period of exposure in relation to prostate cancer development. In addition, it is not yet known to what extent a single phytanic acid measurement reflects the usual circulating concentration or how strongly this is correlated with intra-prostatic fatty acid concentrations. Thus, although moderate correlations were observed with dairy fat measurements taken from the dietary questionnaire (see Chapter 6, section 6.4), further work using a larger sample size (refer to **Table 4.7**) is needed to examine whether a single measure is representative of an individual's long term exposure.

5.6 Conclusion

The findings presented here do not support an association between phytanic acid and risk of prostate cancer. However, the study was underpowered to detect modest risk estimates and consideration of findings from other large cohort studies, especially those with access to repeat blood measurements, are needed in order to determine further the nature of the association between phytanic acid and risk of prostate cancer.

Figure 5:1 Schematic diagram of phytanic acid metabolism from animal sources (ruminants and fish) to human metabolism within sub-cellular peroxisomes

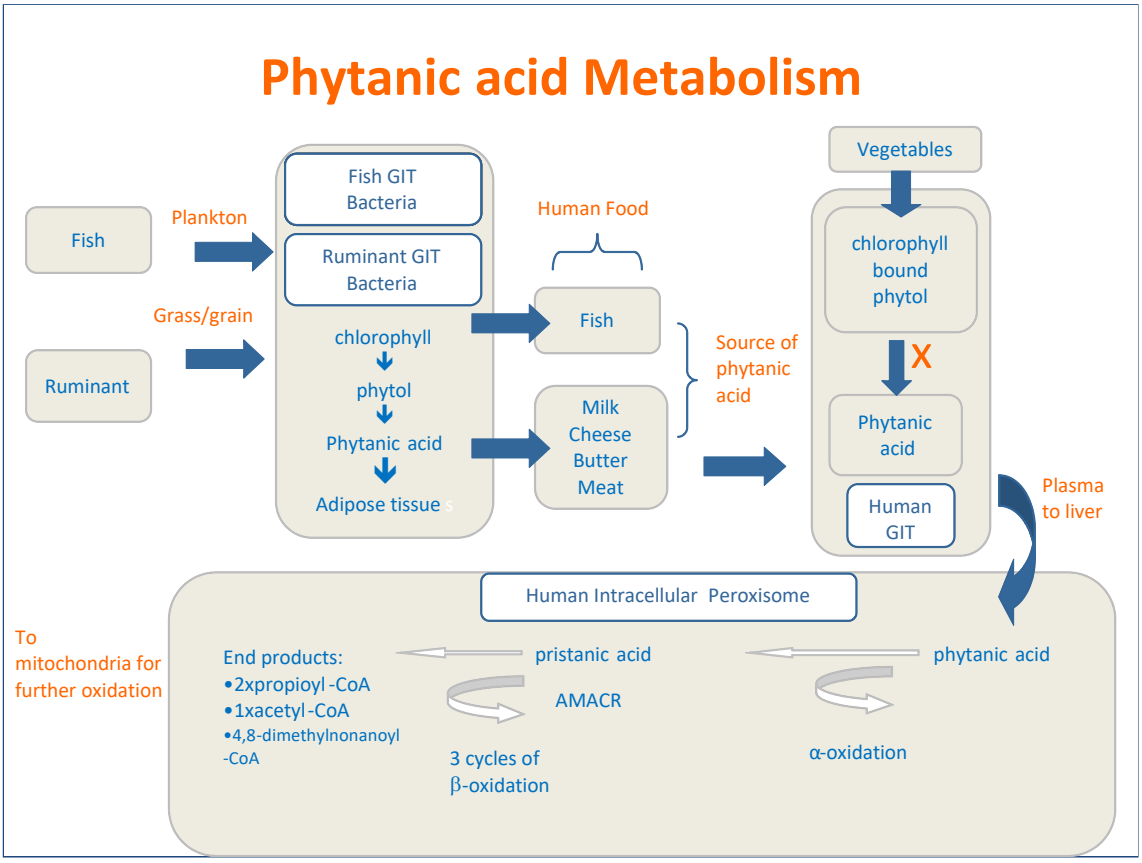


Table 5.1 Baseline characteristics of prostate cancer cases and matched controls in EPIC

Characteristic	Cases		Controls		*P* value[2]
Number	566		566		
Age at blood collection; yrs (mean); [SD]	61.6	[6.4]	61.6	[6.4]	0.06
Age at diagnosis; yrs (mean); [SD]	64.8	[6.3]	-		
Anthropometry; mean [SD]					
Height (cm)	171.8	[6.8]	172.1	[6.9]	0.36
Weight (kg)	78.9	[10.9]	80.2	[11.6]	0.04
BMI (kg/m²)	26.7	[3.4]	27.1	[3.6]	0.08
Smoking; n [%][1]					
Never	187	[33.3]	158	[28.2]	
Former	261	[46.4]	267	[47.7]	
Current	114	[20.3]	135	[24.1]	0.15
Alcohol consumption (g/d); n [%][1]					
< 8	207	[36.6]	218	[38.6]	
8-15	110	[19.5]	120	[21.2]	
16-39	149	[26.4]	128	[22.7]	
≥40	99	[17.5]	99	[17.5]	0.44
Physical Activity: n [%][1]					
inactive	103	[18.5]	84	[15.1]	
moderately inactive	199	[35.7]	179	[32.3]	
moderately active	225	[40.4]	260	[46.9]	
active	30	[5.4]	32	[5.8]	0.06
Marital Status: n [%][1]					
Married or Cohabitating	408	[88.5]	411	[89.7]	
Not married or cohabitating	53	[11.5]	47	[10.3]	0.51
Educational Level: n [%][1]					
Primary or None	201	[37.5]	198	[37.4]	
Secondary	196	[36.6]	214	[40.4]	
Degree	139	[25.9]	118	[22.3]	0.23

[1] Unknown for some participants; percentages exclude unknown values

[2] *P* values relate to likelihood ratio chi-square tests of association for categorical variables, and paired differences tests for continuous variables

Table 5.2 Adjusted geometric mean phytanic acid concentrations and 95% CI among the 561[1] control participants

	Plasma Phytanic Acid Concentration (µmol/L)			
	n	Adjusted geometric mean[2]	*P* for heterogeneity[4]	*P* for trend[5]
Characteristics:				
Age at blood collection (yrs):				
≤54	78	2.96 (2.56, 3.43)		
55 – 59	157	2.51 (2.26, 2.80)		
60 – 64	193	2.49 (2.26, 2.75)		
≥65	133	2.28 (1.96, 2.65)	0.12	0.02
BMI (kg/m²):				
< 25	166	2.41 (2.20, 2.63)		
≥25	395	2.55 (2.41, 2.69)	0.31	
Smoking:[3]				
Never	156	2.58 (2.36, 2.83)		
Former	264	2.48 (2.31, 2.66)		
Current	135	2.42 (2.18, 2.67)	0.61	
Alcohol consumption (g/d):[3]				
< 8	217	2.53 (2.33, 2.74)		
8 – 15	119	2.60 (2.34, 2.90)		
16 – 39	127	2.44 (2.20, 2.71)		
≥40	97	2.42 (2.13, 2.74)	0.81	0.46
Physical activity:[3]				
Inactive	150	2.56 (2.32, 2.84)		
Moderately inactive	164	2.52 (2.30, 2.76)		
Moderately active	129	2.38 (2.15, 2.63)		
Active	107	2.46 (2.20, 2.75)	0.76	0.41
Marital status:[3]				
Married or cohabitating	407	3.03 (2.86, 3.21)		
Not married or cohabitating	47	3.02 (2.53, 3.61)	0.98	
Educational level:[3]				
Primary or Equivalent	196	2.32 (2.13, 2.53)		
Secondary	211	2.50 (2.31, 2.71)		
Degree	118	2.69 (2.41, 3.00)	0.14	
Country:				
Germany	197	2.91 (2.40, 3.53)		
Greece	9	1.48 (0.93, 2.35)		
Italy	62	1.56 (1.12, 2.18)		
The Netherlands	25	3.97 (2.42, 6.49)		
Spain	93	1.28 (0.96, 1.70)		
United Kingdom	175	3.44 (2.66, 4.46)	< 0.0001	
Fasting status:[3]				
< 3	282	2.71 (2.52, 2.93)		
3 – 6	119	2.50 (2.24, 2.79)		
> 6	152	2.13 (1.88, 2.41)	0.01	0.002
Season of blood collection:				
December – February	137	2.29 (2.08, 2.52)		
March – May	164	2.43 (2.22, 2.65)		
June – August	133	2.71 (2.46, 2.99)		
September – November	127	2.65 (2.39, 2.93)	0.06	

[1] Duplicate observations of 5 controls that appear in the data set twice (due to the incidence density sampling protocol) have been removed

[2] Adjusted for age (< 50, 50-54, 55-59, 60-64, 65-69, ≥70 years), centre (15), batch (1-31), month of blood collection (January - December) and time spent fasting (< 3, 3 – 6, > 6 hours), where the adjustment variable is not the variable of interest; with 95% CI in parenthesis

[3] Unknown for some participants; number of unknowns calculated by subtracting the total number of knowns in each group from 561

[4] *P* value refers to tests of heterogeneity between the logarithm of the geometric means in the separate categories calculated by using analysis of variance

[5] *P* value refers to the test for trend obtained by treating the categorical variable as a continuous variable in the model

Table 5.3 Odds ratios (and 95% CI) for prostate cancer by thirds of plasma phytanic acid concentration in EPIC

	Third of Phytanic Acid Concentration			
	1	2	3	
Range (µmol/L)	0.27 – 1.79	1.80 – 3.70	3.71 – 35.13	
Median value (µmol/L)	1.11	2.57	5.25	
Cases/Controls (n)	181/ 189	197/ 189	188/ 188	
				P for trend[4]
OR[1] (95% CI)	1 (ref)	1.12 (0.80 – 1.57)	1.09 (0.74 – 1.60)	0.66
OR[1,2] (95% CI)	1 (ref)	1.14 (0.81 – 1.60)	1.13 (0.76 – 1.68)	0.53
OR[1,3] (95% CI)	1 (ref)	1.14 (0.80 – 1.63)	1.22 (0.82 – 1.84)	0.34

[1] Conditioned by matching factors: study centre, age at recruitment, length of follow-up, time of blood collection and time between blood collection and last consumption of food or drink

[2] Adjusted for month of blood collection

[3] Adjusted for body mass index, smoking, alcohol intake, education, marital status, physical activity and month of blood

[4] *P* values for trend were obtained with the logarithm of plasma phytanic acid concentration replacing the thirds of concentration

Table 5.4 Odds ratios (and 95% CI) for prostate cancer associated with a doubling of phytanic acid concentration in EPIC

	Cases (*n*)	OR[1,2,3] (95% CI) for doubling of concentration	*P* for trend[4]	*P* for heterogeneity[5]
Overall prostate cancer	566	1.05 (0.91, 1.21)	0.53	
Prostate cancer stage[6]				
Localized	255	1.21 (0.95 – 1.55)	0.11	0.22
Advanced	116	0.90 (0.65 – 1.26)	0.54	
Histological grade[7]				
Low grade	381	1.03 (0.86 – 1.23)	0.76	0.64
High grade	69	1.15 (0.69 – 1.93)	0.59	
Age at blood collection				
< 60 years	237	1.02 (0.81 – 1.30)	0.85	0.73
≥ 60 years	329	1.08 (0.89 – 1.30)	0.44	
Age at diagnosis				
< 60 years	103	0.76 (0.53 – 1.08)	0.12	0.02
≥ 60 years	463	1.14 (0.97 – 1.35)	0.11	
Time to diagnosis				
< 4 years	306	0.93 (0.77 – 1.12)	0.42	0.07
≥ 4 years	260	1.26 (0.99 – 1.61)	0.06	
Fasting status				
< 3 hours	283	0.89 (0.73 – 1.09)	0.25	0.04
≥ 3 hours	273	1.27 (1.01 – 1.60)	0.04	

[1] The odds ratio provides an estimate of the incidence rate ratio in the study cohort

[2] Adjusted for month of blood collection

[3] Conditioned by matching factors: study centre, age at recruitment, length of follow-up, time of blood collection and time between blood collection and last consumption of food or drink

[4] *P* values for trend were obtained with the logarithm of plasma phytanic acid concentration

[5] *P* for heterogeneity values relate to likelihood ratio chi-square tests of heterogeneity between trends

[6] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic

[7] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system

Chapter 6 Phytanic acid as a nutritional biomarker of fat from dairy, beef and other sources

6.1 Introduction

To evaluate the relation between usual diet and nutrient status on risk for disease epidemiological studies commonly use dietary intake data collected by assessment methods such as a 24-recall, food frequency questionnaire or a 7 day diet dairy. To overcome some of the methodological limitations of these assessments, prospectively collected and archived biological samples are increasingly used to measure nutritional biomarkers as a more accurate assessment of dietary intake. As discussed in **Chapter 4** (on page 22), the capacity of a nutritional biomarker to reliably estimate the usual intake of a food (or constituent of food) is optimised if there is a single dietary source (which is consumed regularly throughout all seasons) and minimal measurement error (introduced by flawed specimen collection, processing and storage, laboratory error, and within-individual variability) associated with circulating concentrations of the biomarker over the exposure period of interest.

Ecological studies (as outlined in **Chapter 3**, on page 10) have shown positive correlations between dairy consumption and risk for prostate cancer. However, evidence from prospective observational studies of the association between intake of dairy products and prostate cancer risk is inconsistent (9). Dairy products have also been investigated as risk factors in cardiovascular disease (184) as a high intake of saturated fat is often associated with increased low-density lipoprotein cholesterol levels (185, 186), a known risk factor for cardiovascular disease (187). Paradoxically, consumption of dairy products is also associated with an increase in "healthy" high-density lipoprotein cholesterol (185, 188) and a reduction in blood pressure (189).

To understand better the possible role of dairy products and dairy fat in the development of diseases, such as prostate cancer and cardiovascular disease, requires accurate and precise biological measures of dairy constituents, including fatty acids, proteins and calcium, that may affect development of disease via different mechanisms.

As discussed in **Chapter 5** (starting on page 41), phytanic acid is a branched chain fatty acid that is not synthesized endogenously in the human body (163) and concentrations measured in the blood therefore reflect dietary intake, mostly from ruminant products (160, 165-167) such as dairy foods and beef, although it is also present in pelagic fish such as salmon and mackerel (161, 167). Phytanic acid is stable at room temperature, robust to multiple freeze-thaw cycles (141), reliably measured with GC-MS technology (141, 190, 191) and demonstrates moderate reliability over the medium term (as discussed in **Chapter 4**). Furthermore, two small studies have shown a statistically significant positive correlation between phytanic acid and dairy fat intake ($r = 0.68$; $P<0.0001$ (141) and $r = 0.24$; $P =0.01$ (177)). These findings suggest that phytanic acid may be a good biomarker of dairy fat intake.

Levels of saturated fatty acids, pentadecanoic (C15:0) and heptadecanoic (C17:0) acid in subcutaneous adipose tissue (192) and blood (193, 194) have been considered good biomarkers of dairy fat intake, as these fatty acids are synthesised by bacterial flora in the rumen of ruminants and are components of bovine milk (195) and adipose tissue(196, 197). Like phytanic acid, pentadecanoic and heptadecanoic acid cannot be synthesized in the human body. Following ingestion, concentrations of these fatty acids in body compartments vary over time, with concentrations in serum cholesterol representing recent ingestion (a few hours after a meal), concentrations in erythrocyte membranes, serum phospholipids and cholesterol esters representing intake over the previous few months and triacylglcyerols of adipose tissue reflecting longer term intake (over a 2-3 year period) (198). Because of their slow turnover(199, 200), fatty acid concentrations in adipose tissue are considered valid markers of long term dietary exposure (192, 198, 201) however, adipose tissue samples are more difficult and invasive to obtain and not available in most prospective cohort studies.

6.2 Aim of study

Given the widespread use of food frequency questionnaires (FFQ) for dietary assessment and collection of baseline blood samples in epidemiological studies, the aim of this study was to evaluate the correlation between FFQ estimated intake of ruminant products (including dairy and beef) and oily fish and a single plasma measure of phytanic acid concentration. A further aim was to investigate the utility of phytanic acid compared to established biomarkers of dairy fat consumption, pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0).

6.3 Methods

6.3.1 Study population

The methods of recruitment used in the EPIC cohort study have been outlined in detail in **Chapter 2** (starting on page 6). In brief, during recruitment between 1992 and 2000, approximately 360,500 women and 153,400 men (aged 35-70 years), from 23 centres in ten European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom), completed either a country-specific standardized food-frequency questionnaire (FFQ; previous year's intake) or an interview based dietary questionnaire and a standardized lifestyle and personal history questionnaire. In addition, detailed and standardized 24-hour dietary recall (24-HDR) data was collected from a stratified and representative subset (37,000 subjects) of participants by using computerized EPIC-SOFT software.

Approximately 139,600 male participants provided a blood sample and in all recruitment centres, blood samples (>30 mL) were protected from light, and transported to local laboratories for processing. With the exception of the Oxford centre in the United Kingdom (UK), whole blood was transported in the collection syringe to a local laboratory where samples were processed and separated into serum, plasma, red cells and buffy coat fractions within 24-hours of collection, prior to storage at -196°C in liquid nitrogen tanks. An exception to this standardised protocol occurred in the EPIC-Oxford centre (UK), where blood samples were collected from general practitioners and transported by post to a central laboratory in

Norfolk (UK). Sodium citrate anticoagulant was used for all of the plasma samples included in this study.

All participants provided written informed consent and local ethics committees in the participating countries and the Internal Review Board of the International Agency for Research on Cancer (IARC) approved the study.

Of 566 participants from Germany, Greece, Italy, the Netherlands, Spain and the United Kingdom who had been selected as controls for a nested-case control analysis of the association between phytanic acid and risk for prostate cancer, and who were alive and free of cancer (except non-melanoma skin cancer) during the follow-up period, provided a blood sample at recruitment and had available fatty acid measurements for pentadecanoic acid and heptadecanoic acid, 561 men were available for this study. Due to the use of an incidence density sampling protocol 5 duplicate observations, for men who appeared as controls twice, were excluded. Of the 561 men, 540 had completed a FFQ and had nutrient data available however data on some food group variables were not available in some centres.

6.3.2 Laboratory methods

6.3.2.1 Determination of phytanic acid

Plasma phytanic acid concentration was measured in singleton using a gas chromatographic /mass spectrometric (GC/MS) assay established at HFL Sport Science Ltd (Fordham, Cambs, UK), and validated over a concentration range of 0.032 – 9.6 µmol/L. Details of this procedure are provided in **Chapter 5** (on page 45). In brief, determination of phytanic acid involved saponification of 50 µl of plasma using 5% potassium hydroxide in ethanol, followed by liquid/liquid extraction and derivatisation of phytanic acid to tert-butyldimethylsilyl (TBDMS) ether, prior to gas chromatographic-mass spectrometric analysis. A Trace 2000 Series gas chromatograph (ThermoQuest, Waltham, MA, USA), with a BPX5 column (25 m x 0.25 mm, 0.22 µm film thickness; SGE, Ringwood, Victoria, Australia) using helium as the carrier gas, was used for all analyses. Phytanic acid was ionized using electron ionization and detected via selected ion monitoring (SIM) on a MD800 mass spectrometer (Fisons Instruments, later ThermoQuest). The two ions monitored were *m/z* 369 for phytanic acid and *m/z* 372 for the internal standard [3-methyl-$^{2}H_3$]-phytanic acid.

The lower limit of quantification (0.032µmol/L) provided adequate sensitivity to detect the lowest concentration in the study samples, although 42 (3.5%) samples exceeded the validated upper limit (9.6µmol/L). These samples were diluted prior to re-extraction and analysis, as validated in a previous EPIC analysis (141).

Assays were conducted by a laboratory technician, blind to both the case-control and quality control status of the plasma samples. Case-control sets were analyzed in the same batch and blinded duplicate quality control samples were included in each, with a mean intra- and inter-batch coefficient of variation of 1.6% and 6.5%, respectively.

6.3.2.2 Determination of plasma pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0)

The details of the laboratory analysis are outline in detail elsewhere (202). In brief, lipids were extracted from 200 µL plasma with chloroform:methanol (2:1) after the addition of 100 µg butylated hydroxytoluene and 20 µg internal standard (1,2-dipalmitoyl-D62-*sn*-glycero-3-phosphocholine) and phospholipids were isolated by solid-phase extraction by using aminopropyl Supelclean solid-phase extraction tubes (Le-Si; Supelco, Bellefonte, PA).

Fatty acid methyl esters, formed by transmethylation of the phospholipids with METH-Prep (Alltech, Deerfield, IL), were analyzed on an SP-2340 column (30 m x 0.32 mm x 0.2 µm film thickness) with a nonpolar fused silica precolumn (1 m x 0.32 mm internal diameter) installed on an HP 5980 gas chromatograph (Agilent, Palo Alto, CA) and with a flame ionization detector. During all steps of the fatty acid analysis, samples from each case-control set were analyzed within the same batch.

Quality control measures for the assays were conducted using pooled plasma samples and the coefficients of variation for the saturated fatty acids were 17.8% and 12.7%, for pentadecanoic acid and heptadecanoic acid, respectively. All fatty acid analysis was conducted in the Nutrition and Hormones Laboratory at the International Agency for Research on Cancer in Lyon, France.

6.3.3 Statistical analysis

For this analysis, the dietary variables selected from the food frequency questionnaire (FFQ) included beef, fish (excluding shellfish), oily fish (including mackerel and salmon), dairy products (butter, milk, yogurt, dairy desserts and cheese) and fat derived from dairy, butter, beef and fish (% of energy). Red meat was not included in the analysis as it includes non-ruminant meat from pork, which represents 40% or more of red meat intake in most centres, except Italy (203). Dietary data were calibrated using a fixed-effects linear model in which centre and gender-specific 24-hour recall data from an 8% random sample of the cohort (16) were regressed on the FFQ intakes (204). Calibration was performed in order to improve the comparability of dietary data across centres and to correct for measurement error in exposure classification. Dietary intake are expressed as arithmetic mean concentrations and standard deviations although for the correlation analyses dietary variables and plasma fatty acid concentrations were logarithmically transformed to approximate a normal distribution and observations with a zero intake were set to half of the lowest non-zero value prior to log transformation.

Multiple regression analysis was used to determine the influence of different factors including body mass index (BMI), age at blood collection, alcohol intake, month of blood collection, smoking status and country on concentrations of phytanic acid, pentadecanoic acid and heptadecanoic acid. The partial r^2 value, calculated as the ratio of each variable's sum of squares, over the residual sum of squares of the model excluding the independent variable,

indicates the proportion of the variation explained by the model that can be attributed to each independent variable.

The association of plasma phytanic acid, pentadecanoic acid and heptadecanoic acid and dietary intake of ruminant foods and nutrients were examined using partial correlation coefficients, with adjustment for total energy intake (205) age at blood collection, BMI and country of origin and, where appropriate, further adjustment for fat intake from dairy foods (as a percentage of energy intake) and fat intake from beef (as a percentage of energy intake). Spearman correlations were also performed as not all dietary data approximated a normal distribution following logarithmic transformation. Phospholipid fatty acid and diet correlations are presented as concentrations (nmol/L) and as a percent of the total 26 fatty acids that were quantified, on a molar basis (i.e. molar percent). Pentadecanoic acid and heptadecanoic acid represented less than 1% of the total fatty acids in plasma phospholipids (0.16% [SD=0.09] and 0.41% [SD=0.15], respectively).

6.4 Results

The arithmetic mean and standard deviation of intake of calibrated foods and nutrients are presented in **Table 6.1** (on page 65). The large standard deviations observed for some foods, including butter, yoghurt and fish, are related to the presence of non-consumers. Among the non-consumers of certain food groups, 60% of non-consumers of fish (n=14) and 63% of non-consumers of oily-fish (n= 50) were from the UK and 76% (n=36) of butter non-consumers and 85% (n=82) of beef non-consumers were from Spain. Fat intake from dairy products accounted for approximately 8% of total energy intake, while fat from beef and fish contributed less than 5% of total energy intake in this population of European men.

Multiple regression analysis of predictors of phytanic acid, pentadecanoic acid and heptadecanoic acid showed that country was the most important factor for variability of phytanic acid (r^2 =38.2%) although it attributed considerably less variability to pentadecanoic acid (r^2 = 5.00%) and heptadecanoic acid (r^2 = 5.86%). Geometric mean concentrations of phytanic acid, pentadecanoic acid and heptadecanoic acid by country are presented in **Table 6.2** (on page 66). The geometric mean phytanic acid concentration in the UK was 3.5 times higher than in Spain, while the difference in the highest versus lowest concentration for pentadecanoic and heptadecanoic acid was considerably less pronounced, with a 1.5 times higher concentration in the UK compared to Greece and 1.4 times higher concentration in the Netherlands compared to Spain, respectively).

Figure 6:1 and **Figure 6:2** show the geometric mean calibrated dairy fat intake (g/day) and beef fat intake (g/day) plotted against the geometric mean phytanic acid concentration (with adjustment for fat from beef or dairy, as appropriate) for each country (excluding Greece due to unavailable data for beef intake). The lowest intake of dairy fat was observed in Spain (12.2 g/day) and this corresponded to the lowest phytanic acid concentration (1.14 µmol/L). Higher intakes of dairy fat (>20 g/day) corresponded to higher phytanic acid concentration in Germany and the Netherlands with the exception of Italy, that reported relatively high intake

of dairy fat (17.0 g/day) but a relatively low phytanic acid concentration (1.23 µmol/L). This finding is in contrast to the higher phytanic acid concentrations measured for the UK participants (3.67 µmol/L), which had the same level of calibrated dairy fat intake (17.0 g/day; **Figure 6:1**). A similar distribution was observed for fat intake from beef and phytanic acid levels, however there was less variation in beef fat intake and the mean concentration of beef fat intake in all countries was considerably lower than for dairy fat intake (<2 g/day and >12 g/day for beef and dairy fat, respectively; **Figure 6:2**).

Spearman correlations, pearson correlations and partial correlations (with adjustment for total energy intake (205) age at blood collection, body mass index (BMI), alcohol and country of origin and, where appropriate, further adjusted for fat intake from dairy (as a percentage of energy intake) and fat intake from beef (as a percentage of energy intake)) between foods and nutrients and plasma phytanic acid and plasma phospholipid pentadecanoic acid and heptadecanoic acid concentrations are shown in **Table 6.3, Table 6.4** and **Table 6.5,** respectively. Alcohol consumption had little effect on phytanic acid (r^2 =0.07%), although a stronger effect on pentadecanoic acid (r^2 =2.87%) and heptadecanoic acid (r^2 =5.67%). Age at blood collection explained 9%, 12% and 5% of variation in phytanic acid, pentadecanoic acid and heptadecanoic acid, respectively. Smoking, month of blood collection and BMI had little effect on all fatty acid concentrations (r^2 <5%; r^2 information is not shown in the table).

Spearman rank correlation coefficients between foods or nutrients and phytanic acid were strongest for butter (r = 0.56), fat from total dairy (% of energy; r = 0.48; p <0.0001) and fat from butter (% of energy; r=0.57; p <0.0001; see **Table 6.3**; page 68). Similarly, spearman correlations were strongest between pentadecanoic acid and heptadecanoic acid and butter, total dairy fat (% of energy) and fat from butter (% of energy) but of a lower magnitude than those observed for phytanic acid (range 0.19 - 0.30; p <0.0001; see **Table 6.4** and **Table 6.5**). Moderate spearman correlations were observed between each fatty acid and beef (range; r=0.15 - 0.20; p <0.0001) and fat from beef (% energy intake; range; r=0.16 - 032; p <0.0001), although the strongest correlations were evident for phytanic acid (r =0.20 and r = 0.32 for beef and fat from beef (% energy); p <0.0001, respectively).

After adjusting for total energy intake, age at blood collection, BMI, alcohol, country and fat intake from beef (% energy intake), plasma concentrations of phytanic acid were most strongly correlated with total dairy fat (% energy; r = 0.33; p < 0.0001) and butter fat (r = 0.28; p < 0.00001), although moderate correlations were also observed with intake of fat from beef (% energy; r= 0.26; p <0.0001), milk (r = 0.24; p <0.0001) and butter (r = 0.24; p <0.0001).

Overall, stronger correlations were observed between the absolute concentration of pentadecanoic acid and heptadecanoic acid and foods or nutrients than were observed when these fatty acids were measured as a proportion of total fatty acids. Correlations equivalent to those observed for phytanic acid were observed between pentadecanoic aicd and dairy fat (% energy; r = 0.33; p < 0.0001; see **Table 6.4**; page69) and butter fat (r = 0.25; p < 0.00001). There was some evidence for a weak correlation between pentadecanoic acid and total beef

intake (r = 0.11; p = 0.01), and less so with beef fat (% energy; r = 0.07; p = 0.13). Although of a lower magnitude than observed for either phytanic acid or pentadecanoic acid, heptadecanoic acid was observed to have similar correlations for dairy (% energy; r = 0.18; p < 0.0001) and beef fat (% energy; r = 0.20; p < 0.0001; see Table 6.5; page 70). Overall, a higher correlation was observed between phytanic acid and fat from all ruminant products, including dairy and beef, than was observed for pentadecanoic acid or heptadecanoic acid.

6.5 Discussion

In the present study, plasma phytanic acid and plasma phospholipid pentadecanoic and heptadecanoic acid concentrations were correlated with source foods and nutrients, including products containing fat of ruminants or oily fish. Overall, the strongest correlations were observed for total dairy fat and plasma phytanic acid (r =0.33; p<0.0001) and plasma phospholidpid pentadecanoic acid concentration (r =0.33; p<0.0001).

Our finding that plasma phytanic acid concentration was highly correlated with dietary intake of fat from dairy products is consistent with two previous studies (141, 177). Furthermore, differences in phytanic acid concentration between countries corresponded to differences in dairy fat consumption, with the highest intake of fat in Germany (representing 3.8 % of energy intake) and the lowest in Spain (representing 1.2% of energy intake). However, there was little evidence for strong differences in dairy fat intake between Italy and the UK despite a 3-fold difference in mean concentration of phytanic acid. This suggests that a measure of phytanic acid differentiates more precisely different levels of dairy fat consumption compared to dietary assessment with the FFQ.

Although phytanic acid was modestly correlated with fat from beef, it was not correlated with fat from fish or oily fish, which have been reported as sources elsewhere (161, 167, 206, 207). These inconsistencies may be due to a limitation of the food-frequency questionnaire and/or a single measurement of phytanic acid to estimate usual dietary intakes, due to seasonal variation in the phytanic acid content of fish products (influenced by plankton content (161)).

Adjustment for country reduced correlation coefficients for butter and total dairy fat and phytanic acid by 30%. The generally lower correlations between phytanic acid and food items in the adjusted analysis (compared to the Spearman rank correlation or the Pearson correlation) highlight the effect of considerable variation in dairy product intake between countries, with a relatively high consumption of butter amongst EPIC participants in Germany and the UK compared to the Mediterranean countries, consistent with earlier findings in the EPIC population (208). Nonetheless, the 10% stronger correlation between milk and phytanic acid following adjustment for country may reflect less variation in intake between countries and greater variation within countries due to regional differences in intake. As previously reported, milk represents the largest proportion of total dairy consumption in all EPIC countries although it varies considerably by geographical region (208).

For pentadecanoic acid the correlations observed in our study between total dairy fat, fat from butter and butter were in the same range (r = 0.25 - 0.34) as observed in studies that compared concentrations in phospholipids and intake using a 7 day food record (193) and total serum lipids and intake measured by FFQ (194). However, stronger correlations have also been reported between phospholipid pentadecanoic acid concentrations and total dairy and milk intake when two 7 day food records or 14 separate 24-hour recall interviews were used (192, 209). In contrast to a previous study that reported moderate correlations (r = 0.5) between concentrations of phospholipid heptadecanoic acid and total dairy and milk intake (209), in the present study weak correlations were observed with milk intake. Moreover, the strength of the correlation between heptadecanoic acid and total fat from beef and total fat from dairy was of a similar magnitude, albeit modest, indicating that heptadecanioc acid is a less specific marker of dairy fat intake than that of pentadecanoic acid.

It is possible that the correlations observed between the concentration (µmol/L) of fatty acids and dietary intake may in part be driven by the total amount of circulating lipid rather than the individual fatty acid under observation. As such, representation of pentadecanoic acid and heptadecanoic acid as a molecular percentage of total phospholipid fatty acids (taking into account variation in total circulating lipids), may provide a better measure. However, the correlations observed for these fatty acids when measured as a molecular percentage of total fatty acids in plasma phospholipids were, in general, lower than those observed for absolute concentrations of each fatty acid. This disparity may be attributable to the different absolute levels of fatty acids in phospholipids as there was evidence of lower pentadecanoic acid and heptadecanoic acid as a percentage of total fatty acids amongst individuals with higher compared to lower total fatty acid concentrations (results not shown).

Previous studies have reported a moderate effect of alcohol on saturated fatty acid concentrations (210-212) and although the mechanism is unclear, alcohol has been shown to affect the metabolism of lipoproteins (212), to raise HDL-cholesterol and antagonise enzymes involved in fatty acid metabolism. Although adjustment was made for alcohol in these analyses, it is not possible to determine the extent to which variation in fatty acids is attributable to dietary differences between alcohol consumers and non-consumers or the mechanism through which alcohol influences the metabolism and circulating concentrations of fatty acids.

6.5.1 Strengths of the study

The relatively large sample size and diverse dietary patterns of participants across six European countries are strengths of this study. Although stronger correlations have been reported between pentadecanoic acid and heptadecanoic acid in adipose tissue and dietary intake of dairy fat (Pearson r = 0.63 and 0.42 for 15:0 and 17:0, respectively) (192) the use of blood measures meant that the study could be conducted amongst a large subset of individuals with validated and prospectively collected dietary data and biological samples.

6.5.2 Limitations of the study

Direct comparability is not achieved with correlations between plasma fatty acid concentrations and FFQ dietary exposure measures due to a host of factors. Notably, plasma is not a long term storage site for fatty acids and sample handling, storage and laboratory measurements will introduce measurement error. Furthermore, reported dietary intakes are measured with error due to problems with recall, under-and over-reporting, seasonal variation in intake and incomplete information in nutrient databases (213). While calibrated values of dietary intake (informed by the 24 hour recall and FFQ) were used in this analysis, stronger correlations with fatty acid concentrations may have been observed with more accurate dietary data, as previously reported from weighed records (194). A further limitation of this study is lack of information on total circulating fatty acid levels, which meant that molecular percentage comparisons of the correlation between phytanic acid, pentadecanoic acid and heptadecanoic acid and dietary measures could not be performed. It is also notable that the sample size of the current study is small and investigation of these correlations in a larger study is required to determine if phytanic acid is a comparable alternative biomarker of dairy fat intake.

6.6 Conclusions

The results of this study suggest that phytanic acid is a strong candidate for use as a nutritional biomarker of ruminant fat intake, including total dairy and beef fat. Plasma phytanic acid is comparable to pentadecanoic acid (in plasma phospholipids) as a biomarker of milk and total dairy fat consumption. However, pentadecanoic acid is a more specific biomarker of dairy fat alone. Together these findings indicate that phytanic acid is a good biomarker of total ruminant fat intake, with utility as an alternative or complementary measure of dietary exposure. These results will help to inform researchers wishing to use nutritional biomarkers of dietary fat intake.

Table 6.1 Mean (standard deviation) food and nutrient intakes estimated using the food frequency questionnaire (FFQ)

Food/Nutrient FFQ	n	Observed mean [SD]	Calibrated mean [SD][1]
Foods (g/d)			
Butter	540	6.0 [11.0]	10.3 [13.4]
Milk	540	198.6 [212.0]	187.5 [146.4]
Cheese	540	32.4 [32.5]	25.6 [16.1]
Yoghurt	540	39.0 [69.1]	27.0 [33.8]
Beef[3]	531	18.9 [25.5]	15.1 [13.2]
Fish	540	33.3 [37.2]	32.6 [32.9]
Oily fish[4]	454	11.2 [14.1]	13.8 [11.6]
Nutrients (% of energy)			
Fat from dairy products[5]	540	8.0 [4.9]	8.3 [4.5]
Fat from butter	540	1.8 [3.1]	2.8 [3.5]
Fat from beef[3]	531	0.7 [0.9]	0.5 [0.4]
Fat from fish	540	0.6 [0.5]	0.7 [0.5]
Fat from oily fish[4]	445	0.4 [0.5]	0.5 [0.5]

[1] Calibration was performed in order to improve the comparability of dietary data across centres and to correct for measurement error in exposure classification

[2] Ruminant meat includes beef, sheep and goat

[3] Data for beef not available for Greece (n= 9)

[4] Data for oily fish, including mackeral and salmon, missing for some participants (n= 86)

[5] Dairy products include milk, butter, cheese, yoghurt, dairy puddings and cream

Table 6.2 Geometric mean concentration of phytanic acid, pentadecanoic acid and heptadecanoic acid by country

	Number	G
Country		
Germany	192	
Greece	9	
Italy	59	
The Netherlands	24	
Spain	92	
United Kingdom	163	

[1] Plasma fatty acid concentrations were
half of the lowest non-zero value prior to

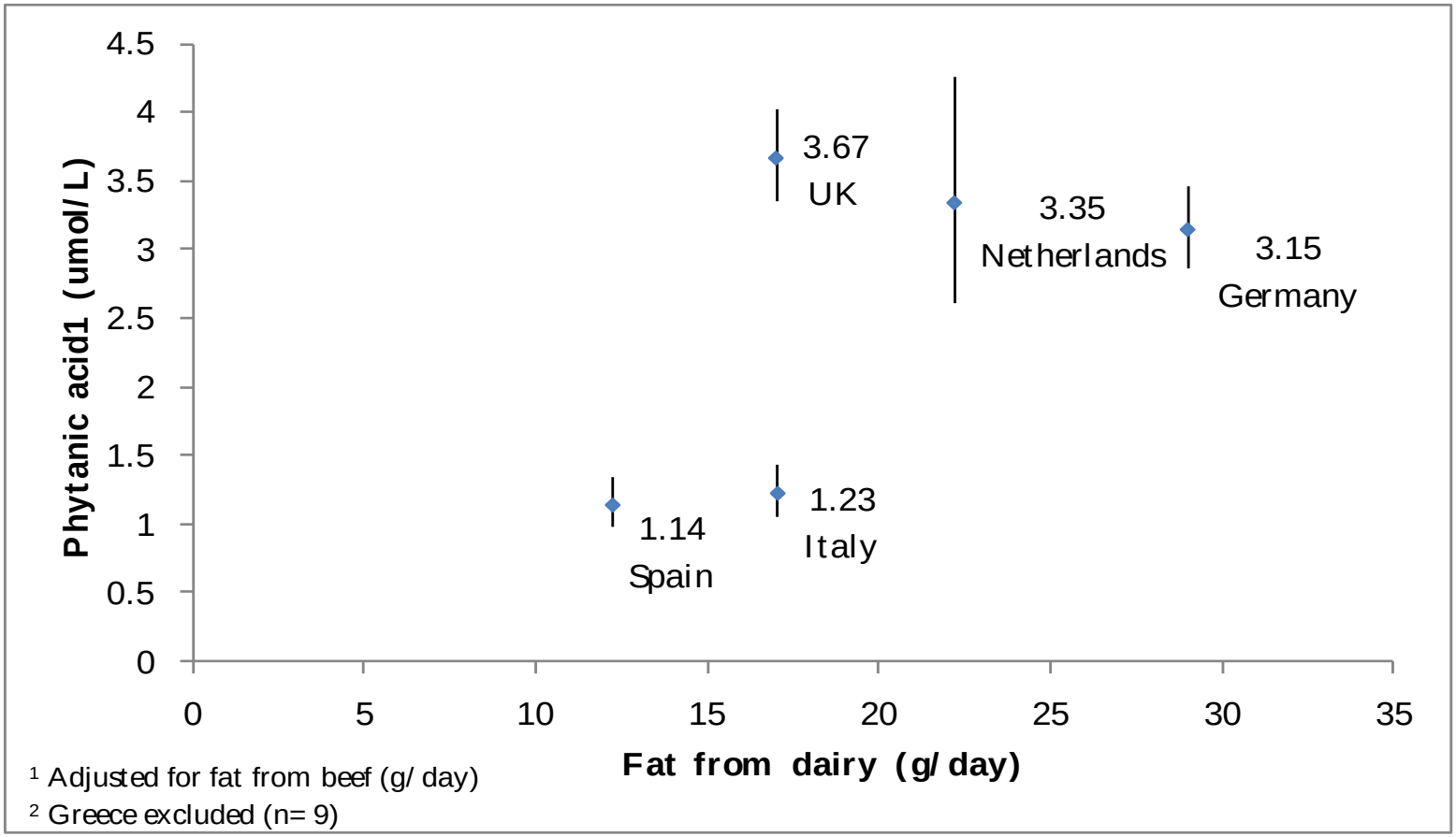


Figure 6:1 Geometric mean dairy fat intake (g/day) by the geometric mean phytanic acid concentration[1,2] (µmol/L)

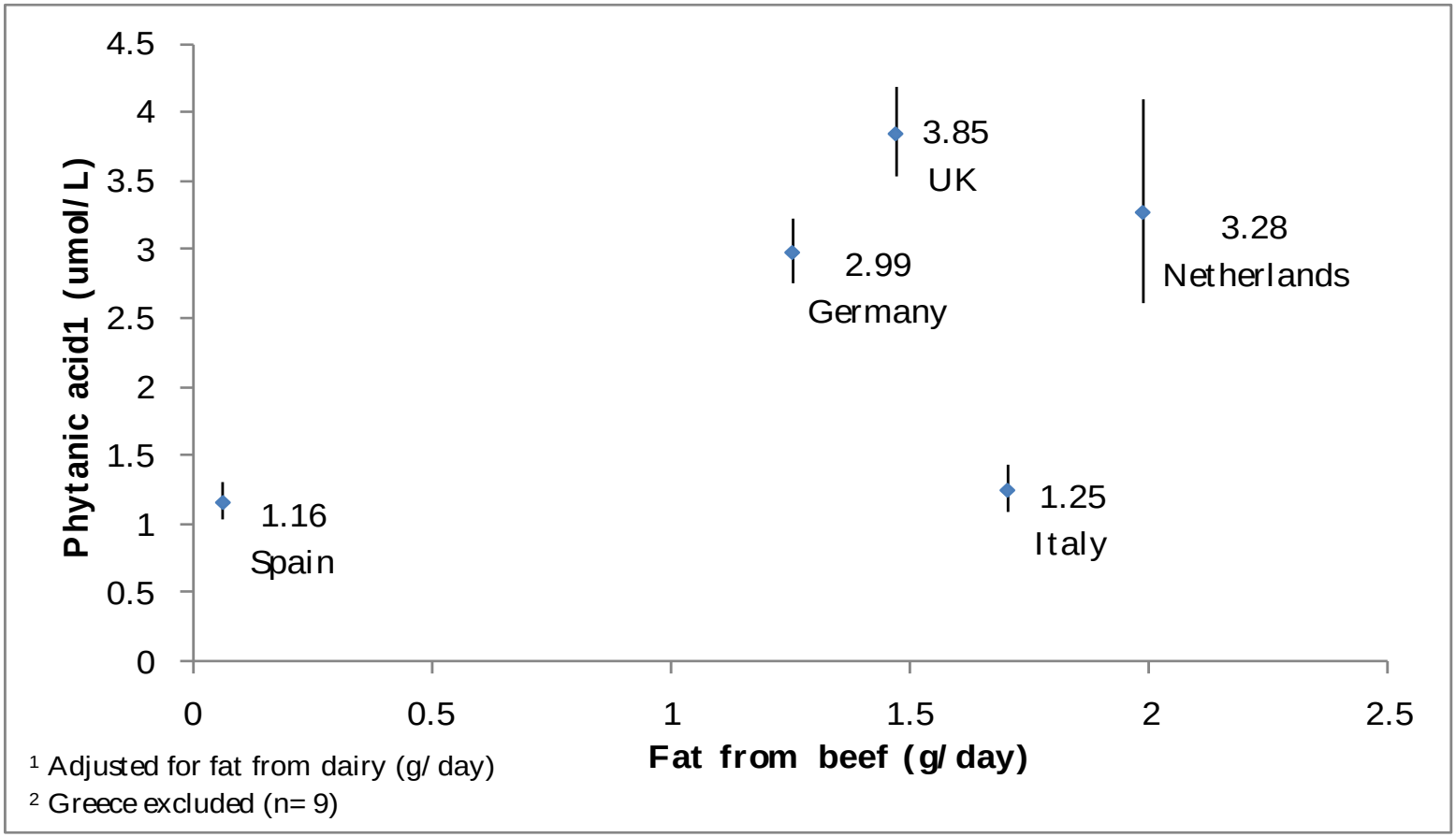


Figure 6:2 Geometric mean beef fat intake (g/day) by the geometric mean phytanic acid concentration[1,2] (µmol/L)

Table 6.3 Associations between food and fat intake acquired from meat, dairy and fish and plasma concentrations of phytanic acid

Food/Nutrient	Phytanic Acid [µmol/L]					
FFQ	Spearman correlation	P value	Unadjusted correlation coefficient [5]	P value	Partial correlation coefficient [1,5]	P value
Foods (g/d)						
Butter	0.56	< 0.0001	0.49	< 0.0001	0.24	< 0.0001
Milk	0.13	0.003	0.19	< 0.0001	0.24	< 0.0001
Cheese	0.01	0.86	0.16	< 0.0001	0.10	0.02
Yoghurt	0.16	0.0002	0.21	< 0.0001	0.07	0.09
Beef[2]	0.20	< 0.0001	0.40	< 0.0001	0.24	< 0.0001
Fish	-0.36	< 0.0001	-0.29	< 0.0001	-0.03	0.52
Oily fish[3]	-0.24	< 0.0001	-0.09	0.006	0.05	0.26
Nutrients (% of energy)						
Fat from Dairy[4]	0.48	< 0.0001	0.46	< 0.0001	0.33	< 0.0001
Fat from butter	0.57	< 0.0001	0.42	< 0.0001	0.28	< 0.0001
Fat from beef[2]	0.32	< 0.0001	0.30	< 0.0001	0.26	< 0.0001
Fat from fish	-0.18	0.0001	-0.13	< 0.0001	0.07	0.10
Fat from oily fish[3]	-0.14	0.003	-0.12	0.001	0.03	0.52

[1] Adjustment for fat from dairy and/or fat from beef (as a percent of total energy), total energy intake, age, alcohol intake, body mass index and country, where appropriate

[2] Data for beef not available for Greece (n= 9)

[3] Data for oily fish, including mackerel and salmon, missing for some participants (n= 86)

[4] Dairy products include milk, butter, cheese, yoghurt, dairy puddings and cream

[5] Correlation coefficients are calculated using the logarithm of the phytanic acid and the logarithm of the calibrated intake of food or calibrated observed intake of a nutrient (as a % of energy intake) in order to better approximate a normal distribution

Table 6.4 Associations between food and fat intake acquired from meat, dairy and fish and plasma concentrations of pentadecanoic acid (C15:0)

Food/Nutrient	Pentadecanoic Acid 15:0							
FFQ	Spearman correlation	P value	Unadjusted correlation coefficient [nmol/L][5]	P value	Partial correlation coefficient [nmol/L][1,5]	P value	Partial correlation coefficient [mol%][1,6]	P value
Foods (g/d)								
Butter	0.30	< 0.0001	0.27	< 0.0001	0.22	< 0.0001	0.01	0.78
Milk	0.12	0.004	0.15	< 0.0001	0.10	0.03	0.07	0.09
Cheese	0.05	0.28	0.12	0.0001	0.13	0.003	-0.02	0.58
Yoghurt	0.06	0.14	0.10	0.001	0.08	0.06	0.00	0.97
Beef[2]	0.15	0.0004	0.18	< 0.0001	0.11	0.01	-0.02	0.72
Fish	-0.13	0.004	-0.08	0.01	0.02	0.67	0.08	0.07
Oily fish[3]	-0.10	0.03	0.00	0.98	0.13	0.01	0.04	0.46
Nutrients (% of energy)								
Fat from Dairy[4]	0.30	< 0.0001	0.31	< 0.0001	0.33	< 0.0001	0.12	0.01
Fat from butter	0.31	< 0.0001	0.23	< 0.0001	0.25	< 0.0001	0.17	0.0001
Fat from beef[2]	0.16	0.0003	0.10	0.001	0.07	0.13	0.03	0.52
Fat from fish	-0.07	0.14	-0.05	0.08	0.03	0.53	0.09	0.04
Fat from oily fish[3]	-0.06	0.22	-0.06	0.10	0.05	0.33	0.07	0.16

[1] Adjustment for fat from dairy and/or fat from beef (as a percent of total energy), total energy intake, age, alcohol intake, body mass index and country, where appropriate

[2] Data for beef not available for Greece (n= 9)

[3] Data for oily fish, including mackerel and salmon, missing for some participants (n= 86)

[4] Dairy products include milk, butter, cheese, yoghurt, dairy puddings and cream

[5] Correlation coefficients are calculated using the logarithm of the concentration of pentadecanoic acid (C15:0) and the logarithm of the calibrated intake of food or calibrated observed intake of a nutrient (as a % of energy intake) in order to better approximate a normal distribution

[6] Correlation coefficients are calculated using the molar percent of pentadecanoic acid (C15:0) and the logarithm of the calibrated intake of food or calibrated observed intake of a nutrient (as a % of energy intake) in order to better approximate a normal distribution

Table 6.5 Associations between food and fat intake acquired from meat, dairy and fish and plasma concentrations of heptadecanoic acid (C17:0)

Food/Nutrient	Heptadecanoic Acid 17:0							
FFQ	Spearman correlation	P value	Unadjusted correlation coefficient [nmol/L][5]	P value	Partial correlation coefficient [nmol/L][1,5]	P value	Partial correlation coefficient [Mol%][1,6]	P value
Foods (g/d)								
Butter	0.19	< 0.0001	0.16	< 0.0001	0.08	0.07	0.01	0.78
Milk	0.09	0.05	0.07	0.02	0.05	0.22	0.07	0.09
Cheese	0.07	0.09	0.10	0.002	0.03	0.45	-0.02	0.58
Yoghurt	0.10	0.02	0.09	0.004	0.03	0.48	0.00	0.97
Beef[2]	0.20	< 0.0001	0.21	< 0.0001	0.18	< 0.0001	-0.02	0.72
Fish	-0.23	0.00000	-0.13	< 0.0001	-0.04	0.34	0.08	0.07
Oily fish[3]	-0.13	0.01	0.03	0.43	0.09	0.06	0.04	0.46
Nutrients (% of energy)								
Fat from Dairy[4]	0.23	< 0.0001	0.24	< 0.0001	0.18	< 0.0001	0.12	0.01
Fat from butter	0.20	< 0.0001	0.20	< 0.0001	0.20	< 0.0001	0.17	0.0001
Fat from beef[2]	0.22	< 0.0001	0.18	< 0.0001	0.20	< 0.0001	0.03	0.52
Fat from fish	-0.12	0.01	-0.04	0.21	0.02	0.61	0.09	0.04
Fat from oily fish[3]	-0.06	0.17	-0.04	0.29	0.03	0.49	0.07	0.16

[1] Adjustment for fat from dairy and/or fat from beef (as a percent of total energy), total energy intake, age, alcohol intake, body mass index and country, where appropriate

[2] Data for beef not available for Greece (n= 9)

[3] Data for oily fish, including mackerel and salmon, missing for some participants (n= 86)

[4] Dairy products include milk, butter, cheese, yoghurt, dairy puddings and cream

[5] Correlation coefficients are calculated using the logarithm of heptadecanoic acid (C 17:0) and the logarithm of the calibrated intake of food or calibrated observed intake of a nutrient (as a % of energy intake) in order to better approximate a normal distribution

[6] Correlation coefficients are calculated using the molar percent of heptadecanoic acid (C 17:0) and the logarithm of the calibrated intake of food or calibrated observed intake of a nutrient (as a % of energy intake) in order to better approximate a normal distribution

Chapter 7 IGF-I and risk of prostate cancer

7.1 Introduction

7.1.1 Physiology of IGF-I

Insulin-like growth factor-1 (IGF-I) is a single chain polypeptide produced primarily by the liver under hormonal stimulation from pituitary growth hormone (GH). The IGF axis is a complex cell regulation system that comprises two ligands, IGF-I and IGF-II, multiple cell-membrane receptors including IGF-I receptor, insulin receptor, IGF-II receptor and hybrid receptors (formed by an insulin half receptor and an IGF-I half receptor), six IGF binding proteins (IGFBP1-6) and a large group of IGFBP proteases and IGFBP-related proteins.

IGF-I mediates most of the anabolic actions of GH, stimulates cell replication, cell proliferation and cell differentiation and inhibits cell death. In addition to its anabolic effects IGF-I exerts insulin-like metabolic effects via inhibition of lipolysis, uptake of amino acids and glucose and protein synthesis (63). IGF-I is produced throughout life largely under the control of hypothalamic growth hormone-releasing hormone (GHRH) and pituitary growth hormone (GH). Production is mostly in the liver, although it is also produced in other organs (214, 215) and exerts its effects in many tissues both locally, by autocrine (within-cell signalling) and paracrine (cell-to-cell signalling) pathways, and systemically via endocrine pathways (63). Although IGF-I is regulated primarily by growth hormone, endogenous (and exogenous) hormones including estrogens, luteinizing hormone and thyroid hormones and other growth factors such as epidermal growth factor, have important influences on the GH-IGF-I axis (216).

IGF-I is secreted into the circulation without forming a tissue store and the majority (>90%) in circulation is bound to IGFBPs and acid-labile subunits in ternary complexes (63). These complexes prolong the half life of IGF-I from 10 minutes to 15 hours (217) and prevent the hypoglycaemic effects of free IGF-I and regulate its bioavailability at the cellular level (218).

At the cellular level, IGF-I's growth-promoting actions are modulated by binding to IGF-I receptors (219). IGF-I receptors are expressed in many tissues and are characterised by two half receptors: a largely extracellular α chain for binding IGF-I and a trans-membrane β chain that possesses a tyrosine kinase domain (involved in the proliferation and differentiation of normal cells during development). In general, the bioavailability of IGF-I is influenced by IGF binding proteins, of which the most abundant binding protein is IGFBP-3 (220). IGFBP-3 regulates the amount of bio-available IGF-I by competitively inhibiting IGF-I/IGF-I-receptor binding and signalling, although IGFBP proteases cleave IGFBPs into fragments with lower binding affinities thereby helping to make IGF-I available for binding (214, 215, 221). Although IGFBPs are usually inhibitory, they can also enhance the effects of IGF-I by ensuring its slow release and protection from proteolytic degradation and by protecting IGF-I receptors from down regulation resulting from high exposure (222).

In mouse studies using IGF-I null (-/-) and wild-type litter mates, IGF-I has been shown to be essential for the normal development of the prostate gland (223) and *in vitro* studies have demonstrated that IGF-I and IGFBPs are synthesized and IGF-I receptors are expressed in normal prostatic tissue (224). The expression of IGF-IR in prostate epithelium is modulated by androgen signalling through the androgen receptor pathway (225, 226). Androgens have also been shown to inhibit the expression of IGFBPs (227).

7.1.2 Determinants of circulating IGF-I concentration

There is evidence to suggest that circulating IGF-I concentrations are modified by a variety of lifestyle and dietary factors, which include potential risk factors for prostate cancer. While associations between IGF-I and some factors are well established the evidence for others is less consistent. A brief summary is provided here.

7.1.2.1 Age and Sex

Circulating IGF-I concentrations are known to increase from birth, with a sharp peak at puberty, followed by a slow decline with increasing age in both men and women (228). However, men have been observed to have higher IGF-I concentrations then women (228-230), and this difference is evident throughout life (124, 231). A recent longitudinal study of long term changes in concentrations confirmed age-related decreases in IGF-I (131), with a reduction of 5.6 and 5.9ng/mL per year of increasing age in Black and White men, respectively ($P < 0.0001$)(131). However, there was no evidence of racial differences in this trend ($P = 0.65$).

7.1.2.2 Ethnicity

Many studies of the determinants of IGF-I have been conducted in relatively homogenous white European populations. Of studies with mixed ethnic populations, few have been of sufficient sample size to examine associations with IGF-I stratified by ethnicity (125, 229, 232-235). Ethnic heterogeneity in circulating IGF-I and IGFBP-3 concentrations and their molar ratio (i.e. IGF-I/IGFBP3) have been reported in some cross-sectional studies (125, 229, 234, 236, 237), although the results are not consistent (232, 235) and findings may be due to chance. Nonetheless, a longitudinal analysis of age, anthropometric and lifestyle factors in black and white men reported differences in the average concentration of IGF-I across three blood collection periods, with lower concentrations observed in Blacks than Whites (β= -17.51ng/mL; $P<0.0001$)(131).

7.1.2.3 Genetics

Twin studies have suggested that 40% of the variation in IGF-I concentration and 60% of the variation in IGFBP3 concentration is attributable to hereditary factors (238-241). A recent genome wide association (GWAS) study among 10,280 Europeans in mid-to late adult life has identified single nucleotide polymorphisms (SNPs), rs11977526, in the IGFBP3 gene region on chromosome 7p12.3, associated with higher IGFBP-3 concentrations ($P = 3.3 \times 10^{-101}$) and lower IGF-I concentrations (after adjustment for IGFBP-3)($P = 1.9 \times 10^{-26}$). Elevated IGF-I and IGFBP-3 concentrations were also associated with a novel and independent locus (rs700752) on chromosome 7p12.3 ($P = 4.9 \times 10^{-9}$ and $P = 4.4 \times 10^{-21}$ for IGF1 and IGFBP, respectively) (242).

While the biological basis for the observed associations with IGF-I concentrations may be due to the effect on circulating IGFBP-3 concentrations, further work is required to determine the direct or indirect mechanisms that link these SNPs with circulating IGF-I concentrations. Furthermore, the functional role of these genetic loci on IGF-I mediated regulation of cell metabolism and growth is yet to be determined.

7.1.2.4 Anthropometry

Epidemiological studies have investigated IGF-I concentrations in relation to anthropometric measures with inconsistent results. Inverse associations of IGF-I with body mass index (BMI) have been reported in many studies (131, 228, 229, 232, 233, 237, 243-249), although positive associations (230, 250-254) and null associations (124, 231, 255) have also been observed. Furthermore, a nonlinear association between BMI and IGF-I concentration has also been reported in many studies, with higher IGF-I concentrations observed amongst those with a mid-range BMI, compared to those with either low or high BMIs (126, 130, 256-259). It is possible that these discrepancies reflect the narrow range of body mass indexes (BMI) in many of the studies and different cut-points used to define BMI categories (259); with non-linear associations detected in studies with narrow cut-points and no association identified in studies that have used broader cut-points (<25, 25-29 and $\geq$30kg/m^2). It has been suggested that blunted GH secretion–the major determinant of IGF-I -may explain the reduction in IGF-I that is observed in obese and undernourished individuals (256, 260). Lower IGF-I concentrations have also been observed among individuals with hepatic steatosis (fatty liver disease) (261), a condition associated with higher BMI independent of alcohol intake (262).

A small number of randomised controlled trials and intervention studies have evaluated the impact of intentional weight loss on risk for cancer, including an assessment of changes in IGF-I concentrations. However, the results of these studies are not consistent as weight loss is associated with reduced IGF-I concentrations in some studies (263, 264) but not all (265).

7.1.2.5 Lifestyle factors: Physical activity, smoking, and alcohol

There is little evidence for an association between alcohol consumption (124, 252, 254) or smoking (124, 230, 252) and IGF-I concentrations. Similarly, the majority of epidemiological evidence does not suggest an association between physical activity and IGF-I concentrations (124, 228-230, 247, 251, 254) although one large American study reported higher IGF-I concentrations amongst women who engage in regular activity (266).

7.1.2.6 Diet

A positive association has been observed between consumption of dairy products or milk and IGF-I concentrations in several cross sectional analyses (258, 267-271). In particular, higher intakes of total and dairy protein and calcium have been associated with elevated concentrations of IGF-I in a large cross sectional study using EPIC data (65). A 1.8% increment increase in dairy protein (as a percentage of total energy intake) and a 417mg increment increase in calcium intake, was associated with a 2.4 and 3.3% increase in IGF-I concentrations ($p < 0.001$), respectively. Overall there was evidence of a positive trend in IGF-I concentrations

across fifths of total and dairy protein, with a difference between the lowest and highest quintile of 7.7 and 7.2% for each macronutrient (% of total energy), respectively (65). There is little evidence to suggest an association between total, saturated and polyunsaturated fat intake and concentrations of IGF-I (65, 229, 231, 258, 269, 270)or between dietary fibre and IGF-I concentrations (65, 231, 258, 272). IGF-I concentrations have been shown to be lower by approximately 13% in vegans compared with meat eaters (P= 0.0006), which suggests a plant based diet may play a role in lowering IGF-I concentrations (273).

7.1.2.7 Calorie restriction and catabolic states

Animal studies have shown 20-50% reductions in circulating IGF-I concentrations following restriction of maintenance energy requirements (between 42 and 50%reduction in calories) for 2 weeks, although following re-feeding ad-libitum for 2 weeks IGF-I concentrations normalised (274, 275). Data from human studies have also shown decreased IGF-I following starvation and sepsis, which are both characterised by GH insensitivity (276). Furthermore, in both animal and human studies dietary protein restriction, independent of reduction in energy intake, is associated with reduced IGF-I concentrations (277, 278) suggesting that protein intake is a strong determinant of IGF-I levels. This finding is supported by findings from epidemiological studies of strong correlations between dietary total and dairy protein and IGF-I concentrations (discussed in section 7.1.2.6).

7.1.3 Mechanisms by which IGF-I may promote cancer

Malignant transformation involves enhanced cell survival and proliferation and the capacity for aberrant cells to escape from cell cycle arrests and apoptotic mechanisms that normally function to terminate their activity (279). IGF-I has been shown to contribute to these transformative processes through increased production, increased IGF-I receptor activity (through IGF-I binding) and decreased IGFBPs to competitively inhibit IGF-I /IGF-I receptor binding.

In mouse models, *lid* mice with a deletion of liver IGF-I have 75% lower circulating IGF-I concentrations than control mice and are resistant to induction of mammary tumours and colon tumours (280, 281). Furthermore, mice fed a restricted calorie diet experienced decreased IGF-I production and reduced incidence and growth of tumours. However replacement of IGF-I in calorie restricted mice deficient in the tumour suppressor gene p53 reversed the effects of calorie restriction (282). Over-expression of IGF-I receptors has also been shown to increase tumour development in the pancreas and mammary glands of mice while IGF-I receptor null mice were observed to have resistance to carcinogenesis (283-285).

In vitro studies have shown increased IGF-I production following reduced oxygen delivery to tumours (which occurs when fast growing tumours outgrow their blood supply). Furthermore, IGF-I has been shown to induce expression of hypoxia inducible factor 1α, which up-regulates vascular endothelial growth factor (VEGF), integral to angiogenesis. Induction of VEGF has been demonstrated in cancers of the colon, lung and thyroid (286-288).

In brief, mechanistic studies have shown that activation of IGF-I receptors and hybrid receptors leads to phosphorylation of insulin receptor substrate 1 which propagates the signal via phosphoinositide 3 kinase and mitogen- phosphorylation at other cytoplasmic tyrosine residues to increase the metastasising capacity of tumour cells (289). Although the effect of binding proteins on IGF-I remains unclear, it is hypothesized that the binding action of IGF-BP2 may serve to provide a continuous bio-available source of IGF-I in the tumour microenvironment (290).

In summary, IGF-I has been shown to influence tumour growth and metastases through its effect on cell proliferation, inhibition of cell death and angiogenesis. Whether IGF-I has a role in proliferation of early cancers or the initial transformation of disease is not yet understood. Given that IGF-I receptor activation (by IGF-I) stimulates pro-survival signalling pathways it is conceivable that high levels of IGF-I in the cellular micro-environment may tip the balance toward the survival of damaged cells that represent a malignant clone (291).

7.1.4 Evidence that circulating IGF-I is involved in prostate cancer progression

7.1.4.1 Experimental studies

Over expression of IGF-I in basal epithelial cells has led to the development of intraepithelial neoplasia in transgenic mice, with hyperplasia of the prostate observed in mice aged 2-3 months and atypical hyperplasia and prostatic intraepithelial neoplasia by 6-7 months of age(292, 293). Other *in vivo* studies have shown up-regulation of both IGF-I and IGF-I receptors during progression to androgen independent growth of LNCaP and LAPC-9 tumours (294). This finding is supported by studies that have demonstrated up-regulated IGFI receptor protein expression and messenger ribonucleic acid (mRNA) in prostate cancer tissue (295, 296). Furthermore, i*n vitro* studies have reported that inhibition of the IGF-I receptor in prostate cancer human xenograft models result in decreased growth in androgen independent and androgen dependent tumours(297). Mice models have also demonstrated substantial reductions in tumour load with reduction of IGF-I receptor activity (298).

Studies have consistently shown that androgen signalling through the androgen receptor result in increased IGF-I receptor protein expression in prostate epithelium, which is associated with increased IGF-I receptor phosphorylation and increased cell proliferation. The capacity of IGF-I signalling to potentiate the transcriptional activity of the androgen receptor in the absence of androgens suggests that the IGF system, in particular the IGF-I receptor, is important for progression of androgen independent prostate cancer (299, 300).

Further evidence of the role of IGF-I in prostate cancer comes from observations of links between genetic variation in the IGF-I gene and risk for prostate cancer. In a large investigation among 2320 patients with prostate cancer and 2290 controls, modest statistically significant elevations in relative risk (Odds ratios ranging from 1.19–1.25; 95% CI ranging from

1.03 – 1.50) were observed for each of four haplotypes[1], which supports a role for inherited variation in IGF1 in the risk of prostate cancer(301).

7.1.4.2 Epidemiological studies

In the past decade numerous prospective cohort studies have used nested case control design studies to examine the possibility that concentrations of IGF-I predict risk of future cancer development. A comprehensive list of the results of published prospective studies of the association of IGF concentrations with risk for prostate cancer is provided in **Table 7.1** (on page 84). IGF-I has been associated with an increased risk for prostate cancer in a pooled reanalysis of worldwide prospective data based on 3,700 men with prostate cancer and 5,200 controls (64). However, most of the cases in this collaborative re-analysis were diagnosed within 6 years of blood collection, limiting the ability to investigate heterogeneity in risk by length of follow-up and leaving open the possibility of reverse causality.

7.2 Aim of study

The aim of this work is to investigate the association between IGF-I concentration and subsequent risk of prostate cancer over the long-term in the European Prospective Investigation into Cancer and Nutrition (EPIC). The EPIC study previously reported a marginally significant increased prostate cancer risk for men in the highest versus lowest third of IGF-I concentration (OR 1.35; 95%CI 0.99 - 1.82; P trend = 0.08) based on 630 cases (and 630 controls), with a median time from blood collection to diagnosis of 3.4 years (range, 0.1 – 8.5 yrs) (12). The results presented in this chapter are an extension of work in EPIC, using a nested case-control design with a total of 1,542 prostate cancer cases (matched with 1,542 control men), with a median time from blood collection to diagnosis of 5.8 years (range 0.1 – 12.8 yrs), with approximately 35% of cases diagnosed more than 7 years after blood collection.

7.3 Methods

7.3.1 Study population

The methods of recruitment used in the EPIC study have been described in chapter 2. In brief, between 1992 and 2000, approximately 360,500 women and 153,400 men were recruited from 23 centres in ten European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom). All participants completed either a standardised food-frequency questionnaire or an interview-based dietary questionnaire, and a detailed lifestyle questionnaire. Approximately 385,700 participants (of which 139,600 were men) provided a blood sample. All participants provided written informed consent and local ethics committees in the participating countries and the Internal Review Board of the International Agency for Cancer Research (IARC) approved the study.

[1] A set of closely linked genetic markers present on one chromosome which tend to be inherited together.

The standardised blood collection protocol involved transportation of whole blood in the collection syringe to a local laboratory where samples were processed and separated into serum, plasma, red cells and buffy coat fractions within 24 hours of collection, prior to placement in 0.5 mL straws for storage at -196°C in liquid nitrogen tanks. Variation to the standardised procedure occurred in the following centres: in the Oxford cohort (UK), samples were sent in the post at ambient temperature to a laboratory in Norfolk (UK), with an average transit time of 1.5 days; in the Copenhagen cohort (Denmark), samples were stored in 1 mL tubes in nitrogen vapour at -150°C, and in the Umeå cohort (Sweden), EDTA plasma (rather than serum) samples were archived and stored at -80°C in electric freezers.

7.3.2 Follow-up for cancer incidence and vital status

Follow-up and case identification was provided through record linkage with population-based cancer registries in Denmark, Italy, the Netherlands, Spain, Sweden and the United Kingdom. In Germany and Greece active follow-up was achieved through contact with study participants and their next of kin and verified through health insurance or medical records and cancer and pathology registries or reports. The sub-cohorts of France and Norway were not included as these countries only recruited women. Prostate cancer cases were defined as code C61 of the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). For each EPIC centre closure dates of the study period were defined as the latest date of complete follow-up for both cancer incidence and vital status: December 2003 for Denmark and the Netherlands, December 2004 for Italy and the United Kingdom, December 2005 for Spain and Sweden and December 2006 for Germany and Greece.

7.3.3 Selection of case and control participants from the study cohort

Cases were defined as men who developed prostate cancer after the date of blood collection and before the end of the study period, as determined by the latest date of follow-up in each study centre. Among participants who had completed a lifestyle and dietary questionnaire, provided a blood sample at recruitment and had no previous cancer diagnosis, 1,874 were diagnosed with incident prostate cancer during the follow-up period. An incidence density sampling protocol was used to select control participants at random from the cohort of men who were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case and who were matched on study centre, length of follow-up, age at blood collection (+/- 6 months), time of blood collection (+/- 1 hour) and duration of fasting at blood collection (< 3, 3 - 6, > 6 hours). Blood samples were available for 1,711 of the 1,874 identified cases, and after exclusion of incomplete case-control sets, 1,542 prostate cancer cases and 1,542 matched controls were available for analysis. This number includes 630 prostate cancer cases from an earlier study (phase 1) and an additional 912 cases with a later diagnosis (phase 2).

Tumour stage information (TNM staging code), derived from pathology reports and cancer registry data, was used to define the following categories: localised (T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised; n = 745), advanced (T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic; n = 309), and unknown stage (n = 488). Information on Gleason score or the World Health Organization

(WHO) grading system was used to generate categories of low grade (Gleason score ≤7, or cases coded as well differentiated or moderately differentiated; n = 848), high grade (Gleason score >7, or cases coded as poorly differentiated or undifferentiated; n = 159), and unknown grade (n = 535).

7.3.4 Laboratory analysis

Serum samples from phase one of the study were assayed in singleton at IARC, Lyon (France) using the DSL-10-5600 ACTIVE® enzyme-linked immunosorbent assay (ELISA) from Diagnostic Systems Laboratories (DSL) (Webster, Texas) as previously reported (3). Serum samples from phase two were assayed in duplicate at the Cancer Epidemiology Unit (CEU) laboratory in Oxford (UK) using the DSL-10-5600 ACTIVE® ELISA from DSL, with the exception of plasma samples from Sweden, which were analysed in singleton by using the automated IDS-iSYS immunoassay system from Immunodiagnostic Systems (IDS) Ltd (Boldon, UK). Both assays included a step to dissociate IGF-I from IGF-I binding proteins prior to measurement. A comparison of 20 samples measured using both the DSL-10-5600 ACTIVE® ELISA and the IDS-iSYS showed relative agreement (r = 0.99), although IGF-I concentrations were on average 23% lower in the IDS-iSYS assay than the DSL-10-5600 ACTIVE® ELISA. The lower limit of detection was <1 nmol/L for the DSL-10-5600 ACTIVE® ELISA and 1.3 nmol/L for the IDS-iSYS immunoassay, adequate to detect the lowest concentration in all study samples.

Laboratory personnel were blind to the case-control status of the samples and each case-control set was analysed in the same batch, together with duplicate quality control samples. For the DSL-10-5600 ACTIVE® ELISA assay, the mean intra- and inter-batch coefficients of variation were 3% and 13% for phase 1 samples and 4.4% and 12.4% for phase 2 samples, respectively; for the IDS-iSYS immunoassay the mean intra- and inter-batch coefficients of variation were 2.3% and 3.2% for phase 2 samples (from Sweden only).

7.3.5 Statistical analysis

Differences in baseline characteristics of case and control participants in matched sets were compared using paired-sample t-tests for continuous variables and conditional logistic regression for categorical variables. Logarithmic transformation of IGF-I values was performed to approximate a normal distribution and box and whisker plots and letter-value displays were used to examine the distribution and to identify possible outliers. Exclusion of observations identified as severe outliers on the logarithmic scale (>3 times the inter-quartile range; n=4) made no material difference to the risk estimates and therefore all observations were included in the final models.

Analysis of variance was used to examine heterogeneity in the geometric mean IGF-I concentration amongst controls by the following variables: phase of study (phase 1, phase 2), country (8), age at blood collection (≤54, 55-59, 60-64, ≥65 years), height (quintiles), weight (quintiles), body mass index (BMI; <24.4, 24.4-26.3, 26.4-28.6, ≥28.7 kg/m^2), smoking (never, past, current, unknown), alcohol consumption (<8, 8-15, 16-39, ≥40 g/day, unknown), physical activity (inactive, moderately inactive, active, unknown), marital status (married/cohabitating,

not married/cohabitating, unknown) and educational attainment (primary or equivalent, secondary, degree level, unknown). Where appropriate, adjustment was made for centre, age at blood collection, alcohol consumption, educational attainment, BMI and assay batch.

Conditional logistic regression models were used to calculate the odds ratios (ORs) for prostate cancer risk by fourths of IGF-I concentration and for a doubling (log2 scale) in IGF-I concentration. Quartile cut-points were defined by the distribution of values among the controls for all cohorts combined with the exception of Sweden, where country-specific cut-points were used to account for the differences in absolute concentrations attributable to the IDS-iSYS immunoassay. Likelihood ratio chi-square tests for linear trend were obtained by replacing the quartiles with the logarithm of IGF-I as a continuous variable in the model.

The effects of potential confounding variables (other than those accounted for in the matched design) were examined by including additional regression terms in the conditional logistic regression model. Adjustment for height, weight, smoking, alcohol consumption, physical activity, marital status and educational attainment made little difference to the risk estimates or their 95% confidence intervals (<10% change). However, all final models included adjustment for age at blood collection to ensure close comparability within matched sets. Separate sensitivity analyses were performed that excluded the Umeå (Sweden) cohort (due to the different assay used for these samples), and that excluded the first four years of follow-up.

Likelihood ratio chi-square tests (based on the difference between the chi-square values from models with and without an interaction term between the linear trend variable and the factor of interest, e.g. stage of disease) were used to examine heterogeneity of linear-trend relative risk estimates for prostate cancer associated with the logarithm of IGF-I concentration by prostate cancer stage (localised, advanced), grade (low, high), time between blood collection and diagnosis (<4, 4-7, >7 years), age at diagnosis (<65, ≥65 years), age at blood collection (<60, ≥60 years), BMI (<25, ≥25 kg/m²), laboratory assay (DSL-10-5600 ACTIVE® ELISA, IDS-iSYS), country (including countries grouped according to a North/South latitude (i.e. Northern countries defined as Denmark, Germany, the Netherlands, Sweden and the UK, and Southern countries defined as Greece, Italy and Spain)).

Owing to the use of incidence density sampling for the selection of controls, the odds ratios presented approximate the incidence rate ratios for IGF-I in relation to prostate cancer in the study cohort (176). All statistical analyses were performed using Stata Statistical Software: Release 12 (College Station, TX, USA: StataCorp LP) and two-tailed tests of statistical significance with p values below 0.05 were considered significant.

7.4 Results

IGF-I measurements were available for 1,542 matched case-control sets: 630 from phase 1 and 912 from phase 2. Of these, 152 were from Denmark, 383 from Germany, 40 from Greece, 145 from Italy, 50 from the Netherlands, 200 from Spain, 192 from Sweden and 380 from the

UK. The mean age at diagnosis was 65.3 years (SD 6.5) and the median time between blood collection and diagnosis was 5.8 years (range: 1 month – 12.8 years). As shown in **Table 7.2**, there were no significant differences between cases and controls according to height, BMI, smoking status, alcohol consumption, physical activity, marital status and educational attainment. However, cases were on average slightly older at blood collection (60.12 versus 60.10 years; $P = 0.03$) and lighter (79.8 versus 80.6 kg; $P = 0.04$) than controls. The geometric mean IGF-I concentration was 3.8% higher in cases than in controls (20.5 versus 19.8 nmol/L; $P = 0.001$). Amongst the cases with information on stage and grade of disease, there was no evidence of heterogeneity by phase of study in the proportion of cases diagnosed with localised or advanced stage disease or in the proportion of cases classified as low or high grade disease (see **Table 7.2;** page 86).

The geometric mean IGF-I concentrations according to baseline characteristics amongst the controls, with adjustment for centre, age at blood collection, alcohol intake, educational attainment and batch, are shown in **Table 7.3**. IGF-I concentration was lower in older men ($P_{trend} <0.0001$) and higher in taller ($P_{trend} = 0.01$) and more educated men ($P_{trend} = 0.01$). There was significant heterogeneity in IGF-I concentration by assay for the IDS-iSYS assay used for Umeå (Sweden) and DSL-10-5600 ACTIVE® ELISA assay used for all other centres ($P_{heterogeneity} < 0.0001$), and by country (among those assayed using the DSL-10-5600 ACTIVE® ELISA), being lowest in Greece (19.5 nmol/L), and highest in the Netherlands (22.8 nmol/L) ($P_{heterogeneity} = 0.002$). There was also evidence of heterogeneity in IGF-I concentration by alcohol consumption ($P_{heterogeneity} = 0.01$), weight ($P_{heterogeneity} = 0.01$) and BMI ($P_{heterogeneity} = 0.003$), although there was no evidence of a trend for these variables ($P_{trend} = 0.08$, $P_{trend} = 0.40$ and $P_{trend} = 0.34$, respectively). IGF-I concentration did not vary according to smoking status or physical activity.

Table 7.4 shows that IGF-I concentration was significantly associated with an increased risk of prostate cancer overall. In comparison to the lowest fourth, the risk of prostate cancer in the highest fourth of IGF-I concentration was 1.69 (95% CI: 1.35, 2.13; $P_{trend} = 0.0002$) after adjusting for age at blood collection. Further adjustment for height, weight, smoking, alcohol intake, marital status, physical activity and educational attainment made little difference to the risk estimate (OR 1.72 (95% CI: 1.36, 2.17); $P_{trend} = 0.0001$; results not shown in table). The odds ratio associated with a doubling of IGF-I concentration was 1.38 (95%CI: 1.17, 1.64); $P_{trend} = 0.0002$).

The associations between IGF-I concentration and risk for prostate cancer subdivided by case participant characteristics are also shown in **Table 7.4**. Higher IGF-I was associated with increased risk for prostate cancer in all subgroups, with the exception of high grade disease. Nonetheless, there was no significant heterogeneity between low and high grade disease in the estimates of linear trends in relative risks. Furthermore, the positive association of higher circulating IGF-I with prostate cancer risk did not differ according to duration of follow-up (ORs for highest vs. lowest quartile for cancer diagnosis <4, 4-7 and >7 years after blood collection were 2.01 (1.35 - 2.99), 1.37 (0.94 - 2.00) and 1.80 (1.17 - 2.77), respectively (P heterogeneity = 0.77)) or by any other subgroup examined, including stage of disease, age at diagnosis, age at

blood collection, assay type, country (including countries grouped according to a North/South latitude), or BMI ($P_{heterogeneity} \geq 0.05$).

There was no evidence of heterogeneity in the trend for the association of IGF-I and risk for prostate cancer between the Umeå cohort (Sweden) and the other centres ($P_{heterogeneity} = 0.46$) and exclusion of case-control sets from Umeå changed the risk estimate by less than 10%.

7.5 Discussion

Our finding that a relatively high concentration of IGF-I is associated with an increased risk for prostate cancer is consistent with results from a pooled re-analysis of individual participant data from 12 prospective studies (which includes phase 1 of this study) (64). This association is evident for both localised and advanced disease and exists over the short and long term. While it is likely that some men with sub-clinical disease at recruitment were included in our study (due to the long lag time between disease onset and clinical symptoms (95)), the positive association observed among men diagnosed with advanced stage disease (for whom nearly 65% were diagnosed more than four years after recruitment) and among men diagnosed more than seven years after blood collection, supports the hypothesis that IGF-I is associated with prostate cancer many years before diagnosis.

IGF-I concentration was also associated with an increased risk of localised prostate cancer. This finding contrasts with those from ProtectT, a large case-control study of approximately 3000 cases and 3000 controls in the UK, which found no association between total IGF-I concentration and the risk for PSA-detected prostate cancer (302). As there is no formal screening programme for prostate cancer in the countries included in the EPIC study, our cases comprise a mixture of PSA detected and clinically-detected disease, and it is possible that the differences between our results and those from ProtecT (and other studies of screen-detected prostate cancer (302)) reflect biological differences in the association of IGF-I with PSA-detected and clinically relevant disease.

The finding that the association between higher IGF-I concentration and prostate cancer risk did not differ significantly by cancer stage is consistent with results from the pooled re-analysis (64) and supports a role for IGF-I in the progression of prostate cancer, conceivably via established mitogenic and anti-apoptotic mechanisms (279). There was no significant difference in the association of IGF-I with prostate cancer by grade of disease, although the risk appeared to be restricted to low-grade disease, with no association for high grade disease, which is also consistent with the pooled re-analysis (64) and more recent findings from a large prospective study (303). However, these results should be interpreted with caution due to small numbers of cases in the high grade subgroup and the potential for misclassification error due to changes in grading practices over time (104-106) that may obscure the distinction between low and high grade disease.

7.5.1 Strengths of the study

Strengths of this study are the prospective nature of the design, the moderately long length of follow-up and large sample size which meant that the sub-group investigations were, in most instances, adequately powered to detect modest risk estimates. The association between IGF-I and risk for prostate cancer remained significant after excluding the first four years of follow-up (thereby reducing the possibility of reverse causality) and the sub-cohort with deviations from the standardised assay method. There was minimal laboratory measurement error and the distribution of our IGF-I values amongst controls is comparable with that from other studies in healthy male adults (125, 302). Previous studies have shown relatively high within-individual reproducibility for IGF-I measured in two samples collected up to 5 years apart (Spearman rank correlations of 0.7 (127). 0.66(304) and 0.87(305)), however measurement error and attenuation of risk estimates associated with a single sample would suggest that the true association per increment increase in IGF-I concentration with prostate cancer risk may be substantially stronger than that observed in our study.

7.5.2 Limitations of the study

Comparisons of our findings based on men of white European origin with those in other ethnic groups are difficult to interpret as studies to date in Asian and African-American populations have been too small to detect modest risk estimates (306, 307). We did not measure IGF-I binding proteins (IGFBPs) in our extended study and although they are known to influence the bioavailability of IGF-I (63), there is evidence to suggest that the association between IGF-I concentration and risk for prostate cancer is not materially affected by adjustment for IGFBP-3, the major IGF-I binding protein in the circulation (64). The extent to which circulating IGF-I concentrations (influenced by anthropometric, diet and lifestyle factors (65, 216, 258, 273)) reflect activity at the tissue level is difficult to determine, as the bio-availability of IGF-I is modulated by interactions between growth hormone, insulin, IGFBPs and IGF-I receptors (63, 308). In future, investigation of the association between circulating IGF-I concentrations and expression of biomarkers at the tissue level may determine further the nature of the association of IGF-I with prostate cancer risk. Moreover, information from studies in a younger population is needed to determine the impact of long term exposure to IGF-I levels and to elucidate the most important time period of IGF-I exposure in relation to prostate cancer development.

7.6 Conclusion

In conclusion, our results suggest that circulating concentrations of IGF-I in middle to late adulthood are strongly associated with subsequent prostate cancer risk over the relatively long term.

Table 7.1 Prospective studies of circulating IGF-I concentration and risk of prostate cancer

Author, Year, Study	Design	Number (case/control)	Blood	IGFI assay	Matched	Adjustments	OR/RR for IGF-I	95% CI	P trend
Chan, J; 1998 Physicians Health Study, USA	NCC	152/ 152	EDTA	ELISA	smoking, duration of follow-up and age (+/-1yr)	unadjusted adjusted for height, weight, BMI, other hormones	highest vs lowest quartile 2.41 highest vs. lowest quartile 4.32	(1.23 -4.74) (1.76 -10.6)	0.006 0.001
Schaefer, C; 1998 KPMCP, USA	Population based cohort study	45/720	serum	RIA	-	adjusted for age	highest vs lowest quartile 0.81	(0.36 -1.81)	0.74
Harman, S; 2000 Baltimore Longitudinal Study on Ageing, USA	NCC	72/127	serum	RIA	age (+/-2yrs), duration follow-up	adjusted for age and visit date	highest vs lowest tertile 3.11	(1.11 -8.74)	< 0.05
Stattin, P; 2000 Northern Sweden Health and Disease Cohort Study, Sweden	population based intervention study	149/298	EDTA	IRMA	age (+/-1 yr), date of completion of questionnaire (+/-1yr) & place of residence	adjusted for age and fasting status adjusted for age, fasting, BMI & smoking	highest vs lowset quartile 1.57 highest vs lowest quartile 1.72	(0.88 -2.81) (0.93 -3.19)	0.02 0.006
Lacey, J; 2001 Washington County Sperm Bank, USA	Population based cohort study	30/60	serum	ELISA	age (+/-1yr), race and date of blood draw	adjusted for age at blood collection	highest vs lowest quartile 0.7	(0.2 -2.3)	0.5
Chan, J; 2002 Physicians Health Study, USA	NCC	379/383	serum	ELISA	age and smoking status	adjusted for IGFBP-3	highest vs lowest quartile (advanced stage) 5.1	(2.0 -13.2)	0.002
Woodson, K; 2003 ATBC, USA	RCT	100/400 freq matched	serum	ELISA	age (+/-5yrs), intervention group and time between blood draw (+/-6months)	adjusted for age, intervention group and BMI	highest vs lowest quartile 0.52	(0.23 -1.16)	0.16
Oliver, S; 2004 PROTECT, UK	NCC within RCT	176/324	serum	ELISA	age (+/-2 yrs), recruitment centre and date of recruitment	unadjusted adjusted for smoking and IGFBP-3	highest vs lowest quartile 2.34 highest vs lowest quartile 3.00	(1.26 -4.34) (1.50-6.01)	0.02 0.005
Stattin, P; 2004 Northern Sweden Health and Disease Cohort Study	NCC	281/560	EDTA	IRMA	age (+/-6months) and date of recruitment (+/-2 months)	adjusted for age	highest vs lowest quartile 1.67 highest vs lowest quartile (advanced stage) 2.87	(1.02 -2.71) (1.01 -8.12)	0.05 0.1
Chen, C; 2005 Cardiovascular Health Study	Population based cohort study	174/174	plasma	IRMA	age (+/-4yrs), race, year of recruitment and year blood collection	Unadjusted (but matched) Adjusted insulin, BMI & IGFBP-3	highest vs lowest quartile 0.67 highest vs lowest quartile 0.77	(0.37 -1.25) (0.33 -1.84)	0.2 0.45

Abbreviations: NCC = nested case control study; RCT = random ised controled trial; BMI refers to body mass index; ELISA = Enzyme-linked immunosorbent assay; EDTA = Ethylenediaminetetraacetic acid; IGF-I = insulin like growth factor-I; IGFPB-3 = nsulin like growth factor binding protein-3; RIA = radioimmunoassay; IRMA = immunoradiometric assay; PSA = prostate specific antigen.

Table 7.1(continued) Prospective studies of circulating IGF-I concentration and risk of prostate cancer

Author, Year, Study	Design	Number (case/control)	Blood	IGF I test	Matched	Adjustments	OR/RR for IGF-I	95% CI	P trend
Meyer, F; 2005 SU.VI.MAX trial	Popn based RCT	100/400		immunoassay	age	unadjusted adjusted intervention. IGFs, smok, alcohol, BMI	highest vs lowest quartile 1.34 highest vs lowest quartile 1.8	(0.68 -2.65) (0.76 -4.27)	0.31 0.13
Platz, E; 2005 Health Professionals Follow-up Study, USA	NCC	462/462		ELISA	age, time of day, year, season of blood collection and PSA	unadjusted adjusted for IGFBP-3	highest vs lowest quartile 1.37 highest vs lowest quartile 1.17	(0.92 -2.03) (0.69 -1.99)	0.05 0.29
Morris, JK; 2006 British United Provident Association (BUPA), UK	NCC	141/423	serum	ELISA	age and duration of blood storage	-	highest vs lowest quartile 1.37	(0.76 -2.49)	0.62
Severi, G; 2006 Melbourne Collaborative Cohort Study, Australia	Case-cohort design	524/1826	plasma	ELISA	-	adjusted for left censoring at the age at baseline	highest vs lowest quartile 1.07	(0.79 -1.46)	0.5 0.008
Weiss, J; 2007 Prostate, Lung, Colorectal & Ovarian Cancer Screening Trial (PLCO), USA	NCC in RCT	727/887	Serum	ELISA	age at entry, time since initial screen and year of blood collection	unadjusted but matched matched and adjusted for IGFBP-3	highest vs lowest quartile 1.14 highest vs lowest quartile 1.12	(0.86 -1.51) (0.79 -1.6)	0.18 0.28
Allen, N; 2007 European prospective Investigation into cancer and nutrition (EPIC)	NCC	630/630	serum	ELISA	study center, age at recruitment(+/-6mnths), time of day and duration of fasting at blood collection	unadjusted adjusted for IGFBP-3	highest vs lowest tertile 1.35 highest vs lowest tertile 1.39	(0.99 -1.82) (1.02 -1.89)	0.08 0.12
Bonugian, M ; 2008 The Multiethnic Case-Control Study, USA	NCC	96/416	serum	ELISA	ethnicity, region and 5 year age category	-	highest vs lowest quartile 1.26	(0.66 -2.41)	> 0.05
Mucci, L ; 2010 Physicians Health Study	NCC	545/545	serum	ELISA (free IGF-I)	age (+/-1 yr or 5 yrs) and smoking status	adjusted age, smok, height and BMI	highest vs lowest quartile 0.9	(0.6 -1.3)	0.78
Pham , X; 2010 Japan Collaborative Cohort Study (JACC), Japan	NCC	914/2739 incident cases	serum	IRMA	study area, gender and age	unadjusted 1st model:adjusted BMI, smok, alcohol 2nd model:adjusted also for IGF-II and IGFBP-3	highest vs lowest quartile 0.86 highest vs lowest quartile 0.86 highest vs lowest quartile 0.93	(0.69 -1.08) (0.68 -1.08) (0.70 -1.27)	0.08 0.07 0.5
Gill, K; 2010 The Multiethnic Cohort (MEC), USA	NCC	467/934	serum	ELISA	study location, ethnicity, age at blood collection, date and time of day of blood collection and fasting status	adjusted BMI, family istory, years of education and time fasted at blood collection	highest vs lowest quartile 1.23	(0.85 -1.78)	0.62
Nimptsch, K; 2011 Health Professionals Follow-up Study, USA	NCC	1,331/1,331	plasma	ELISA	year of birth (+/-1yr), PSA test before blood draw, time of blood draw (12am -9am , 9am -12pm , 12-4pm , 4-12am), season (Wint, Spr, Sum , Fall) and year(exact)	unadjusted adjusted for IGFBP-3	highest vs lowest quartile 1.41 highest vs lowest quartile 1.23	(1.12-1.78) (0.92-1.64)	0.001 0.06

Abbreviations: NCC= nested case control study; RCT= random ised controled trial; BMI refers to body mass index; ELISA= Enzyme-linked immunosorbent assay; EDTA= Ethylenediaminetetraacetic acid; IGF-I= insulin like growth factor-I; IGFPB-3= nsulin like growth factor binding protein-3; RIA= radioimmunoassay; IRMA= immunoradiometric assay; PSA= prostate specific antigen.

Table 7.2 Characteristics of 1,542 prostate cancer cases and 1,542 control subjects in EPIC overall and subdivided into study phase

	Overall			Phase 1			Phase 2		
	Cases	Controls	*P* value[2]	Cases	Controls	*P* value[2]	Cases	Controls	*P* value[2]
	n= 1,542	n= 1,542		n= 630	n= 630		n= 912	n= 912	
Age at blood collection; yrs(mean); [SD]	60.12 [6.5]	60.10 [6.5]	0.03	61.5 [6.2]	61.4 [6.2]	0.07	59.2 [6.5]	59.2 [6.5]	0.23
Anthropometry: mean [SD]									
Height (cm)	173.0 [6.9]	173.2 [6.9]	0.29	172.2 [6.7]	172.5 [7.0]	0.43	173.5 [7.0]	173.9 [6.9]	0.46
Weight (kg)	79.8 [11.4]	80.6 [12.1]	0.04	79.3 [11.2]	80.6 [12.2]	0.05	80.1 [11.5]	80.7 [12.1]	0.30
BMI (kg/m²)	26.7 [3.5]	26.9 [3.6]	0.1	26.8 [3.5]	27.1 [3.7]	0.09	26.6 [3.4]	26.7 [3.6]	0.45
Smoking; n [%] [1]									
Never	518 [34.2]	489 [32.2]		197 [31.5]	168 [27.0]		321 [36.3]	321 [35.8]	
Former	662 [43.7]	640 [42.2]		282 [45.1]	281 [45.2]		380 [42.8]	359 [40.1]	
Current	334 [22.1]	389 [25.6]	0.06	147 [23.5]	173 [27.8]	0.12	187 [21.1]	216 [24.1]	0.27
Alcohol consumption; (g/d); n [%] [1]									
< 8	621 [40.3]	623 [40.5]		223 [35.5]	223 [35.5]		398 [43.6]	400 [43.9]	
8-15	301 [19.6]	311 [20.2]		121 [19.3]	134 [21.3]		180 [19.7]	177 [19.4]	
16-39	354 [23.0]	370 [24.0]		169 [26.9]	155 [24.7]		185 [20.3]	215 [23.6]	
≥40	264 [17.1]	236 [15.3]	0.57	115[18.3]	116 [18.5]	0.72	149 [16.3]	120 [13.2]	0.13
Physical activity; n [%] [1]									
Inactive	245 [18.4]	221 [16.6]		124 [20.0]	107 [17.3]		121 [17.1]	114 [16.1]	
Moderately inactive	425 [32.0]	415 [31.2]		218 [35.1]	199 [32.1]		207 [29.2]	216 [30.5]	
Active	660 [49.6]	693 [52.1]	0.25	279 [44.9]	314 [50.7]	0.06	381 [53.7]	379 [53.5]	0.86
Marital status; n [%] [1]									
Married or cohabitating	1023 [88.0]	1030 [88.7]		391 [87.5]	396 [89.2]		632 [88.4]	634 [88.4]	
Not married or cohabitating	139 [12.0]	131 [11.3]	0.60	56 [12.5]	48 [10.8]	0.39	83 [11.6]	83 [11.6]	1.00
Educational level; n [%] [1]									
Primary or equivalent	561 [38.1]	579 [39.3]		232 [38.7]	231 [39.0]		329 [37.7]	348 [39.5]	
Secondary	552 [37.5]	556 [37.7]		215 [35.8]	224 [37.8]		337 [38.6]	332 [37.6]	
Degree	360 [24.4]	339 [23.0]	0.55	153 [25.5]	137 [23.1]	0.54	207 [23.7]	202 [22.9]	0.49
IGF-I concentration (nmol/L)									
Geometric mean [95% CI][3]	20.5 [20.2, 20.9]	19.8 [19.5, 20.1]	0.001	21.9 [21.2, 22.5]	21.3 [20.7, 21.9]	0.23	19.7 [19.4, 12.0]	18.8 [18.5, 19.1]	< 0.0001
Country; n [%]									
Denmark	152 [9.9]	152 [9.9]		79 [12.5]	79 [12.5]		73 [8.0]	73 [8.0]	
Germany	383 [24.8]	383 [24.8]		186 [29.5]	186 [29.5]		197 [21.6]	197 [21.6]	
Greece	40 [2.6]	40 [2.6]		9 [1.4]	9 [1.4]		31 [3.4]	31 [3.4]	
Italy	145 [9.4]	145 [9.4]		60 [9.5]	60 [9.5]		85 [9.3]	85 [9.3]	
Netherlands	50 [3.2]	50 [3.2]		25 [4.0]	25 [4.0]		25 [2.7]	25 [2.7]	
Spain	200 [13.0]	200 [13.0]		93 [14.8]	93 [14.8]		107 [11.7]	107 [11.7]	
Sweden	192 [12.5]	192 [12.5]		-	-		192 [21.1]	192 [21.1]	
United Kingdom	380 [24.6]	380 [24.6]		178 [28.3]	178 [28.3]		202 [22.2]	202 [22.2]	
Cases only; n [%]									
Time to diagnosis (years)									
< 4	457 [29.6]			374 [59.4]			83 [9.1]		
4-7	560 [36.3]			228 [36.2]			332 [36.4]		
> 7	525 [34.1]			28 [4.4]			497 [54.5]		
Age at diagnosis, years; mean [SD]	65.3 [6.5]			64.4 [6.2]			65.9 [6.6]		
Stage; n [%] [1,4]									
Localised	745 [71.7]			303 [68.7]			442 [72.1]		
Advanced	309 [29.3]			138 [31.3]			171 [27.9]		
Grade; n [%] [1,5]									
Low	848 [84.2]			385 [84.6]			463 [83.9]		
High	159 [15.8]			70 [15.4]			89 [16.1]		

[1] Unknown for some participants; percentages exclude unknown values

[2] *P* values relate to likelihood ratio chi-square tests of association for categorical variables, and paired differences tests for continuous variables

[3] Adjusted for age (< 50, 50 - 54, 55 - 59, 60 - 64, 65 - 69, ≥70 years), centre (1-18), batch (1 - 78), alcohol intake (< 8, 8-15, 16-39, ≥40 g/ day, unknown), educational attainment (primary school, secondary school, higher education/degree, unknown) and body mass index (quartile cut-points: < 24.4, 24.4-, 26.4-, 28.7- kg/m²); with 95% CI in parenthesis

[4] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic

[5] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system

Table 7.3 Adjusted geometric mean IGF-I concentrations and 95% CI among 1,542 control participants according to baseline characteristics

		Adjusted geometric mean IGF-I		
	n	(nmol/L)[1] [95% CI]	*P* for heterogeneity[3]	*P* for trend[4]
Characteristics:				
Phase 1	630	20.1 [18.3, 22.0]		
Phase 2	912	19.6 [18.4, 20.8]	0.74	
Assay Type				
DSL-10-5600 ACTIVE® ELISA	1350	20.9 [20.3, 21.5]		
IDS-iSYS immunoassay	192	13.3 [11.1, 15.9]	< 0.0001	
Age at blood collection (yrs):				
≤54	308	21.6 [20.8, 22.4]		
55 – 59	446	20.5 [19.8 , 21.1]		
60 – 64	520	19.3 [18.7, 19.8]		
≥65	268	17.7 [16.8, 18.7]	< 0.0001	< 0.0001
Height (cm)				
< 167.1	309	19.2 [18.5, 20.0]		
167.1 –	310	19.3 [18.6, 20.0]		
171.5 –	332	19.9 [19.2, 20.6]		
175.0 –	311	19.9 [19.1, 20.6]		
≥179.0	280	20.7 [19.9, 21.5]	0.07	0.01
Weight (kg)				
< 71.0	312	18.7 [18.1, 19.4]		
71.0 –	308	20.6 [19.9, 21.4]		
76.5 –	312	20.0 [19.3 , 20.8]		
82.5 –	303	20.0 [19.3, 20.8]		
≥89.5	307	19.5 [18.8, 20.2]	0.01	0.40
BMI (kg/m²):				
≤24.9	485	19.6 [19.0, 20.1]		
25-29.9	793	20.3 [19.8, 20.7]		
≥30	264	18.7 [18.0, 19.5]	0.003	0.34
Smoking:[2]				
Never	489	20.1 [19.6, 20.8]		
Former	640	19.7 [19.2, 20.2]		
Current	389	19.5 [18.8, 20.1]	0.32	
Alcohol consumption (g/d):[2]				
< 8	623	19.8 [19.3, 20.4]		
8 – 15	311	20.4 [19.6, 21.1]		
16 – 39	370	20.0 [19.3, 20.7]		
≥40	236	18.5 [17.7, 19.3]	0.01	0.08
Physical activity:[2]				
Inactive	221	20.7 [19.7, 21.7]		
Moderately inactive	415	20.2 [19.6, 20.9]		
Active	693	20.4 [19.9, 20.9]	0.75	0.46
Educational level:[2]				
Primary or Equivalent	579	19.2 [18.7, 19.7]		
Secondary	556	19.9 [19.4, 20.5]		
Degree	339	20.5 [19.7, 21.2]	0.03	0.01
Country:				
Denmark	152	19.9 [18.4, 21.4]		
Germany	383	19.8 [19.0, 20.6]		
Greece	40	19.5 [17.5, 21.7]		
Italy	145	20.6 [19.3, 22.1]		
Netherlands	50	22.8 [20.8, 25.1]		
Spain	200	20.5 [19.5, 21.7]		
Sweden	192	13.9 [12.2, 15.8]		
United Kingdom	380	22.3 [21.2, 23.5]	0.002[5]	

[1] Adjusted for age (< 50, 50-54, 55-59, 60-64, 65-69, ≥70 years), centre (1-18), batch (1-78), alcohol consumption (< 8, 8-15, 16-39, ≥40 g/day, unknown), educational attainment (primary school, secondary school, higher education/degree, unknown) and body mass index (quartile cut-points: < 24.4, 24.4–, 26.4–, 28.7– kg/m²), where the adjustment variable is not the variable of interest; with 95% CI in parenthesis

[2] Unknown for some participants; number of unknowns calculated by subtracting the known total number in each group from 1,542

[3] *P* value refers to tests of heterogeneity between the logarithm of the geometric means in the separate categories (excluding unknowns) calculated by using analysis of variance

[4] *P* value refers to the test for trend obtained by treating the categorical variable as a continuous variable in the model

[5] *P* value refers to tests of heterogeneity between the logarithm of the geometric means amongst countries assayed using the DSL-10-5600 ACTIVE® ELISA (i.e. excluding Umeå, Sweden, assayed using the IDS-iSYS immunoassay)

Table 7.4 Odds ratios (95% CI) for prostate cancer associated with quartile concentrations of IGF-I or a doubling in IGF-I concentration in the European Prospective Investigation into Cancer and Nutrition

		Fourth of circulating IGF-I concentration[1]						
		Q1	Q2	Q3	Q4	OR for doubling[2]	*P* for trend[3]	*P* for heterogeneity[4]
Overall prostate cancer[2]	Cases/controls (*n*)	297/386	375/385	419/386	451/385	1,542/1,542		
	OR [95% CI]	1 (ref)	1.31 [1.05, 1.62]	1.51 [1.21, 1.88]	1.69 [1.35, 2.13]	1.38 [1.17, 1.64]	0.0002	
Prostate cancer stage[2,5]								
Localised stage	Cases/controls (*n*)	146/196	186/195	208/174	205/180	745/745		
	OR [95% CI]	1 (ref)	1.29 [0.95, 1.76]	1.69 [1.24, 2.30]	1.65 [1.19, 2.27]	1.40 [1.10, 1.78]	0.01	
Advanced stage	Cases/Controls (*n*)	57/83	84/68	71/77	97/81	309/309		0.84
	OR [95% CI]	1 (ref)	2.05 [1.22, 3.44]	1.58 [0.95, 2.63]	2.15 [1.26, 3.68]	1.46 [1.01, 2.12]	0.04	
Histological grade[2,6]								
Low grade	Cases/controls (*n*)	165/208	197/212	218/196	268/232	848/848		
	OR [95% CI]	1 (ref)	1.19 [0.89, 1.59]	1.46 [1.09, 1.96]	1.57 [1.17, 2.10]	1.36 [1.09, 1.69]	0.01	
High grade	Cases/controls (*n*)	37/45	46/34	40/36	36/44	159/159		0.19
	OR [95% CI]	1 (ref)	1.79 [0.90, 3.56]	1.48 [0.75, 2.94]	1.05 [0.52, 2.10]	0.95 [0.58, 1.55]	0.84	
Time to diagnosis[2]								
< 4 years	Cases/controls (*n*)	93/136	88/90	107/90	169/141	457/457		
	OR [95% CI]	1 (ref)	1.57 [1.03, 2.39]	1.96 [1.29, 2.99]	2.01 [1.35, 2.99]	1.39 [1.08, 1.80]	0.01	
4–7 years	Cases/controls (*n*)	122/145	135/139	154/139	149/137	560/560		
	OR [95% CI]	1 (ref)	1.13 [0.80, 1.61]	1.33 [0.94, 1.90]	1.37 [0.94, 2.00]	1.29 [0.96, 1.71]	0.08	0.77
> 7 years	Cases/controls (*n*)	82/105	152/156	158/157	133/107	525/525		
	OR [95% CI]	1 (ref)	1.30 [0.89, 1.89]	1.39 [0.95, 2.05]	1.80 [1.17, 2.77]	1.50 [1.04, 2.16]	0.03	

[1] Quartile cut-points (16.5, 20.6 and 25.3 nmol/L) were defined among control participants for all cohorts combined except Umeå, Sweden where country specific cut-points (13.2, 16.0 and 18.8 nmol/L) were used to account for variation in IGF-I concentrations attributable to the assay method

[2] Conditioned by matching factors: study centre, age at recruitment, time of blood collection, follow-up time and duration of fasting at blood collection and adjusted for age at blood collection

[3] *P* values for trend were obtained by replacing the categorical variable with the logarithm of IGF-I concentration

[4] *P* for heterogeneity values relate to likelihood ratio chi-square tests of heterogeneity between trends (using the logarithm of IGF-I)

[5] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic

[6] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system

Table 7.4(continued) Odds ratios (95% CI) for prostate cancer associated with quartile concentrations of IGF-I or a doubling in IGF-I concentration in the European Prospective Investigation into Cancer and Nutrition

		Fourth of circulating IGF-I concentration[1]						
		Q1	Q2	Q3	Q4	OR for doubling[2]	*P* for trend[3]	*P* for heterogeneity[4]
Age at diagnosis [2]								
< 65 years	Cases/controls (*n*)	106/139	166/165	176/185	255/214	703/703		
	OR [95% CI]	1 (ref)	1.33 [0.95, 1.86]	1.29 [0.92, 1.81]	1.67 [1.19, 2.34]	1.26 [0.99, 1.60]	0.06	
≥65 years	Cases/controls (*n*)	191/247	209/220	243/201	196/171	839/839		0.33
	OR [95% CI]	1 (ref)	1.28 [0.96, 1.70]	1.67 [1.25, 2.22]	1.67 [1.22, 2.28]	1.50 [1.18, 1.92]	0.001	
Age at blood collection [2]								
< 60 years	Cases/controls (*n*)	113/144	185/184	194/215	262/211	754/754		
	OR [95% CI]	1 (ref)	1.29 [0.93, 1.79]	1.18 [0.86, 1.64]	1.67 [1.21, 2.32]	1.34 [1.05, 1.70]	0.02	
≥60 years	Cases/controls (*n*)	184/242	190/201	225/171	189/174	788/788		0.73
	OR [95% CI]	1 (ref)	1.28 [0.96, 1.72]	1.87 [1.38, 2.53]	1.65 [1.19, 2.28]	1.43 [1.12, 1.81]	0.003	
Assay type [2]								
IDS-iSYS immunoassay	Cases/controls (*n*)	35/48	47/48	36/48	74/48	192/192		
(Umeå, Sweden only)	OR [95% CI]	1 (ref)	1.35 [0.75, 2.42]	1.01 [0.54, 1.88]	2.19 [1.21, 3.97]	1.64 [0.97, 2.76]	0.06	
DSL-10-5600 ACTIVE® ELISA	Cases/controls (*n*)	262/338	328/337	383/338	377/337	1,350/1,350		0.46
(all centres except Umeå)	OR [95% CI]	1 (ref)	1.30 [1.03, 1.65]	1.57 [1.24, 1.98]	1.61 [1.26, 2.07]	1.35 [1.13, 1.62]	0.001	

[1] Quartile cut-points (16.5, 20.6 and 25.3 nmol/L) were defined among control participants for all cohorts combined except Umeå, Sweden where country specific cut-points (13.2, 16.0 and 18.8 nmol/L) were used to account for variation in IGF-I concentrations attributable to the assay method

[2] Conditioned by matching factors: study centre, age at recruitment, time of blood collection, follow-up time and duration of fasting at blood collection and adjusted for age at blood collection

[3] *P* values for trend were obtained by replacing the categorical variable with the logarithm of IGF-I concentration

[4] *P* for heterogeneity values relate to likelihood ratio chi-square tests of heterogeneity between trends (using the logarithm of IGF-I)

[5] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic

[6] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system

Chapter 8 Folate and vitamin B_{12} and risk for prostate cancer

8.1 Introduction

The development of cancer is believed to be a result of molecular and genetic alterations, of which DNA damage is a component (309). The plausible biological association between folate and vitamin B_{12} and carcinogenesis relates to their role in DNA methylation, repair and synthesis, as abnormal DNA methylation and subsequent aberrant gene expression has been demonstrated in several types of cancer (87).

8.1.1 Sources of folate and vitamin B_{12}

8.1.1.1 Folate

Folate is a water soluble B vitamin acquired in the diet from leafy vegetables, fruits, pulses and nuts, where it is present as pteroylpolyglutamates, of which approximately 50% are bio-available (310). In the UK National Diet and Nutritional survey folate intake was highest from cereals (33%) and vegetables (27%), although it is also acquired from animal foods (20%), beer (8%) and nuts(3%) (311). Heat from food processing and cooking can inactivate pterolpolyglutamates and result in losses of 50-95% of folate in food (312).

Folic acid is a synthetic form of folate with almost 100% bio-availability and commonly used in supplements and the fortification of foods. Folic acid supplements are widely used to prevent and treat folate deficiency in groups at risk and also to prevent adverse events associated with anti-folate medication such as methotrexate (used in the treatment of some cancers, psoriasis and rheumatoid arthritis). Folic acid fortification of foods is practiced in several countries, with national fortification of cereals-grain products introduced in the United States and Canada in the mid-1990s (313, 314). The rationale for folic acid fortification is prevention of neural tube defects in developing embryos (315) and the reduced incidence of open neural tube defects in recent years indicates efficacy of this strategy (122, 316). Nonetheless, many countries, including the UK, have not chosen this general food fortification strategy, mainly due to the unknown effects of augmented folic acid intake on the general population outside the target group of fertile women. Concerns exist regarding masking of vitamin B_{12} deficiency in the older population and a possible risk of cancer development (317).

8.1.1.2 Vitamin B_{12}

Vitamin B_{12}, also known as cobalamin, is synthesized by certain algae, fungi and bacteria and sources in a human diet are from products of animal origin in which vitamin B_{12} has accumulated, either from ingestion of vitamin B_{12} containing micro-organisms or by bacterial synthesis within the animal's gastrointestinal system (318). The meat and offal of ruminants are particularly rich sources of vitamin B_{12}, with the highest content in the liver and kidneys. It is also present in shell-fish and chicken and to a lesser extent in eggs and dairy products such as milk and cheese (319). In the circulation vitamin B_{12} is bound to transcobalamin (comprising holotranscobalamin and apotranscobalamin) and haptocorrin proteins, in an approximate

20:80 ratio, although only the holotranscobalamin bound component is available to cells via receptor mediated uptake (320).

8.1.2 Determinants of circulating folate and vitamin B_{12} concentrations

Levels of circulating folate and vitamin B_{12} are influenced by dietary intake, gastrointestinal absorption, medications, genotype and lifestyle factors such as smoking and alcohol consumption (319, 321-323).

Excessive alcohol consumption has been shown to impair re-absorption of folate (322) however moderate intake does not appear to have an effect (324). Tobacco smoking has been associated with reduced red blood cell and plasma folate status in several studies (325-328), although there is limited evidence for an effect on plasma vitamin B_{12} concentrations (328). Methylenetetrahydrofolate reductase (MTHFR) genotype is a strong determinant of folate (329), with mutation of 677C to 677T decreasing the enzyme's activity, which causes 16-24% lower folate concentrations compared with the wild-type over a range of folate intakes (323).

Absorption of vitamin B_{12} in the ileum of the small intestine is mediated by intrinsic factor, a glycoprotein synthesised and secreted by parietal cells of the gastric mucosa after food consumption, and which has been shown to decrease with increasing age (330). Malabsorption of vitamin B_{12} occurs in individuals with reduced gastric acid secretion (HCL), gastric pathology or lack of intrinsic factor (330). Among healthy adults body stores of vitamin B_{12} are maintained due to efficient entero-hepatic circulation and recycling from bile and intestinal secretions and depletion requires prolonged periods (years) of zero intake(318).

8.1.3 Physiology of folate and vitamin B_{12}

Folate and vitamin B_{12} maintain DNA stability through their ability to donate one carbon units for cellular metabolism. **Figure 8:1** on page 107 (adapted from Van Guelpen (331)) shows the reactions involved in donation of folate methyl groups to nucleotide synthesis and methylation reactions. Initially folate is converted to 5,10-methylenetetrahydrofolate (5,10-mehthyleneTHF), which is a cofactor in the synthesis of nucleotides, purine and thymidylate, the structural units of deoxyribonucleic acid (DNA).

Following an irreversible reaction with the enzyme, MTHFR, the product of the reaction, 5-methyltetrahydrofolate (5-methyl THF) donates methyl groups for re-methylation of homocysteine to methionine. This re-methylation reaction is catalysed by the enzyme methionine synthetase, although vitamin B_{12} is also an essential cofactor. Methionine is converted to S-adenysl methionine (SAM), the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation (332). Vitamin B_{12} also has a role in nucleic acid metabolism via its role in the conversion of 5-methyl THF to tetrahydrofolate (THF), which is required for thymidine synthesis. The conversion of deoxyuridine monophsphate (dUMP) to deoxythymidylic acid (dTMP) is essential in normal DNA repair and synthesis.

8.1.4 Pathophysiology of folate and vitamin B_{12}

Under conditions of folate deficiency, dUMP accumulates and as a result uracil becomes incorporated in DNA instead of thymine. This alteration leads to point mutations and the generation of single- and double- stranded DNA breaks, chromosome breakage and micronucleus formation (333, 334).

When vitamin B_{12} is deficient, homocysteine accumulates and SAM synthesis and DNA methylation are reduced. DNA methylation is considered important for maintaining DNA and chromosome stability (335) and genomic hypomethylation has been shown in cancer patients (336) and cell culture (337). Furthermore, hypermethylation of tumour suppressor genes which leads to silencing of gene expression has also been shown to have a role in carcinogenesis of the prostate (338, 339).

Elevated homocysteine has been associated with increased risk for cardiovascular disease (340) and folate has also been widely used in patients with cardiovascular diseases to lower homocysteine. However, recent data from two meta-analyses of 8 (341) and 13 (342) randomized controlled trials (RCT), do not show a benefit of folic acid supplementation (taken for 2 -7 years and a median duration of 5 years) on the risk for cardiovascular events (341, 342)(although a subsequently published study suggests that concomitant aspirin treatment may reduce any protective affect associated with lowered homocysteine concentrations(343)).

In summary, deficient folate and vitamin B_{12} states may lead to increased DNA damage and altered DNA methylation (events considered important in cancer development (344)) and although mechanisms by which folate depletion disrupts DNA integrity are well characterized, the down-stream effects of these molecular changes are less clear. In particular, little is known of the gene pathways through which folate depletion may enhance carcinogenesis of organs such as the prostate. Nonetheless the association between folate and vitamin B12 and carcinogenesis appears complex, as very high concentrations of folate and vitamin B12 may also increase risk for prostate cancer via hypermethylation of DNA. It has been speculated that the timing and dose of folate levels is important in cancer development, with low folate conducive to tumour initiation but high folate contributing to tumour progression once malignant transformation has occurred and a proliferating neoplasm is established (345)(discussed further in Section 8.1.5.1).

8.1.5 Evidence that folate and vitamin B_{12} are involved in cancer development

8.1.5.1 Experimental studies

Experimental studies in rodents who are genetically predisposed to developing colo-rectal cancer suggest that the timing of folate administration during cancer progression can modify outcomes. Folate given prior to the development of pre-neoplastic lesions (including polyps in the colon) has been shown to prevent tumour development while folate given in the presence of early lesions has been shown to increase the rate of tumour progression (346-348).

Furthermore, supra-physiological doses of folate have been reported to promote tumour growth in animal studies (88). Whilst these findings suggest that any effect of folate administration on cancer development may differ according to the timing and dose of folate administration (349), they need to be interpreted within the totality of the evidence and require confirmation in human studies.

8.1.5.2 Longitudinal studies in humans

Folate and vitamin B_{12} have been implicated in increased all cause cancer incidence and mortality (91, 350), although not consistently (341), and there is also some suggestion that the direction of the association with risk may differ by cancer type. Elevated folate intake has been associated with increased risk for oesophageal, gastric and pancreatic cancer (351), although a reduced risk of colo-rectal cancer (352). There is evidence that risk for prostate cancer increases with folic acid supplementation (353, 354), and some evidence from prospective observational studies for increased prostate cancer risk with elevated folate and vitamin B_{12}(92).

The results from prospective epidemiological studies of the association of circulating folate and vitamin B_{12} and prostate cancer risk are summarised in **Table 8.1**. In brief, a study nested within the Alpha-Tocopherol, Beta-Carotene Lung Cancer Prevention (ATBC) trial (based on 224 cases), found no association between circulating folate and vitamin B_{12} and prostate cancer (RR=1.20; 95% CI 0.74-1.94 and RR= 1.12; 95% CI 0.7-1.78 for highest versus lowest serum concentrations of folate and B12, respectively)(355), although a nested case control study within the Northern Sweden Health and Disease Cohort (based on 254 cases) found that high plasma levels of both folate and B_{12} were associated with a statistically significant increased risk of prostate cancer ((RR=1.6; 95% CI 1.03-2.49 and RR= 2.63; 95% CI 1.61-4.29 for highest versus lowest serum concentrations of folate and B_{12}, respectively) (356). Elevated serum concentrations of vitamin B_{12} have also been associated with a statistically significant increased risk of advanced stage prostate cancer within a nested case control study of the EPIC cohort (RR=1.69; 95% CI 1.05-2.72)(146).

A pooled analysis of individual patient data from eight randomised controlled trials (RCT) of B-vitamin supplements in populations with cardiovascular disease, observed no effect on overall cancer incidence (RR 1.05; 95% CI 0.98-1.13), although cancer specific results were not reported (341). However, a subsequent aggregate meta-analysis of six folic acid supplementation RCTs reported a risk ratio for incident cancer of 1.21(95% CI 1.05-1.39) (357). Although information regarding heterogeneity of rate ratios was not provided, inclusion of the study by Cole et al (353) is likely to have a strong impact on these results, as among the six trials this is the only study that reported a significant increase in cancer incidence.

In 2012 a systematic review and meta-analysis, of data from 10 RCTs, and which included a longer follow-up time and possibly a more diverse population than the earlier pooled analysis, reported a borderline statistically significant increased cancer incidence after folic acid supplementation (RR 1.07; 95% CI 1.00-1.14) (91). In addition, a prostate cancer specific meta-

analysis of six RCTs showed an elevated prostate cancer incidence amongst the folic acid supplement group (RR 1.24; 95% CI 1.03-1.49). While some heterogeneity was evident between these studies (I^2=17%), the increased risk remained after omitting an outlying study from the pooled effect estimate (RR 1.19; 95% CI 1.01-1.39, I^2=0%) (91).

A meta-analysis of circulating concentrations of folate and vitamin B_{12} and risk for prostate cancer in prospective observational studies (although it also includes the placebo arms of two RCTs (350, 354)) reported a modest increased risk of prostate cancer per 10nmol/L increment increase in folate (Fixed effects pooled estimate OR = 1.19; 95% CI 1.03-1.37) and per 100pmol/L increment increase in vitamin B_{12} (Fixed effects pooled estimate OR = 1.10 (95%CI 1.03-1.18) (92)).

While the two recent meta-analyses of RCTs (91, 357) are consistent with the aggregate meta-analysis of prospective observational studies (92) on serum folate and prostate cancer risk, the follow-up time of most RCTs may be too limited to adequately capture the time frame for cancer development and may not therefore reflect the long-term risk associated with folic acid supplementation. Large scale prospective studies with prolonged follow-up time are needed in order to understand better the association of a range of circulating levels on prostate cancer risk.

8.2 Aim of study

The aim of this study is to investigate the association between circulating folate and vitamin B_{12} concentrations and risk for prostate cancer. This work is an extension of earlier research (146), using an expanded nested case control study of 1,480 men with prostate cancer (who have a mean time from blood collection to diagnosis of 6.2 years (SD 3.2) and 1,775 matched control participants. Also reported here are the results from a study of the association between dietary folate and vitamin B_{12} intake and prostate cancer risk using available dietary data from 142,252 men, including 4,728 incident prostate cancer cases, who are participating in the EPIC cohort study. A further aim is to include a meta-analysis of prospective data on the association between circulating concentrations of folate and vitamin B_{12} and risk of prostate cancer, including the results of the present analysis which comprises the largest prospective study to date.

8.3 Methods

8.3.1 Study cohort

The European Prospective Investigation into Cancer and Nutrition is a multi-centre, international prospective study designed to investigate the associations between lifestyle and dietary factors and risk for cancer. The methods of recruitment have been described previously in **Chapter 2**, section 2.2 on page 6 (and published by Riboli, 2002(13)) In brief, between 1992 and 2000, approximately 360,500 women and 153,400 men, mostly aged 35-70 years, were recruited from 23 centres in ten European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom). All

participants completed a standardized food frequency questionnaire or an interview based dietary questionnaire, and a detailed lifestyle questionnaire. Approximately 385,700 participants (139,600 men) also provided a blood sample. The standardised blood sampling protocol was followed in all centres except Oxford (UK), Copenhagen (Denmark) and Umeå (Sweden), and involved delivery of whole blood in the collection syringe to a local laboratory where samples were processed and separated into serum, plasma, red cells and buffy coat fractions within 24-hours of collection, prior to placement in 0.5mL straws for storage at -196˚C in liquid nitrogen tanks or at -80 in freezers in Umeå. In the Copenhagen cohort samples were stored in 1 mL tubes in nitrogen vapour at -150°C and in the Umeå cohort (Sweden), EDTA plasma (rather than serum) samples were archived and stored at -80°C in electric freezers. Blood samples from the Oxford cohort were sent in the post at ambient temperature to a laboratory in Norfolk, with an average transit time of 1.5 days.

Dietary folate and vitamin B_{12} intake was calculated by multiplying the nutrient content of each food of a specific portion size by the frequency of consumption as stated on the food frequency questionnaire. To improve the comparability of dietary data across centres and to correct for measurement error in exposure classification, calibration was performed using a fixed-effects linear model in which centre and gender-specific 24-hour recall data (collected via interviewer using a computerized method) from an 8% random sample of the cohort were regressed on the food frequency questionnaire intakes (15, 204). Information on supplements was not used in the calculation of dietary intake.

The present cohort study includes male participants from Denmark, Germany, Greece, Italy, the Netherlands, Spain, Sweden and the United Kingdom (UK). The nested case control study does not include Denmark (serum was not available) or the sub-cohorts in France and Norway (these cohorts only included women). Almost all participants are of white European origin and, with the exception of the health conscious Oxford sub-cohort (recruited throughout the UK via General Practices and the Vegetarian Society), were recruited from the population residing in defined geographical areas.

All participants provided written informed consent and local ethics committees in the participating countries and the Internal Review Board of the International Agency for Research on Cancer (IARC) approved the study.

8.3.2 Follow-up and case identification from the study cohort

Follow-up was provided through record linkage with population-based cancer registries in Denmark, Italy, the Netherlands, Spain, Sweden and the United Kingdom. In Germany and Greece active follow-up was achieved through study subjects and their next of kin and verified through health insurance or medical records and cancer and pathology registries or reports. Participants with active follow-up were censored at the last known contact or at the date of diagnosis of cancer, death, emigration or the end of the follow-up period, whichever came first.

Prostate cancer cases were defined as code C61 according to the 10th revision of the International Statistical Classification of Injuries, Disease and Deaths (ICD-10). Cases were defined as men who developed prostate cancer after the date of recruitment and before the end of the study period, as determined by the latest date of follow-up in each study centre. Tumour stage information (TNM staging score), derived from pathology reports and cancer registry data, was used to define the following categories: localized (T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localized; Total n =1,727), advanced (T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic; n = 935), and unknown stage (Total n = 2,066). Information on Gleason score or the World Health Organization (WHO) grading system was used to define low grade (Gleason score $\leq$7, or cases coded as well differentiated or moderately differentiated; Total n = 1,863), high grade (Gleason score >7, or cases coded as poorly differentiated or undifferentiated; Total n = 382), and unknown grade (Total n = 2,483).

Men were excluded from the analysis if they had a previous diagnosis of cancer, if the date of cancer diagnosis or end of follow-up was missing, if they did not have dietary or other lifestyle data, if they reported an improbable total energy intake (in the top or bottom 1% of the ratio of estimated energy intake to energy requirement), if they were aged less than 20 years at recruitment or censored at recruitment. Overall, 142,252 men were available for analysis from a total of 148,016 men in the original dataset.

8.3.3 Selection of case and control participants for the nested case control study

For the nested case control study data from 867 cases that were included in a previous study (phase 1) were combined with data from 613 extra cases who were subsequently diagnosed with prostate cancer (phase 2), and for whom a blood sample and information on date of blood collection were available. After exclusion of cases for which a matched control sample was not available, folate and vitamin B_{12} measurements were available in 855 and 854 phase 1 sets, respectively, and all of the 613 phase 2 sets. Cases were matched to a single control (with the exception of Umeå in Sweden where 2 controls were used) on study centre, length of follow-up, age at blood collection (+/- 6 months), time of blood collection (+/- 1 hour) and duration of fasting at blood collection (< 3, 3 - 6, > 6 hours). An incidence density sampling protocol was used to select control participants at random from the cohort of men who were alive and free of cancer (except non-melanoma skin cancer) at the time of the diagnosis of the index case.

8.3.4 Laboratory analysis

Folate and vitamin in B_{12} were measured at Bevital (Bergen, Norway) in singleton using a *Lactobacillus casei* and *Lactobacillus leichmannii* microbiological assay, respectively, adapted to a microtiter plate format and performed by a robotic workstation (Microlab AT plus2; Hamilton Bondaduz). The lower limits of quantification for folate and vitamin B_{12} (2 nmol/L and 30 pmol/L, respectively) provided adequate sensitivity to detect the lowest concentration in the study samples. Assays were conducted by a laboratory technician, blind to both the case-control and quality control status of the serum samples. Case-control sets were analyzed

in the same batch and blinded duplicate quality control samples were included in each, with mean intra- and inter-batch coefficients of variation of 6.0% and 6.3% (phase 1) and 10.3% and 12.5% (phase 2)for folate and 5.4% and 6.7%(phase 1) and 4.0% and 11.7% (phase 2) for vitamin B_{12}.

8.3.5 Statistical analysis

8.3.5.1 Cohort study

Age standardised rates were calculated for each country as the weighted average of the ratio of cases to person years in each of 6 attained age groups (<50, 50-54, 55-59, 60-64, 65-69, 70+ years), with weights chosen according to the European standard population.

Cox proportional hazards models, stratified by centre and age at entry (<50, 50-54, 55-59, 60-64, 65-69, 70+ years) and with attained age as the underlying time variable, were used to determine relative risks and 95% CI for the association between prostate cancer and dietary intake of folate and vitamin B_{12}. Proportional hazards assumptions were tested using Schoenfield residuals and no evidence of non-proportionality was observed. Each nutrient intake approximated a normal distribution and analyses were performed using categorical variables (based on quintiles of the observed distribution among non-cases across all EPIC centres combined, with the lowest category as the reference group) and as continuous variables (using calibrated intake values, with nutrient increments set at 65.30 µg/day for folate and 2.41 µg/day for vitamin B_{12} (corresponding to one standard deviation in intake).

A complete set of data was available for all variables with the exception of smoking, physical activity, education and marital status (1.2%, 2.2%, 2.7% and 30.9% missing values, respectively; marital status data was not collected from participants in Denmark and Spain). All missing values were assigned to a separate stratum for each variable. All Cox models included adjustment for energy intake (kJ/day; fifths), but adjustment for educational level (no degree, degree, unknown), smoking status (never, former, current, unknown), marital status (married/cohabitating, not married/cohabitating, unknown), height (continuous) and weight (continuous) made little difference to the risk estimates or their 95% confidence intervals, and were not included in the final model as these factors did not confound the association. Tests for interaction between folate and vitamin B_{12} intake and BMI, alcohol consumption and smoking on prostate cancer risk were examined.

Tests for linear trend across categories of intake were conducted by including the median value of each fifth as a continuous variable; tests for trend were also calculated by including the continuous term in the model, where appropriate. Hazard ratios (HR) and 95% CI for the association between prostate cancer risk and nutrient intakes were also calculated according to subgroups of grade and stage of disease, age at diagnosis, time between recruitment and diagnosis and country. Tests for heterogeneity between subgroups (after exclusion of the unknown category) were obtained by fitting stratified Cox proportional hazard models based on competing risks, comparing the risk coefficients and standard errors in the subgroups of stage (advanced, localised), grade (high, low), age at diagnosis (<65, $\geq$ 65 years) and time to diagnosis (< 4, $\geq$ 4 years)(358). Heterogeneity of risk estimates across countries was evaluated

by using likelihood ratio tests. Sensitivity analyses excluding the first 4 years of follow up were conducted to investigate the possibility of reverse causality and evaluate the impact on risk estimates.

Correlations between nutrient intakes estimated by the food frequency questionnaire (an insufficient number of men in the nested data set had 24-hour recall data) and serum concentrations of folate and vitamin B_{12} were examined using partial correlation coefficients, with adjustment for total energy intake (205).

8.3.5.2 Nested case control study

Logarithmic transformation of serum folate and vitamin B_{12} values were performed to approximate a normal distribution. To identify possible outliers, the distributions of values for each analyte were examined using box and whiskers plots and letter-value displays. A small proportion of observations were identified as severe outliers on the logarithmic scale (> 3 times the inter-quartile range). However all values were considered within a biologically plausible range and sensitivity analyses excluding these observations made no material difference to the risk estimates, and all observations were included in the final models.

Analysis of variance was used to examine the association between serum folate and vitamin B_{12} concentrations and participant characteristics (case-control status, country, centre, fasting status, month of blood collection, age at blood collection, body mass index (BMI), smoking status, alcohol consumption, physical activity, marital status and educational attainment). Where appropriate, adjustment was made for centre, age at blood collection, month of blood collection and laboratory batch.

Conditional logistic regression models were used to calculate the odds ratios (ORs) for overall prostate cancer risk by fifths of folate and vitamin B_{12} concentration, with cut points defined by the distribution amongst control participants for all centres combined. Tests for linear trend were calculated using the quintile median value of each analyte and treating that value as a continuous variable. Due to the large differences in absolute folate levels by country (see Table 8.7), with Sweden representing over 80% of the bottom quintile, the association between folate and risk for prostate cancer was also analysed using country specific cut points (defined by the distribution amongst control participants for the specific country) and an overall test for trend was obtained by assigning to each category the numerical values 1, 2, 3, 4 and 5. A likelihood ratio X^2 test was used to examine heterogeneity of trends in prostate cancer risk by country. A fixed effects model, assigning a weight to each country (equivalent to the inverse of its variance) was used to obtain the most precise estimate of the population effect per quintile increment increase.

The effects of potential confounding variables (other than those accounted for in the matched design) were examined by including additional regression terms in the conditional logistic regression model. As for the cohort analysis of dietary intake, this NCC analysis also showed

that additional adjustment for weight (continuous), height (continuous), smoking (never, past, present, unknown), alcohol consumption (<8, 8- 15, 16 - 39, ≥ 40 g/day), physical activity (inactive, moderately inactive, moderately active, active, unknown), marital status (married/cohabitating, not married/cohabitating, unknown), educational attainment (primary or equivalent, secondary, degree level) made no appreciable difference to the risk estimate (<5% change) or its 95% confidence interval and were not included in the final model. However, all final models included adjustment for age at blood collection to ensure close comparability within matched sets.

Likelihood ratio χ^2 tests were used to examine heterogeneity of trends in prostate cancer risk associated with a doubling (log 2) of folate and vitamin B_{12} concentration by phase of study (phase 1, phase 2), cancer stage (localized, advanced), cancer grade (low, high), age at blood collection (< 65, ≥ 65 years), age at diagnosis (< 65, ≥ 65 years), time between blood collection and diagnosis (< 4, 4-7, >7years), country and BMI (< 25, ≥ 25 kg/m²). Separate sensitivity analyses were performed that excluded: the Oxford cohort (with a different blood collection protocol); the Swedish cohort (with low folate concentrations); and the first four years of follow-up. Exclusion of these subgroups made no appreciable difference to risk estimates and all observations were included in the final models.

A meta-analysis of associations of circulating folate and vitamin B_{12} concentrations and prostate cancer risk was conducted by combining log OR estimates for each micronutrient from the current study, together with estimates from individual prospective studies, as summarised in a previously published meta-analysis (92) and a subsequent relevant publication of the association of serum folate and risk for prostate cancer (359). All prospective observational studies were identified by searching Medline and Scopus databases using the search terms “folate”, “folic acid”, “vitamin B_{12}” and “cobalamin” together with the MeSH term “prostatic neoplasms” and text term “prostate cancer”. Log OR per unit increase in micronutrient concentrations (10 nmol/L for folate and 100 pmol/L for vitamin B_{12}) were calculated for categorical data by using the mean or median exposure in each quantile, as described by Chêne and Thompson (360) or the range of exposure in each quantile (when the mean or median were not reported), as described by Greenland and Longnecker (361), and reported in more detail elsewhere (92). Where provided, the most completely adjusted model estimate was used from each study. The influence of each study on the estimate of effect was determined by the change in the pooled point estimates following exclusion of any one study. A fixed-effect model (assuming that all studies in the meta-analysis share a common (true) effect size) that assigns a weight to each study equivalent to the inverse of each study’s variance was used to obtain the most precise estimate of the population effect. A random-effects meta-analysis was also performed because studies differed in the mix of participants and one cannot discount the possibility of different effect sizes in different populations. However, the results did not differ substantially from the fixed effects model and are not presented here. The symmetry of a funnel plot was visualised to assess the validity of the meta-analysis and to ascertain the presence of bias (in particular, publication bias that occurs when there is an association of publication probability with the statistical significance of study results). In the presence of bias an asymmetrical funnel plot is observed (362, 363). A formal statistical test for funnel plot asymmetry (such as the “metabias” command in STATA) was not applied due to the small number of studies included (<10) and the relatively low power of the

meta-analysis (362). False-positive results have also been reported in the presence of substantial between-study heterogeneity (as observed in the present meta-analysis) (362).

All statistical analyses were performed using STATA 10 statistical software (StataCorp LP, College Station, TX, USA) and two-tailed tests of statistical significance with p values below 0.05 were considered significant.

8.4 Results

8.4.1 Cohort study

After an average of 11.4 (SD=2.9) years follow-up, 4,728 men were diagnosed with prostate cancer among the 142,252 participants, with a total of 1,624,628 person-years follow up time. As shown in **Table** 8.2 (on page 109), there was significant variation in the intake of folate and vitamin B_{12} by country (p for heterogeneity =<0.0001), with the highest median 24-hr recall intake of folate in the United Kingdom (334.5 µg/day) and the lowest intake observed in Sweden (230.3 µg/day); the highest intake of vitamin B_{12} was in Spain (6.4 µg/day) and the lowest intake in the Greece (3.3 µg/day). Across all countries the intake of vitamin B_{12} and folate followed a relatively normal distribution.

The results in **Table 8.3** (on page 110) show the hazard ratios (HR) and 95% CI for prostate cancer by quintile of observed dietary folate and vitamin B_{12} intake, stratified by centre and age at entry (<50, 50-54, 55-59, 60-64, 65-69, 70+ years) and with adjustment for energy intake (fifths). There was no evidence of an association between quintiles of intake of folate or vitamin B_{12} and risk for prostate cancer overall (Adjusted HR for highest vs. lowest fifth: 1.02; 95% CI 0.90, 1.12; P trend = 0.57 for folate, and 0.97; 95% CI 0.87, 1.09; P trend = 0.42 for vitamin B_{12}.

Hazard ratios for prostate cancer in relation to calibrated intakes of dietary folate and vitamin B_{12} (on a continuous scale) are shown in **Table 8.4** (on page 111). There was no association between calibrated folate and vitamin B_{12} intake and risk for prostate cancer overall or by each subgroup of grade, stage of disease, time from recruitment to diagnosis or age at diagnosis (P trend >0.05) and no evidence of heterogeneity in risk across subgroups (P heterogeneity >0.05). There was also no difference in the association with prostate cancer risk by country of origin (results not shown). There was no evidence of effect modification in the association between vitamin B_{12} or folate and any of the co-factors, including alcohol intake, BMI and smoking status, that were included in the complete multivariate model (results not shown). Sensitivity analyses excluding the first 4 years of follow-up (n= 727 cases excluded) made no material difference to the risk estimates (Table 8.4).

Partial correlation coefficients for serum concentrations and calibrated dietary intake of folate and vitamin B_{12} among control men were 0.33 and 0.20 (p <0.00001), respectively after adjustment for energy intake.

8.4.2 Nested case control study

The characteristics of cases and controls in the nested-case control data set are shown in **Table 8.5**. There were no significant differences between cases and controls according to height, BMI, smoking status, alcohol consumption, physical activity, marital status and educational attainment. However, cases were slightly older than controls at blood collection (59.8 vs. 59.2 years; p =0.04).

The geometric mean concentrations of serum folate and vitamin B_{12} according to baseline characteristics amongst control participants, with adjustment for centre, age at blood collection, alcohol intake, month of blood collection and batch are shown in **Table 8.6**. Folate concentration was 11.7% lower in inactive compared with active men (P_{trend} = 0.02) and 5% lower in lighter men (BMI < 25) compared with heavier men (BMI $\geq$ 25; P_{trend} = 0.02). There was no association between circulating concentrations of folate and other factors including phase of study, age at blood collection, alcohol consumption, marital status, educational attainment and month of blood collection ($P_{heterogeneity}$ > 0.05). Vitamin B_{12} concentration was 12.6% lower in men aged ≥65 y compared with those aged ≤54 y (P_{trend} < 0.0001) but there was no evidence of significant heterogeneity in vitamin B_{12} concentration by alcohol consumption, BMI, smoking, alcohol consumption, marital status, physical activity, month of blood collection or educational attainment ($P_{heterogeneity}$ > 0.05).

The distribution of serum folate and vitamin B_{12} concentrations amongst control participants by phase of study and country are shown in **Table 8.7** (on page 114). Overall, concentrations of both micronutrients varied significantly by country, with the highest geometric mean folate concentration observed in men from the UK (16.3 nmol/L) and the lowest in men from Sweden (6.0 nmol/L; P heterogeneity< 0.0001). The highest mean vitamin B_{12} concentration was observed in participants from Spain (388.0 pmol/L) and the lowest amongst those from Germany (316.1 pmol/L; P heterogeneity <0.0001). There was no evidence of significant heterogeneity in folate or vitamin B_{12} concentrations by phase of study (P heterogeneity by study phase > 0.05; results not shown), although very low concentrations were observed in blood samples from the Swedish cohort in phase 1.

The risk of prostate cancer by fifths of folate and vitamin B12 concentration are shown in **Table 8.8** (on page 114) for each phase separately and combined. Overall, there was no evidence of an association between folate and vitamin B_{12} concentrations and prostate cancer risk in each study phase or when the data were combined from both study phases. Compared with men in the lowest fifth of folate, men in the second to fifth quintiles all had elevated odds ratios, with an odds ratio of 1.47 for the top fifth (95% CI 1.07, 2.01), but with no evidence of a trend (P trend = 0.28). For vitamin B_{12}, men in the highest fifth of circulating concentration had an odds ratio of 1.14 (95% CI 0.90, 1.46; P trend = 0.67). Similar to the analysis of dietary intake and risk, adjustment for potential confounding factors made no material difference to the risk estimates of circulating biomarkers on risk (<10% change in risk estimates), and apart

from age at blood collection, were not included in the final models (fully adjusted results not shown).

As shown in **Table 8.9** there was no evidence of an association between folate (per quintile increment increase) and risk for prostate cancer by country or overall (combining data from all countries) using country specific cut points; OR for highest vs. lowest fifth = 1.19 (95% CI; 0.94, 1.49; p trend= 0.21). There was also no evidence for heterogeneity in the trends across the quintiles by country (P heterogeneity=0.91) as shown in **Figure 8:2.**

Odds ratios associated with a doubling of serum folate and vitamin B_{12} by subgroup characteristics are shown in **Table 8.10.** Consistent with the categorical analysis, there was no evidence of an association between circulating folate and vitamin B_{12} concentrations and risk for prostate cancer when analysed on a continuous scale (OR $_{phase\ 1\&2}$ = 1.05: 95% CI 0.95, 1.15; P trend= 0.33 for folate, and OR $_{phase\ 1\&2}$ = 1.05: 95% CI 0.92, 1.21; P trend= 0.47 for vitamin B_{12}). There was marginal evidence of statistical heterogeneity in the association between vitamin B_{12} and prostate cancer risk by stage of disease (P heterogeneity = 0.05), with a stronger association for advanced disease (OR $_{phase1\&2}$ = 1.35; CI 95% 0.97, 1.87; P = 0.07) compared with localised disease (OR $_{phase1\&2}$ = 0.92; CI 95% 0.74, 1.14; P = 0.43). However, the increased risk among men with advanced disease was restricted to the phase 1 study (OR $_{phase\ 1}$ = 1.94: 95% CI 1.09, 3.44; P= 0.02), with no association observed in phase 2 (OR $_{phase\ 2}$ = 1.08 95%CI 0.69, 1.68; P= 0.73; results not shown in table). There was no evidence of heterogeneity in the association of circulating folate or vitamin B_{12} with risk by grade of disease, time from recruitment to diagnosis, age at diagnosis, age at blood collection, country or phase of study (P heterogeneity >0.05).

Figure 8:3 - Figure 8:7(page 107 to 123), show the results of a meta-analysis of the association of circulating folate and vitamin B_{12} concentration and risk for prostate cancer, combining the results from the current analysis with those from 7 other studies to include approximately 3,500 cases (92). **Figure 8:3** shows that there is no association between circulating folate concentration and risk for prostate cancer (OR per 10 nmol/L increment: 1.02, 95%CI 0.98, 1.07; P = 0.35). The two largest contributing studies were ProtecT (I^2 46.8%), published by Collin, 2010 (92), and the updated EPIC analysis, as reported in this chapter (I^2 = 46.8%). As shown in **Figure 8:4**, exclusion of the ProtecT study (with a cross-sectional case-control study design) made very little difference to the overall association (OR 1.03; 95% CI 0.97, 1.10; P = 0.31).

For vitamin B_{12}, six studies were included in the meta-analysis (as shown in **Figure 8:5**). Overall, there was a significant positive association between circulating vitamin B_{12} concentration and prostate cancer risk (OR 1.05; 95% CI 1.02, 1.09 per 100 pmol/L; P = 0.02), of which the current EPIC study contributed the most data (I^2=68%). However, there was considerable heterogeneity between studies (I^2 = 56.8%; P heterogeneity = 0.04)) that was largely attributable to one study (Hultdin, 2005) that included men from the Västerbotten Intervention Project and the MONICA study in Sweden (356). Exclusion of this study, as shown

in **Figure 8:6,** substantially reduced the between-studies heterogeneity with little impact on the modest association with risk (OR 1.04; 95%CI 1.00. 1.08; P = 0.03). However, following exclusion of the Collin study (92) (cross-sectional case-control design) a statistically significant association with risk was no longer evident (OR 1.03; 95%CI 0.99. 1.07; p=0.12; **Figure 8:7**). **Figure 8:8** and **Figure 8:9** show asymmetrical funnel plots for folate and vitamin B_{12} that is suggestive of bias in the meta-analysis. For folate the risk estimates in the smaller studies by Rossi, Hultdin and Weinstein (with < 300 cases per study) differ from the two larger studies by Collin and Price (with ~1,460 cases in each study). These small study effects may occur due to publication bias, less rigorous methodology in smaller studies, sampling variation or chance.

8.5 Discussion

In this large prospective cohort study of European men neither dietary intake nor circulating concentrations of folate and vitamin B_{12} were associated with risk for prostate cancer, although combining the data from EPIC with that of the other studies suggests a weak association between vitamin B_{12} and risk cannot be ruled out.

Evidence from prospective studies of an association between dietary intake of folate and vitamin B_{12} on subsequent risk of prostate cancer is sparse. The observed null association for dietary folate intake and risk for prostate cancer in the current study is consistent with data from the American Cancer Society Cancer Prevention Study II Nutrition Cohort study and the Melbourne Collaborative Cohort Study, the only other cohort studies that have published on this association (Mutivariate RR = 1.03, 95% CI 0.94, 1.13 and RR = 1.00, 95% CI 0.83, 1.22 for highest vs. lowest quintile, respectively) (364, 365). Our finding of no association for vitamin B_{12} intake and prostate cancer risk is consistent with the Melbourne Collaborative Cohort Study (RR = 0.92, 95% CI 0.76, 1.11 for highest vs. lowest quintile) but contrasts with the positive association observed in the ATBC trial among Finnish smokers (RR= 1.36; 95%CI 1.14, 1.96 for highest vs. lowest quintile; P trend = 0.01) (366), although it is possible that the findings of that study may not be representative of the general population. Statistically significant interactions between alcohol and smoking status and risk for prostate cancer were not observed in our study which is in contrast to previous reports (355, 365).

The prospective design of the EPIC study minimizes the potential for recall bias in the dietary assessment and the use of validated dietary instruments, including a food frequency questionnaire and 24-hour recall to calibrate dietary intakes across a common reference scale (as discussed in Chapter 2, section 2.3.1, page 6) adjusts for systematic over- or under-estimation of dietary intake (17). However, as all dietary assessment instruments are prone to measurement error, prospective studies that have used blood concentrations as nutritional biomarkers of dietary exposure may provide more precise estimates of risk.

The null findings for a doubling of circulating concentrations of folate and risk for overall prostate cancer is consistent with the results of the meta-analysis presented here, and most (350, 354, 355, 367) but not all (356) individual studies. Nonetheless, in the quintile analysis there was a suggestion of a threshold effect for folate, with higher risk estimates observed in

the upper three quintiles compared to the lowest quintile, although there was no evidence of a linear trend. Separate analyses by country were performed however the numbers in each category were small and there was not sufficient additional evidence to support a threshold effect for folate. While absolute concentrations varied between countries there was no evidence that the association with risk differed by country. Taken together these findings do not support a relation between either the absolute concentration of folate or the relative concentration of folate and risk for prostate cancer.

For vitamin B_{12}, our extended analysis in EPIC found no overall association with prostate cancer risk, and although there was some evidence of a small difference in the association of vitamin B_{12} and risk by stage of disease, the stronger association for advanced disease was not statistically significant and was restricted to phase 1 of the study, as previously published (146). Given the relatively small sample size of the phase 1 analysis (with 154 advanced cases), this finding was most likely due to chance. The only other study to investigate heterogeneity in risk by disease stage also reported no difference in the association of vitamin B_{12} (or folate) with prostate cancer risk, although the number of advanced stage cases was small (n = 163), limiting the power to detect modest risk estimates (92).

The meta-analysis of 6 prospective studies reported here found a small increased risk of 5% associated with a 100 pmol/L unit increase in vitamin B_{12} concentration. This association was marginally attenuated following exclusion of the Hultdin study (that contributed to the significant between-studies heterogeneity in the association (see Figure 8:6)) and no longer evident following exclusion of the Collin study. Although the results from the EPIC study show no strong evidence of heterogeneity in the association by time between blood collection and diagnosis (< 4, 4-7, >7years; P heterogeneity >0.5), suggesting that reverse causation may not be of real importance, given the latent period of prostate cancer may exceed 10 years (discussed in Chapter 3 section3.3), longer follow-up with sensitivity analysis excluding the first 10 years is required to exclude the possibility that men without prostate cancer have different associations with vitamin B_{12} concentrations compared to men with prostate cancer. Given the cross-sectional design of the Collin study (with blood collected at the time of prostate cancer diagnosis), it is possible that the positive association may reflect reverse-causation, as prostate tumour cells have been shown to have an increased demand for vitamin b12 due to up-regulation of methionine synthase activity in the biosynthesis of polyamines (368, 369).

Confounding or effect modification by BMI, alcohol intake and smoking status was not observed for circulating concentrations of folate or vitamin B_{12}, consistent with other studies (92, 355, 356).

The modest correlation observed between folate and vitamin B_{12} intake, as assessed by the FFQ, and circulating concentrations ($r = 0.33$ and $r = 0.20$ for folate and vitamin B_{12}) is of a similar magnitude to previous reports (355, 370-372), although stronger correlations have also been observed in other studies (373, 374). The lower correlations observed in the current study are likely to be partly attributable to error in dietary measurement, as data on

supplementation was not collected. However, unmeasured factors known to affect nutrient status will also play a role in the discrepancy between dietary and blood measures. Furthermore, the poor reliability obtained for circulating concentrations of folate and vitamin B_{12} in a subset of EPIC participants (discussed in Chapter 4, section 4.5) suggests that a single blood measure may not capture usual exposure levels and therefore may not correlate well with FFQ measures.

Individual patient data was not used in the meta-analysis, hence the calculated risk estimates for folate and vitamin B_{12} for all prospective studies combined are subject to the assumptions and adjustments of each study. To understand better these associations, and to avoid the limitations of relying on point estimates to report an association, pooled re-analysis of individual participant data across studies is required.

8.5.1 Strengths of the study

Strengths of the current analyses include the large sample size, sufficiently powered to examine associations by sub-group, for which detailed dietary data and blood measured are available. The considerable variation in dietary intake and circulating concentrations of folate and vitamin B_{12} in participants with diverse dietary patterns enabled evaluation of risk relationships across a wide range of exposure levels. The prospective design of both studies minimizes the impact of disease on risk estimates and the length of follow-up permitted investigation for evidence of heterogeneity in the association with risk by time from recruitment to diagnosis and to perform sensitivity analyses excluding early follow-up, the Oxford cohort (with different blood handling procedures) and the Swedish cohort (with very low folate concentrations); although these exclusions made no appreciable difference to risk estimates and all data were include in the final analysis.

8.5.2 Limitations of the study

The absence of data on folate and vitamin B_{12} supplement use in this analysis may have led to underestimation of dietary intake in some individuals. Furthermore, marked variation in the frequency and type of supplement use by country has been previously demonstrated in the EPIC study (375) and this may bias comparisons of dietary intakes across countries. Concentrations of holotranscobalim (the bio-available component of vitamin B_{12} in the circulation) were not available in this study, and this protein may provide a more sensitive marker than total vitamin B_{12} for classifying vitamin B_{12} status, particularly in individuals with deficient levels (320).

The poor repeatability of folate and vitamin B_{12} indicate that a single measurement of circulating concentrations may not characterise adequately an individual's usual blood concentration (ICC for folate = 0.18; ICC for vitamin B_{12} =0.34) thereby reducing the ability to detect associations with disease risk where only small measures of effect are anticipated. Transport of whole blood samples in the Oxford cohort via post may have altered concentrations as substantial decreases in folate (up to 15% within 24 hours and 30% over 3 days)(147, 148, 376, 377) and elevated vitamin B_{12} concentrations (up to 27%; due to leaching

from red blood cells) have been observed with prolonged serum-clot contact time (147, 376, 377), although exposure to light appears to have little impact on concentrations (378). Circulating concentrations of folate fluctuate with changes in intake, making it difficult to distinguish transitory reduction in intake from long term exposure to low levels (379). A measure of red blood cell (RBC) folate concentration may provide a better estimate of habitual dietary intake as the lifespan of a RBC is approximately 120 days (123).

In future, large scale mendelian randomization studies (using yet to be established genetic variants that affect circulating concentrations of folate and vitamin B_{12} without directly affecting risk for prostate cancer or modifying the effect of folate and vitamin B_{12} on prostate cancer risk) to estimate the causal effect of phenotypes on risk for prostate cancer may provide an alternative and useful way of overcoming some of the limitations of estimating the association between circulating folate and vitamin B12 and risk for disease (380), although these studies are subject to other limitations (381).

8.6 Conclusion

Folate and vitamin B_{12} intake and circulating concentrations are not associated with risk for prostate cancer in this study of European men. However, a small significant association was observed between elevated vitamin B_{12} concentration and risk for prostate cancer in a meta-analysis using aggregate risk estimates. This finding should be interpreted with caution due to heterogeneity in risk estimates between studies. Overall, these findings emphasise the need for pooling individual participant data from world-wide prospective studies in order to increase the precision for identification of what may be modest risk estimates for folate and vitamin B_{12}.

Figure 8:1 Folate and vitamin B_{12} metabolism

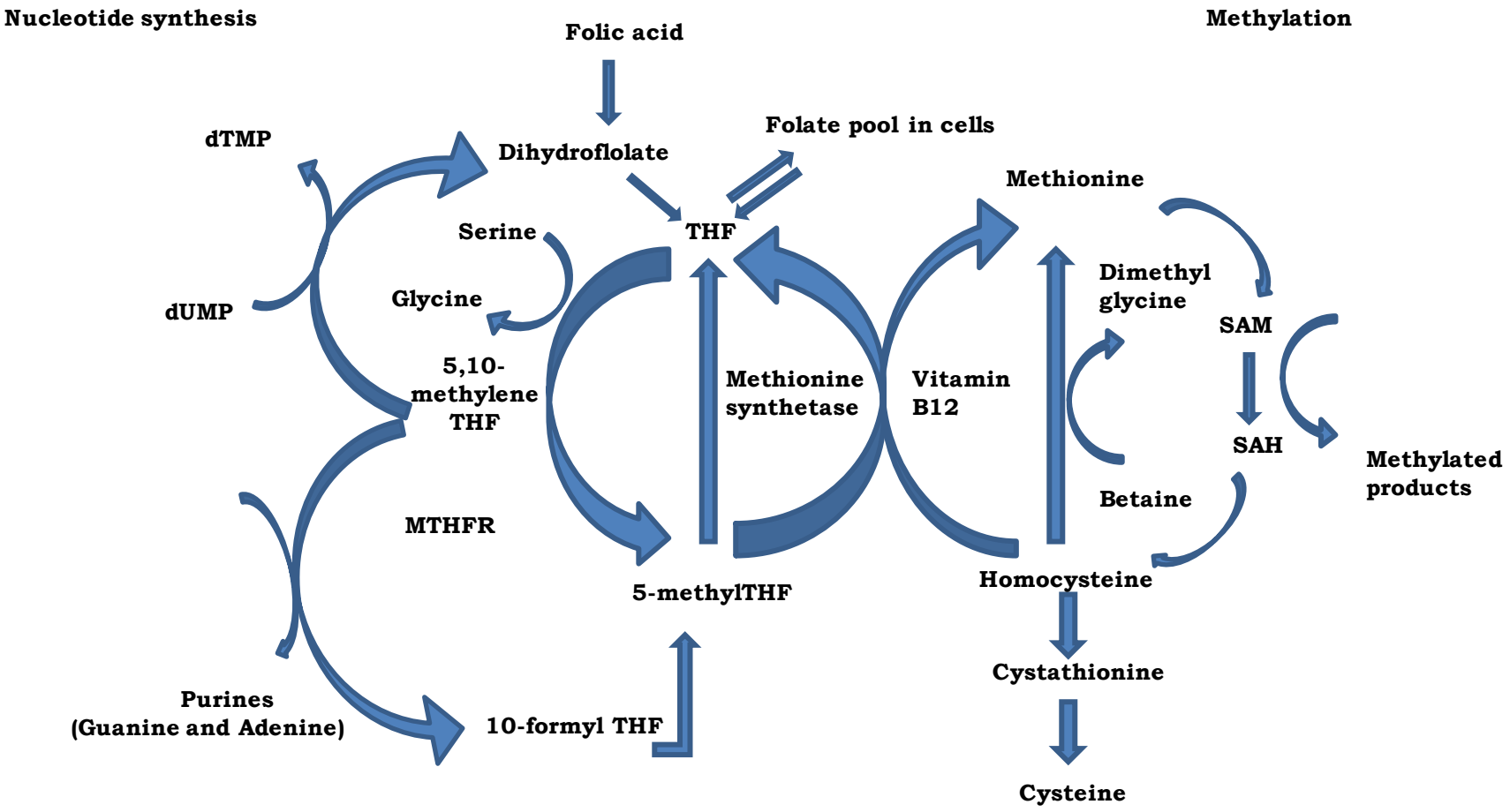


Table 8.1 Prospective observational studies of the association between circulating folate and vitamin B_{12} and risk for prostate cancer

Author, Year, Study	Design	Number (case/control)	Blood	Assay	Matching Criteria	Adjustments	Odds ratios [95% CI]	P trend
Folate Studies								
Weinstein, S; 2003 ATBC study	NCC in RCT	232/464	Serum	Radioassay, Bio-Rad Laboratories, Richmond, CA	clinic, intervention group, date blood collection, age and serum availability	No	highest vs lowest quartile 1.20 [0.74 – 1.94]	0.52
Hultdin, J; 2005 Northern Sweden Health and Disease Cohort (NSHDC)	NCC	254/514	Heparin plasma	Quantaphase II radioassay, Biorad, CA	age, recruitment date	No	highest vs lowest quartile 1.60 [1.03 – 2.49]	0.02
Rossi, E; 2006 Busselton, Western Australia	Cohort	52 /466	Serum	Automated microbiological assay	No	smoking, alcohol, BMI	continuous non-transformed scale 1.18 [0.90 – 1.51]	0.24
Johansson, M; 2008 European Investigation into Cancer and Nutrition (EPIC)	NCC	869/ 1,174	Serum	Lactobacillus casei microbiological assay, Bevital, Norway	study centre, age at enrolemnt, time of day of blood collection, duration of follow-up and time since last	smoking, alcohol intake, BMI, marital status, educational attainment, physical activity	Unadjusted model: highest vs lowest quartile 1.23 [0.84 – 1.81] Adjusted model: highest vs lowest quartile 1.30 [0.88 – 1.93]	0.62 0.46
Collin, S; 2010 ProtecT	NCC	1,461/1,507	Plasma	Lactobacillus casei microbiological assay, Perkin-Elmer Life and Analytical Sciences	5-year age group and recruitment centre	age	highest vs lowest quartile 1.01 [0.82 – 1.24]	0.7
Beilby, J; 2010	NCC in RCT	96/225	Serum	Automated immunoassay Architect i2000, Abbott, Chigago, USA	No	No	highest vs lowest tertile 1.09 [0.48 – 2.46] continuous log scale 0.99 [0.56 – 1.77]	0.83 0.83
Vitamin B_{12}								
Weinstein, S; 2003 ATBC study	NCC in RCT	232/464	Serum	Radioassay, Bio-Rad Laboratories, Richmond, CA	clinic, intervention group, date blood collection, age and serum availability	No, other than matching	highest vs lowest quartile 1.12 [0.70 – 1.78]	0.81
Hultdin, J; 2005 Northern Sweden Health and Disease Cohort	NCC	254/514	Heparin plasma	Quantaphase II radioassay, Biorad, CA	age, recruitment date	No, other than matching	highest vs lowest quartile 2.63 [1.61 – 4.29]	< 0.001
Johansson, M; 2008 European Investigation into Cancer and Nutrition (EPIC)	NCC	869/ 1,174	Serum	Lactobacillus leichmannii microbiological assay, Bevital, Norway	study centre, age at enrolemnt, time of day of blood collection, duration of follow-up	smoking, alcohol intake, BMI, marital status, educational attainment, physical activity	Unadjusted model: highest vs lowest quartile 1.16 [0.85 – 1.58] Adjusted model: highest vs lowest quartile 1.19 [0.87 – 1.63] Unadjusted model advanced disease: doubling in concentration 1.69 [1.05 -2.72]	0.21 0.16
Collin, S; 2010 ProtecT	NCC	1,461/1,507	Plasma	Lactobacillus casei microbiological assay, Perkin-Elmer Life and Analytical Sciences	5-year age group and recruitment centre	age	highest vs lowest quartile 1.17 [0.95 – 1.43]	0.06

Abbreviations: NCC = nested case control study; RCT = randomised controled trial; BMI refers to body mass index.

Table 8.2 Characteristics of participants in the European Prospective Investigation into Cancer and Nutrition (EPIC) stratified by country

	Denmark	Germany	Greece	Italy	Netherlands	Spain	Sweden	UK	Total
Number of Men	26,294	21,172	10,806	14,029	9,637	15,148	22,313	22,853	142,252
Person-years	291,838.9	212,888.1	100,175.8	162,332.0	116,901.7	186,327.7	296,483.2	257,680.9	1,624,628
Prostate cancer cases (n)	937	796	78	293	147	347	1,389	741	4,728
Localised/advanced (n)[1]	376/312	435/148	32/21	52/54	23/54	201/26	330/163	278/157	1727/935
Low/high grade (n)[1]	78/111	417/200	29/17	131/97	77/28	190/94	328/195	130/123	1380/865
Age at recruitment[2]	56.0 (50.0 – 64.0)	52.5 (41.0 – 63.0)	51.0 (33.0 – 72.0)	49.0 (38.0 – 62.0)	43.0 (23.0 – 58.0)	49.0 (40.0 – 63.0)	51.0 (30.0 – 68.0)	52.0 (29.0 – 73.0)	52.0 (33.0 – 67.0)
Weight (kg)[2]	81.8 (65.1 – 105.0)	82.0 (65.5 – 104.4)	80.0 (62.0 – 102.0)	76.61 (61.5 – 97.0)	80 (63.1 – 102.0)	80.4 (65.2 – 100.0)	79.5 (63.5 – 101.5)	77.0 (61.24 – 98.6)	79.8 (63.4 – 102.0)
Height (cm)[2]	176.5 (166.0 – 187.5)	175.4 (164.8 – 186.6)	170.0 (159.0 – 182.0)	171.5 (161.0 – 183.0)	178.5 (166.5 – 191.0)	169.0 (158.9 – 179.5)	177.0 (167.0 – 188.0)	175.8 (164.4 – 186.5)	175.0 (162.8 – 187.0)
BMI (kg/m^2)[2]	26.2 (21.5 – 33.0)	26.6 (21.8 – 33.4)	27.6 (22.3 – 34.3)	26.0 (21.5 – 32.3)	25.2 (20.3 – 31.7)	28.2 (23.5 – 34.3)	25.3 (20.7 – 31.6)	25.1 (20.4 – 31.4)	26.1 (21.2 – 32.9)
Incidence rate[3]	35.6	38.7	8.0	23.0	23.0	23.0	47.1	24.4	30.0
Median Daily intake[4]									
Energy (kJ/day)	10,538.2	10,063.6	8,359.1	10,539.8	11,088.4	10,815.5	9,619.7	9,528.7	10,104.6
Vit B12 intake (µg/day)	6.2	5.6	3.3	4.6	4.1	6.4	5.6	4.3	5.2
Folate intake (µg/day)	292.8	243.5	275.8	268.9	275.9	290.9	230.3	334.5	264.9
Alcohol (g/day)	24.4	19.8	9.7	21.7	12.5	21.1	0.003	7.0	12.9

[1]Stage and grade unknown for some cases

[2]Median age and 5-95th percentile in parenthesis

[3]Age standardised incidence rates per 10,000 person-years were calculated by using a weighted average of the incidence rates in each of 4 attained age groups (50-54, 55-59, 60-64 and 65-69 years) with weights based on the European standard population

[4]Median intake estimated from 24-hour recall in the calibration sample of non-case participants, (n= 12,318 (8.7%)); includes non-consumers; used as a more accurate estimate of variation in dietary intake than values derived from the food frequency questionnaires

Table 8.3 Hazard ratios (HR) and 95% confidence intervals (CI) for prostate cancer among 142,252 men in the EPIC cohort by quintiles of dietary intake of folate and vitamin B_{12}

	HR per quintile[3] increase in observed intake					
	Q1	Q2	Q3	Q4	Q5	P for trend[4]
Folate						
Median intake from 24hr recall(µg/day)	250.8	259.6	259.7	271.3	290.3	
Number of cases/non-cases	1,138/27,505	1,026/27,505	943/27,505	888/27,505	733/27,504	
HR [95% CI][1]	1 [ref]	1.01 [0.93, 1.10]	1.02 [0.93, 1.11]	1.09 [0.99, 1.20]	1.05 [0.94, 1.16]	0.19
HR [95% CI][1,2]	1 [ref]	1.02 [0.93, 1.11]	1.02 [0.93, 1.13]	1.08 [0.97, 1.21]	1.02 [0.90, 1.16]	0.57
Vitamin B_{12}						
Median intake from 24hr recall(µg/day)	3.67	4.85	5.21	6.05	6.98	
Number of cases/non-cases	770/27,505	919/27,505	1,052/27,505	985/27,505	1,002/27,504	
HR [95% CI][1]	1 [ref]	0.98 [0.89, 1.08]	1.07 [0.97, 1.18]	0.97 [0.88, 1.07]	1.01 [0.91, 1.12]	0.92
HR [95% CI][1,2]	1 [ref]	0.98 [0.89, 1.09]	1.06 [0.96, 1.18]	0.95 [0.86, 1.06]	0.97 [0.87, 1.09]	0.42

[1] All models are stratified by centre and adjusted for age at study entry (< 50, 50 – 54, 55 – 59, 60 – 64, 65 – 69, ≥ 70 years).

[2] Adjusted for energy intake (fifths).

[3] Cutpoints defined by the quintiles among non-case participants for all centres combined and correspond to < 228.3, 228.3–, 277.7–, 327.6–, > 396.6 µg/day for folate and < 4.22, 4.22–, 5.78–, 7.44–, > 9.79 µg/day for vitamin B_{12}.

[4] Tests for linear trend were obtained by assigning to each category the quintile median value and treating that value as a continuous variable.

Table 8.4 Hazard ratios (HRs) and 95% confidence intervals (CI) for prostate cancer among 142,252 men in the EPIC cohort by dietary intake of folate and vitamin B_{12}

	Folate					B12				
	Number of cases	HR per unit increase in calibrated intake[2]	95% CI	*P* for trend[3]	*P* for heterogeneity in trend[4]	Number of cases	HR per unit increase in calibrated intake[2]	95% CI	*P* for trend[3]	*P* for heterogeneity in trend[4]
Total prostate cancer[1]	4,728	1.00	[0.93, 1.07]	0.91		4,728	0.98	[0.93, 1.03]	0.43	
Grade of PrCa[1,5]										
Low	1,863	1.02	[0.91, 1.13]	0.77		1,863	0.97	[0.89, 1.05]	0.38	
High	382	0.99	[0.78, 1.24]	0.90	0.82	382	1.01	[0.85, 1.19]	0.94	0.66
Stage of PrCa[1,6]										
Localised	1,727	1.04	[0.93, 1.16]	0.49		1,727	1.01	[0.93, 1.09]	0.82	
Advanced	935	0.98	[0.84, 1.14]	0.77	0.52	935	0.97	[0.86, 1.08]	0.54	0.53
Age at Diagnosis[1]										
< 65yrs	1,694	0.99	[0.88, 1.10]	0.79		1,694	1.04	[0.96, 1.12]	0.35	
≥ 65yrs	3,034	1.00	[0.92, 1.09]	0.94	0.80	3,034	0.95	[0.89, 1.01]	0.09	0.07
Time to diagnosis[1]										
< 4 years	727	1.01	[0.85, 1.21]	0.91		727	0.98	[0.86, 1.11]	0.71	
≥ 4 years	4,001	1.02	[0.95, 1.10]	0.61	0.93	4,001	0.98	[0.93, 1.04]	0.52	0.93

[1] All models are stratified by centre and adjusted for age at study entry (< 50, 50 – 54, 55 – 59, 60 – 64, 65 – 69, ≥ 70 years) and adjusted for calibrated energy intake (fifths).

[2] A standard deviation increment increase in intake of folate or vitamin B_{12} of 65.3 µg/day and 2.4 µg/day, respectively.

[3] Tests for linear trend were based on a standard deviation increment increase in folate (65.3 µg/day) or vitamin B12 (2.4 µg/day) intake.

[4] *P* for heterogeneity values relate to likelihood ratio chi-square tests of heterogeniety between trends.

[5] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system.

[6] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic.

Table 8.5 Characteristics of 1,480 prostate cancer cases and 1,777 control participants in EPIC with measurement of circulating folate and vitamin B_{12} concentrations

	Overall		
	Cases	Controls	*P* value [2]
	1,480	1,777	
Characteristics:			
Anthropometry: mean [SD]			
Height (cm)	172.9 [6.9]	173.6 [7.0]	0.33
Weight (kg)	79.7 [11.3]	80.3 [11.7]	0.15
Age at blood collection; yrs (mean); [SD]	59.8 [6.8]	59.2 [6.8]	0.04
BMI (kg/m²); mean [SD]	26.6 [3.4]	26.7 [3.5]	0.29
Smoking: n [%] [1]			
Never	503 [34.8]	655 [37.9]	
Former	611 [42.3]	653 [37.8]	
Current	331 [22.9]	422 [24.4]	0.68
Alcohol consumption (g/d); n [%] [1]			
< 8	420 [36.3]	424 [36.7]	
8-15	230 [19.9]	240 [20.7]	
16-39	293 [25.3]	303 [26.2]	
≥40	214 [18.5]	190 [16.4]	0.62
Physical activity; n [%] [1]			
Inactive	189 [16.6]	176 [15.5]	
Moderately inactive	371 [32.5]	360 [31.6]	
Active	581 [50.9]	602 [52.9]	0.47
Marital status; n [%] [1]			
Married or cohabitating	870 [69.6]	918 [59.7]	
Not married or cohabitating	380 [30.4]	619 [40.3]	0.44
Educational level; n [%] [1]			
Primary or Equivalent	634 [45.1]	868 [51.1]	
Secondary	452 [32.2]	491 [28.9]	
Degree	319 [22.7]	339 [20.0]	0.58
Cases Only			
Time to Diagnosis (yrs); n [%]			
< 2	145 [9.8]		
2 - 3	224 [15.1]		
4 - 5	171 [11.6]		
6 - 7	179 [12.1]		
≥8	761 [51.4]		
Age at Diagnosis; mean [SD]	65.8 [6.7]		
Stage; n [%] [1,3]			
Localised	679 [45.9]		
Advanced	273 [18.5]		
Grade; n [%] [1,4]			
Low	949 [64.1]		
High	171 [11.6]		

[1] Unknown for some participants; percentage calculations excluded unknown values

[2] *P* values relate to a likelihood ratio chi-square test of difference for categorical variables or a weighted paired sample t test for continuous variables

[3] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic

[4] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system

Table 8.6 Adjusted geometric mean folate and vitamin B_{12} concentrations [95% CI] among control participants according to baseline characteristics

	Serum Folate Concentration (nmol/L)				Serum Vitamin B_{12} Concentration (pmol/L)			
	n	Adjusted geometric mean [95% CI][1]	*P* for heterogeneity	*P* for trend[4]	*n*	Adjusted geometric mean [95% CI][1]	*P* for heterogeneity[3]	*P* for trend[4]
Characteristics:								
Age at blood collection (yrs):								
≤54	422	10.3 [9.8, 10.9]			422	349.0 [337.0, 361.5]		
55 – 59	520	10.8 [10.3, 11.4]			527	330.9 [320.6, 341.6]		
60 – 64	558	10.7 [10.3, 11.3]			550	330.0 [320.0, 340.2]		
≥65	258	10.7 [9.9, 11.7]	0.53	0.59	258	304.9 [288.5, 322.3]	0.001	0.001
BMI (kg/m²):								
< 25	592	10.3 [9.9, 10.8]			591	327.9 [318.5, 337.6]		
≥25	1159	10.9 [10.5, 11.2]	0.07	0.02	1157	332.4 [325.6, 339.3]	0.46	0.67
Smoking:[2c]								
Never	646	11.1 [10.7, 11.6]			650	327.1 [318.1, 336.3]		
Former	647	10.8 [10.3, 11.2]			642	340.1 [330.6, 349.9]		
Current	418	10.3 [9.7, 10.8]	0.06		418	328.0 [316.8, 339.5]	0.13	
Alcohol consumption (g/d):[2c]								
< 8	415	14.7 [13.9, 15.6]			419	326.7 [314.9, 338.9]		
8 – 15	238	14.7 [13.7, 15.8]			237	332.8 [317.3, 349.2]		
16 – 39	299	15.0 [14.1, 16.1]			298	337.8 [323.5, 352.8]		
≥40	188	14.2 [13.1, 15.5]	0.79	0.39	186	307.7 [290.9, 325.4]	0.06	0.19
Physical activity:[2c]								
Inactive	175	13.6 [12.4, 14.8]			175	319.8 [301.8, 338.8]		
Moderately inactive	355	14.5 [13.6, 15.4]			354	331.7 [318.8, 345.1]		
Active	593	15.4 [14.7, 16.1]	0.04	0.02	592	329.3 [319.4, 339.5]	0.59	0.80
Marital status:[2a,b]								
Married or cohabitating	907	10.4 [10.0, 10.9]			903	320.9 [311.7, 330.4]		
Not married or cohabitating	614	10.1 [9.5, 10.7]	0.42		616	328.1 [315.8, 340.8]	0.44	
Educational level:[2c]								
Primary or Equivalent	860	10.2 [9.8, 10.6]			859	335.6 [327.1, 344.3]		
Secondary	481	10.8 [10.2, 11.3]			484	329.2 [318.1, 340.7]		
Degree	338	11.0 [10.4, 11.7]	0.09		335	324.4 [311.5, 337.7]	0.39	
Month of blood collection:								
December – February	450	10.7 [10.2, 11.3]			450	329.0 [318.3, 340.0]		
March – May	527	10.6 [10.1, 11.1]			529	333.9 [323.9, 344.2]		
June – August	283	11.1 [10.4, 11.9]			287	333.0 [319.2, 347.4]		
September – November	498	10.4 [10.0, 11.0]	0.45		491	328.2 [317.9, 338.8]	0.85	

[1] Adjusted for age (< 50, 50 – 54, 55 – 59, 60 – 64, 65 – 69, ≥ 70 years), centre (1-18), batch (1 - 12 folate and 1 - 14 vitamin B12) and month of blood collection (January -December), where the adjustment variable is not the variable of interest; with 95% CI in parenthesis

[2a] Unknown for some participants; [2b] Denmark and Spain were excluded as marital status data is unavailable for these centres

[3] *P* value refers to tests of heterogeneity between the logarithm of the geometric means in the separate categories (excluding unknowns) calculated by using analysis of variance

[4] *P* value refers to the test for trend obtained by treating the categorical variable as a continuous variable in the model

Table 8.7 Geometric mean [95% CI] folate and vitamin B_{12} concentrations by phase of study and country among control participants in the European Prospective Investigation into Cancer and Nutrition (EPIC)

	Germany	Greece	Italy	Netherlands	Spain	Sweden	UK	Total	P for heterogeneity[3]
Folate									
Phase 1[1] (n)	176	9	60	22	90	616	172	1145	
Geometric mean folate (nmol/L)[2] [95% CI]	14.8 [13.2, 16.7]	12.3 [8.35, 18.0]	9.90 [8.10, 12.11]	10.9 [8.46, 14.1]	14.0 [11.9, 16.4]	5.9 [5.5, 6.3]	16.2 [14.3, 18.2]	9.4 [9.1, 9.7]	< 0.0001
Phase 2[1] (n)	169	30	83	23	107	-/-	201	613	< 0.0001
Geometric mean folate (nmol/L)[2] [95% CI]	15.70 [14.4, 17.1]	13.0 [10.7, 15.8]	11.7 [10.4, 13.1]	10.8 [8.62, 13.5]	15.5 [13.9, 17.2]	-/-	16.7 [15.4, 18.2]	15.1 [14.4, 15.7]	
Combined phase 1 & 2[1] (n)	345	39	143	45	197	616	373	1758	
Geometric mean folate (nmol/L)[2] [95% CI]	15.25 [14.3, 16.3]	12.3 [10.3, 14.7]	11.0 [10.0, 12.2]	10.7 [9.03, 12.6]	14.7 [13.5, 16.1]	6.0 [5.6, 6.3]	16.3 [15.2, 17.5]	9.4 [9.1, 9.7]	< 0.0001
Vitamin B_{12}						-/-			
Phase 1[1] (n)	170	9	61	24	91	615	174	1144	
Geometric mean vitamin B_{12} (pmol/L)[2] [95% CI]	289.7 [268.6, 312.4]	280.0 [220.2, 356.0]	300.8 [265.3, 341.1]	280.0 [240.0, 326.7]	359.0 [324.9, 396.7]	331.8 [318.4, 345.8]	292.6 [271.8, 314.9]	315.2 [308.7, 321.8]	< 0.0001
Phase 2[1] (n)	169	30	83	23	107	-/-	201	613	
Geometric mean vitamin B_{12} (pmol/L)[2] [95% CI]	340.0 [320.6, 360.7]	353.1 [308.0, 404.8]	335.0 [308.5, 363.7]	344.2 [294.7, 402.0]	406.4 [377.8, 437.1]	-/-	357.8 [337.6, 379.3]	356.7 [346.0, 367.8]	0.003
Combined phase 1 & 2[1] (n)	339	39	144	47	198	615	375	1757	
Geometric mean vitamin B_{12} (pmol/L)[2] [95% CI]	316.1 [302.3, 330.5]	323.7 [287.9, 364.0]	321.9 [301.3, 343.9]	317.3 [285.0, 353.2]	388.0 [366.5, 410.8]	331.2 [317.8, 345.2]	322.4 [307.8, 337.7]	330.3 [324.6, 336.1]	< 0.0001

[1] Duplicate observations of 5 controls that appear in the data set twice (because of the incidence densisty sampling protocol) were removed

[2] Adjusted for age (< 50, 50 - 54, 55 - 59, 60 - 64, 65 - 69, ≥ 70 years), batch (1 - 12 folate and 1 - 14 vitamin B_{12}) and month of blood collection (January - December), where the adjustment variable is not the variable of interest; with 95% CI in parenthesis

[3] *P* value refers to tests of heterogeneity between the logarithm of the geometric means in the separate categories calculated by using analysis of variance

Table 8.8 Odds ratios (ORs) and 95% confidence intervals [95% CI] for prostate cancer by quintiles of circulating folate and vitamin B_{12} concentration in the EPIC cohort

	Fifth of circulating folate concentration[1] (nmol/L)					
	Q1	Q2	Q3	Q4	Q5	P for trend[3]
Phase 1						
Cases/controls (n)	161/337	180/280	193/191	155/165	166/177	
OR [95% CI][2]	1 [ref]	1.22 [0.92, 1.62]	1.67 [1.19, 2.34]	1.38 [0.96, 1.99]	1.40 [0.96, 2.03]	0.48
Phase 2						
Cases/controls (n)	12/17	63/72	163/160	194/190	181/174	
OR [95% CI][2]	1 [ref]	1.27 [0.56, 2.91]	1.51 [0.69, 3.31]	1.52 [0.68, 3.39]	1.56 [0.70, 3.46]	0.42
Phase 1 and 2						
Cases/controls (n)	173/354	243/352	356/351	349/355	347/351	
OR [95% CI][2]	1 [ref]	1.23 [0.95, 1.61]	1.58 [1.18, 2.12]	1.44 [1.06, 1.97]	1.47 [1.07, 2.01]	0.28

	Fifth of circulating vitamin B_{12} concentration[1] (pmol/L)					
	Q1	Q2	Q3	Q4	Q5	P for trend[3]
Phase 1						
Cases/controls (n)	181/251	203/249	184/235	143/234	143/180	
OR [95% CI][2]	1 [ref]	1.23 [0.93, 1.63]	1.25 [0.94, 1.66]	1.04 [0.76, 1.42]	1.28 [0.93, 1.75]	0.34
Phase 2						
Cases/controls (n)	96/100	114/103	123/118	114/120	166/172	
OR [95% CI][2]	1 [ref]	1.15 [0.78, 1.70]	1.08 [0.75, 1.56]	0.99 [0.68, 1.46]	0.99 [0.68, 1.44]	0.64
Phase 1 and 2						
Cases/controls (n)	277/351	317/352	307/353	257/354	309/352	
OR [95% CI][2]	1 [ref]	1.20 [0.96, 1.51]	1.19 [0.95, 1.49]	1.03 [0.81, 1.31]	1.14 [0.90, 1.46]	0.67

[1] Quintile cutpoints were defined among control participants for all cohorts combined; corresponding to < 5.59, 5.59 –, 8.72 –, ≥12.7 nmol/L for folate and < 250.6, 250.6 –, 308.4 –, 367.3 –, ≥441.1 pmol/L for vitamin B12

[2] Conditioned by matching factors: study centre, length of follow-up, age at recruitment, time of blood collection and duration of fasting at blood collection and adjusted for age at blood collection

[3] Tests for linear trend were obtained by assigning to each category the quintile median value and treating that value as a continuous variable.

Table 8.9 Odds ratios (ORs) and 95% confidence intervals [95% CI] for prostate cancer by quintiles of circulating folate concentration in the EPIC cohort using country specific quintile cutpoints

	Germany			Greece			Italy			Netherlands		
Folate	Median [nmol/L]	OR[2] [95% CI]		Median [nmol/L]	OR[2] [95% CI]		Median [nmol/L]	OR[2] [95% CI]		Median [nmol/L]	OR[2] [95% CI]	
Quintile												
1	8.67	1		7.91	1		5.66	1		6.63	1	
2	12.04	1.22	0.78, 1.92	10.66	1.98	0.37, 10.51	8.52	0.95	0.42, 2.12	8.14	0.53	0.12, 2.42
3	15.26	0.81	0.49, 1.33	12.40	0.94	0.14, 6.17	10.35	1.15	0.51, 2.60	10.11	0.68	0.15, 3.12
4	19.93	0.97	0.61, 1.57	15.46	3.12	0.60, 16.24	13.74	1.76	0.77, 4.01	13.32	1.78	0.47, 6.81
5	31.73	1.08	0.66, 1.74	22.59	0.52	0.12, 2.35	23.38	0.99	0.44, 2.21	21.21	1.13	0.28, 4.57
P trend[3]		0.90			0.90			0.58			0.37	

	Spain			Sweden			UK				Total[5]	
Folate	Median [nmol/L]	OR[2] [95% CI]		Median [nmol/L]	OR[2] [95% CI]		Median [nmol/L]	OR[2] [95% CI]		P for heterogeneity[4]	OR[2] [95% CI]	
Quintile												
1	7.88	1		3.40	1		8.24	1			1	
2	11.09	1.41	0.75, 2.64	4.61	1.09	0.70, 1.69	11.87	1.13	0.71, 1.80		1.14	0.91, 1.42
3	14.64	1.39	0.73, 2.66	5.59	0.89	0.57, 1.40	15.04	1.01	0.63, 1.62	0.91	0.96	0.76, 1.21
4	18.61	1.13	0.58, 2.22	7.08	1.12	0.72, 1.73	21.42	0.93	0.57, 1.50		1.13	0.90, 1.42
5	26.67	1.03	0.53, 2.02	14.69	1.39	0.88, 2.17	38.28	1.34	0.84, 2.15		1.19	0.94, 1.49
P trend[3]		0.80			0.18			0.44			0.21	

[1]Quintile cutpoints were defined among control participants using country specific cutpoints; corresponding to 10.27, 13.75, 17.03 and 24.12 nmol/L for Germany, 8.66, 11.37, 13.26 and 19.97 nmol/L for Greece, 7.47, 9.31, 11.47 and 16.88 nmol/L for Italy, 7.14, 8.65, 11.34 and 17.05 nmol/L for Netherlands, 9.55, 12.76, 16.39 and 22.69 nmol/L for Spain, 4.05, 5.12, 6.16 and 8.03 nmol/L for Sweden and 10.13, 13.44, 17.46 and 26.70 nmol/L for UK.

[2] Conditioned by matching factors: study centre, age at recruitment, time of blood collection and duration of fasting at blood collection and adjusted for age at blood collection.

[3]Test for linear trend for each country was obtained by assigning to each category the quintile median value and treating that value as a continuous variable.

[4] P for heterogeneity values relate to likelihood ratio chi-square tests of heterogeneity between trends.

[5]Test for linear trend combining all the data was obtained by assigning to each category the numerical values 1, 2, 3, 4 and 5.

Table 8.10 Odds ratios (ORs) and 95% confidence intervals [95% CI] for prostate cancer associated with a doubling of circulating folate and vitamin B_{12} concentration in the EPIC study

	Folate					Vitamin B_{12}				
	Number of cases/controls	OR[1]	95% CI	P for trend[3]	P for heterogeneity in trend[4]	Number of cases/controls	OR[1]	95% CI	P for trend[3]	P for heterogeneity in trend[4]
Total Prostate Cancer[2]	1,468/1,763	1.05	[0.95, 1.15]	0.33		1,467/1,762	1.05	[0.92, 1.21]	0.47	
Stage[2,5]										
Localised stage	673/787	1.07	[0.94, 1.23]	0.30		676/790	0.92	[0.74, 1.14]	0.43	
Advanced stage	271/320	1.08	[0.89, 1.31]	0.43	0.97	266/315	1.35	[0.97, 1.87]	0.07	0.05
Grade of Prostate Cancer[2,6]										
Low grade	940/1,187	1.05	[0.94, 1.17]	0.40		938/1,186	1.03	[0.87, 1.23]	0.69	
High grade	171/204	1.06	[0.82, 1.38]	0.66	0.94	170/203	0.94	[0.64, 1.38]	0.76	0.67
Time to Diagnosis (years)[2]										
< 4	361/389	0.95	[0.81, 1.13]	0.59		359/387	1.10	[0.82, 1.46]	0.53	
4–7	541/618	1.16	[0.99, 1.36]	0.06		542/618	1.06	[0.84, 1.35]	0.62	
> 7	566/756	1.02	[0.88, 1.19]	0.78	0.22	566/757	1.02	[0.83, 1.25]	0.85	0.91
Age at diagnosis (years)[2]										
< 65	657/804	1.06	[0.93, 1.22]	0.38		656/802	1.04	[0.84, 1.30]	0.71	
≥65	811/959	1.04	[0.92, 1.17]	0.56	0.76	811/960	1.07	[0.90, 1.27]	0.46	0.80
Age at blood collection (years)[2]										
< 60	750/952	1.08	[0.95, 1.23]	0.22		749/951	1.10	[0.90, 1.34]	0.35	
≥60	718/811	1.01	[0.88, 1.15]	0.89	0.43	718/811	1.01	[0.84, 1.22]	0.89	0.58

[1] The odds ratio for a doubling of the concentration in folate and vitamin B_{12} (calculated on the log2 scale).

[2] Conditioned by matching factors: study centre, age at recruitment, time of blood collection and duration of fasting at blood collection and adjusted for age at blood collection.

[3] P values for trend were obtained with the logarithm of serum folate and vitamin B_{12} concentrations.

[4] P for heterogeneity values relate to likelihood ratio chi-square tests of heterogeneity between trends.

[5] Localised stage includes T0 or T1 or T2 and N0 or NX and M0, or stage coded in the recruitment centre as localised and advanced stage includes T3 or T4 and/or N1+ and/or M1, or stage coded in the recruitment centre as metastatic.

[6] Low grade includes Gleason score ≤7, or cases coded as well differentiated or moderately differentiated according to the World Health Organization (WHO) grading system and high grade includes Gleason score > 7, or cases coded as poorly differentiated or undifferentiated according to the WHO grading system.

Figure 8:2 Fixed effects meta-analysis of the association of circulating folate and risk for prostate cancer by EPIC country

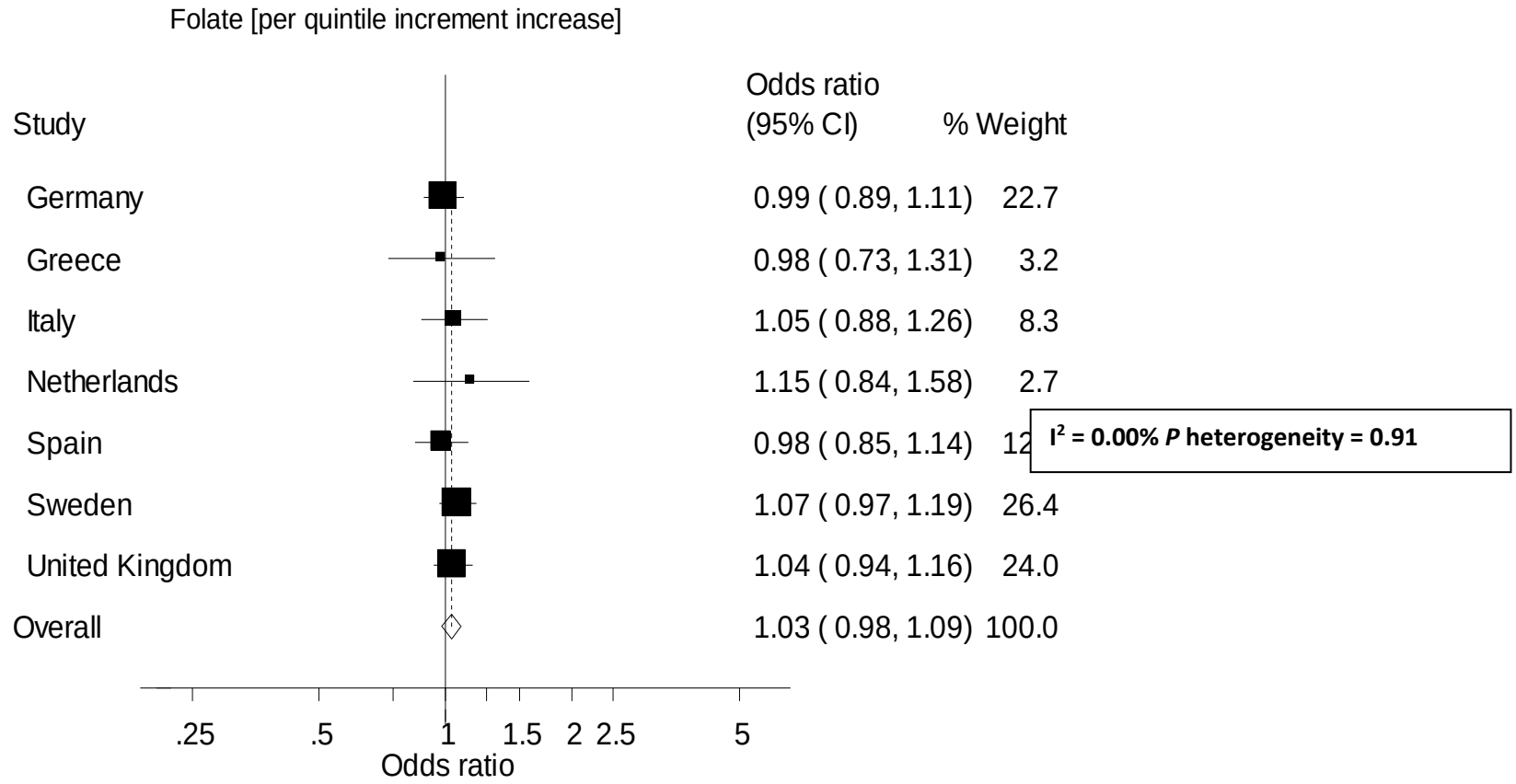


Figure 8:3 Fixed effects meta-analysis of the association of circulating folate and risk for prostate cancer

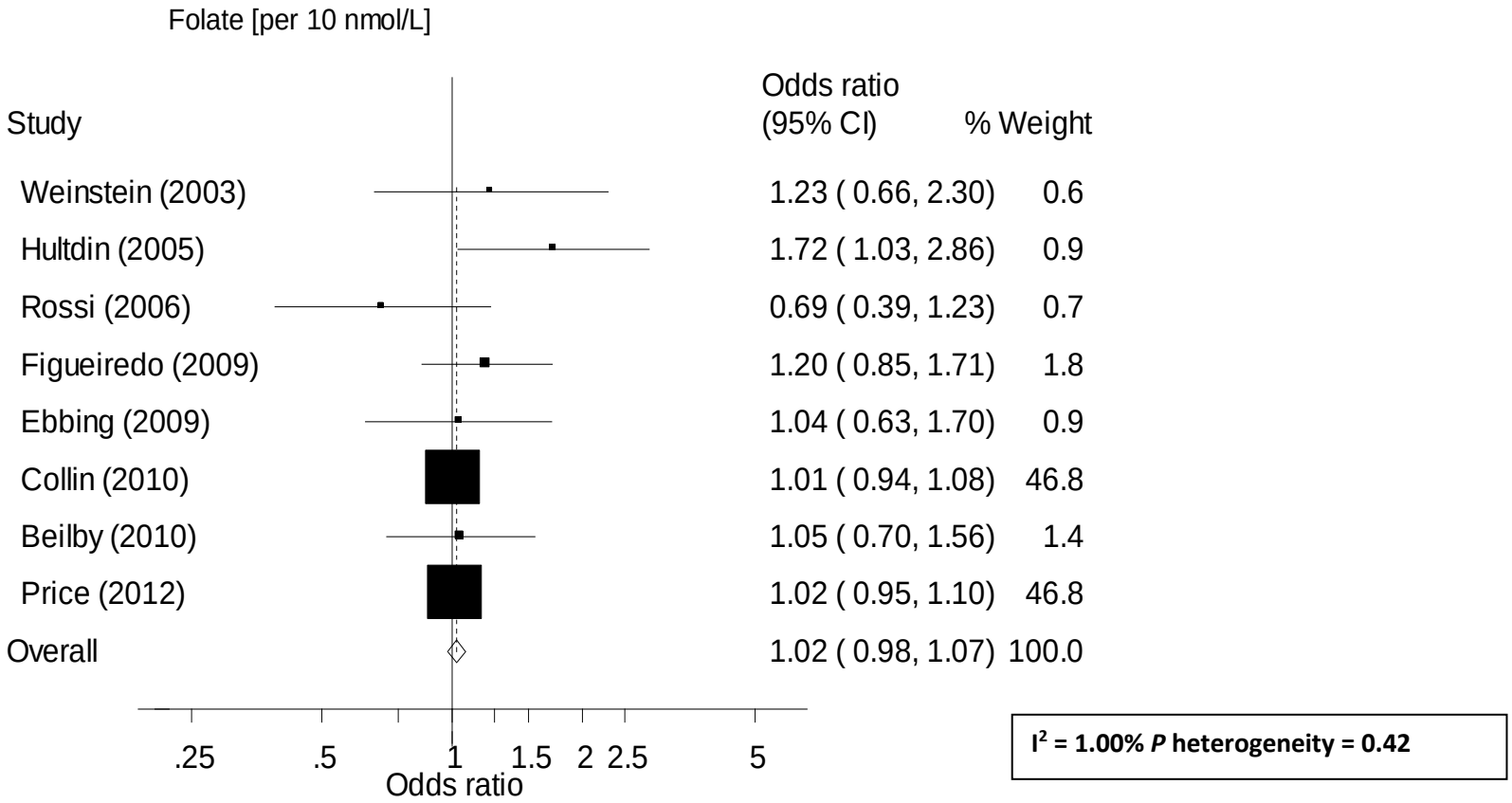


Figure 8:4 Fixed effects meta-analysis of the association of circulating folate and risk for prostate cancer in prospective studies, excluding Collin, 210

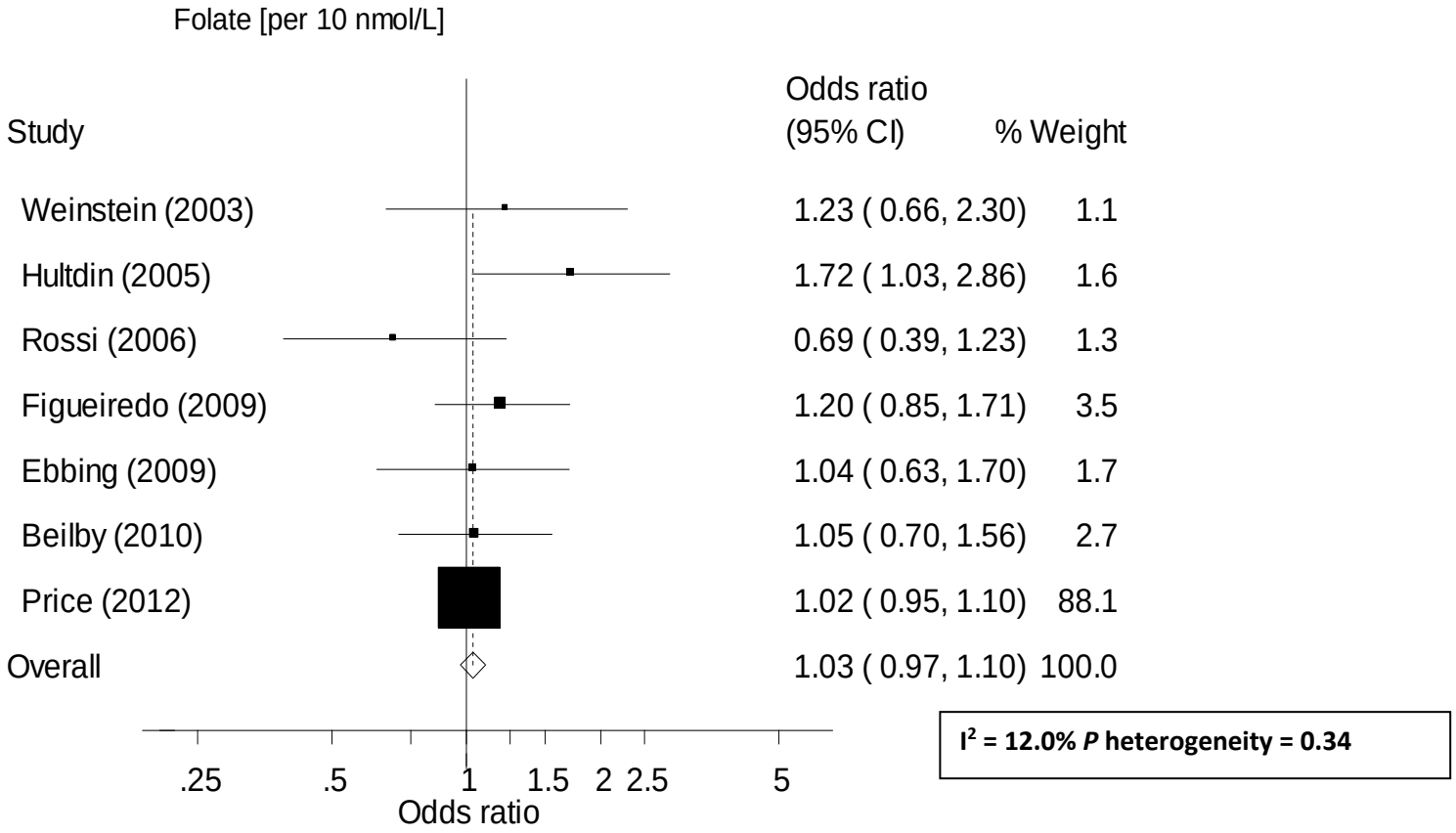


Figure 8:5 Fixed effects meta-analysis of the association of circulating vitamin B_{12} and risk for prostate cancer

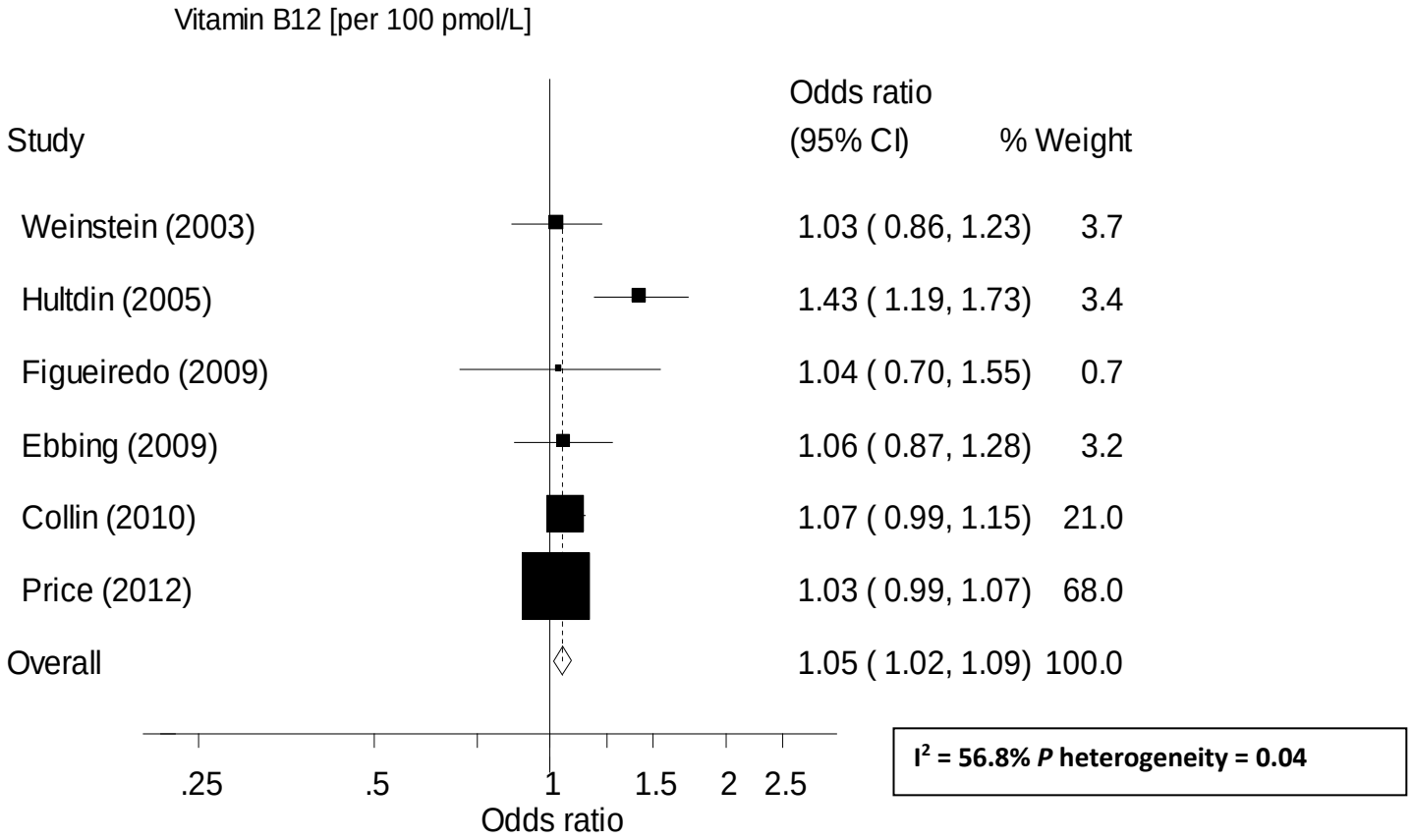


Figure 8:6 Fixed effects meta-analysis of the association of circulating vitamin B_{12} and risk for prostate cancer, excluding Hultdin, 2005

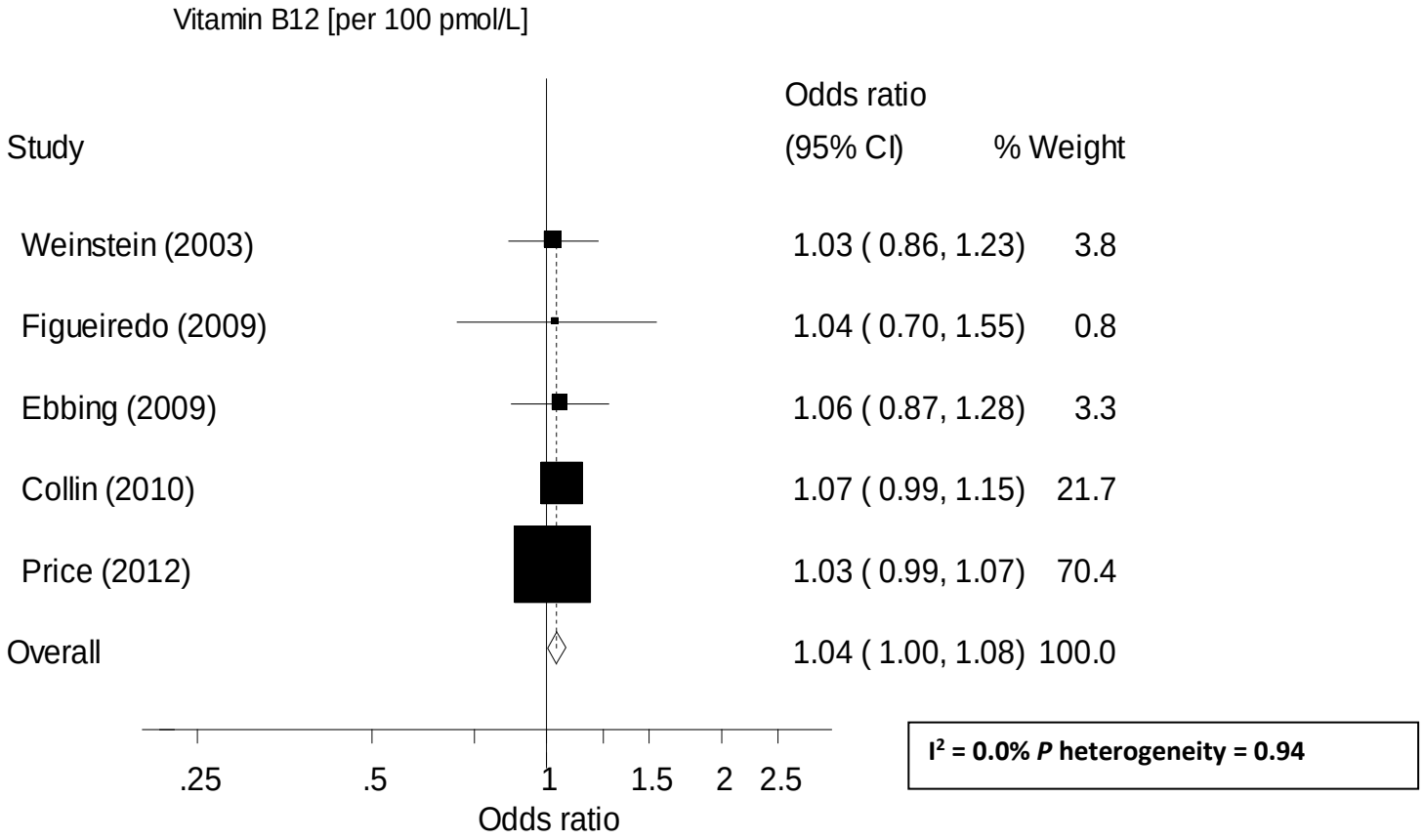


Figure 8:7 Fixed effects meta-analysis of the association of circulating vitamin B_{12} and risk for prostate cancer in prospective studies excluding Hultdin, 2005 and Collin, 2010

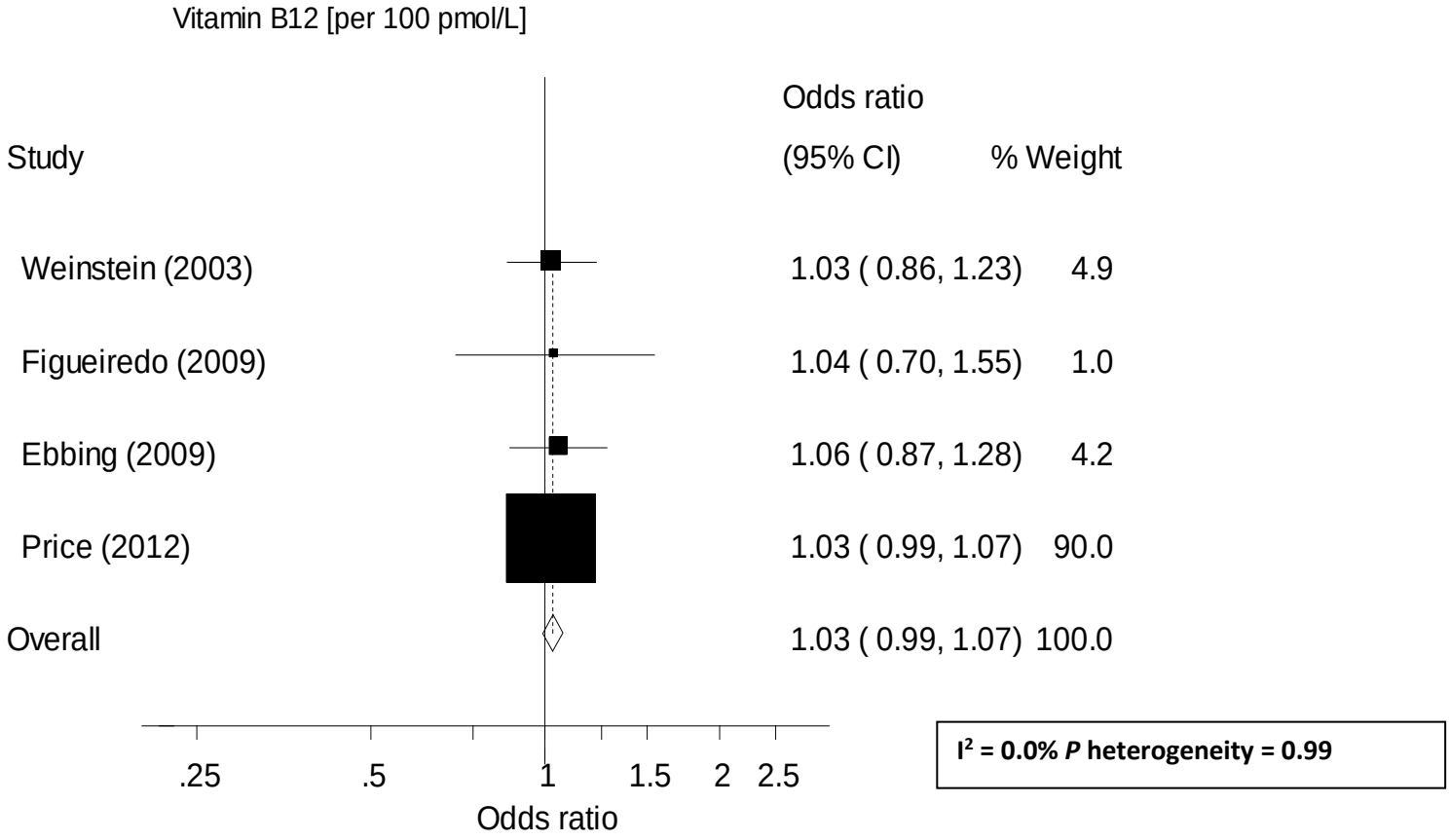


Figure 8:8 Funnel plot of 8 studies of the association of circulating folate and risk for prostate cancer

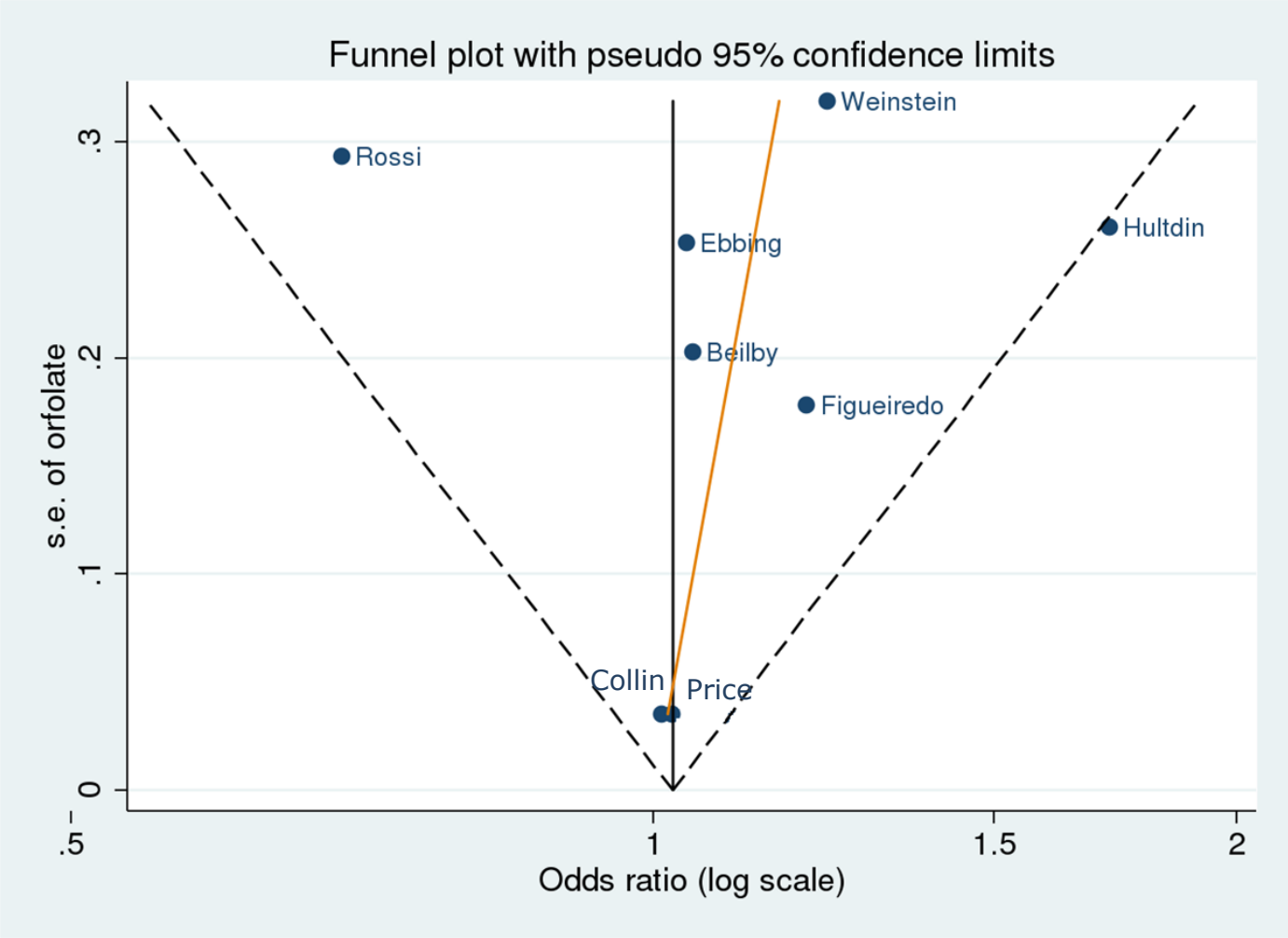
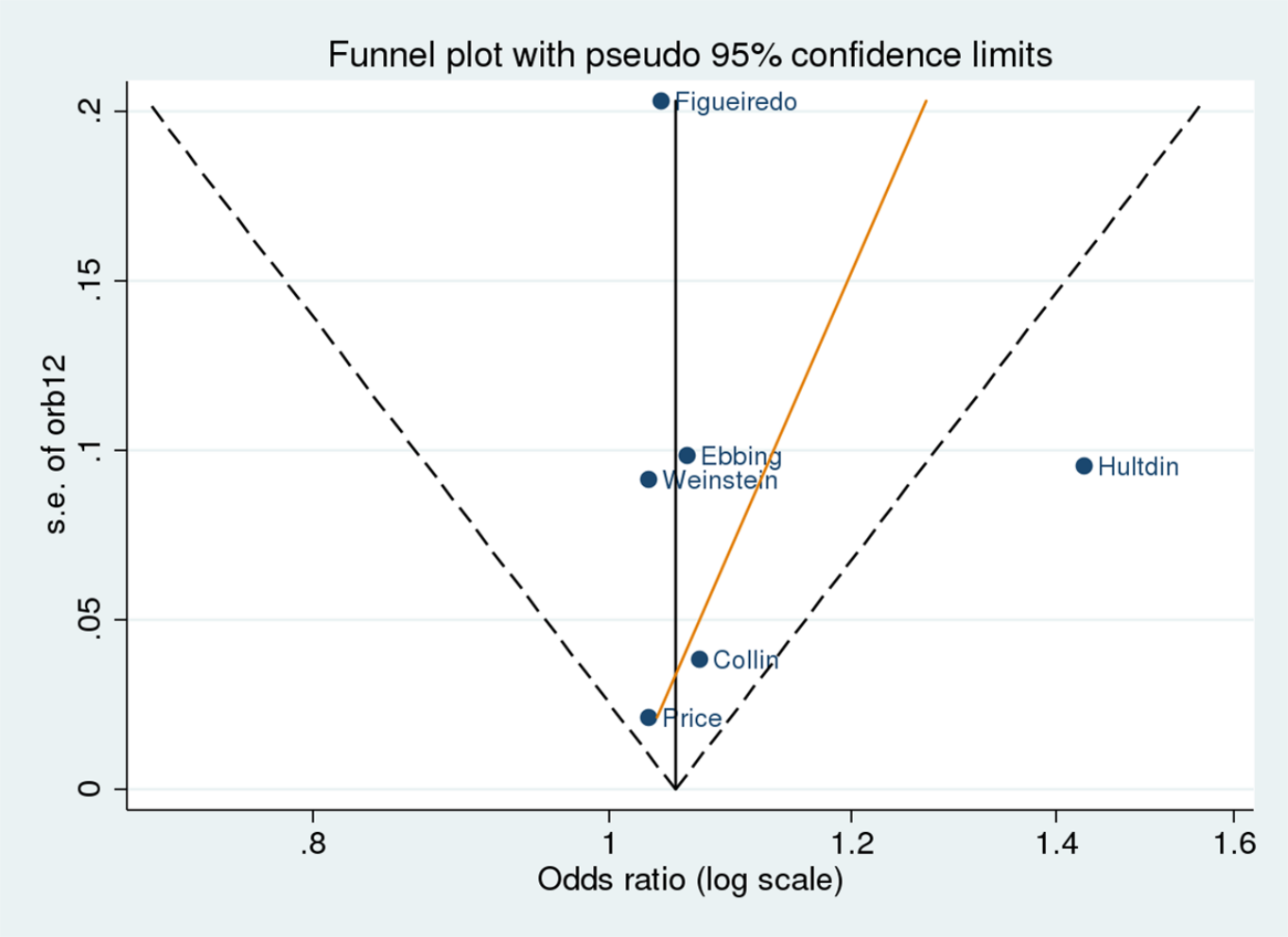


Figure 8:9 Funnel plot of 6 studies of the association of circulating vitamin B_{12} and risk for prostate cancer



Chapter 9 Discussion

9.1 Overview

Evidence from ecological and migrant studies supports a role for a Western diet (high in fat and animal products) in the development of prostate cancer. However, prospective epidemiological investigations have not shown convincing evidence for an association between diet and risk for prostate cancer. Lack of consistent findings may be related to error associated with measurement of habitual dietary intake using traditional survey methods. Validated nutritional biomarkers provide more objective and precise measures of specific nutrients and food constituents, while minimizing error typically associated with respondent recall (manifested typically as underestimated energy and dietary fat intake and an overestimated intake of perceived healthy components of the diet (11)). Nonetheless, nutritional biomarkers must offer reliable estimates of habitual dietary exposure if they are to be useful in the investigation of association with subsequent cancer risk.

9.2 Aim of study

The main aim of this thesis was to investigate nutritional and hormonal risk factors for prostate cancer using validated biological markers of folate, vitamin B_{12} and phytanic acid intake and endogenous IGF-I activity. A subsidiary aim was to utilise the long-term follow-up and large sample size of the EPIC study to differentiate prostate cancer outcomes by classification of disease according to grade (high and low grade disease), stage (localised and advanced) and time to diagnosis from study entry (thereby reducing the risk of reverse causality).

9.3 Main findings from this thesis

9.3.1 Phytanic acid and risk for prostate cancer

Observation of a strong correlation between phytanic acid concentration and fat intake from dairy and beef (as presented in Chapter 6) indicate that this fatty acid, while not a specific biomarker of dairy fat, is a good biomarker of ruminant fat intake, with utility as an alternative or complimentary measure of dietary ruminant fat exposure. However, there was no evidence of an association between circulating phytanic acid concentration and risk for prostate cancer overall or by subgroups of grade or stage of disease (outlined in detail in Chapter 5). These findings are the first published results from a prospective investigation of the association between phytanic acid and risk for prostate cancer and consideration of findings from other large cohorts with repeat measures of phytanic acid (taken 5 - 10 years apart) are required in order to determine further the nature of the association.

9.3.2 IGF-I and risk for prostate cancer

A strong positive association was observed between IGF-I concentration and risk for prostate cancer and there was no evidence of heterogeneity in the association in all sub-group analyses including grade and stage of disease, age at blood collection, time to diagnosis from blood collection and age at diagnosis. While an association was not observed for IGF-I and high

grade disease, the small number of cases in this group meant that the study was underpowered to detect modest risk estimates. These findings presented in Chapter 7 are consistent with those of a pooled analysis of individual patient data from 8 prospective studies and provide strong evidence for an association between IGF-I and risk for prostate cancer.

9.3.3 Folate and vitamin B12 and risk for prostate cancer

The findings presented in Chapter 8 do not provide evidence of an association for either dietary intake or circulating concentrations of folate or vitamin B_{12} and risk for overall prostate cancer in the EPIC study. There was also no evidence of statistically significant interactions between folate or vitamin B_{12} and prostate cancer risk by grade or stage of disease, duration of time between recruitment and diagnosis or alcohol intake. The results of the meta-analysis of prospective studies including the EPIC data did not provide evidence for an association between folate concentrations and prostate cancer risk but there was evidence to support a 4% higher risk per 100 pmol/L increase in circulating vitamin B_{12}. Although of a lower magnitude, this finding is consistent with that of an earlier meta-analysis of prospective data (92); strengthening the evidence for a modest increased risk of prostate cancer with elevated vitamin B_{12} concentration.

9.3.4 Folate, IGF-I, phytanic acid and vitamin B_{12}: reliability studies

Repeat blood samples, available in a subset of individuals, permitted evaluation of the reliability (over a 6 year period), of a single measure of folate, IGF-I, phytanic acid and vitamin B_{12}. The results of these analyses, presented in Chapter 4, contextualise the findings of the relation with disease risk and raise important questions about the suitability of single blood measures to adequately quantify exposure for folate and vitamin B_{12}, given the low reliability measures of 0.18 and 0.34 obtained for each analyte, respectively. As expected for each biomarker, the reliability studies show that estimates of usual concentrations based on a single blood measurement weaken the ability to detect associations with disease risk and, where small effect sizes are anticipated, may bias associations toward the null. Multiple blood samples taken several years apart are needed to account for within-person variation in blood concentrations (attributable to a multitude of factors including changes in diet over time, fasting status at blood collection and season of blood collection) to better characterise relationships between biomarkers of nutritional and hormonal exposure and disease risk.

9.4 Findings in context

The findings presented in this thesis (Chapter 4 to 8) add new information to the literature on the associations between nutritional biomarkers and risk for prostate cancer. The EPIC study now has an average follow-up time of 11.4 years, allowing us to investigate bias resulting from reverse causality (which is problematic for the interpretation of results from prospective studies with a shorter duration of follow-up). A pooled re-analysis of worldwide prospective data based on 3,700 men with prostate cancer and 5,200 controls showed that IGF-I was associated with a modest risk of prostate cancer (64). However, most of the cases in this collaborative re-analysis were diagnosed within 6 years of blood collection, limiting the ability to investigate heterogeneity in risk by length of follow-up and leaving open the possibility of reverse causality. The results from the analysis presented in this thesis confirm that circulating

concentrations of IGF-I in middle to late adulthood are strongly associated with subsequent prostate cancer risk over the relatively long term. Given these findings are based on a single blood sample it is anticipated that the true association is substantially stronger (up to two-fold higher based on the reliability study presented in Chapter 4). As IGF-I is the only potentially modifiable risk factor so far identified, research into the effects of reducing circulating IGF-I levels on subsequent prostate cancer risk is warranted.

The small significant association observed between elevated vitamin B_{12} concentration and risk for prostate cancer observed in the meta-analysis using aggregate risk estimates requires confirmation using individual level participant data (where uniform methods can be used to provide more precise estimates of the relative risk). A better understanding of the relation between folate and prostate cancer risk would also be achieved with an individual participant meta-analysis, to ensure consistency in inclusion criteria and definitions for exposure and outcome variables and to ensure standardised adjustment for confounding variables across studies (382).

9.5 Strengths of study

Strengths of the EPIC study include the prospective design, large sample size and availability of information about potential risk factors that may confound observed associations, including age, smoking and alcohol consumption and detailed data about lifestyle and dietary habits. The combination of a large sample size and the long duration of follow-up permitted sub-analyses excluding participants diagnosed with cancer within a short duration of blood collection, whilst maintaining adequate power to detect modest risk estimates. The heterogeneity of dietary intake amongst 10 European countries is reflected in the wide range of biomarker concentrations, thereby maximising statistical power to detect an association if one exists.

Blood samples were collected at recruitment in the majority (80%) of male participants, enhancing the internal validity of the findings, and with a few exceptions (discussed below in the limitations section), a standardised protocol was used for sample collection, processing, labelling and storage. This approach minimised pre-analytical variation and ensured correct specimen identification at retrieval. An incidence density sampling protocol was used to select control participants from the same study centre thereby avoiding introduction of systematic differences with cases in the time of storage of blood samples. All cases and controls were analysed after stratification by centre to take into account systematic differences in sampling procedures or exposure measurement between the EPIC recruitment centres.

All assays were conducted by trained laboratory personnel blind to the case control status of samples and quality control (QC) samples were routinely included in all batches, with calculation of intra-and intra-batch coefficients of variation. QC pooled blood samples were used in all analyses included in this thesis and standardised QC samples from the United Kingdom National External Quality Assessment Service (NEQAS) were used in the IGF-I assays in phase 2 to ensure the results of our study are comparable with other studies. In all

biomarker assays, the analytical reference ranges of the laboratory methods were adequate to detect all concentrations, including the very low levels of folate observed in Sweden and low vitamin B_{12} amongst vegans.

9.6 Limitations of study

The analyses included in this thesis were sufficiently large in size and adequately powered to detect modest risk estimates for total prostate cancer however there were insufficient numbers of high grade cases to perform meaningful sub-analyses and test for heterogeneity by grade of disease (especially after inclusion of multiple potential confounding variables in the model).

For the analyses included in this thesis information on diet and potential confounding variables was taken from the recruitment questionnaire and it is possible that some exposures (such as body weight and smoking status) changed over the duration of follow-up. However adjusting for potential confounders made no material difference to the overall associations, so the extra error introduced by using a single measurement is unlikely to have a large effect on the relative risk estimates. There is little information collected on socioeconomic status (except level of education) which limits investigation of associations between nutritional factors and prostate cancer stratified according to level of deprivation, which may be important given that men of low socioeconomic status are more likely to have a delayed diagnosis, a more limited diagnostic workup, and less invasive treatments (33, 383). Information on supplement use in the EPIC study is not collected in all centres, so the contribution made from supplements to overall phytanic acid, folate and vitamin B_{12} concentrations and dietary intake cannot be investigated in the associations with risk for prostate cancer. Although information on personal medical history was collected in EPIC, family history of prostate cancer was not asked specifically so it was not possible to control for this confounding variable in the analyses presented here.

Prostate cancer diagnosis and tumour stage and grade information was derived from pathology reports and cancer registry data, thereby avoiding bias and error associated with self-report. However these data were not standardised across EPIC centres and are likely to be affected by changes in diagnostic practices over time and between countries. The TNM system and the Gleason score were both devised over 40 years ago (102, 103), and although historically TNM stage information was limited to findings from physical examination (including digital rectal examination) and radiography, in recent years advanced diagnostic techniques such as ultrasound and bone imaging have lead to marked improvements in the quality of stage information over time. The TNM score from pathology specimens is often higher (i.e. more aggressive) than that based on clinical examination alone however the contribution made to TNM score from clinical and pathology staging has not been accounted for in the classification of disease in the EPIC study and this may vary across centres.

In regard to the histological grading of prostate cancer, the volume and quality of tissue available for histopathology and Gleason grading has changed over time with the current

practice of ultrasound guided needle biopsy at diagnosis rather than the previous practice of tissue collection via radical prostatectomy at surgery. This change in diagnostic practice has also led to a trend toward allocation of higher grades to cancers that would have previously been classified as less-aggressive forms of the disease (102, 104, 105), with implications for the classification of cancers in EPIC due to its prolonged follow-up and lack of standardisation of histopathology specimens.

It is also notable that widespread prostate cancer screening has increased detection of early disease and revealed heterogeneous patterns of cancer that were unapparent when the Gleason criterion was first devised. To accommodate this change, scoring recommendations have been updated at intervals; mostly recently in 2005 (106). Prior to 2005, the overall Gleason score was generated using the two most predominant Gleason grades. However, the current recommendation is to use the most predominant grade and the tertiary grade. The tertiary grade represents the highest grade, independent of the amount of tumour present (105). This change in grading criteria has further contributed to the upward trend in Gleason scoring over time (105).

Unlike some other studies, such as ProtecT in the UK, in the EPIC cohort screening with PSA, digital rectal examination, diagnostic imaging and prostatic biopsy were not used at recruitment and men who enrolled were assumed to be healthy and free from cancer. However, given the long latent period of prostate cancer, it is possible that some of the cases diagnosed after recruitment may have harboured sub-clinical disease at enrolment and during follow-up and will therefore be misclassified as controls in the study. While this is not necessarily a problem for investigating risk factors associated with progression of clinically relevant (i.e. aggressive) prostate cancer, it does limit meaningful investigation of risk factors associated with disease initiation. Furthermore, data on history of PSA testing or PSA test results at the time of diagnosis for men participating in the EPIC cohort are not available. The absence of this data does not allow for investigation of heterogeneity in PSA testing uptake between different centres or sub-analyses according to PSA level at diagnosis. Nonetheless, annual rates of PSA testing in middle aged men within the participating countries is relatively low; in England and Wales 6% of men receive PSA testing and in Italy, the Netherlands and Spain the rates are 6%, 7% and 9%, respectively (24, 384-386). These proportions are low compared with approximately 38% in the white population of the United States (55, 387) where prospective cohort studies have a lower proportion of advanced stage disease being diagnosed (100). This highlights the importance of European cohorts, such as EPIC, in the investigation of risk factors for advanced disease.

Standardised blood collection, processing and transport procedures were implemented in most centres (as discussed in section 9.5), however deviations to the protocol occurred in the Oxford, Sweden and Denmark cohorts. The transport of Oxford blood samples in the post may have led to degradation of biomarkers that are sensitive to delayed processing, such as folate (discussed in Chapter 4, section 4.6). To this extent the results of the reliability studies, performed on blood from Oxford participants may lack validity, highlighting the need for a

larger study using a subset of participants from across EPIC countries in order to understand better the reliability of single estimates to measures usual concentrations of these biomarkers.

IGF-I assays were not completed using the same laboratory methods because the DSL ELISA assay method was no longer available at the time of the extended study. Steps were taken to ensure that a comparable assay was used for the remaining samples but there was evidence of difference in the detection limit and measured levels with the different assays. However, despite differences in the absolute levels between the two assays, analyses stratified by assay type showed no differences in the association and all case-control sets were measured using the same assay method.

The EPIC study is conducted in 10 European countries and the majority of participants are of white European origin hence the findings of the studies outlined in this thesis may not be generalisable to other ethnic groups.

9.7 Recommendations for future research

Implicit in the work of this thesis, investigating the associations between circulating concentrations of folate, IGF-I, phytanic acid and vitamin B_{12} and risk for prostate cancer, is an assumption that a measure of circulating concentrations is a useful surrogate of bio-available concentrations at the level of the prostate. However, there are few investigations of the correlation between circulating concentrations of nutritional biomarkers and activity at the tissue level, and such studies have been performed in a very limited numbers of samples (388). Moreover, to the author's knowledge, there are no published data of the association between tissue concentrations of the biomarkers under study in this thesis and risk for prostate cancer. To determine if circulating concentrations of these biomarkers mirror activity at the cellular level within the prostate gland requires further investigation.

With increasing utilisation of biomarkers in epidemiological research there are opportunities for collaborative studies using individual participant data. In 2008 the Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) was initiated and it has grown into a 54-member consortium, representing over 225 associated organisations (mostly biobanks) from 30 countries. This collaborative initiative aims to harmonise cohorts by making data and materials rapidly and widely available to researchers, providing resources and operational concepts to improve the quality and sustainability of biobanks and, outlining standard operating procedures and codes of conduct (389).

Pooling data increases statistical power, enabling investigation of associations amongst subgroups, has the advantage of standardisation of the modelling of the exposure and confounding variables (creating the same categories and adjustments) in order to remove potential sources of heterogeneity that affect the comparability of studies and provides a wider range of biomarker concentrations from different populations with varied dietary habits (390). However, if individual level meta-analyses are to provide more reliable and precise

estimates of effect it is imperative that attention be given to methodological processes including blood collection procedures, time between blood collection and freezing of samples, temperature during storage and laboratory assays to ensure that variation in biomarkers attributable to these processes is minimized and observed differences in biomarker concentrations between populations reflect true differences (390). While retrospective pooling projects offer more robust assessment of exposure-disease relations, future pooling projects of biomarker studies would ideally be conceived prior to embarking on individual studies in order to limit differences in the design. This would extend to conducting analyses in the same laboratory, with the same assay methods, during the same time period and with inclusion of blinded quality control samples.

Data pooling for reliability assessment of biomarkers on large numbers is also needed to determine the extent to which a single measure adequately quantifies an individual's usual exposure. Repeat measurement of biomarkers on a large scale will allow for quantitative correction for regression dilution bias that results from the combined effects of measurement error (i.e. short term-biological variability (including seasonal variation) and longer-term within person differences that may include a change in diet, physical activity, medication or disease status (391)). Where multiple measures on participants are not available in all of the pooled studies a representative subsample of each study could be used to determine the reliability of the biomarker and derivation of a correction coefficient to apply to a summary estimate (358). Furthermore, if standardised quality control samples were used, as provided by the National External Quality Assessment Service (NEQAS) in the UK, differences in measurements between laboratories could be quantified and used to adjust values from each study to a common scale (390, 392).

With mounting epidemiological evidence to suggest a role of IGF-I in prostate cancer development, further research is warranted to determine the effect of modifying circulating IGF-I concentrations through medical intervention or modified lifestyle. Evidence from large scale randomised controlled trials of the effect of reducing IGF-I concentration on the risk of prostate cancer and robust meta-analyses of these studies are required to generate a more precise estimate of modest differences in prostate cancer risk associated with levels of IGF-I exposure. Studies have investigated the interaction of IGF-I with IGF-I binding proteins and IGF-I receptors, although the extent to which binding proteins alter the association between IGF-I and risk requires further study.

In future, pooling data from large Mendelian randomisation studies of genetic variants known to modify exposure levels may help to determine better the effects of lifelong differences in folate, IGF-I, phytanic acid and vitamin B_{12} concentrations on the risk of prostate cancer.

Better characterisation of prostate cancer according to aggressiveness of disease is needed to reduce error associated with the outcome measurement and for understanding possible etiological differences. Multi-site prospective studies with access to long term follow-up would benefit from standardisation of histopathology in order to define more precisely disease

end-points, taking into account varied health-seeking behaviours by individuals, health care delivery differences between countries and changes in standards of health care over time. Identification of diagnostic and prognostic biomarkers of prostate cancer, that clearly differentiate aggressive from non- aggressive disease, is also warranted, and where much current research is focused.

9.8 Conclusion

In conclusion, there is convincing evidence that IGF-I is an important and potentially modifiable risk factor for prostate cancer many years before diagnosis. There is limited evidence of an association between circulating folate and vitamin B_{12} concentrations and risk for prostate cancer and no convincing evidence for an association between phytanic acid and risk for prostate cancer. Future studies should, where possible, incorporate multiple blood samples taken several years apart to better characterise long term relationships between these biomarkers of nutritional and hormonal exposure and disease risk and pool individual participant data from prospective studies for meta-analysis to strengthen the power to detect modest associations.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010;60(5):277-300.

2. Armstrong B, Doll R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. Int J Cancer 1975;15(4):617-31.

3. Haenszel W, Kurihara M. Studies of Japanese migrants. I. mortality from cancer and other diseases among Japanese in the United States. J Natl Cancer Inst 1968;40(1):43-68.

4. Whittemore AS, Kolonel LN, Wu AH, John EM, Gallagher RP, Howe GR, et al. Prostate cancer in relation to diet, physical activity, and body size in blacks, whites, and asians in the United States and canada. J Natl Cancer Inst 1995;87(9):652-61.

5. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles county. Br J Cancer 1991;63(6):963-6.

6. Chan JM, Gann PH, Giovannucci EL. Role of diet in prostate cancer development and progression. Journal of Clinical Oncology 2005;23(32):8152-60.

7. Dagnelie PC, Schuurman AG, Goldbohm RA, Van Den Brandt PA. Diet, anthropometric measures and prostate cancer risk: A review of prospective cohort and intervention studies. BJU Int 2004;93(8):1139-50.

8. Key TJ, Allen NE, Spencer EA, Travis RC. The effect of diet on risk of cancer. Lancet 2002;360(9336):861-8.

9. World Cancer Research Fund/American Institute for Cancer Research. Food, nutrition, physical activity and the prevention of cancer: A global perspective. 1st ed. Washington: American Institute for Cancer Research; 2007.

10. Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG, et al. The influence of finasteride on the development of prostate cancer. New England Journal of Medicine 2003;349(3):215-24.

11. Bingham SA. Biomarkers in nutritional epidemiology. Public Health Nutr 2002;5(6 A):821-7.

12. Allen NE, Key TJ, Appleby PN, Travis RC, Roddam AW, Rinaldi S, et al. Serum insulin-like growth factor (IGF)-I and IGF-binding protein-3 concentrations and prostate cancer risk: Results from the European prospective investigation into cancer and nutrition. Cancer Epidemiology Biomarkers and Prevention 2007;16(6):1121-7.

13. Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, et al. European prospective investigation into cancer and nutrition (EPIC): Study populations and data collection. Public Health Nutr 2002;5(6 B):1113-24.

14. Margetts BM, Pietinen P. European prospective investigation into cancer and nutrition: Validity studies on dietary assessment methods. International Journal of Epidemiology 1997;26(SUPPL. 1).

15. Slimani N, Ferrari P, Ocké M, Welch A, Boeing H, Van Liere M, et al. Standardization of the 24-hour diet recall calibration method used in the European prospective investigation into cancer and nutrition (EPIC): General concepts and preliminary results. Eur J Clin Nutr 2000;54(12):900-17.

16. Slimani N, Kaaks R, Ferrari P, Casagrande C, Clavel-Chapelon F, Lotze G, et al. European prospective investigation into cancer and nutrition (EPIC) calibration study: Rationale, design and population characteristics. Public Health Nutr 2002;5(6 B):1125-45.

17. Ferrari P, Kaaks R, Fahey MT, Slimani N, Day NE, Pera G, et al. Within- and between-cohort variation in measured macronutrient intakes, taking account of measurement errors, in the European prospective investigation into cancer and nutrition study. Am J Epidemiol 2004;160(8):814-22.

18. Rosner B, Willett WC, Spiegelman D. Correction of logistic regression relative risk estimates and confidence intervals for systematic within-person measurement error. Statistics in Medicine 1989;8(9):1051-69.

19. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61(2):69-90.

20. GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide IARC CancerBase no. 5. version 2.0, IARCPress, lyon, 2004 [homepage on the Internet]. IARC CancerBase No. 5. 2004.

21. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. Ca-A Cancer Journal for Clinicians 2005;55(2):74-108.

22. Etzioni R, Penson DF, Legler JM, Di Tommaso D, Boer R, Gann PH, et al. Overdiagnosis due to prostate-specific antigen screening: Lessons from U.S. prostate cancer incidence trends. J Natl Cancer Inst 2002;94(13):981-90.

23. Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. International Journal of Cancer 2000;85(1):60-7.

24. Melia J, Moss S, Johns L. Rates of prostate-specific antigen testing in general practice in England and wales in asymptomatic and symptomatic patients: A cross-sectional study. BJU International 2004;94(1):51-6.

25. Sirovich BE, Schwartz LM, Woloshin S. Screening men for prostate and colorectal cancer in the United States: Does practice reflect the evidence? Journal of the American Medical Association 2003;289(11):1414-20.

26. Beaulac JA, Fry RN, Onysko J. Lifetime and recent prostate specific antigen (PSA) screening of men for prostate cancer in canada. Can J Public Health 2006;97(3):171-6.

27. Andriole GL, Levin DL, Crawford ED, Gelmann EP, Pinsky PF, Chia D, et al. Prostate cancer screening in the prostate, lung, colorectal and ovarian (PLCO) cancer screening trial: Findings from the initial screening round of a randomized trial. J Natl Cancer Inst 2005;97(6):433-8.

28. Schröder FH, Hugosson J, Roobol MJ, Tammela TLJ, Ciatto S, Nelen V, et al. Screening and prostate-cancer mortality in a randomized European study. New Engl J Med 2009;360(13):1320-8.

29. Baade PD, Youlden DR, Krnjacki LJ. International epidemiology of prostate cancer: Geographical distribution and secular trends. Mol Nutr Food Res 2009;53(2):171-84.

30. Hankey BF, Feuer EJ, Clegg LX, Hayes RB, Legler JM, Prorok PC, et al. Cancer surveillance series: Interpreting trends in prostate Cancer—Part I: Evidence of the effects of screening in recent prostate cancer incidence, mortality, and survival rates. J Natl Cancer Inst 1999;91(12):1017-24.

31. Collin M, Martin RM, Metcalfe C, Gunnell D, Albertsen PC, Neal D, et al. Prostate-cancer mortality in the USA and UK in 1975-2004: An ecological study. The Lancet Oncology 2008;9(5):445-52.

32. Shavers VL, Brown ML. Racial and ethnic disparities in the receipt of cancer treatment. J Natl Cancer Inst 2002;94(5):334-57.

33. Ward E, Jemal A, Cokkinides V, Singh GK, Cardinez C, Ghafoor A, et al. Cancer disparities by race/ethnicity and socioeconomic status. Ca-A Cancer Journal for Clinicians 2004;54(2):78-93.

34. Cannon L, Bishop DT, Skolnick M, Hunt S, Lyon JL, Smart CR. Genetic epidemiology of prostate cancer in the utah mormon genealogy. Inheritance of Susceptibility to Cancer in Man 1982:47-69.

35. Steinberg GD, Carter BS, Beaty TH, Childs B, Walsh PC. Family history and the risk of prostate cancer. Prostate 1990;17(4):337-47.

36. Carter BS, Beaty TH, Steinberg GD, Childs B, Walsh PC. Mendelian inheritance of familial prostate cancer. Proceedings of the National Academy of Sciences of the United States of America 1992;89(8):3367-71.

37. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, et al. Environmental and heritable factors in the causation of cancer: Analyses of cohorts of twins from Sweden, Denmark, and Finland. New England Journal of Medicine 2000;343(2):78-85.

38. Kiciński M, Vangronsveld J, Nawrot TS. An epidemiological reappraisal of the familial aggregation of prostate cancer: A meta-analysis. PLoS ONE 2011;6(10).

39. Johns LE, Houlston RS. A systematic review and meta-analysis of familial prostate cancer risk. BJU Int 2003;91(9):789-94.

40. Bruner DW, Moore D, Parlanti A, Dorgan J, Engstrom P. Relative risk of prostate cancer for men with affected relatives: Systematic review and meta-analysis. Int J Cancer 2003;107(5):797-803.

41. Zeegers MPA, Jellema A, Ostrer H. Empiric risk of prostate carcinoma for relatives of patients with prostate carcinoma. Cancer 2003;97(8):1894-903.

42. Ben-Shlomo Y, Evans S, Ibrahim F, Patel B, Anson K, Chinegwundoh F, et al. The risk of prostate cancer amongst black men in the United Kingdom: The PROCESS cohort study. European Urology 2008;53(1):99-105.

43. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008 v1.2, cancer incidence and mortality worldwide: IARC CancerBase no. 10 [internet].Lyon, France: International agency for research on cancer; 2010. available from: Http://globocan.iarc.fr, accessed on 23/02/2012. 2010.

44. Bratt O, Garmo H, Adolfsson J, Bill-Axelson A, Holmberg L, Lambe M, et al. Effects of prostate-specific antigen testing on familial prostate cancer risk estimates. J Natl Cancer Inst 2010;102(17):1336-43.

45. Haiman CA, Chen GK, Blot WJ, Strom SS, Berndt SI, Kittles RA, et al. Genome-wide association study of prostate cancer in men of African ancestry identifies a susceptibility locus at 17q21. Nat Genet 2011;43(6):570-3.

46. Eeles RA, Kote-Jarai Z, Al Olama AA, Giles GG, Guy M, Severi G, et al. Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. Nat Genet 2009;41(10):1116-21.

47. Eeles RA, Kote-Jarai Z, Giles GG, Olama AAA, Guy M, Jugurnauth SK, et al. Multiple newly identified loci associated with prostate cancer susceptibility. Nature Genetics 2008;40(3):316-21.

48. Yeager M, Orr N, Hayes RB, Jacobs KB, Kraft P, Wacholder S, et al. Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. Nature Genetics 2007;39(5):645-9.

49. Gudmundsson J, Sulem P, Gudbjartsson DF, Blondal T, Gylfason A, Agnarsson BA, et al. Genome-wide association and replication studies identify four variants associated with prostate cancer susceptibility. Nat Genet 2009;41(10):1122-6.

50. Gudmundsson J, Sulem P, Manolescu A, Amundadottir LT, Gudbjartsson D, Helgason A, et al. Genome-wide association study identifies a second prostate cancer susceptibility variant at 8q24. Nat Genet 2007;39(5):631-7.

51. Gudmundsson J, Sulem P, Rafnar T, Bergthorsson JT, Manolescu A, Gudbjartsson D, et al. Common sequence variants on 2p15 and Xp11.22 confer susceptibility to prostate cancer. Nat Genet 2008;40(3):281-3.

52. Takata R, Akamatsu S, Kubo M, Takahashi A, Hosono N, Kawaguchi T, et al. Genome-wide association study identifies five new susceptibility loci for prostate cancer in the Japanese population. Nat Genet 2010;42(9):751-4.

53. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, et al. Finding the missing heritability of complex diseases. Nature 2009;461(7265):747-53.

54. Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 2010;19(8):1893-907.

55. Etzioni R, Berry KM, Legler JM, Shaw P. Prostate-specific antigen testing in black and white men: An analysis of medicare claims from 1991-1998. Urology 2002;59(2):251-5.

56. Coleman MP, Quaresma M, Berrino F, Lutz J-, De Angelis R, Capocaccia R, et al. Cancer survival in five continents: A worldwide population-based study (CONCORD). The Lancet Oncology 2008;9(8):730-56.

57. Al Olama AA, Kote-Jarai Z, Giles GG, Guy M, Morrison J, Severi G, et al. Multiple loci on 8q24 associated with prostate cancer susceptibility. Nat Genet 2009;41(10):1058-60.

58. Liu H, Wang B, Han C. Meta-analysis of genome-wide and replication association studies on prostate cancer. Prostate 2011;71(2):209-24.

59. Marker PC, Donjacour AA, Dahiya R, Cunha GR. Hormonal, cellular, and molecular control of prostatic development. Dev Biol 2003;253(2):165-74.

60. Roddam AW, Allen NE, Appleby P, Key TJ. Endogenous sex hormones and prostate cancer: A collaborative analysis of 18 prospective studies. Journal of the National Cancer Institute 2008;100(3):170-83.

61. Ntais C, Polycarpou A, Ioannidis JPA. Association of the CYP17 gene polymorphism with the risk of prostate cancer: A meta-analysis. Cancer Epidemiol Biomarkers Prev 2003;12(2):120-6.

62. Wang F, Zou Y-, Feng X-, Su H, Huang F. CYP17 gene polymorphisms and prostate cancer risk: A meta-analysis based on 38 independent studies. Prostate 2011;71(11):1167-77.

63. Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: Biological actions. Endocr Rev 1995;16(1):3-34.

64. Roddam AW, Allen NE, Appleby P, Key TJ, Ferrucci L, Carter HB, et al. Insulin-like growth factors, their binding proteins, and prostate cancer risk: Analysis of individual patient data from 12 prospective studies. Ann Intern Med 2008;149(7):461-71.

65. Crowe FL, Key TJ, Allen NE, Appleby PN, Roddam A, Overvad K, et al. The association between diet and serum concentrations of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 in the European prospective investigation into cancer and nutrition. Cancer Epidemiol Biomarkers Prev 2009;18(5):1333-40.

66. Doll R, Peto R. The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 1981;66(6):1191-308.

67. Gann PH, Hennekens CH, Sacks FM, Grodstein F, Giovannucci EL, Stampfer MJ. Prospective study of plasma fatty acids and risk of prostate cancer. J Natl Cancer Inst 1994;86(4):281-6.

68. Michaud DS, Augustsson K, Rimm EB, Stampfer MJ, Willet WC, Giovannucci E. A prospective study on intake of animal products and risk of prostate cancer. Cancer Causes Control 2001;12(6):557-67.

69. Chan JM, Stampfer MJ, Ma J, Gann PH, Gaziano JM, Giovannucci EL. Dairy products, calcium, and prostate cancer risk in the physicians' health study. Am J Clin Nutr 2001;74(4):549-54.

70. Berndt SI, Carter HB, Landis PK, Tucker KL, Hsieh LJ, Metter EJ, et al. Calcium intake and prostate cancer risk in a long-term aging study: The baltimore longitudinal study of aging. Urology 2002;60(6):1118-23.

71. Rodriguez C, Jacobs EJ, Patel AV, Calle EE, Feigelson HS, Fakhrabadi-Shokoohi D, et al. Jewish ethnicity and prostate cancer mortality in two large US cohorts. Cancer Causes Control 2002;13(3):271-7.

72. Tseng M, Breslow RA, Graubard BI, Ziegler RG. Dairy, calcium, and vitamin D intakes and prostate cancer risk in the national health and nutrition examination epidemiologic follow-up study cohort. Am J Clin Nutr 2005;81(5):1147-54.

73. Kesse E, Bertrais S, Astorg P, Jaouen A, Arnault N, Galan P, et al. Dairy products, calcium and phosphorus intake, and the risk of prostate cancer: Results of the french prospective

SU.VI.MAX (supplémentation en vitamines et minéraux antioxydants) study. Br J Nutr 2006;95(3):539-45.

74. Ahn J, Albanes D, Peters U, Schatzkin A, Lim U, Freedman M, et al. Dairy products, calcium intake, and risk of prostate cancer in the prostate, lung, colorectal, and ovarian cancer screening trial. Cancer Epidemiol Biomarkers Prev 2007;16(12):2623-30.

75. Mitrou PN, Albanes D, Weinstein SJ, Pietinen P, Taylor PR, Virtamo J, et al. A prospective study of dietary calcium, dairy products and prostate cancer risk (finland). Int J Cancer 2007;120(11):2466-73.

76. Rohrmann S, Platz EA, Kavanaugh CJ, Thuita L, Hoffman SC, Helzlsouer KJ. Meat and dairy consumption and subsequent risk of prostate cancer in a US cohort study. Cancer Causes Control 2007;18(1):41-50.

77. Kurahashi N, Inoue M, Iwasaki M, Sasazuki S, Tsugane S. Dairy product, saturated fatty acid, and calcium intake and prostate cancer in a prospective cohort of Japanese men. Cancer Epidemiol Biomarkers Prev 2008;17(4):930-7.

78. Allen NE, Key TJ, Appleby PN, Travis RC, Roddam AW, Tjønneland A, et al. Animal foods, protein, calcium and prostate cancer risk: The European prospective investigation into cancer and nutrition. Br J Cancer 2008;98(9):1574-81.

79. Thornburg T, Turner AR, Chen YQ, Vitolins M, Chang B, Xu J. Phytanic acid, AMACR and prostate cancer risk. Future Oncology 2006;2(2):213-23.

80. Wierzbicki AS, Lloyd MD, Schofield CJ, Feher MD, Gibberd FB. Refsum's disease: A peroxisomal disorder affecting phytanic acid α-oxidation. Journal of Neurochemistry 2002;80(5):727-35.

81. Wierzbicki AS, Mayne PD, Lloyd MD, Burston D, Mei G, Sidey MC, et al. Metabolism of phytanic acid and 3-methyl-adipic acid excretion in patients with adult refsum disease. Journal of Lipid Research 2003;44(8):1481-8.

82. Van Den Brink DM, Wanders RJA. Phytanic acid: Production from phytol, its breakdown and role in human disease. Cellular and Molecular Life Sciences 2006;63(15):1752-65.

83. Jiang Z, Fanger GR, Woda BA, Banner BF, Algate P, Dresser K, et al. Expression of α-methylacyl-CoA racemase (P504S) in various malignant neoplasms and normal tissues: A study of 761 cases. Hum Pathol 2003;34(8):792-6.

84. Jiang Z, Woda BA, Rock KL, Xu Y, Savas L, Khan A, et al. P504S: A new molecular marker for the detection of prostate carcinoma. Am J Surg Pathol 2001;25(11):1397-404.

85. Beach R, Gown AM, De Peralta-Venturina MN, Folpe AL, Yaziji H, Salles PG, et al. P504S immunohistochemical detection in 405 prostatic specimens including 376 18-gauge needle biopsies. Am J Surg Pathol 2002;26(12):1588-96.

86. Kumar-Sinha C, Shah RB, Laxman B, Tomlins SA, Harwood J, Schmitz W, et al. Elevated α-methylacyl-CoA racemase enzymatic activity in prostate cancer. Am J Pathol 2004;164(3):787-93.

87. Fenech M. The role of folic acid and vitamin B12 in genomic stability of human cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2001;475(1-2):57-67.

88. Ulrich CM, Potter JD. Folate and cancer - timing is everything. J Am Med Assoc 2007;297(21):2408-9.

89. Ulrich CM, Potter JD, Ulrich N. Folate supplementation: Too much of a good thing? Cancer Epidemiol Biomarkers Prev 2006;15(2):189-93.

90. Kim Y-. Folate: A magic bullet or a double edged sword for colorectal cancer prevention? Gut 2006;55(10):1387-9.

91. Wien TN, Pike E, Wisloff T, Staff A, Smeland S, Klemp M. Cancer risk with folic acid supplements: A systematic review and meta-analysis. BMJ Open 2012;2(1):e000653.

92. Collin SM, Metcalfe C, Refsum H, Lewis SJ, Zuccolo L, Smith GD, et al. Circulating folate, vitamin B12, homocysteine, vitamin B12 transport proteins, and risk of prostate cancer: A case-control study, systematic review, and meta-analysis. Cancer Epidemiology Biomarkers & Prevention 2010;19(6):1632-42.

93. Riboli E. Nutrition and cancer: Background and rationale of the European prospective investigation into cancer and nutrition (EPIC). Ann Oncol 1992;3(10):783-91.

94. Breslow N, Chan CW, Dhom G. Latent carcinoma of prostate at autopsy in seven areas. International Journal of Cancer 1977;20(5):680-8.

95. Etzioni R, Cha R, Feuer EJ, Davidov O. Asymptornatic incidence and duration of prostate cancer. American Journal of Epidemiology 1998;148(8):775-85.

96. Stattin P, Holmberg E, Johansson J-, Holmberg L, Adolfsson J, Hugosson J. Outcomes in localized prostate cancer: National prostate cancer register of sweden follow-up study. J Natl Cancer Inst 2010;102(13):950-8.

97. Sánchez-Chapado M, Olmedilla G, Cabeza M, Donat E, Ruiz A. Prevalence of prostate cancer and prostatic intraepithelial neoplasia in caucasian mediterranean males: An autopsy study. Prostate 2003;54(3):238-47.

98. Akazaki K, Stemmermann GN. Comparative study of latent carcinoma of the prostate among Japanese in japan and hawaii. J Natl Cancer Inst 1973;50(5):1137-42.

99. Draisma G, Etzioni R, Tsodikov A, Mariotto A, Wever E, Gulati R, et al. Lead time and overdiagnosis in prostate-specific antigen screening: Importance of methods and context. J Natl Cancer Inst 2009;101(6):374-83.

100. Platz EA, De Marzo AM, Giovannucci E. Prostate cancer association studies: Pitfalls and solutions to cancer misclassification in the PSA era. Journal of Cellular Biochemistry 2004;91(3):553-71.

101. Draisma G, Boer R, Otto SJ, van der Cruijsen IW, Damhuis RAM, Schröder FH, et al. Lead times and overdetection due to prostate-specific antigen screening: Estimates from the European randomized study of screening for prostate cancer. J Natl Cancer Inst 2003;95(12):868-78.

102. Schroder FH, Hermanek P, Denis L, Fair WR, Gospodarowicz MK, Pavone-Macaluso M. The TNM classification of prostate cancer. Prostate 1992;20(SUPPL. 4):129-38.

103. Gleason DF. Histologic grading of prostate cancer: A perspective. Hum Pathol 1992;23(3):273-9.

104. Albertsen PC, Hanley JA, Barrows GH, Penson DF, Kowalczyk PDH, Sanders MM, et al. Prostate cancer and the will rogers phenomenon. J Natl Cancer Inst 2005;97(17):1248-53.

105. Epstein JI, Allsbrook Jr. WC, Amin MB, Egevad LL, Bastacky S, López Beltrán A, et al. The 2005 international society of urological pathology (ISUP) consensus conference on gleason grading of prostatic carcinoma. Am J Surg Pathol 2005;29(9):1228-42.

106. Schmidt C. Gleason scoring system faces change and debate. J Natl Cancer Inst 2009;101(9):622-9.

107. Giovannucci E, Liu Y, Platz EA, Stampfer MJ, Willett WC. Risk factors for prostate cancer incidence and progression in the health professionals follow-up study. International Journal of Cancer 2007;121(7):1571-8.

108. Park S, Murphy SP, Wilkens LR, Stram DO, Henderson BE, Kolonel LN. Calcium, vitamin D, and dairy product intake and prostate cancer risk: The multiethnic cohort study. Am J Epidemiol 2007.

109. Albertsen PC, Hanley JA, Gleason DF, Barry MJ. Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. J Am Med Assoc 1998;280(11):975-80.

110. Tworoger SS, Hankinson SE. Use of biomarkers in epidemiologic studies: Minimizing the influence of measurement error in the study design and analysis. Cancer Causes Control 2006;17(7):889-99.

111. Potischman N. Biologic and methodologic issues for nutritional biomarkers. Journal of Nutrition 2003;133(3 SUPPL.).

112. Frost C, Thompson SG. Correcting for regression dilution bias: Comparison of methods for a single predictor variable. Journal of the Royal Statistical Society. Series A: Statistics in Society 2000;163(2):173-89.

113. Rosner B, Spiegelman D, Willett WC. Correction of logistic regression relative risk estimates and confidence intervals for measurement error: The case of multiple covariates measured with error. American Journal of Epidemiology 1990;132(4):734-45.

114. Frost C, White IR. The effect of measurement error in risk factors that change over time in cohort studies: Do simple methods overcorrect for 'regression dilution'? International Journal of Epidemiology 2005;34(6):1359-68.

115. Phillips AN, Smith GD. How independent are ”independent” effects? relative risk estimation when correlated exposures are measured imprecisely. Journal of Clinical Epidemiology 1991;44(11):1223-31.

116. Phillips AN, Smith GD. Bias in relative odds estimation owing to imprecise measurement of correlated exposures. Statistics in Medicine 1992;11(7):953-61.

117. Sander G. Statistical uncertainty due to misclassification: Implications for validation substudies. J Clin Epidemiol 1988;41(12):1167-74.

118. Clayton PE, Banerjee I, Murray PG, Renehan AG. Growth hormone, the insulin-like growth factor axis, insulin and cancer risk. Nat Rev Endocrionol 2011;7(1):11-24.

119. Adlercreutz H. Phyto-oestrogens and cancer. Lancet Oncol 2002;3(6):364-73.

120. Larsson SC, Giovannucci E, Wolk A. Folate and risk of breast cancer: A meta-analysis. J Natl Cancer Inst 2007;99(1):64-76.

121. Price AJ, Allen NE, Appleby PN, Crowe FL, Jenab M, Rinaldi S, et al. Plasma phytanic acid concentration and risk of prostate cancer: Results from the European prospective investigation into cancer and nutrition. Am J Clin Nutr 2010;91(6):1769-76.

122. Wolff T, Witkop CT, Miller T, Syed SB. Folic acid supplementation for the prevention of neural tube defects: An update of the evidence for the U.S. preventive services task force. Ann Intern Med 2009;150(9):632-9.

123. Gibson RS. Assessment of folate and vitamin B12 status. In: Principles of Nutritional Assessment. Second ed. ; 2005. p. 595-633.

124. Goodman-Gruen D, Barrett-Connor E. Epidemiology of insulin-like growth factor-I in elderly men and women: The rancho bernardo study. Am J Epidemiol 1997;145(11):970-6.

125. Platz EA, Pollak MN, Rimm EB, Majeed N, Tao Y, Willett WC, et al. Racial variation in insulin-like growth factor-1 and binding protein-3 concentrations in middle-aged men. Cancer Epidemiology Biomarkers and Prevention 1999;8(12):1107-10.

126. Kaaks R, Lukanova A. Energy balance and cancer: The role of insulin and insulin-like growth factor-I. Proc Nutr Soc 2001;60(1):91-106.

127. Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M, et al. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. Journal of the National Cancer Institute 2002;94(14):1099-106.

128. Muti P, Quattrin T, Grant BJB, Krogh V, Micheli A, Schünemann HJ, et al. Fasting glucose is a risk factor for breast cancer. Cancer Epidemiology Biomarkers & Prevention 2002;11(11):1361-8.

129. Borofsky ND, Vogelman JH, Krajcik RA, Orentreich N. Utility of insulin-like growth factor-1 as a biomarker in epidemiologic studies. Clinical Chemistry 2002;48(12):2248-51.

130. Lukanova A, Lundin E, Zeleniuch-Jacquotte A, Muti P, Mure A, Rinaldi S, et al. Body mass index, circulating levels of sex-steroid hormones, IGF-I and IGF-binding protein-3: A cross-sectional study in healthy women. European Journal of Endocrinology 2004;150(2):161-71.

131. Gapstur SM, Kopp P, Brian C-H. Chiu, Gann PH, Colangelo LA, Liu K. Longitudinal associations of age, anthropometric and lifestyle factors with serum total insulin-like growth factor-I and IGF binding protein-3 levels in black and white men: The CARDIA male hormone study. Cancer Epidemiol Biomarkers Prev 2004;13(12):2208-16.

132. Missmer SA, Spiegelman D, Bertone-Johnson ER, Barbieri RL, Pollak MN, Hankinson SE. Reproducibility of plasma steroid hormones, prolactin, and insulin-like growth factor levels

among premenopausal women over a 2- to 3-year period. Cancer Epidemiology Biomarkers and Prevention 2006;15(5):972-8.

133. Severi G, Morris HA, MacInnis RJ, English DR, Tilley WD, Hopper JL, et al. Circulating insulin-like growth factor-I and binding protein-3 and risk of prostate cancer. Cancer Epidemiology Biomarkers and Prevention 2006;15(6):1137-41.

134. Weiss JM, Huang W-, Rinaldi S, Fears TR, Chatterjee N, Chia D, et al. IGF-I and IGFBP-3: Risk of prostate cancer among men in the prostate, lung, colorectal and ovarian cancer screening trial. International Journal of Cancer 2007;121(10):2267-73.

135. Baglietto L, English DR, Hopper JL, Morris HA, Tilley WD, Giles GG. Circulating insulin-like growth factor-I and binding protein-3 and the risk of breast cancer. Cancer Epidemiology Biomarkers and Prevention 2007;16(4):763-8.

136. Chia VM, Newcomb PA, White E, Zheng Y, Potter JD, Lampe JW. Reproducibility of serum leptin, insulin-like growth factor-I, and insulin-like growth factor-binding protein-3 measurements. Horm Res 2008;69(5):295-300.

137. McKinley MC, Strain JJ, McPartlin J, Scott JM, McNulty H. Plasma homocysteine is not subject to seasonal variation. Clin Chem 2001;47(8):1430-6.

138. Lacher DA, Hughes JP, Carroll MD. Estimate of biological variation of laboratory analytes based on the third national health and nutrition examination survey. Clin Chem 2005;51(2):450-2.

139. Kant AK, Graubard BI. Ethnic and socioeconomic differences in variability in nutritional biomarkers. Am J Clin Nutr 2008;87(5):1464-71.

140. Bowman GL, Shannon J, Ho E, Traber MG, Frei B, Oken BS, et al. Reliability and validity of food frequency questionnaire and nutrient biomarkers in elders with and without mild cognitive impairment. Alzheimer Dis Assoc Disord 2011;25(1):49-57.

141. Allen NE, Grace PB, Ginn A, Travis RC, Roddam AW, Appleby PN, et al. Phytanic acid: Measurement of plasma concentrations by gas-liquid chromatography-mass spectrometry analysis and associations with diet and other plasma fatty acids. Br J Nutr 2008;99(3):653-9.

142. Liu K, Stamler J, Dyer A. Statistical methods to assess and minimize the role of intra-invididual variability in obscuring the relationship between dietary lipids and serum cholesterol. J Chronic Dis 1978;31(6-7):399-418.

143. Sempos CT, Johnson NE, Smith EL, Gilligan C. Effects of intraindividual and interindividual variation in repeated dietary records. Am J Epidemiol 1985;121(1):120-30.

144. Shoukri MM, Asyali MH, Donner A. Sample size requirements for the design of reliability study: Review and new results. Stat Methods Med Res 2004;13(4):251-71.

145. Travis RC, Spencer EA, Allen NE, Appleby PN, Roddam AW, Overvad K, et al. Plasma phyto-oestrogens and prostate cancer in the European prospective investigation into cancer and nutrition. Br J Cancer 2009;100(11):1817-23.

146. Johansson M, Appleby PN, Allen NE, Travis RC, Roddam AW, Egevad L, et al. Circulating concentrations of folate and vitamin B12 in relation to prostate cancer risk: Results from the

European prospective investigation into cancer and nutrition study. Cancer Epidemiol Biomarkers Prev 2008;17(2):279-85.

147. Drammeh BS, Schleicher RL, Pfeiffer CM, Jain RB, Zhang M, Phuong HN. Effects of delayed sample processing and freezing on serum concentrations of selected nutritional indicators. Clin Chem 2008;54(11):1883-91.

148. Van Eijsden M, Van Der Wal MF, Hornstra G, Bonsel GJ. Can whole-blood samples be stored over 24 hours without compromising stability of C-reactive protein, retinol, ferritin, folic acid, and fatty acids in epidemiologic research? Clin Chem 2005;51(1):230-2.

149. Gilsing AMJ, Crowe FL, Lloyd-Wright Z, Sanders TAB, Appleby PN, Allen NE, et al. Serum concentrations of vitamin B12 and folate in british male omnivores, vegetarians and vegans: Results from a cross-sectional analysis of the EPIC-oxford cohort study. Eur J Clin Nutr 2010;64(9):933-9.

150. Dunn JE. Cancer epidemiology in populations of the United States--with emphasis on hawaii and california--and japan. Cancer Res 1975;35(11 Pt. 2):3240-5.

151. Gao X, LaValley MP, Tucker KL. Prospective studies of dairy product and calcium intakes and prostate cancer risk: A meta-analysis. Journal of the National Cancer Institute 2005;97(23):1768-77.

152. Hsing AW, McLaughlin JK, Schuman LM, Bjelke E, Gridley G, Wacholder S, et al. Diet, tobacco use, and fatal prostate cancer: Results from the lutheran brotherhood cohort study. Cancer Res 1990;50(21):6836-40.

153. Giovannucci E, Rimm EB, Colditz GA, Stampfer MJ, Ascherio A, Chute CC, et al. A prospective study of dietary fat and risk of prostate cancer. J Natl Cancer Inst 1993;85(19):1571-9.

154. Schuurman AG, Van Den Brandt PA, Dorant E, Goldbohm RA. Animal products, calcium and protein and prostate cancer risk in the netherlands cohort study. Br J Cancer 1999;80(7):1107-13.

155. Rodriguez C, McCullough ML, Mondul AM, Jacobs EJ, Fakhrabadi-Shokoohi D, Giovannucci EL, et al. Calcium, dairy products, and risk of prostate cancer in a prospective cohort of United States men. Cancer Epidemiol Biomarkers Prev 2003;12(7):597-603.

156. Koh KA, Sesso HD, Paffenbarger RS, Lee I-. Dairy products, calcium and prostate cancer risk. Br J Cancer 2006;95(11):1582-5.

157. Hansen RP, Shorland FB. The branched-chain fatty acids of butterfat. II. the isolation of multi-branched C20 saturated fatty acid fraction. Biochem J 1952;50(3):358-60.

158. Hansen RP, Shorland FB. The branched-chain fatty acids of butterfat. 3. further investigations on a multibranched C20 saturated fatty acid fraction. Biochem J 1953;55(4):662-3.

159. Lough AK. Blood lipids. 4. the isolation of 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid) from ox-plasma lipids. Biochem J 1964;91(3):584-8.

160. Hansen RP. Occurrence of 3,7,11,15-tetramethylhexadecanoic acid in ox perinephric fat. Chem Ind 1965;7:303-4.

161. Ackman RG, Hooper SN. Examination of isoprenoid fatty acids as distinguishing characteristics of specific marine oils with particular reference to whale oils. Comp Biochem Physiol 1968;24(2):549-65.

162. Steinberg D, Avigan J, Mize C, Eldjarn L, Try K, Refsum S. Conversion of U-C14-phytol to phytanic acid and its oxidation in heredopathia atactica polyneuritiformis. Biochem Biophys Res Commun 1965;19(6):783-9.

163. Baxter JH. Absorption of chlorophyll phytol in normal man and in patients with refsum's disease. J Lipid Res 1968;9(5):636-41.

164. Eldjarn L, Try K, Stokke O, Munthe-Kaas AW, Refsum S, Steinberg D, et al. Dietary effects on serum-phytanic-acid levels and on clinical manifestations in heredopathia atactica polyneuritiformis. Lancet 1966;1(7439):691-3.

165. Sonneveld W, Begemann PH, van Beers GJ, Keuning R, Schogt JCM. 3,7,11,15-tetramethylhexadecanoic acid, a constituent of butterfat. J Lipid Res 1962;3(3):351-5.

166. Lough AK. The phytanic acid content of the lipids of bovine tissues and milk. Lipids 1977;12(1):115-9.

167. Brown PJ, Mei G, Gibberd FB, Burston D, Mayne PD, McClinchy JE, et al. Diet and refsum's disease. the determination of phytanic acid and phytol in certain foods and the application of this knowledge to the choice of suitable convenience foods for patients with refsum's disease. J Hum Nutr Diet 1993;6(4):295-305.

168. Wills AJ, Manning NJ, Reilly MM. Refsum's disease. QJM 2001;94(8):403-6.

169. Mize CE, Herndon JH,Jr, Blass JP, Milne GW, Follansbee C, Laudat P, et al. Localization of the oxidative defect in phytanic acid degradation in patients with refsum's disease. J Clin Invest 1969;48(6):1033-40.

170. Britton TC, Gibberd FB, Clemens ME, Billimoria JD, Sidey MC. The significance of plasma phytanic acid levels in adults. J Neurol Neurosurg Psychiatry 1989;52(7):891-4.

171. Verhoeven NM, Jakobs C. Human metabolism of phytanic acid and pristanic acid. Progress in Lipid Research 2001;40(6):453-66.

172. Jiang Z, Wu C-, Woda BA, Dresser K, Xu J, Fanger GR, et al. P504S/ α -methylacyl-CoA racemase: A useful marker for diagnosis of small foci of prostatic carcinoma on needle biopsy. Am J Surg Pathol 2002;26(9):1169-74.

173. Rubin MA, Zhou M, Dhanasekaran SM, Varambally S, Barrette TR, Sanda MG, et al. α -Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. JAMA 2002;287(13):1662-70.

174. Mobley JA, Leav I, Zielie P, Wotkowitz C, Evans J, Lam Y-, et al. Branched fatty acids in dairy and beef products markedly enhance α -methylacyl-CoA racemase expression in prostate cancer cells in vitro. Cancer Epidemiol Biomarkers Prev 2003;12(8):775-83.

175. Zomer AWM, VanDerSaag PT, Poll-The BT. Phytanic and pristanic acid are naturally occuring ligands. Advances in Experimental Medicine and Biology 2003;544:247-54.

176. Pearce N. What does the odds ratio estimate in a case-control study? Int J Epidemiol 1993;22(6):1189-92.

177. Xu J, Thornburg T, Turner AR, Vitolins M, Case D, Shadle J, et al. Serum levels of phytanic acid are associated with prostate cancer risk. Prostate 2005;63(3):209-14.

178. Steinberg D, Avigan J, Mize CE, Baxter JH, Cammermeyer J, Fales HM, et al. Effects of dietary phytol and phytanic acid in animals. J Lipid Res 1966;7(5):684-91.

179. Baxter JH, Steinberg D, Mize CE, Avigan J. Absorption and metabolism of uniformly 14C-labeled phytol and phytanic acid by the intestine of the rat studied with thoracic duct cannulation. Biochim Biophys Acta 1967;137(2):277-90.

180. Cohn JS, McNamara JR, Cohn SD, Ordovas JM, Schaefer EJ. Postprandial plasma lipoprotein changes in human subjects of different ages. J Lipid Res 1988;29(4):469-79.

181. Fielding BA, Callow J, Owen RM, Samra JS, Matthews DR, Frayn KN. Postprandial lipemia: The origin of an early peak studied by specific dietary fatty acid intake during sequential meals. Am J Clin Nutr 1996;63(1):36-41.

182. Zheng SL, Chang B-, Faith DA, Johnson JR, Isaacs SD, Hawkins GA, et al. Sequence variants of α -methylacyl-CoA racemase are associated with prostate cancer risk. Cancer Res 2002;62(22):6485-8.

183. FitzGerald LM, Thomson R, Polanowski A, Patterson B, McKay JD, Stankovich J, et al. Sequence variants of α -methylacyl-CoA racemase are associated with prostate cancer risk: A replication study in an ethnically homogeneous population. Prostate 2008;68(13):1373-9.

184. Elwood PC, Pickering JE, Ian Givens D, Gallacher JE. The consumption of milk and dairy foods and the incidence of vascular disease and diabetes: An overview of the evidence. Lipids 2010;45(10):925-39.

185. Mensink RP, Zock PL, Kester ADM, Katan MB. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. Am J Clin Nutr 2003;77(5):1146-55.

186. Grundy SM, Denke MA. Dietary influences on serum lipids and lipoproteins. J Lipid Res 1990;31(7):1149-72.

187. Efficacy and safety of cholesterol-lowering treatment: Prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. The Lancet 2005;366(9493):1267-78.

188. Temme EHM, Mensink RP, Hornstra G. Comparison of the effects of diets enriched in lauric, palmitic, or oleic acids on serum lipids and lipoproteins in healthy women and men. Am J Clin Nutr 1996;63(6):897-903.

189. Griffith LE, Guyatt GH, Cook RJ, Bucher HC, Cook DJ. The influence of dietary and nondietary calcium supplementation on blood pressure: An updated metaanalysis of randomized controlled trials. Am J Hypertens 1999;12(1 I):84-92.

190. Takemoto Y, Suzuki Y, Horibe R, Shimozawa N, Wanders RJA, Kondo N. Gas chromatography/mass spectrometry analysis of very long chain fatty acids, docosahexaenoic

acid, phytanic acid and plasmalogen for the screening of peroxisomal disorders. Brain and Development 2003;25(7):481-7.

191. Vreken P, van Lint AEM, Bootsma AH, Overmars H, Wanders RJA, van Gennip AH. Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas chromatography–electron impact mass spectrometry. Journal of Chromatography B: Biomedical Sciences and Applications, 1998;713(2):281-7.

192. Wolk A, Vessby B, Ljung H, Barrefors P. Evaluation of a biological marker of dairy fat intake. Am J Clin Nutr 1998;68(2):291-5.

193. Smedman AE, Gustafsson I, Berglund LG, Vessby BO. Pentadecanoic acid in serum as a marker for intake of milk fat: Relations between intake of milk fat and metabolic risk factors. Am J Clin Nutr 1999;69(1):22-9.

194. Brevik A, Veierod MB, Drevon CA, Andersen LF. Evaluation of the odd fatty acids 15:0 and 17:0 in serum and adipose tissue as markers of intake of milk and dairy fat. Eur J Clin Nutr 2005;59(12):1417-22.

195. Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM, Dewhurst RJ. Factors affecting odd- and branched-chain fatty acids in milk: A review. Anim Feed Sci Technol 2006;131(3-4):389-417.

196. Nelson ML, Busboom JR, Cronrath JD, Falen L, Blankenbaker A. Effects of graded levels of potato by-products in barley- and corn-based beef feedlot diets: I. feedlot performance, carcass traits, meat composition, and appearance. J Anim Sci 2000;78(7):1829-36.

197. Boles JA, Bowman JG, Surber LMM, Boss DL. Effects of barley variety fed to steers on carcass characteristics and color of meat. J Anim Sci 2004;82(7):2087-91.

198. Arab L. Biomarkers of fat and fatty acid intake. Journal of Nutrition 2003;133(3 SUPPL.).

199. Katan MB, Deslypere JP, Van Birgelen APJM, Penders M, Zegwaard M. Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: An 18-month controlled study. J Lipid Res 1997;38(10):2012-22.

200. HIRSCH J, FARQUHAR JW, AHRENS Jr. EH, PETERSON ML, STOFFEL W. Studies of adipose tissue in man. A microtechnic for sampling and analysis. Am J Clin Nutr 1960;8:499-511.

201. Baylin A, Mi KK, Donovan-Palmer A, Siles X, Dougherty L, Tocco P, et al. Fasting whole blood as a biomarker of essential fatty acid intake in epidemiologic studies: Comparison with adipose tissue and plasma. Am J Epidemiol 2005;162(4):373-81.

202. Crowe FL, Allen NE, Appleby PN, Overvad K, Aardestrup IV, Johnsen NF, et al. Fatty acid composition of plasma phospholipids and risk of prostate cancer in a case-control analysis nested within the European prospective investigation into cancer and nutrition. Am J Clin Nutr 2008;88(5):1353-63.

203. Linseisen J, Kesse E, Slimani N, Bueno-de-Mesquita HB, Ocké MC, Skeie G, et al. Meat consumption in the European prospective investigation into cancer and nutrition (EPIC) cohorts: Results from 24-hour dietary recalls. Public Health Nutr 2002;5(6 B):1243-58.

204. Ferrari P, Day NE, Boshuizen HC, Roddam A, Hoffmann K, Thiébaut A, et al. The evaluation of the diet/disease relation in the EPIC study: Considerations for the calibration and the disease models. Int J Epidemiol 2008;37(2):368-78.

205. Willet WC. Implications of total energy intake for epidemiologic analyses. In: MacManon B, editor. Nutritional Epidemiology. 1st ed. New York: Oxford University Press, Inc; 1990. p. 245-71.

206. Duncan WR, Garton GA. The fatty acid composition and intramolecular structure of triglycerides derived from different sites in the body of the sheep. J Sci Food Agric 1967;18(3):99-102.

207. Downing DT. Branched-chain fatty acids in lipids of the newly born lamb. J Lipid Res 1964;5(2):210-5.

208. Hjartaker A, Lagiou A, Slimani N, Lund E, Chirlaque MD, Vasilopoulou E, et al. Consumption of dairy products in the European prospective investigation into cancer and nutrition (EPIC) cohort: Data from 35 955 24-hour dietary recalls in 10 European countries. Public Health Nutr 2002;5(6B):1259-71.

209. Wolk A, Furuheim M, Vessby B. Fatty acid composition of adipose tissue and serum lipids are valid biological markers of dairy fat intake in men. J Nutr 2001;131(3):828-33.

210. Saadatian-Elahi M, Slimani N, Chajès V, Jenab M, Goudable J, Biessy C, et al. Plasma phospholipid fatty acid profiles and their association with food intakes: Results from a cross-sectional study within the European prospective investigation into cancer and nutrition. Am J Clin Nutr 2009;89(1):331-46.

211. Rosell M, Johansson G, Berglund L, Vessby B, De Faire U, Hellénius M-. The relation between alcohol intake and physical activity and the fatty acids 14:0, 15:0 and 17:0 in serum phospholipids and adipose tissue used as markers for dairy fat intake. Br J Nutr 2005;93(1):115-21.

212. Hannuksela ML, Liisanantti MK, Savolainen MJ. Effect of alcohol on lipids and lipoproteins in relation to atherosclerosis. Crit Rev Clin Lab Sci 2002;39(3):225-83.

213. Byers T, Gieseker K. Issues in the design and interpretation of studies of fatty acids and cancer in humans. Am J Clin Nutr 1997;66(6 SUPPL.):1541S-7S.

214. Arany E, Afford S, Strain AJ, Winwood PJ, Arthur MJP, Hill DJ. Differential cellular synthesis of insulin-like growth factor binding protein-1 (IGFBP-1) and IGFBP-3 within human liver. J Clin Endocrinol Metab 1994;79(6):1871-6.

215. Chin E, Zhou J, Dai J, Baxter RC, Bondy CA. Cellular localization and regulation of gene expression for components of the insulin-like growth factor ternary binding protein complex. Endocrinology 1994;134(6):2498-504.

216. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst 2000;92(18):1472-89.

217. Guler H-, Zapf J, Schmid C, Froesch ER. Insulin-like growth factors I and II in healthy man. estimations of half-lives and production rates. Acta Endocrinol 1989;121(6):753-8.

218. Ballard FJ, Knowles SE, Walton PE, Edson K, Owens PC, Mohler MA, et al. Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I), IGF-II and des(1-3)IGF-I in rats. J Endocrinol 1991;128(2):197-204.

219. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, et al. Insulin-like growth factor I receptor primary structure: Comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J 1986;5(10):2503-12.

220. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 2002;23(6):824-54.

221. Rajah R, Bhala A, Nunn SE, Peehl DM, Cohen P. 7S nerve growth factor is an insulin-like growth factor-binding protein protease. Endocrinology 1996;137(7):2676-82.

222. Conover CA, Powell DR. Insulin-like growth factor (IGF)-binding protein-3 blocks IGF-I-induced receptor down-regulation and cell densitization in cultured bovine fibroblasts. Endocrinology 1991;129(2):710-6.

223. Ruan W, Powell-Braxton L, Kopchick JJ, Kleinberg DL. Evidence that insulin-like growth factor I and growth hormone are required for prostate gland development. Endocrinology 1999;140(5):1984-9.

224. Cohen P, Peehl DM, Lamson G, Rosenfeld RG. Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins in primary cultures of prostate epithelial cells. J Clin Endocrinol Metab 1991;73(2):401-7.

225. Pandini G, Mineo R, Frasca F, Roberts Jr. CT, Marcelli M, Vigneri R, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res 2005;65(5):1849-57.

226. Plymate SR, Tennant MK, Culp SH, Woodke L, Marcelli M, Colman I, et al. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF-I on proliferation and AR activity between localized and metastatic tumors. Prostate 2004;61(3):276-90.

227. Nickerson T, Pollak M, Huynh H. Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2,3,4 and 5. Endocrinology 1998;139(2):807-10.

228. Parekh N, Roberts CB, Vadiveloo M, Puvananayagam T, Albu JB, Lu-Yao GL. Lifestyle, anthropometric, and obesity-related physiologic determinants of insulin-like growth factor-1 in the third national health and nutrition examination survey (1988-1994). Ann Epidemiol 2010;20(3):182-93.

229. DeLellis K, Rinaldi S, Kaaks RJ, Kolonel LN, Henderson B, Le Marchand L. Dietary and lifestyle correlates of plasma insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3): The multiethnic cohort. Cancer Epidemiol Biomarkers Prev 2004;13(9):1444-51.

230. Chang S, Wu X, Yu H, Spitz MR. Plasma concentrations of insulin-like growth factors among healthy adult men and postmenopausal women: Associations with body composition, lifestyle, and reproductive factors. Cancer Epidemiol Biomarkers Prev 2002;11(8):758-66.

231. Probst-Hensch NM, Wang H, Goh VHH, Seow A, Lee H-, Yu MC. Determinants of circulating insulin-like growth factor I and insulin-like growth factor binding protein 3 concentrations in a cohort of singapore men and women. Cancer Epidemiology Biomarkers and Prevention 2003;12(8):739-46.

232. Tricoli JV, Winter DL, Hanlon AL, Raysor SL, Watkins-Bruner D, Pinover WH, et al. Racial differences in insulin-like growth factor binding protein-3 in men at increased risk of prostate cancer. Urology 1999;54(1):178-82.

233. Cruickshank JK, Heald AH, Anderson S, Cade JE, Sampayo J, Riste LK, et al. Epidemiology of the insulin-like growth factor system in three ethnic groups. Am J Epidemiol 2001;154(6):504-13.

234. Faupel-Badger JM, Berrigan D, Ballard-Barbash R, Potischman N. Anthropometric correlates of insulin-like growth factor 1 (IGF-1) and IGF binding protein-3 (IGFBP-3) levels by Race/Ethnicity and gender. Ann Epidemiol 2009;19(12):841-9.

235. Berrigan D, Potischman N, Dodd KW, Hursting SD, Lavigne J, Barrett JC, et al. Race/ethnic variation in serum levels of IGF-I and IGFBP-3 in US adults. Growth Horm IGF Res 2009;19(2):146-55.

236. Schutte AE, Huisman HW, Van Rooyen JM, Malan L, Malan NT, Fourie CMT, et al. A significant decline in IGF-I may predispose young Africans to subsequent cardiometabolic vulnerability. J Clin Endocrinol Metab 2010;95(5):2503-7.

237. Slattery ML, Baumgartner KB, Byers T, Guiliano A, Sweeney C, Herrick J, et al. Genetic, anthropometric, and lifestyle factors associated with IGF-1 and IGFBP-3 levels in hispanic and non-hispanic white women. Cancer Causes Control 2005;16(10):1147-57.

238. Hong Y, Pedersen NL, Brismar K, Hall K, De Faire U. Quantitative genetic analyses of insulin-like growth factor I (IGF-I), IGF-binding protein-1, and insulin levels in middle-aged and elderly twins. J Clin Endocrinol Metab 1996;81(5):1791-7.

239. Verhaeghe J, Loos R, Vlietinck R, Van Herck E, Van Bree R, De Schutter A-. C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in cord serum of twins: Genetic versus environmental regulation. AM J OBSTET GYNECOL 1996;175(5):1180-8.

240. Harrela M, Koistinen H, Kaprio J, Lehtovirta M, Tuomilehto J, Eriksson J, et al. Genetic and environmental components of interindividual variation in circulating levels of IGF-I, IGF-II, IGFBP-1, and IGFBP-3. J Clin Invest 1996;98(11):2612-5.

241. Souren NY, Paulussen ADC, Loos RJF, Gielen M, Beunen G, Fagard R, et al. Anthropometry, carbohydrate and lipid metabolism in the east flanders prospective twin survey: Heritabilities. Diabetologia 2007;50(10):2107-16.

242. Kaplan RC, Petersen A-, Chen M-, Teumer A, Glazer NL, DÖring A, et al. A genome-wide association study identifies novel loci associated with circulating IGF-I and IGFBP-3. Hum Mol Genet 2011;20(6):1241-51.

243. Gram IT, Norat T, Rinaldi S, Dossus L, Lukanova A, Téhard B, et al. Body mass index, waist circumference and waist-hip ratio and serum levels of IGF-I and IGFBP-3 in European women. Int J Obes 2006;30(11):1623-31.

244. Sesti G, Sciacqua A, Cardellini M, Marini MA, Maio R, Vatrano M, et al. Plasma concentration of IGF-I is independently associated with insulin sensitivity in subjects with different degrees of glucose tolerance. Diabetes Care 2005;28(1):120-5.

245. Irwin ML, McTiernan A, Bernstein L, Gilliland FD, Baumgartner R, Baumgartner K, et al. Relationship of obesity and physical activity with C-peptide, leptin, and insulin-like growth factors in breast cancer survivors. Cancer Epidemiol Biomarkers Prev 2005;14(12):2881-8.

246. Sandhu MS, Gibson JM, Heald AH, Dunger DB, Wareham NJ. Association between insulin-like growth factor-I:Insulin-like growth factor-binding protein-1 ratio and metabolic and anthropometric factors in men and women. Cancer Epidemiol Biomarkers Prev 2004;13(1):166-70.

247. Allen NE, Appleby PN, Kaaks R, Rinaldi S, Davey GK, Key TJ. Lifestyle determinants of serum insulin-like growth-factor-I (IGF-I), C-peptide and hormone binding protein levels in british women. Cancer Causes Control 2003;14(1):65-74.

248. London SJ, Yuan J-, Travlos GS, Gao Y-, Wilson RE, Ross RK, et al. Insulin-like growth factor I, IGF-binding protein 3, and lung cancer risk in a prospective study of men in china. J Natl Cancer Inst 2002;94(10):749-54.

249. Copeland KC, Colletti RB, Devlin JT, McAuliffe TL. The relationship between insulin-like growth factor-I, adiposity, and aging. Metab Clin Exp 1990;39(6):584-7.

250. Schoen RE, Schragin J, Weissfeld JL, Thaete FL, Evans RW, Rosen CJ, et al. Lack of association between adipose tissue distribution and IGF-1 and IGFBP-3 in men and women. Cancer Epidemiol Biomarkers Prev 2002;11(6):581-6.

251. Landin-Wilhelmsen K, Wilhelmsen L, Lappas G, Rosen T, Lindstedt G, Lundberg P, et al. Serum insulin-like growth factor I in a random population sample of men and women: Relation to age, sex, smoking habits, coffee consumption and physical activity, blood pressure and concentrations of plasma lipids, fibrinogen, parathyroid hormone and osteocalcin. CLIN ENDOCRINOL 1994;41(3):351-7.

252. Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, et al. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. J Natl Cancer Inst 1999;91(7):620-5.

253. Probst-Hensch NM, Yuan J-, Stanczyk FZ, Gao Y-, Ross RK, Yu MC. IGF-1, igf-2 and IGFBP-3 in prediagnostic serum: Association with colorectal cancer in a cohort of chinese men in shanghai. Br J Cancer 2001;85(11):1695-9.

254. Teramukai S, Rohan T, Eguchi H, Oda T, Shinchi K, Kono S. Anthropometric and behavioral correlates of insulin-like growth factor I and insulin-like growth factor binding protein 3 in middle-aged Japanese men. Am J Epidemiol 2002;156(4):344-8.

255. Ben-Shlomo Y, Holly J, McCarthy A, Savage P, Davies D, Gunnell D, et al. An investigation of fetal, postnatal and childhood growth with insulin-like growth factor I and binding protein 3 in adulthood. Clin Endocrinol 2003;59(3):366-73.

256. Gianotti L, Broglio F, Ramunni J, Lanfranco F, Gauna C, Benso A, et al. The activity of GH/IGF-I axis in anorexia nervosa and in obesity: A comparison with normal subjects and patients with hypopituitarism or critical illness. Eat Weight Disord 1998;3(2):64-70.

257. Maccario M, Tassone F, Grottoli S, Rossetto R, Gauna C, Ghigo E. Neuroendocrine and metabolic determinants of the adaptation of GH/IGF-I axis to obesity. Ann Endocrinol 2002;63(2 I):140-4.

258. Holmes MD, Pollak MN, Willett WC, Hankinson SE. Dietary correlates of plasma insulin-like growth factor I and insulin-like growth factor binding protein 3 concentrations. Cancer Epidemiology Biomarkers and Prevention 2002;11(9):852-61.

259. Crowe FL, Key TJ, Allen NE, Appleby PN, Overvad K, Grønbæk H, et al. A cross-sectional analysis of the associations between adult height, BMI and serum concentrations of IGF-I and IGFBP-1 -2 and -3 in the European prospective investigation into cancer and nutrition (EPIC). Ann Hum Biol 2011;38(2):194-202.

260. Veldhuis JD, Iranmanesh A. Physiological regulation of the human growth hormone (GH)-insulin-like growth factor type I (IGF-I) axis: Predominant impact of age, obesity, gonadal function, and sleep. Sleep 1996;19(10 SUPPL.):S221-4.

261. Völzke H, Nauck M, Rettig R, Dörr M, Higham C, Brabant G, et al. Association between hepatic steatosis and serum IGF1 and IGFBP-3 levels in a population-based sample. Eur J Endocrinol 2009;161(5):705-13.

262. Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol 2006;40(SUPPL. 1):S5-S10.

263. Bougoulia M, Triantos A, Koliakos G. Effect of weight loss with or without orlistat treatment on adipocytokines, inflammation, and oxidative markers in obese women. Hormones (Athens) 2006;5(4):259-69.

264. Kaaks R, Lonning, Laron. Nutrition, insulin, IGF-1 metabolism and cancer risk: A summary of epidemiological evidence. ; 2004 [cited 23 March 2009].

265. Belobrajdic DP, Frystyk J, Jeyaratnaganthan N, Espelund U, Flyvbjerg A, Clifton PM, et al. Moderate energy restriction-induced weight loss affects circulating IGF levels independent of dietary composition. Eur J Endocrinol 2010;162(6):1075-82.

266. Lukanova A, Toniolo P, Akhmedkhanov A, Hunt K, Rinaldi S, Zeleniuch-Jacquotte A, et al. A cross-sectional study of IGF-I determinants in women. Eur J Cancer Prev 2001;10(5):443-52.

267. McGreevy KM, Hoel BD, Lipsitz SR, Hoel DG. Impact of nutrients on insulin-like growth factor-I, insulin-like growth factor binding protein-3 and their ratio in African american and white males. Public Health Nutr 2007;10(1):97-105.

268. Morimoto LM, Newcomb PA, White E, Bigler J, Potter JD. Variation in plasma insulin-like growth factor-1 and insulin-like growth factor binding protein-3: Personal and lifestyle factors (United States). Cancer Causes Control 2005;16(8):917-27.

269. Gunnell D, Oliver SE, Peters TJ, Donovan JL, Persad R, Maynard M, et al. Are diet-prostate cancer associations mediated by the IGF axis? A cross-sectional analysis of diet, IGF-1 and IGFBP-3 in healthy middle-aged men. Br J Cancer 2003;88(11):1682-6.

270. Giovannucci E, Pollak M, Liu Y, Platz EA, Majeed N, Rimm EB, et al. Nutritional predictors of insulin-like growth factor I and their relationships to cancer in men. Cancer Epidemiology Biomarkers and Prevention 2003;12(2):84-9.

271. Ma J, Giovannucci E, Pollak M, Chan JM, Gaziano JM, Willett W, et al. Milk intake, circulating levels of insulin-like growth factor-I, and risk of colorectal cancer in men. J Natl Cancer Inst 2001;93(17):1330-6.

272. Baibas N, Bamia C, Vassilopoulou E, Sdrolias J, Trichopoulou A, Trichopoulos D. Dietary and lifestyle factors in relation to plasma insulin-like growth factor I in a general population sample. Eur J Cancer Prev 2003;12(3):229-34.

273. Allen NE, Appleby PN, Davey GK, Kaaks R, Rinaldi S, Key TJ. The associations of diet with serum insulin-like growth factor I and its main binding proteins in 292 women meat-eaters, vegetarians, and vegans. Cancer Epidemiol Biomarkers Prev 2002;11(11):1441-8.

274. Maxwell A, Butterwick R, Yateman M, Batt RM, Cotterill A, Camacho-Hübner C. Nutritional modulation of canine insulin-like growth factors and their binding proteins. J Endocrinol 1998;158(1):77-85.

275. Maxwell A, Butterwick R, Batt RM, Camacho-Hübner C. Serum insulin-like growth factor (IGF)-I concentrations are reduced by short-term dietary restriction and restored by refeeding in domestic cats (felis catus). J Nutr 1999;129(10):1879-84.

276. Thissen J-, Underwood LE, Ketelslegers J-. Regulation of insulin-like growth factor-I in starvation and injury. Nutr Rev 1999;57(6):167-76.

277. Sanchez-Gomez M, Malmlöf K, Mejia W, Bermudez A, Ochoa MT, Carrasco-Rodriguez S, et al. Insulin-like growth factor-I, but not growth hormone, is dependent on a high protein intake to increase nitrogen balance in the rat. Br J Nutr 1999;81(2):145-52.

278. Clemmons DR, Van Wyk JJ. Factors controlling blood concentration of somatomedin C. Clin Endocrinol Metab 1984;13(1):113-43.

279. Grimberg A. Mechanisms by which IGF-I may promote cancer. Cancer Biol Ther 2003;2(6):630-5.

280. Olivo-Marston SE, Hursting SD, Lavigne J, Perkins SN, Maarouf RS, Yakar S, et al. Genetic reduction of circulating insulin-like growth factor-1 inhibits azoxymethane-induced colon tumorigenesis in mice. Mol Carcinog 2009;48(12):1071-6.

281. Wu Y, Cui K, Miyoshi K, Hennighausen L, Green JE, Setser J, et al. Reduced circulating insulin-like growth factor I levels delay the onset of chemically and genetically induced mammary tumors. Cancer Res 2003;63(15):4384-8.

282. Dunn SE, Kari FW, French J, Leininger JR, Travlos G, Wilson R, et al. Dietary restriction reduces insulin-like growth factor I levels, which modulates apoptosis, cell proliferation, and tumor progression in p53- deficient mice. Cancer Res 1997;57(21):4667-72.

283. Jones RA, Campbell CI, Gunther EJ, Chodosh LA, Petrik JJ, Khokha R, et al. Transgenic overexpression of IGF-IR disrupts mammary ductal morphogenesis and induces tumor formation. Oncogene 2007;26(11):1636-44.

284. Lopez T, Hanahan D. Elevated levels of IGF-1 receptor convey invasive and metastatic capability in a mouse model of pancreatic islet tumorigenesis. Cancer Cell 2002;1(4):339-53.

285. Sell C, Rubini M, Rubin R, Liu J-, Efstratiadis A, Baserga R. Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor. Proc Natl Acad Sci U S A 1993;90(23):11217-21.

286. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression,

which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 2002;277(41):38205-11.

287. Tang Y, Zhang D, Fallavollita L, Brodt P. Vascular endothelial growth factor C expression and lymph node metastasis are regulated by the type I insulin-like growth factor receptor. Cancer Res 2003;63(6):1166-71.

288. Bustin SA, Dorudi S, Phillips SM, Feakins RM, Jenkins PJ. Local expression of insulin-like growth factor-I affects angiogenesis in colorectal cancer. Tumor Biol 2002;23(3):130-8.

289. Brodt P, Fallavollita L, Khatib A-, Samani AA, Zhang D. Cooperative regulation of the invasive and metastatic phenotypes by different domains of the type I insulin-like growth factor receptor β subunit. J Biol Chem 2001;276(36):33608-15.

290. So AI, Levitt RJ, Eigl B, Fazli L, Muramaki M, Leung S, et al. Insulin-like growth factor binding protein-2 is a novel therapeutic target associated with breast cancer. Clin Cancer Res 2008;14(21):6944-54.

291. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8(12):915-28.

292. DiGiovanni J, Kiguchi K, Frijhoff A, Wilker E, Bol DK, Beltrán L, et al. Deregulated expression of insulin-like growth factor 1 in prostate epithelium leads to neoplasia in transgenic mice. Proc Natl Acad Sci U S A 2000;97(7):3455-60.

293. DiGiovanni J, Bol DK, Wilker E, Beltrán L, Carbajal S, Moats S, et al. Constitutive expression of insulin-like growth factor-1 in epidermal basal cells of transgenic mice leads to spontaneous tumor promotion. Cancer Res 2000;60(6):1561-70.

294. Nickerson T, Chang F, Lorimer D, Smeekens SP, Sawyers CL, Pollak M. In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR). Cancer Res 2001;61(16):6276-80.

295. Hellawell GO, Turner GDH, Davies DR, Poulsom R, Brewster SF, Macaulay VM. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. Cancer Research 2002;62(10):2942-50.

296. Ryan CJ, Haqq CM, Simko J, Nonaka DF, Chan JM, Weinberg V, et al. Expression of insulin-like growth factor-1 receptor in local and metastatic prostate cancer. Urologic Oncology: Seminars and Original Investigations 2007;25(2):134-40.

297. Wu JD, Odman A, Higgins LM, Haugk K, Vessella R, Ludwig DL, et al. In vivo effects of the human type I insulin-like growth factor receptor antibody A12 on androgen-dependent and androgen-independent xenograft human prostate tumors. Clin Cancer Res 2005;11(8):3065-74.

298. Wu JD, Haugk K, Coleman I, Woodke L, Vessella R, Nelson P, et al. Combined in vivo effect of A12, a type 1 insulin-like growth factor receptor antibody, and docetaxel against prostate cancer tumors. Clin Cancer Res 2006;12(20 PART 1):6153-60.

299. Mohler JL, Gregory CW, Ford III H, Kim D, Weaver CM, Petrusz P, et al. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004;10(2):440-8.

300. Gregory CW, He B, Johnson RT, Ford OH, Mohler JL, French FS, et al. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. Cancer Res 2001;61(11):4315-9.

301. Cheng I, Stram DO, Penney KL, Pike M, Le Marchand L, Kolonel LN, et al. Common genetic variation in IGF1 and prostate cancer risk in the multiethnic cohort. J Natl Cancer Inst 2006;98(2):123-34.

302. Rowlands MA, Holly JM, Gunnell D, Donovan J, Lane JA, Hamdy F, et al. Circulating insulin-like growth factors and IGF-binding proteins in PSA-detected prostate cancer: The large case-control study ProtecT. Cancer Res 2012.

303. Nimptsch K, Platz EA, Pollak MN, Kenfield SA, Stampfer MJ, Willett WC, et al. Plasma insulin-like growth factor 1 is positively associated with low-grade prostate cancer in the health professionals follow-up study 1993-2004. Int J Cancer 2011;128(3):660-7.

304. Platz EA, Pollak MN, Leitzmann MF, Stampfer MJ, Willett WC, Giovannucci E. Plasma insulin-like growth factor-1 and binding protein-3 and subsequent risk of prostate cancer in the PSA era. Cancer Causes and Control 2005;16(3):255-62.

305. Kaaks R, Toniolo P, Akhmedkhanov A, Lukanova A, Biessy C, Dechaud H, et al. Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. J Natl Cancer Inst 2000;92(19):1592-600.

306. Gill JK, Wilkens LR, Pollak MN, Stanczyk FZ, Kolonel LN. Androgens, growth factors, and risk of prostate cancer: The multiethnic cohort. Prostate 2010;70(8):906-15.

307. Borugian MJ, Spinelli JJ, Sun Z, Kolonel LN, Oakley-Girvan I, Pollak MD, et al. Prostate cancer risk in relation to insulin-like growth factor (IGF)-I and IGF-binding protein-3: A prospective multiethnic study. Cancer Epidemiology Biomarkers and Prevention 2008;17(1):252-4.

308. Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, et al. Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. International Journal of Biochemistry and Cell Biology 1996;28(6):619-37.

309. Vineis P, Perera F. Molecular epidemiology and biomarkers in etiologic cancer research: The new in light of the old. Cancer Epidemiol Biomarkers Prev 2007;16(10):1954-65.

310. Pfeiffer CM, Rogers LM, Bailey LB, Gregory III JF. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual- label stable-isotope protocol. Am J Clin Nutr 1997;66(6):1388-97.

311. Ruston D, Hoare J, Henderson L, Gregory J, editors. The national diet & nutrition survey: Adults aged 19 to 64 years nutritional status (anthropometry and blood analytes), blood pressure and physical activity. London: ; 2004.

312. Sauberlich HE. Bioavailability of vitamins. Prog Food Nutr Sci 1985;9(1-2):1-33.

313. Pfeiffer CM, Johnson CL, Jain RB, Yetley EA, Picciano MF, Rader JI, et al. Trends in blood folate and vitamin B-12 concentrations in the United States, 1988-2004. Am J Clin Nutr 2007;86(3):718-27.

314. Oakley Jr. GP, Tulchinsky TH. Folic acid and vitamin B12 fortification of flour: A global basic food security requirement. Public Health Rev 2010;32(1):284-95.

315. Wald N, Sneddon J, Densem J, Frost C, Stone R. Prevention of neural tube defects: Results of the medical research council vitamin study. Lancet 1991;338(8760):131-7.

316. Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong L-C. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. J Am Med Assoc 2001;285(23):2981-6.

317. Reynolds E. Vitamin B12, folic acid, and the nervous system. Lancet Neurol 2006;5(11):949-60.

318. Scott JM. Folate and vitamin B12. Proc Nutr Soc 1999;58(2):441-8.

319. Watanabe F. Vitamin B12 sources and bioavailability. Exp Biol Med 2007;232(10):1266-74.

320. Nexo E, Hoffmann-Lücke E. Holotranscobalamin, a marker of vitamin B-12 status: Analytical aspects and clinical utility. Am J Clin Nutr 2011;94(1):359S-65S.

321. Gregory JF,III. Case study: Folate bioavailability. J Nutr 2001;131(4):1376S-1382.

322. Mason JB, Choi S-. Effects of alcohol on folate metabolism: Implications for carcinogenesis. Alcohol 2005;35(3):235-41.

323. De Bree A, Verschuren WMM, Bjoørke-Monsen A-, Van der Put NMJ, Heil SG, Trijbels FJM, et al. Effect of the methylenetetrahydrofolate reductase 677C→T mutation on the relations among folate intake and plasma folate and homocysteine concentrations in a general population sample. Am J Clin Nutr 2003;77(3):687-93.

324. Payette H, Gray-Donald K. Dietary intake and biochemical indices of nutritional status in an elderly population, with estimates of the precision of the 7-d food record. Am J Clin Nutr 1991;54(3):478-88.

325. Piyathilake CJ, Hine RJ, Dasanyake AP, Richards EW, Freeberg LE, Vaughn WH, et al. Effect of smoking on folate levels in buccal mucosal cells. Int J Cancer 1992;52(4):566-9.

326. Piyathilake CJ, Macaluso M, Hine RJ, Richards EW, Krumdieck CL. Local and systemic effects of cigarette smoking on folate and vitamin B- 12. Am J Clin Nutr 1994;60(4):559-66.

327. Piyathilake CJ, Macaluso M, Hine RJ, Vinter DW, Richards EW, Krumdieck CL. Cigarette smoking, intracellular vitamin deficiency, and occurrence of micronuclei in epithelial cells of the buccal mucosa. Cancer Epidemiol Biomarkers Prev 1995;4(7):751-8.

328. Gabriel HE, Crott JW, Ghandour H, Dallal GE, Choi S-, Keyes MK, et al. Chronic cigarette smoking is associated with diminished folate status, altered folate form distribution, and increased genetic damage in the buccal mucosa of healthy adults. Am J Clin Nutr 2006;83(4):835-41.

329. van de Rest O, Durga J, Verhoef P, Melse-Boonstra A, Brants HAM. Validation of a food frequency questionnaire to assess folate intake of dutch elderly people. Br J Nutr 2007;98(5):1014-20.

330. Carmel R. Cobalamin, the stomach, and aging. Am J Clin Nutr 1997;66(4):750-9.

331. Van Guelpen B. Folate in colorectal cancer, prostate cancer and cardiovascular disease. Scand J Clin Lab Invest 2007;67(5):459-73.

332. Zingg J, Jones P. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis 1997;18(5):869-82.

333. Blount BC, Ames BN. DNA damage in folate deficiency. Baillieres Clin Haematol 1995;8(3):461-78.

334. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. Proceedings of the National Academy of Sciences of the United States of America 1997;94(7):3290-5.

335. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature 1998;395(6697):89-93.

336. Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128(7):1204-12.

337. Duthie SJ, Narayanan S, Blum S, Pirie L, Brand GM. Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. Nutr Cancer 2000;37(2):245-51.

338. Henrique R, Jerónimo C. Molecular detection of prostate cancer: A role for GSTP1 hypermethylation. Eur Urol 2004;46(5):660-9.

339. Jerónimo C, Henrique R, Hoque MO, Mambo E, Ribeiro FR, Varzim G, et al. A quantitative promoter methylation profile of prostate cancer. Clin Cancer Res 2004;10(24):8472-8.

340. Graham IM, Daly LE, Refsum HM, Robinson K, Brattström LE, Ueland PM, et al. Plasma homocysteine as a risk factor for vascular disease. JAMA: The Journal of the American Medical Association 1997;277(22):1775-81.

341. Clarke R, Halsey J, Lewington S, Lonn E, Armitage J, Manson JE, et al. Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality: Meta-analysis of 8 randomized trials involving 37,485 individuals. Arch Intern Med 2010;170(18):1622-31.

342. Lee M, Hong K-, Chang S-, Saver JL. Efficacy of homocysteine-lowering therapy with folic acid in stroke prevention: A meta-analysis. Stroke 2010;41(6):1205-12.

343. Wald DS, Morris JK, Wald NJ. Reconciling the evidence on serum homocysteine and ischaemic heart disease: A meta-analysis. PLoS ONE 2011;6(2).

344. Duthie SJ. Folate and cancer: How DNA damage, repair and methylation impact on colon carcinogenesis. J Inherit Metab Dis 2011;34(1):101-9.

345. Kim Y-. Role of folate in colon cancer development and progression. J Nutr 2003;133(11 SUPPL. 1).

346. Le Leu RK, Young GP, McIntosh GH. Folate deficiency reduces the development of colorectal cancer in rats. Carcinogenesis 2000;21(12):2261-5.

347. Song J, Sohn K-, Medline A, Ash C, Gallinger S, Kim Y-. Chemopreventive effects of dietary folate on intestinal polyps in apc+/- Msh2-/- mice. Cancer Res 2000;60(12):3191-9.

348. Song J, Medline A, Mason JB, Gallinger S, Kim Y-. Effects of dietary folate on intestinal tumorigenesis in the apc(min) mouse. Cancer Res 2000;60(19):5434-40.

349. Kim Y-. Folate, colorectal carcinogenesis, and DNA methylation: Lessons from animal studies. Environ Mol Mutagen 2004;44(1):10-25.

350. Ebbing M, Bønaa KH, Nygård O, Arnesen E, Ueland PM, Nordrehaug JE, et al. Cancer incidence and mortality after treatment with folic acid and vitamin B_{12}. JAMA - Journal of the American Medical Association 2009;302(19):2119-26.

351. Larsson SC, Giovannucci E, Wolk A. Folate intake, MTHFR polymorphisms, and risk of esophageal, gastric, and pancreatic cancer: A meta-analysis. Gastroenterology 2006;131(4):1271-83.

352. Kennedy DA, Stern SJ, Moretti M, Matok I, Sarkar M, Nickel C, et al. Folate intake and the risk of colorectal cancer: A systematic review and meta-analysis. Cancer Epidemiol 2011;35(1):2-10.

353. Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, et al. Folic acid for the prevention of colorectal adenomas: A randomized clinical trial. JAMA 2007;297(21):2351-9.

354. Figueiredo JC, Grau MV, Haile RW, Sandler RS, Summers RW, Bresalier RS, et al. Folic acid and risk of prostate cancer: Results from a randomized clinical trial. J Natl Cancer Inst 2009;101(6):432-5.

355. Weinstein SJ, Hartman TJ, Stolzenberg-Solomon R, Pietinen P, Barrett MJ, Taylor PR, et al. Null association between prostate cancer and serum folate, vitamin B6, vitamin B12, and homocysteine. Cancer Epidemiol Biomarkers Prev 2003;12(11):1271-2.

356. Hultdin J, Van Guelpen B, Bergh A, Hallmans G, Stattin P. Plasma folate, vitamin B12, and homocysteine and prostate cancer risk: A prospective study. International Journal of Cancer 2005;113(5):819-24.

357. Baggott JE, Oster RA, Tamura T. Meta-analysis of cancer risk in folic acid supplementation trials. Cancer Epidemiol 2012;36(1):78-81.

358. Smith-Warner SA, Spiegelman D, Ritz J, Albanes D, Beeson WL, Bernstein L, et al. Methods for pooling results of epidemiologic studies: The pooling project of prospective studies of diet and cancer. Am J Epidemiol 2006;163(11):1053-64.

359. Beilby J, Ambrosini GL, Rossi E, De Klerk NH, Musk AW. Serum levels of folate, lycopene, β-carotene, retinol and vitamin e and prostate cancer risk. Eur J Clin Nutr 2010;64(10):1235-8.

360. Chêne G, Thompson SG. Methods for summarizing the risk associations of quantitative variables in epidemiologic studies in a consistent form. Am J Epidemiol 1996;144(6):610-21.

361. Greenland S, Longnecker MP. Methods for trend estimation from summarized dose-response data, with applications to meta-analysis. Am J Epidemiol 1992;135(11):1301-9.

362. Harbord RM, Harris RJ, Sterne JAC. Updated tests for small-study effects in meta-analyses. Stata J 2009;9(2):197-210.

363. Egger M, Smith GD, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. Br Med J 1997;315(7109):629-34.

364. Stevens VL, Rodriguez C, Pavluck AL, McCullough ML, Thun MJ, Calle EE. Folate nutrition and prostate cancer incidence in a large cohort of US men. Am J Epidemiol 2006;163(11):989-96.

365. Bassett JK, Severi G, Hodge AM, Baglietto L, Hopper JL, English DR, et al. Dietary intake of B vitamins and methionine and prostate cancer incidence and mortality. Cancer Causes Control 2012;23(6):855-63.

366. Weinstein SJ, Stolzenberg-Solomon R, Pietinen P, Taylor PR, Virtamo J, Albanes D. Dietary factors of one-carbon metabolism and prostate cancer risk. Am J Clin Nutr 2006;84(4):929-35.

367. Rossi E, Hung J, Beilby JP, Knuiman MW, Divitini ML, Bartholomew H. Folate levels and cancer morbidity and mortality: Prospective cohort study from busselton, Western Australia

. Ann Epidemiol 2006;16(3):206-12.

368. Kenyon SH, Nicolaou A, Ast T, Gibbons WA. Stimulation in vitro of vitamin B12-dependent methionine synthase by polyamines. Biochem J 1996;316(2):661-5.

369. Goodwin AC, Jadallah S, Toubaji A, Lecksell K, Hicks JL, Kowalski J, et al. Increased spermine oxidase expression in human prostate cancer and prostatic intraepithelial neoplasia tissues. Prostate 2008;68(7):766-72.

370. Adimora AA, Schoenbach VJ, Martinson FEA, Stancil TR, Donaldson KH. Comparing biological measurements of vitamin C, folate, alpha-tocopherol and carotene with 24-hour dietary recall information in nonhispanic blacks and whites. Ann Epidemiol 2001;11(6):406-16.

371. Fraser GE, Franke AA, Jaceldo-Siegl K, Bennett H. Reliability of serum and urinary isoflavone estimates. Biomarkers 2010;15(2):135-9.

372. Verkleij-Hagoort AC, de Vries JHM, Stegers MPG, Lindemans J, Ursem NTC, Steegers-Theunissen RPM. Validation of the assessment of folate and vitamin B12 intake in women of reproductive age: The method of triads. Eur J Clin Nutr 2007;61(5):610-5.

373. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA: The Journal of the American Medical Association 1993;270(22):2693-8.

374. Jacques PF, Sulsky SI, Sadowski JA, Phillips JC, Rush D, Willett WC. Comparison of micronutrient intake measured by a dietary questionnaire and biochemical indicators of micronutrient status. Am J Clin Nutr 1993;57(2):182-9.

375. Skeie G, Braaten T, Hjartåker A, Lentjes M, Amiano P, Jakszyn P, et al. Use of dietary supplements in the European prospective investigation into cancer and nutrition calibration study. Eur J Clin Nutr 2009;63(SUPPL. 4):S226-38.

376. Zhang DJ, Elswick RK, Miller WG, Bailey JL. Effect of serum-clot contact time on clinical chemistry laboratory results. Clin Chem 1998;44(6):1325-33.

377. Chu SY, MacLeod J. Effect of three-day clot contact on results of common biochemical tests with serum. Clin Chem 1986;32(11):2100.

378. Clement NF, Kendall BS. Effect of light on vitamin B12 and folate. Lab Med 2009;40(11):657-9.

379. Bailey LB. Folate status assessment. J Nutr 1990;120(11 SUPPL.):1508-11.

380. Collin SM, Metcalfe C, Palmer TM, Refsum H, Lewis SJ, Smith GD, et al. The causal roles of vitamin B 12 and transcobalamin in prostate cancer: Can mendelian randomization analysis provide definitive answers? Int J Mol Epidemiol Genet 2011;2(4):316-27.

381. Davey Smith G, Ebrahim S. 'Mendelian randomization': Can genetic epidemiology contribute to understanding environmental determinants of disease? International Journal of Epidemiology 2003;32(1):1-22.

382. Lewington S, Bragg F, Clarke R. A review on metaanalysis of biomarkers:Promises and pitfalls. Clin Chem 2012.

383. Rapiti E, Fioretta G, Schaffar R, Neyroud-Caspar I, Verkooijen HM, Schmidlin F, et al. Impact of socioeconomic status on prostate cancer diagnosis, treatment, and prognosis. Cancer 2009;115(23):5556-65.

384. D'Ambrosio G, Samani F, Cancian M, De Mola C. Practice of opportunistic prostate-specific antigen screening in italy: Data from the health search database. European Journal of Cancer Prevention 2004;13(5):383-6.

385. Otto SJ, Van Der Cruijsen IW, Liem MK, Korfage IJ, Lous JJ, Schröder FH, et al. Effective PSA contamination in the Rotterdam section of the European randomized study of screening for prostate cancer. International Journal of Cancer 2003;105(3):394-9.

386. Páez A, Luján M, Llanes L, Romero I, De la Cal MA, Miravallesc E, et al. PSA-use in a spanish industrial area. European Urology 2002;41(2):162-6.

387. Etzioni R, Gulati R, Falcon S, Penson DF. Impact of PSA screening on the incidence of advanced stage prostate cancer in the United States: A surveillance modeling approach. Medical Decision Making 2008;28(3):323-31.

388. Tomaszewski JJ, Cummings JL, Parwani AV, Dhir R, Mason JB, Nelson JB, et al. Increased cancer cell proliferation in prostate cancer patients with high levels of serum folate. Prostate 2011;71(12):1287-93.

389. Wichmann H-, Kuhn KA, Waldenberger M, Schmelcher D, Schuffenhauer S, Meitinger T, et al. Comprehensive catalog of European biobanks. Nat Biotechnol 2011;29(9):795-7.

390. Key TJ, Appleby PN, Allen NE, Reeves GK. Pooling biomarker data from different studies of disease risk, with a focus on endogenous hormones. Cancer Epidemiol Biomarkers Prev 2010;19(4):960-5.

391. Blood cholesterol and vascular mortality by age, sex, and blood pressure: A meta-analysis of individual data from 61 prospective studies with 55 000 vascular deaths. Lancet 2007;370(9602):1829-39.

392. Stanczyk FZ, Lee JS, Santen RJ. Standardization of steroid hormone assays: Why, how, and when? Cancer Epidemiol Biomarkers Prev 2007;16(9):1713-9.

Appendix

9.9 EPIC baseline questionnaire