

GENETICS OF THE SLEEP APNOEA/HYPOPNOEA SYNDROME

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Doctor of Medicine (MD)

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2004

I declare that the work in this thesis was composed by myself and is based on studies done by myself with assistance as detailed within the Acknowledgments.

The work was performed at the Scottish National Sleep Laboratory (Royal Infirmary Edinburgh), University of Edinburgh and the Wellcome Trust Clinical Research Facility between November 2001 and May 2003 whilst I was the recipient of a Long-Term Training Fellowship (European Respiratory Society), a Helen Bearpark Scholarship (Australasian Sleep Association) and an Australian Federation of University Women Fellowship.

This thesis has not been submitted in whole or in part in candidature for any other degree, postgraduate diploma or professional qualification.

Renata Ludmila Riha

21.07.2003

For my family and for Heather and Katharina

| <u>TABLE OF CONTENTS</u> | <u>Page</u> |
|--|--------------------|
| Table of Tables | x |
| Table of Figures | xiii |
| Abbreviations | xiv |
| Acknowledgements | xvii |
| Abstract of Thesis | xix |
| <u>Chapter 1</u> | 1 |
| <u>The Obstructive Sleep Apnoea/Hypopnoea Syndrome – An Overview</u> | |
| 1.0 Definition of the Obstructive Sleep Apnoea/Hypopnoea Syndrome | 2 |
| 1.1 Epidemiology of OSAHS | 3 |
| 1.2 Pathophysiology of OSAHS | 4 |
| 1.3 Risk factors for OSAHS | 9 |
| 1.4 Clinical features of OSAHS | 11 |
| 1.5 Morbidity and mortality associated with OSAHS | 13 |
| 1.6 Assessment of OSAHS | 15 |
| 1.6.1 Sleep disordered breathing | 15 |
| 1.6.2 Daytime Sleepiness | 16 |
| 1.7 Treatment of OSAHS | 18 |
| 1.7.1 Conservative measures | 19 |
| 1.7.2 Continuous positive airways pressure (CPAP) | 20 |
| 1.7.3 Oral Appliances | 21 |
| 1.7.4 Surgery | 22 |
| 1.7.5 Pharmacotherapy | 23 |
| 1.8 The OSAHS phenotype | 24 |

| | <u>Page</u> |
|--|-------------|
| 1.9 Concluding remarks | 31 |
| <u>Chapter 2</u> | |
| <u>Candidate Genes in OSAHS</u> | |
| 2.0 Introductory Remarks | 32 |
| 2.1 Is the Obstructive Sleep Apnoea/Hypopnoea Syndrome Hereditary? | 32 |
| 2.2 Craniofacial Morphology | 37 |
| 2.2.1 Craniofacial Development | 37 |
| 2.2.2 Postnatal Facial Growth | 43 |
| 2.2.3 Determinants of Skeletal Growth | 46 |
| Vitamin D Receptor | 47 |
| Beta-2-adrenergic receptor | 49 |
| Growth Hormone | 50 |
| Insulin – like Growth Factor | 51 |
| Insulin-like Growth Factor Receptor | 54 |
| Growth Hormone Receptor | 55 |
| 2.2.4 Summary of Craniofacial Factors | 56 |
| 2.3 Obesity | 56 |
| 2.4 Sleepiness | 64 |
| 2.4.1 The Role of Cytokines | 64 |
| 2.4.2 Hypocretin (Orexin) | 69 |
| 2.5 The Upper Airway in OSAHS | 70 |
| 2.5.1 Regulation of Upper Airway Tone | 70 |
| 2.5.2 Neuropathy in the Upper Airway – its role in OSAHS | 76 |
| 2.5.3 Disorders of Connective Tissue | 80 |

| | <u>Page</u> |
|--|-------------|
| 2.6 Control of Ventilation | 81 |
| 2.7 Apolipoprotein E – a role in OSAHS? | 85 |
| 2.8 Hypertension in OSAHS | 92 |
| 2.9 Methods of Genetic Studies | 95 |
| 2.10 AIMS OF THE THESIS | 99 |
| 2.11 Summary | 111 |
| <u>Chapter 3</u> | |
| <u>Method</u> | |
| 3.0 Introduction | 112 |
| 3.1.1 Recruitment of subjects | 112 |
| 3.1.2 Data collection | 112 |
| 3.1.3 Additional Subjects without a diagnosis of OSAHS | 115 |
| 3.1.4 Phenotyping | 117 |
| 3.1.5 Blood donors | 118 |
| 3.2 Cephalometry | 118 |
| 3.2.1 Technique of cephalometry | 118 |
| 3.2.2 Method of measurement and recording of results | 119 |
| Radiology | 119 |
| Measurement | 121 |
| 3.2.3 Reproducibility | 124 |
| 3.3 Polysomnography | 126 |

| | <u>Page</u> |
|---|--------------------|
| 3.3.1 Technique of measurement and measurement parameters recorded | 126 |
| Home – based Sleep Studies | 126 |
| In-lab Polysomnography | 127 |
| 3.3.2 Sleep staging and scoring of respiratory events | 127 |
| Hypoxaemia | 134 |
| 3.3.3 Reproducibility | 134 |
| 3.4 DNA Extraction | 136 |
| 3.4.1 Blood collection and storage | 136 |
| 3.4.2 Method of DNA extraction | 136 |
| Blood Donor Panels | 138 |
| 3.5 Genotyping | 138 |
| 3.5.1 Polymerase Chain Reaction – Restriction Fragment Length Polymorphism Analysis (PCR-RFLP) | 139 |
| Preparation of the PCR Reaction Mixture | 140 |
| 3.5.2. Allelic Discrimination Analysis using TaqMan® | 142 |
| Assay Design | 144 |
| 3.6 Data Collection and Statistical Analysis | 145 |
| <u>Chapter 4</u> | |
| <u>Results</u> | |
| 4.0 Characteristics of the Study Population | 148 |
| 4.1 Cephalometry | 149 |
| Skeletal Class | 155 |
| 4.2 Blood Donor Controls | 156 |
| 4.3 Apolipoprotein E | 156 |

| | <u>Page</u> |
|---|--------------------|
| 4.4 Results for TaqMan Analyses | 161 |
| 4.5 Tumour Necrosis Factor- Alpha | 163 |
| 4.6 Growth Hormone Receptor | 164 |
| 4.7 Serotonin Receptor- 2A Gene | 166 |
| 4.8 Beta- 2 Adrenoceptor | 167 |
| 4.9 Conclusion | 169 |
| <u>Chapter 5</u> | |
| <u>Discussion</u> | |
| 5.0 Introduction | 170 |
| 5.1 Characteristics of the study population | 171 |
| 5.1.1. Subjects with and without OSAHS | 171 |
| 5.1.2. Phenotyping | 173 |
| <u>5.2 Cephalometry</u> | 175 |
| 5.2.1 Cephalometric Findings in Apnoeic vs. Non-apnoeic Snorers | 176 |
| 5.2.2 The Associations of Obesity and Cephalometric findings in OSAHS | 178 |
| 5.2.3 The Effects of Age on Cephalometric findings in OSAHS | 181 |
| 5.2.4 The Effects of Gender on Cephalometric findings in OSAHS | 182 |
| 5.2.5 Genetic Factors affecting the Craniofacial Complex in OSAHS | 183 |
| 5.2.6 Other considerations | 185 |
| 5.2.7 Limitations of the cephalometric Study | 187 |
| 5.3 Genotyping | 188 |
| 5.3.1 DNA Extraction | 188 |

| | <u>Page</u> |
|--|--------------------|
| 5.3.2 PCR-RFLP | 188 |
| 5.3.3 TaqMan – based Allelic Discrimination | 188 |
| 5.4 Apolipoprotein E | 190 |
| 5.5 Tumour Necrosis Factor Alpha | 193 |
| 5.6 Growth Hormone Receptor | 200 |
| 5.7 Serotonin Receptor - 2A | 205 |
| 5.8 Beta-2 Adrenergic Receptor | 210 |
| 5.9 Concluding Remarks | 214 |
| <u>Chapter 6</u> | |
| 6.1 Conclusions | 215 |
| 6.2 Future Directions | 220 |
| 6.2.1 Phenotyping | 220 |
| 6.2.2 Craniofacial Development | 221 |
| 6.2.3 Obesity | 221 |
| 6.2.4 The Upper Airway | 221 |
| 6.2.5 Cytokines, Sleep and Ageing | 222 |
| 6.3 Summary | 223 |
| <u>References</u> | 224 |
| Appendix 1: Epworth Sleepiness Questionnaire | |
| Appendix 2: Enrolment questionnaire | |
| Appendix 3: Sibling enrolment questionnaire | |
| Appendix 4: Enrolment questionnaire and letter | |
| Appendix 5: Scottish Sleep Centre questionnaire | |

Appendix 6: DNA extraction protocols

Appendix 7: Publications and Presentations arising from the thesis

TABLE OF TABLES

| | <u>Page</u> |
|--|-------------|
| Table 1: Age and gender specific prevalence rates of the apnoea and hypopnoea index based on polysomnographic results for a sample of 1,050 men and 1,098 women from the Vitoria - Gasteiz region (Basque country, Spain) | 27 |
| Table 2: Age and gender specific prevalence rates of the apnoea and hypopnoea index based on polysomnographic results for a sample of 352 men and 250 women from Wisconsin (United States of America) | 28 |
| Table 3: Prevalence rates of the apnoea and hypopnoea index by age based on polysomnographic results for a sample of 741 men from two counties in Southern Pennsylvania (United States of America) | 28 |
| Table 4: Mean Epworth Sleepiness Score in a Normal or Control Population according to Age and Region | 29 |
| Table 5: Mean Epworth Sleepiness Score in a Population of Drivers by Age | 30 |
| Table 6: Classification of AHI according to Gender and Age | 30 |
| Table 7: Prevalence of Sleep Disordered Breathing among First-degree Relatives of Proband with OSAHS | 35 |
| Table 8: Signalling and transcription factors involved in the developing face (not exhaustive) | 42 |
| Table 9: WHO classification of weight in adults according to BMI | 57 |
| Table 10: Neurotransmitters that influence energy expenditure and appetite | 58 |
| Table 11: Positive association studies of candidate genes for weight loss or weight gain in humans | 63 |
| Table 12: Pharyngeal nerve lesions in snorers and patients with OSAHS | 77 |
| Table 13: Ventilatory control abnormalities in patients with SDB, family members and controls | 82 |
| Table 14: Gene deletion models in transgenic mice and effects on ventilatory response to chemical challenges | 83 |

| | <u>Page</u> |
|--|-------------|
| Table 15: Distribution of ApoE allelic frequencies in a European population | 86 |
| Table 16: Distribution of ApoE genotypes and alleles in OSA and Controls | 89 |
| Table 17: Study designs for the identification of disease genes | 96 |
| Table 18: Findings from studies of the relationship between -308 genotype and TNF- α production by stimulated peripheral blood mononuclear cells | 100 |
| Table 19: Study results using reporter gene constructs to investigate the transcriptional effects of the -308 G/A polymorphism | 100 |
| Table 20: DNA sequence variants identified in the human 5HTR2A gene | 105 |
| Table 21: Presence or absence of OSAHS phenotype using scores derived for SDB and sleepiness | 117 |
| Table 22: Abbreviation of Cephalometric Distances and Angles | 123 |
| Table 23: Inter and Intra-rater Reproducibility for Cephalometric Measurements | 125 |
| Table 24: Scoring reproducibility for AHI, REM-sleep time, TST and Sleep Efficiency | 135 |
| Table 25: PCR-RFLP pattern for ApoE using <i>HaeII</i> and <i>AflIII</i> | 140 |
| Table 26: SNPs for which probes and primers were designed | 144 |
| Table 27: Characteristics of the Study Population (n=228) | 148 |
| Table 28: Characteristics of the Study Population (n=228) | 149 |
| Table 29: Bony measurements and angles for dentate non-snoring and snoring males and females with and without OSAHS. | 151 |
| Table 30: Significant Comparisons in Cephalometric Variables between Obese and Non-Obese Men and Women with and without OSAHS | 154 |
| Table 31: Significant Comparisons in Cephalometric Variables between Male Sibs discordant for a Diagnosis of OSAHS | 154 |

| | <u>Page</u> |
|---|-------------|
| Table 32: Distribution of Skeletal Classes between Male and Female Dentate Subjects | 155 |
| Table 33: Mean Age and Age Range of Anonymous Blood Donors | 156 |
| Table 34: Characteristics of the Study Population including Apolipoprotein E Genotype and Allelic Frequencies | 158 |
| Table 35: Characteristics of the Study Population divided along an AHI of greater than or less than 15/hr | 159 |
| Table 36: Study Subjects according to Apolipoprotein E4 carriage | 160 |
| Table 37: Allelic Frequencies in the Study Population vs. a UK population | 160 |
| Table 38: Allelic and Genotype Frequencies for TNF- α (-308) A/G Polymorphism in Subjects with definite OSAHS vs. those without | 164 |
| Table 39: Allelic and Genotype Frequencies for +561 (T/G) GHR Polymorphism in Subjects with definite OSAHS vs. those without. | 165 |
| Table 40: Carriage of the +561 G GHR allele in subjects with a BMI greater than or equal to 30 kg/m ² | 166 |
| Table 41: Allelic and Genotype Frequencies for 5HTR2A promoter polymorphism C/T Subjects with definite OSAHS vs. those without. | 167 |
| Table 42: Characteristics of the Study Population with and without definite OSAHS (n=138) | 168 |
| Table 43: Allelic and Genotype Frequencies for G+79C ADRB-2 Polymorphism in Subjects with definite OSAHS vs. those without | 168 |
| Table 44: Distribution of the C+79G ADRB-2 Polymorphism in Subjects with definite OSAHS vs. those without | 169 |
| Table 45: Summary of p-values for allelic distribution, minor allele carriage and genotype for subjects with and without OSAHS for the genes TNF- α , GHR, 5HTR2A and ADRB-2. | 170 |

TABLE OF FIGURES

| | |
|--|-----|
| Fig 1: Sites of Obstruction in the Upper Airway (denoted by shaded area) | 5 |
| Fig 2: Classification of the Upper Airway after Mallampati | 12 |
| Fig 3: Processes involved in facial development | 41 |
| Fig 4: Facial Profiles (from left to right): retrognathic (skeletal class II); orthognathic (skeletal class I) and prognathic (skeletal class III) | 45 |
| Fig 5: Short and long-term factors influencing energy homeostasis | 59 |
| Fig 6: Factors involved in sleep promotion and regulation | 65 |
| Fig 7: Diagram representing the neuronal circuitry thought to be involved in the pontine regulation of REM sleep and generation of postural motor atonia. | 72 |
| Fig 8: Technique of performing cephalometry | 120 |
| Fig 9: Cephalometric Points of Reference | 122 |
| Fig 10: Cephalometric Planes and Angles | 122 |
| Fig 11: Example of Awake EEG | 128 |
| Fig 12: Example of Stage 2 Sleep | 129 |
| Fig 13: Example of Stage 4 Sleep | 130 |
| Fig 14: Example of REM Sleep | 131 |
| Fig 15: Example of a Hypopnoea on the Compumedics™ W-Series® Split Screen | 133 |
| Fig 16: 318 base pair APOE sequence amplified during PCR-RFLP | 140 |
| Fig 17: Chemistry of the TaqMan-based Allelic Discrimination Analysis | 142 |
| Fig 18: Gel showing RFLP patterns for APOE using <i>HaeII</i> and <i>AflIII</i> restriction enzymes | 157 |
| Fig 19: Plot of the 5HTR2A promoter polymorphism C/T after TaqMan analysis | 162 |
| Fig 20: Plot of the ADRB2 polymorphism C+79G after TaqMan analysis | 162 |

ABBREVIATIONS

5-HT_{2A} – serotonin receptor 2A

A - adenine

AASM – American Association of Sleep Medicine

AD – autosomal dominant

ADRB – beta-adrenergic receptor

AHI – apnoea/ hypopnoea index

AHI – apnoea/hypopnoea index

APOE – apolipoprotein Epsilon

AR – autosomal recessive

ASDA – American Sleep Disorders Association

BMI – body mass index

BMI – body mass index (kg/m²)

C - cytosine

CI – confidence interval

COPD – chronic obstructive pulmonary disease

CPAP – continuous positive airway pressure

DBP – diastolic blood pressure

DNA – deoxyribonucleic acid

ECACC – European Collection of Cell Cultures

ECG - electrocardiogram

EEG – electro-encephalogram

EMG - electromyogram

EOG – electro-oculogram

ESS – Epworth Sleepiness Score

G - guanine

GHR – growth hormone receptor

HeLa – epithelial carcinoma cell line

KV - kilovoltage

MSLT – multiple sleep latency test

MWT – multiple wakefulness test

NHMRC – national health and medical research council (Australia)

NREM – non-rapid eye movement sleep

OR – odds ratio

OSAHS – obstructive sleep apnoea/hypopnoea syndrome

OSAHS – obstructive sleep apnoea/hypopnoea syndrome

PCR – polymerase chain reaction

PSG – polysomnogram

QTL – quantitative trait locus

REM – rapid eye movement sleep

RFLP – restriction fragment length polymorphism

SaO₂ – oxygen saturation when awake

SASA – Scottish Association for Sleep Apnoea

SBP – systolic blood pressure

SDB – sleep disordered breathing

SIDS – sudden infant death syndrome

SNP – single nucleotide polymorphism

SNP – single nucleotide polymorphism

SpO₂ – oxygen saturation during sleep

T - thymidine

TBE – Tris-borate EDTA

TE – Tris-EDTA

TIA – transient ischaemic attack

TNF – tumour necrosis factor

TST – total sleep time

UARS – upper airway resistance syndrome

ACKNOWLEDGMENTS

My sincere thanks go to Professor Neil Douglas whose vision made possible this study. I am indebted to him for his support, encouragement and patience. His incisive and constructive logic have allowed me to hone my own critical faculties in the area of medical research and his faith in the steady progress of science and my own abilities has been invaluable. I am also grateful to Dr Shona Kerr who has so generously given of her time in supporting this study and for reviewing, discussing and reading the thesis as it progressed.

Throughout the course of this project, I was assisted by a number of colleagues and collaborators. Dr Pirkko Brander, who was responsible for the data collection in the early part of the study, laid the foundations of further work carried out in this thesis. Also, without the assistance of Marjorie Vennelle with cephalometric analysis or the SIDS study, this project would not be in its present form. I am grateful to Dr Nigel McArdle for some of the data collection prior to my arrival in Edinburgh and to the unceasing labours of the sleep technologists, Sian Finch, Laura Bolton and Elizabeth Hill who spent many hours scoring sleep studies.

The night staff and technologists of the Sleep Centre assisted greatly by obtaining overnight polysomnography and kept me on the straight and narrow with patient appointments. I am grateful also to the secretaries of the unit, Maggie Osburn, Margaret Smith and Liz Kennedy who inducted me into the arcane mysteries of data management in the Sleep Centre and were of invaluable help in chasing patient records. Carol Hoy, Joan Elder, Jane Krumins and Sandra White helped with patient recruitment. Peter Wraith provided invaluable assistance with IT support. Dr Heather Engleman undertook not only statistical coaching, but also assisted with practical

issues. Her unconditional friendship, generosity and support kept me going and remain priceless.

I am grateful to the Wellcome Trust Core Research Facility for taking on the project under the leadership of Professor David Porteous & Dr Shona Kerr. Stuart Bayliss and Angie Fawkes performed the TaqMan analyses and patiently explained and revised the data with me. I will not forget Alison Condie who first taught me how to extract DNA, to perform PCR and who ran the Apolipoprotein aspects of the study with me. Catriona Graham helped with statistical analyses of the genetic components of the thesis. Special thanks also go to Adrian Edwards at the DNA Extraction Core Laboratory in the Wellcome Trust Centre for Human Genetics, Oxford for assistance with DNA extraction and transport. Cephalometry was performed in the Department of Radiology at the Royal Infirmary Edinburgh. In particular, I should like to thank Nicola Yorkston and Jan Palmer who explained the technique and procedure of cephalometric roentgenograms. Dr Ian Yang, my friend and colleague of many years who took time out of his own busy schedule to read my final chapters, deserves my sincerest gratitude. Although the assistance listed above is material, many of my colleagues and collaborators provided the intangible elements of solidarity, friendship and good humour that made my stay in Edinburgh unforgettable. Many of them have become close personal friends. For their camaraderie and their support, I also thank Eileen Dolan, Dr Thomas Mackay, Dr Virna Teixeira, Dr Mohamed Al-Abri, Lynne Anderson, Dr Chung Yao Hsu and Bilgay Izci. Finally, none of the studies in this thesis could have been performed without the altruism of their participants who proved that on the whole, the Scots are a warm and generous people.

ABSTRACT OF THE THESIS

The obstructive sleep apnoea/hypopnoea syndrome (OSAHS) affects approximately 2 - 4% of the middle-aged population. OSAHS is characterised by obstruction of the upper airway during sleep, resulting in repetitive breathing pauses accompanied by both oxygen desaturations and arousals from sleep. OSAHS results in sleepiness and daytime cognitive impairment and is an independent risk factor for hypertension. A number of studies have shown that OSAHS runs in families and that this can occur without obesity. Possible mechanisms for this familial predisposition include craniofacial structure, differential deposition of adipose tissue, differential susceptibility to sleepiness and genetically determined differences in upper airway control. This thesis examined possible candidate genes that might contribute towards the development of OSAHS. The genes of interest included tumour necrosis factor - alpha (potential associations with ageing, hypercytokinaemia in OSAHS, obesity and sleepiness), Apolipoprotein E (associations with the development of cerebrovascular disease), the serotonin receptor 2A (modulation of upper airway muscle tone and response to selective serotonin re-uptake inhibitors), beta-2 adrenoreceptor (growth, fat metabolism and blood pressure) and the growth hormone receptor (influence on postnatal bone growth and height including the craniofacial complex). 557 consecutive subjects with a diagnosis of OSAHS were approached at the Scottish Sleep Centre. 104 subjects (all Caucasian) were recruited together with 107 of their siblings as well as an additional 17 unrelated subjects without OSAHS and underwent overnight polysomnography and cephalometry. Blood was taken for DNA analysis. Subjects were classified as having definite OSAHS (n=110), indeterminate status (n=34) or not having OSAHS (n=83) based on their apnoea/hypopnoea

frequency and sleepiness as measured by the Epworth Sleepiness Score. DNA was extracted using standard techniques and polymorphisms in the candidate genes were examined using allelic discrimination testing with TaqMan™. The Apolipoprotein E4 polymorphisms were determined using polymerase chain reaction, restriction fragment length polymorphism. DNA from 192 random, healthy UK blood donors (assumed not to have OSAHS) was used as an additional control.

Differences between subjects with and without OSAHS were as expected: there were over twice as many men in the OSAHS group compared to the non-OSAHS group ($p < 0.0001$) and systolic blood pressure was significantly higher ($p = 0.002$) in the OSAHS group. Furthermore, the OSAHS group were more obese ($p < 0.0001$) and had a greater neck circumference ($p < 0.0001$) than the non-OSAHS group.

Cephalometry revealed that both male and female apnoeics had significantly lower-set hyoid bones than non-apnoeic snorers ($p = 0.01$ and $p = 0.038$ respectively). In male subjects with OSAHS, a smaller mandible and lower-set hyoid were the most important characteristics distinguishing siblings with from sibs without OSAHS, independently of age and BMI. However, age, sex, BMI and edentulism were found to influence craniofacial parameters in both groups.

For the genetic analyses, the Apo E e4 allele (examined in 73 subjects) was not associated significantly with a diagnosis of OSAHS. The single nucleotide polymorphisms examined in each of the 4 other candidate genes in this study are summarised in the following table [the p-value is for the analysis comparing subjects with OSAHS ($n=110$) to those without it ($n=275$)].

Table: Genotype and Allele Frequencies for Single Nucleotide Polymorphisms in 4 Candidate Genes for OSAHS

| Gene | Allele Distribution | Minor Allele Carriage | Minor Genotype |
|------------------|---------------------|-----------------------|----------------|
| TNF-a (-308 A/G) | A/G* | A* | A/A |
| GHR (+561 T/G) | G/T* | G | G/G* |
| ADRB-2 (C+79G) | G/C | C | C/C* |
| 5-HT-2a (C/T) | C/T | C | C/C |

* $p < 0.02$ after correction for multiple comparisons

In addition, the TNF-a -308 A allele showed significant association with the OSAHS phenotype when comparing siblings discordant for carriage of this allele. The increased prevalence of some of the minor polymorphisms in the study population with OSAHS suggested there may be abnormalities in metabolism and the regulation of growth, which may directly contribute to its aetiology.

These preliminary findings would require exploration in other populations, but are compatible with OSAHS being a polygenic disorder. This thesis highlights that there is much to be done in our search for relevant genetic factors that will lead to a greater understanding of this complex, chronic and very common disease.

CHAPTER 1

THE OBSTRUCTIVE SLEEP APNOEA/HYPOPNOEA SYNDROME – AN OVERVIEW

Sleep apnoea had been recognised throughout human history, dating as far back as the 4th Century BC. Numerous reports throughout the 19th Century and the early part of the 20th Century gave way to systematically conducted studies on patients with OSAHS and related syndromes (Lavie 1984; Kryger 1983).

The obstructive sleep apnoea/hypopnoea syndrome (OSAHS) was first properly documented in neurophysiological sleep laboratories using techniques developed for the investigation of other conditions such as depression and narcolepsy. OSAHS was first described as such in 1965 (Gastaut, Tasinari & Duron, 1965; Jung&Kuhlo 1965) and since that time there has been an explosion in the facilities for its diagnosis and treatment as well as a rapid advancement in our understanding of its far-reaching consequences.

OSAHS is a common condition affecting approximately 0.3 - 4% of the middle-aged population (Douglas 2002) and is defined on the basis of symptoms of daytime sleepiness and objective measures of disordered breathing during sleep. Characteristic of OSAHS is obstruction of the upper airway during sleep, resulting in repetitive breathing pauses accompanied by oxygen desaturation and arousal from sleep. This results in diurnal sleepiness leading to cognitive impairment. Furthermore, OSAHS has been recognised as an independent risk factor for hypertension.

This chapter focuses on definitions of OSAHS, provides a general overview of its epidemiology, aetiologies and treatment and concludes by defining the OSAHS phenotype that will be used in this thesis.

1.0 Definition of the Obstructive Sleep Apnoea/Hypopnoea Syndrome

OSAHS is the result of recurrent upper airways narrowing during sleep leading to recurrent arousal from sleep with consequent sleep fragmentation and recurrent transient hypertension among the important physiological effects.

The episodes of pharyngeal obstruction are termed apnoea if complete and associated with no airflow and hypopnoea if partial. The term OSAHS is thus defined as a combination of relevant symptoms with significant numbers of apnoeas and hypopnoeas during sleep. The term apnoea is defined, in adults, as the cessation of airflow for a minimum of 10 seconds. Apnoeas/hypopnoeas are often, but not always, associated with an electroencephalographic arousal at their termination and with a 2 – 4% drop in oxygen saturation (American Association of Sleep Medicine Task Force 1999). Some controversy remains with respect to a standardised definition of hypopnoea, but in our centre this is a 50% reduction in thoraco-abdominal movement for at least 10 seconds from the preceding stable baseline when asleep, a definition since adopted by the American Academy of Sleep Medicine (1999).

Brief consideration should also be given to the term upper airways resistance syndrome (UARS) which is technically defined as the coexistence of daytime sleepiness with increased respiratory effort during periods of increased upper airway resistance but without accompanying hypopnoeas or apnoeas. It is increasingly considered to be part of the spectrum on OSAHS rather than a separate condition in its own right (Douglas 2000) and its frequency is highly dependent on the recording techniques used to detect hypopnoeas.

1.1 Epidemiology of OSAHS

Reporting of OSAHS was initially confined to case series, but more recently, large-scale epidemiological studies have attempted to answer questions on the incidence and prevalence of OSAHS. However, few of these have used ideal methods and it is therefore difficult to draw firm conclusions from them.

Generally, the best-conducted studies in terms of method and rigor of technique employed have found a prevalence of OSAHS in the middle-aged **male** population of 0.3 – 4% (Stradling and Crosby 1991; Young et al 1993; Bearpark et al 1995; Jennum and Sjol 1992). The study showing the lowest quoted prevalence may have underestimated the occurrence of OSAHS in the population by using oximetry alone, with ≥ 5 4% dips in SpO₂ per hour, as an initial screening measure (Stradling and Crosby 1991), whilst those studies showing the highest prevalences may be overestimating the prevalence by including central apnoeas and breath holds occurring during wakefulness (Jennum and Sjol 1992) using inductance plethysmography at home. Assessment of sleepiness differed between the studies on account of using self-devised questionnaires specific to each study. Fewer studies exist examining the prevalence of OSAHS in **women**, but it is probably half that of men at 0.5 – 1% (Young et al 1993; Jennum and Sjol 1992).

OSAHS occurs throughout the entire lifespan – from neonates to the elderly. In adults, the frequency of disordered breathing during sleep increases with age and is poorly associated with an increased incidence of daytime sleepiness or other symptoms of OSAHS (Ancoli-Israel et al 1985; Duran et al 2001; Bixler et al 1998).

1.2 Pathophysiology of OSAHS

The pathogenesis of obstructive sleep apnoea (OSA) has been a matter of intense investigation since the 1970's. Although, the precise mechanisms have not been entirely elucidated, three fundamental features have been proven beyond doubt (Kuna and Remmers 2000):

1. The pharynx is the site of upper airway obstruction during sleep
2. Patients with OSA commonly have anatomical abnormalities of the upper airway when awake
3. During inspiration, the size of the pharyngeal lumen depends on the balance between narrowing forces resulting from intrapharyngeal suction pressure and dilating forces generated principally by pharyngeal muscles.

The sites of upper airway narrowing can be broadly classified into three regions (Fig 1.): the retropalatal region; the retroglossal region and the hypopharyngeal region.

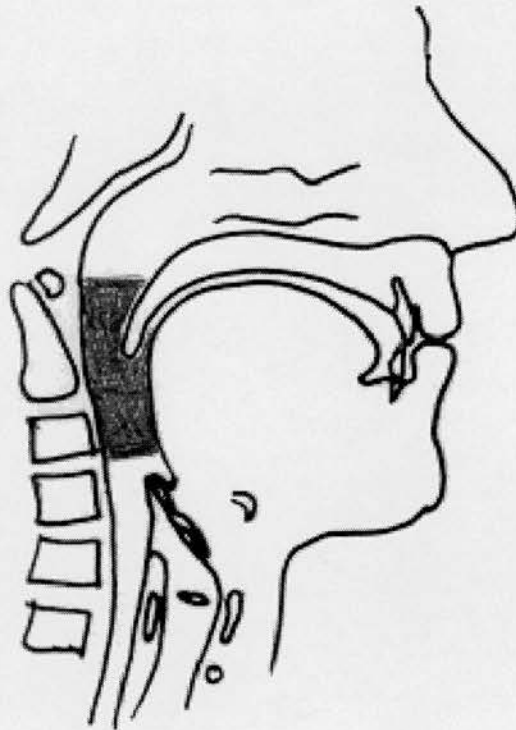


Fig1. Sites of Obstruction in the Upper Airway (denoted by shaded area)

The key pathophysiological feature in OSAHS is the occurrence of upper airway obstruction during sleep which does not occur during wakefulness. Although the precise pathogenetic mechanisms remain incompletely understood, they are thought to be the result of the following:

1. Upper airway calibre is reduced and pharyngeal wall compliance increased as a result of reduced tonic upper airway dilating muscle activity (Tangel et al 1991). The decrease in calibre results in an increase in upper airway resistance of variable degree. This is not observed in the healthy non-snorers,

rather it is a phenomenon observed in those who snore or have sleep apnoea (Badr 1999). Increased pharyngeal wall compliance during sleep in snorers is manifested by the occurrence of inspiratory flow limitation as flow plateaus during inspiration, despite continuous generation of subatmospheric intraluminal pressure. The combination of inspiratory flow limitation and increased resistance leads to an increased work of breathing, hypoventilation and frequent arousal from sleep.

2. Normally, the ventilatory controls system is able to compensate for added loads and this is essential for the preservation of chemoreceptor homeostasis. Immediate compensation is compromised during NREM sleep and thus, resistive loading leads to a decrease in tidal volume and minute ventilation and then alveolar hypoventilation with subsequent hypercapnia. The latter restores ventilation towards normal levels. In awake humans, application of negative pressure to the upper airway elicits reflex activation of the genioglossus or the tensor palatini muscles leading to dilation of the upper airway. This reflex is absent during NREM sleep (Wheatley et al 1993). Thus, the failure of immediate load compensation results in hypoventilation and a subsequent increase in respiratory muscle activity (Henke et al 1991).
3. The hypocapnic apnoeic threshold is an important determinant of OSAHS. When the wakefulness stimulus to breathe is lost during sleep, ventilation in NREM sleep becomes critically dependent on chemoreceptor stimuli. When PaCO_2 falls below a highly reproducible hypocapnic apnoeic threshold (Skatrud et al 1983), central apnoea develops. Hypocapnia is thought to be the most important inhibitory factor during NREM sleep. REM sleep differs

in that peripheral atonia is accompanied by augmented inspiratory medullary neuronal activity and the REM sleep EEG shares many features of the awake EEG. It has as yet not been established whether hypocapnia inhibits ventilation during REM sleep.

4. Central apnoea leads to pharyngeal narrowing or occlusion which adds an obstructive component to central apnoea and requires a higher drive to resume ventilation (Badr et al 1995). In order to resume rhythmic breathing, an elevation of PaCO₂ above eupnoeic levels is necessary to overcome the inertia of the ventilatory control system (Leevers et al 1993). Thus, the combination of elevated PaCO₂ and upper airway obstruction leads to arousal and hyperventilation which causes subsequent apnoea (Xie et al 2001).
5. Upper Airway Patency during sleep is determined by a number of factors. There is evidence that the pharyngeal airway is smaller during wakefulness in patients with OSAHS compared to those without it (Schwab et al 1993a & b; 1995). The airway in OSAHS narrows laterally in sleep compared to normals. Pharyngeal patency during sleep is a function of transmural pressure across the pharyngeal wall and the compliance of the pharyngeal wall. Negative intraluminal pressure, generated by thoracic pump muscle activity, is thought to cause UA obstruction in subjects with OSAHS (Morrell et al 1998). The increase in air velocity resulting from inspiratory narrowing during sleep is thought to cause decreased intraluminal pressure according to the Bernoulli principle – intraluminal pressure becomes more negative and causes greater collapse of the upper airway. This is the most widely accepted theory for

apnoea generation but there is no direct evidence from sleeping human studies.

Extraluminal pressures are also thought to influence upper airway patency including passive gravitational forces generated by craniofacial structure or adipose tissue surrounding the UA (Haponik et al 1983; Horner et al 1989 a&b; Shelton et al 1993)

Intrinsic stiffness of the pharyngeal wall also determines its degree of collapsibility during sleep and upper airway dilating muscles are presumed critical to the regulation of this. However there is conflicting evidence regarding the exact mechanism whereby upper airway muscles affect pharyngeal compliance:

- a. The pharyngeal airway is more collapsible in dead infants (Reed et al 1985) but not in paralysed dogs (Fouke et al 1986).
- b. Patients with OSAHS have increased activity of the genioglossus muscle during wakefulness and sleep (Mezzanotte et al 1992; Suratt et al 1988).
- c. Hypocapnic central apnoea results in greater upper airway narrowing in subjects with OSAHS relative to normals (Badr et al 1995).
- d. Compliance at the level of the retropalatal airway is decreased during REM sleep relative to NREM sleep which may be due to increased vascular perfusion of the pharynx during REM sleep (Rowley et al 1998).

Thoracic caudal traction may also play a role in UA patency during sleep. The UA is connected to the thoracic cage and the mediastinum by several structures and

increased lung volume during inspiration is associated with increased UA calibre in awake human beings as a result of thoracic inspiratory activity providing caudal traction on the UA (Van de Graaff 1988). This occurs independently of UA dilation muscle activity. This mechanism has been shown in sleeping subjects to reduce upper airway resistance and increase retropalatal airway size when end-expiratory lung volume was increased by passive inflation (Begle et al 1991).

Surface mucosal factors are also thought to influence airway patency during sleep (Olson et al 1988) and this may be an important pathogenetic mechanism in OSAHS subjects with a lot of mucosal inflammation from repeated trauma. Jokic et al (1998) showed that pharyngeal mucosal surface tension is associated with decreased AHI in sleeping people but the relative contribution of this mechanism to the generation of UA dysfunction in OSAHS is yet to be determined.

In summary, UA occlusion is the result of an interaction between multiple anatomic and physiological abnormalities which involve a small, highly compliant pharynx, central breathing instability leading to reduced ventilatory motor output to UA dilators and collapsing transmural pressure. Although sub-atmospheric intraluminal pressure contributes to the generation of a collapsing transmural pressure, it cannot be construed of as the sole mechanism of UA obstruction during sleep.

1.3 Risk factors for OSAHS

The pathophysiology of OSAHS cannot be seen in isolation as a dysfunction of upper airway muscles alone, but as a number of interrelated pathologies and risk factors. The strongest risk factors for OSAHS are obesity and ageing (Strohl and

Redline 1996; Levinson et al 1993; Rajala et al 1991; Dealberto et al 1994; Grunstein et al 1993; Katz et al 1990; Davies et al 1990).

Morbid obesity defined as a body mass index (BMI) of $> 30\text{kg/m}^2$, is present in 60 – 90% of patients with OSAHS. Central obesity, characterised by a high waist:hip ratio or increased neck circumference probably is probably better correlated with OSAHS. OSAHS is more common in men and the risk of OSAHS is higher in females who are obese and postmenopausal (Wilhoit and Suratt 1987; Guilleminault et al 1988; Richman et al 1994; Dancey et al 2001).

OSAHS is also associated with craniofacial abnormalities such as retrognathia. Jaw abnormalities are more important in thinner OSAHS patients (Lowe et al 1986, 1995, 1996; Ferguson et al 1995; Partinen et al 1988).

A familial component has been identified and this is further discussed in Chapter 2 of this thesis. Additionally, race and certain congenital conditions such as Marfan's syndrome, Down's syndrome and the Pierre-Robin syndrome predispose to the development of OSAHS (Bassiri and Guilleminault 2000). Acquired conditions such as acromegaly, hypothyroidism and menopause are also associated with OSAHS (Bassiri and Guilleminault 2000).

OSAHS is exacerbated by alcohol ingestion - thought to reduce the activity of the genioglossus muscle, thereby leading to upper airway collapse (Taasan et al 1981; Krol et al 1984; Scrima et al 1982; Scanlan et al 2000). Further exacerbation can occur as a result of sedative use, sleep deprivation, tobacco use and supine posture (Wetter et al 1994; Bliwise et al 1988; Roth et al 1985). Reduced nasal patency, due to congestion or anatomical defects as well as respiratory allergies can also significantly contribute to OSAHS (Lavie et al 1983).

1.4 Clinical features of OSAHS

The symptoms of OSAHS can be conveniently divided into those manifesting during sleep and those present whilst the patient is awake. The most common complaint of patients with OSAHS is excessive daytime somnolence often associated with fatigue. This may range from subtle to severe and impact on driving alertness, result in intellectual impairment, personality changes and mood disturbances, and most commonly, depression. Other symptoms suggestive of OSAHS include morning headaches, decreased libido or impotence and decreased dexterity (Bassiri and Guilleminault 2000; Douglas 2002). Nocturnal symptoms are sometimes apparent to the patient but generally, are reported by a bed partner as most patients with OSAHS will be oblivious to their behaviour whilst asleep. The most common symptoms reported by bed partners include snoring, snorting, choking attacks terminating a snore and witnessed apnoeas. Although an absence of snoring does not exclude a diagnosis of OSAHS or upper airway resistance, virtually all patients with this condition snore. Apnoeic episodes are reported by about 75% of bed partners (Hoffstein and Szalai 1993). During mild apnoeic episodes, there may be preserved respiratory effort whilst in severe cases there may be a brief cessation of any thoracoabdominal movement whatsoever. Gasps, chokes, snorts, vocalisations or brief awakenings generally terminate apnoeic episodes. Bed partners will generally report a sudden cessation of snoring followed by a loud snort and a resumption of snoring. Some patients will awaken after such a sequence, however, most do not and are unaware of their sleep disordered breathing (Bassiri and Guilleminault 2000; Douglas 2000). Those who are aware of frequent events leading to awakening can try

to delay and prevent themselves from falling asleep, thus presenting with a 'paradoxical' insomnia. Other symptoms commonly reported during sleep include nocturia (Kales 1985), diaphoresis, gastro-oesophageal reflux and drooling (ASDA 1997; Kales 1985). Many patients awaken in the morning complaining of a dry mouth. A number of clinical features are associated with OSAHS; however, the predictive value of any single one is limited in confirming the diagnosis. It has been shown that clinical impression alone has a sensitivity of 60% and specificity of 63% (Hoffstein and Szalai 1993). The principal abnormalities include obesity (BMI > 28 kg/m) and enlarged neck circumference (>40 cm) (Kushida et al 1997). Dental abnormalities identified include narrow mandible, narrow maxilla, dental overjet and retrognathia, cross-bite and dental malocclusion (Class 2). Nasal problems include enlarged nasal turbinates and deviated nasal septum. The vast majority of aberrations occurs in the pharynx and includes high and narrow hard palate, elongated and low-lying uvula, prominent tonsillar pillars, enlarged tonsils and adenoids and macroglossia (Bassiri and Guilleminault 2000; Douglas 2002). An effective way of clinically categorising abnormalities of the upper airway involves the use of the Mallampati score (see Figure 2).

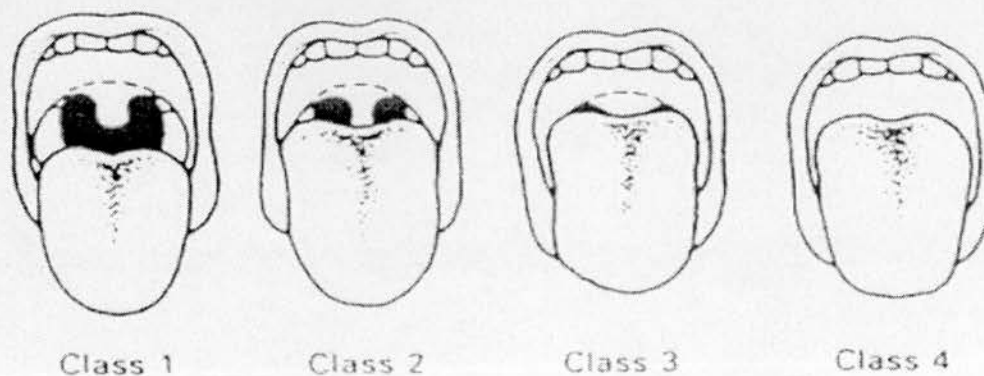


Fig 2. Classification of the Upper Airway after Mallampati (*Can Anaesth Soc J* 1983; 30: 316 – 317)

Nevertheless, history and clinical examination alone (including blood pressure and BMI) can predict the presence of OSAHS in only 50% of patients attending a sleep disorders clinic (Hoffstein and Szalai 1993). Definitive diagnosis requires overnight investigation of breathing pattern.

1.5 Morbidity and mortality associated with OSAHS

Untreated OSAHS can contribute to the development or progression of other disorders. OSAHS has now been shown to be a cause for systemic hypertension (Faccenda et al 2001; Pepperell et al 2002; Hla et al 2002; Sanner et al 2002; Becker et al 2003; Lavie et al 2000) and there is accumulating evidence suggesting that it can also cause pulmonary hypertension (Blankfield et al 2000; Sajkov et al 2002). OSAHS is associated with ischaemic heart disease (Kiely and McNicholas 2000). A recent study has shown that atrial pacing may ameliorate sleep apnoea present in patients with significant cardiac rhythm disturbances (Garrigue et al 2002), but this study has not been replicated.

Disordered breathing during sleep has been found to be a significant clinical feature in a proportion of patients with cerebrovascular disease – stroke and transient ischaemic attacks. However, studies are contradictory, with some showing no increase in OSAHS in those with TIA (McArdle et al 2003), whilst others show a high prevalence of OSAHS in those with stroke (see Neau et al 2003 for review). Two studies have been published so far looking at the effectiveness of treating OSAHS in stroke with a significant reduction in sleep-disordered breathing and improvement in quality of life (Sandberg et al 2001; Wessendorf et al 2001). Patients with OSAHS and moderate to severe co-existent lung disease such as COAD are

more likely to develop type II respiratory failure that will improve with treatment of the obstructive apnoeas (de Miguel et al 2002; Mansfield and Naughton 1999). Likewise, nocturnal asthma may be worsened by sleep apnoea (Chan et al 1988; Guilleminault et al 1988) and treatment may lead to improvement

OSAHS leads to neuropsychological impairment that includes deficits in attention, concentration, vigilance, manual dexterity, visuomotor skills, memory, verbal fluency and executive function (Engleman et al 2000). Perhaps the most important complication of OSAHS, and the one which has the greatest impact from the public health perspective, is driving accidents. Over a third of patients with OSAHS report having had had an accident or near-miss accident on account of falling asleep whilst driving (Engleman et al 1997). There is also objective evidence of 1.3 – 12 fold increases in accident rates among those with sleep apnoea (Teran-Santos et al 1999; Young et al 1997; Horstman et al 2000; Barbe et al 1998; Findley et al 1998; George and Smiley 1999; Lloberes et al 2000) and accident rates in OSAHS patients have been found to be 1.3-7 times higher than those in the general population (Barbe et al 1998; Findley et al 1998; George and Smiley 1999). Vigilance testing and driving simulators in studies assessing driving performance in patients with OSAHS reveal that performance is markedly reduced and the impairment is not just limited to periods when patients actually fall asleep but also when they are awake due to reduced vigilance. There is also evidence that OSAHS patients have a 50% increased risk of work place accidents (Ulfberg et al 2000; Lindberg et al 2001).

There is no evidence related to mortality specifically as a result of OSAHS. Only one small, non-randomised study with 54% follow-up of patients has examined the impact of CPAP therapy on survival of patients with OSAHS (He et al 1988). There

was a suggestion that survival was improved by CPAP but this must be viewed in the context of the study's significant methodological limitations.

1.6 Assessment of OSAHS

As stated in 1.5 above, definitive diagnosis of OSAHS requires objective recording and measurement of sleep and breathing during the night in addition to a measure of daytime sleepiness (objective or subjective) and other symptoms.

1.6.1 Sleep disordered breathing

An objective measure of sleep disordered breathing at night is generally required to confirm the diagnosis of OSAHS. The method most widely used, and which is considered by some to be the 'gold standard' for diagnosis despite limited evidence, is overnight polysomnography (PSG). The American Academy of Sleep Medicine Task Force published indications for PSG in 1997 and measurement techniques and syndrome definitions in 1999. Most PSG studies monitor the following routinely: nasal and/or oral airflow; thoracoabdominal movement; snoring; electroencephalogram (EEG); electro-oculogram (EOG); electro-myogram (EMG); and oxygen saturation. Signal collection and interpretation is usually computerised, but manual scoring of the trace should still be performed using guidelines for interpretation of the EEG published in 1968 by Rechtschaffen and Kales, and the AASM criteria (1999) and Gould et al (1988) for scoring of respiratory and other events.

Full night PSG is generally performed, but split-night studies are also used (Pepperell et al 2002) in which the first half of the study night is used for diagnosis and the

second half to monitor treatment response using continuous positive airway pressure (see 1.7.2 below).

A more recent introduction to the assessment of sleep-disordered breathing has been cardiorespiratory monitoring alone. This involves the measurement of airflow, respiratory effort, oxygen saturation and heart rate, but no EEG. The great advantages of these systems are price and portability and the ability of patients to monitor themselves at home. Ferber et al (1994) reviewed seven studies on portable ambulatory monitoring systems and reported a sensitivity of 78 - 100% and a specificity of 67 - 100% in comparison to in-lab PSG, although this is of course not a gold standard..

Overnight oximetry is sometimes used as a screening test for identifying patients who are at risk of having significant OSAHS but should never be seen as a substitute for in-lab PSG or home cardiorespiratory monitoring. There are severe limitations inherent in this technique used in isolation including the inability to detect apnoeas or hypopnoeas not associated with oxygen desaturation and the upper airway resistance syndrome. Furthermore, nocturnal oxygen desaturation may be related to sleep hypoventilation without associated upper airways obstruction e.g. in COPD, severe kyphoscoliosis, muscular dystrophy and morbid obesity and in the setting of periodic breathing associated with severe heart failure.

1.6.2 Daytime Sleepiness

Sleepiness is difficult to define and is the subject of intense discussion (see Cluydts et al 2002 for review). For the purposes of this thesis, sleepiness will be regarded as 'normal' sleepiness (result of the normal circadian rhythm) and 'pathological'

sleepiness (result of altered sleep scheduling) (Moldofsky 1992). Pathological sleepiness can be further subdivided into 'habitual' (e.g. as the result of recurring precipitants of sleepiness such as OSA) or 'occasional' (e.g. as the result of jet lag or medication).

Three methods of classifying sleepiness can be utilised as proposed by Cluydts (2002):

1. Inferring sleepiness from behavioural measures, e.g. observation of yawning frequency, actigraphy, facial expression or performance tests such as the driving simulator, psychomotor vigilance tests and reaction time tests
2. Self-evaluation of sleepiness by rating scales, e.g. the Stanford Sleepiness Scale to measure sleepiness at a given instant, Epworth Sleepiness Score (ESS) to measure sleepiness averaged over a month.
3. Direct electrophysiological measures e.g. multiple sleep latency test (Thorpy 1992) and multiple wakefulness test (Doghramji et al 1997); pupillometry and cerebral evoked potentials.

Probably the most widely used and best-validated scale assessing daytime sleepiness is the Epworth Sleepiness Score (see Appendix 1) first devised in 1991 by Johns. Its advantages include ease of administration and low cost. It assesses global level of sleepiness and is independent of short-term variations in sleepiness with the time of day and also inter-day variations (Johns 1994). The ESS aims at measuring the general level of daytime sleepiness as a stable individual characteristic and has a satisfactory test-retest reliability (Johns 1992). The ESS is also able to discriminate between normal and pathological sleepiness (Johns 1991). The accuracy of the ESS depends on the awareness of subjects falling asleep and this may not always be the

case (Reyner and Horne 1998). Rating of the subject's sleepiness by a significant other may be more precise (Kingshott et al 1995). The ESS does not strongly correlate with more objective measures of daytime sleepiness such as the MSLT or MWT (Benbadis et al 1999; Johns 2000) but this is in keeping with the fact that sleepiness is not a unitary concept. The ESS reproducibly reflects changes in sleepiness with therapy in OSAHS (see Patel et al 2003 for review). Because of its reliability and its ability to differentiate between abnormal and normal levels of sleepiness as well as for its ease of administration, the ESS is the measure of sleepiness used in this thesis.

1.7 Treatment of OSAHS

A number of modalities have been employed in the treatment of OSAHS, each with inherent limitations. Some measures are more effective than others in reducing or abolishing OSAHS and are not suited to all individuals. If the primary goal in treatment is considered to be the reduction of the AHI to 5 events per hour or less, then continuous positive airway pressure (CPAP) is the most effective form (Lojander et al 1996; Wright and White 2000). However, it is obtrusive and often poorly tolerated (see Engleman and Wild 2003 for review). Surgery of the upper airway and face (e.g. uvulopalatopharyngoplasty, maxillo-mandibular advancement osteotomy, hyoid myotomy with suspension, laser-assisted uvulopalatoplasty), can improve OSAHS dramatically but is relevant in only a minority of patients. Results are not often sustained in the long-term and use of other devices subsequently is more difficult. Oral appliances are useful in many patients but are generally not

sufficiently effective in the moderate or severe categories of OSAHS (Wright and White 2000). Pharmacotherapy has no major role (Smith et al 2002).

1.7.1 Conservative measures

These measures, generally addressing lifestyle modification, are probably the simplest, cheapest and least effective method for the long-term treatment of OSAHS. They are also the most frequently recommended in a clinical practice setting and include advice re: weight loss; exercise; sleep hygiene; avoidance of smoking and alcohol and soporific medications.

Weight loss is recommended on the basis that it should decompress the upper airway and promote its patency, especially in the context of worsening of OSAHS with weight gain. Uncontrolled studies have suggested that it may be effective (Lojander 1998; Noseda 1996; Smith 1985). Exercise is recommended as an adjunct to weight loss and also to alter sleep structure.

Improvement in sleep hygiene includes measures to improve the sleep environment, avoiding stimulants before bedtime, avoiding daytime naps etc. Few studies have been conducted examining sleep hygiene and its effect on sleep apnoea (Redline et al 1998) but sleep deprivation has been shown to increase upper airway collapsibility (Series 1994). Avoiding of excessive alcohol in the evenings has been shown to be important (Issa 1982; Scrima 1982; Mitler et al 1988). Smokers have a four-to five-fold higher risk in having at least moderate OSA (Wetter et al 1994). Sleep position has also been looked at in terms of modifying the degree of OSA experienced (Isono et al 2002).

However, there are no randomised controlled trials looking at the effects of these lifestyle modifications in the context of OSAHS (Shneerson and Wright 2001) and thereby a lack of evidence as to their true and sustained effectiveness. Nevertheless, as they are non-invasive they can continue to be recommended.

1.7.2 Continuous positive airways pressure (CPAP)

Nasal CPAP therapy was first described in 1981 by Sullivan et al. and has since become the 'gold standard' for the treatment of OSAHS. CPAP acts as a pneumatic splint, preventing collapse of the pharyngeal airway. It acts by elevating the pressure in the oropharyngeal airway and reversing the transmural pressure gradient across the pharyngeal airway. One important factor, which determines the effectiveness of the system, is that the apparatus providing the pressure at the nasal airway must have the capacitance to maintain any given pressure during inspiration. Generally, CPAP is commenced under supervision in a hospital-based setting. The optimal pressure (cmH₂O) is titrated to reduce the AHI to less than five ideally. Once the correct CPAP level is reached and the airway is open, sleep should no longer be fragmented by repetitive arousals. It is well known that the response to CPAP therapy in patients treated in this fashion is generally very good in terms of the amelioration of daytime sleepiness and impaired daytime function – however, there are few controlled studies that show this. A recent review of randomised placebo controlled trials included only twelve such studies on the basis of methodological limitation in the others (White et al 2002) but concluded nevertheless that CPAP (when compared to placebo) resulted in significant improvements in objective and subjective sleepiness, measures of oxygenation and mean arterial blood pressure as well as improvements in health

status and the mental health role. A more extensive review by the NHMRC in Australia (2000) came to the same conclusions.

CPAP is not without its problems and side-effects which include: nasal congestion, nasal dryness, epistaxis, skin abrasions, conjunctivitis from air leak, chest discomfort, aerophagy, sinus discomfort, noise levels, partner intolerance and inconvenience. All these factors may affect compliance and usage – an issue discussed in detail in the reviews by Engleman and Wild (2003), Douglas and Engleman (1998), McArdle et al (2000) and Hoy et al (1999).

The newer forms of intelligent CPAP, which are also in use, are based on flow limitation detection with a few in use that are based on the principles of the forced oscillation technique for the measurement of complex resistances in the airway (DuBois et al 1956). They enable treatment pressure to be adjusted to the actual requirement to keep the upper airway patent. Two objectives are thus possible with these devices: the determination of the pressure level for fixed CPAP home treatment (autotitrating system) or the replacing fixed CPAP by automatically varying pressure during the entire treatment period (autoadjusting system). The latter is hoped to reduce pressure-associated side effects, which may compromise patient compliance. A recent randomised crossover trial comparing APAP with CPAP showed that both systems were equally effective in treating sleep apnoea (Randerath et al 2001).

1.7.3 Oral Appliances

There are two forms of oral appliance: those, which hold the tongue forward, and the much commoner group which reposition the mandible and the tongue in a forward position during sleep. Lowe (2000) has produced a full review of the types of oral

appliances available on the market and their comparative effectiveness. In 1995, the American Sleep disorders Association (ASDA) reviewed the available literature on oral appliances and recommended that they be used in the treatment of simple snoring or mild sleep apnoea, being reserved for the treatment of moderate or severe sleep apnoea only in instances where CPAP therapy had failed or was declined by the patient. Unfortunately, poor study design in a number of trials looking at the effectiveness of oral appliances in comparison to CPAP or placebo alone has made their role in day-to-day clinical practice difficult to evaluate. The first paper to be published using the randomised, controlled crossover design of mandibular advancement splint vs. placebo suggested that it is an effective treatment in some patients with OSA, including those patients with moderate or severe OSA (Mehta et al 2001). The results of further trials are currently awaited.

1.7.4 Surgery

Surgery for sleep apnoea is a complex and controversial field and encompasses procedures carried out by surgeons attached to the subspecialties of Otolaryngology, Maxillofacial surgery and specialised surgery of the gastrointestinal system (bariatric surgery). There are no randomised controlled trials assessing the effectiveness in the long-term of these methods and often they are best applied on a situational basis and adapted to the patient's anatomy, e.g. if the patient has severe ankylosis of the temporomandibular joint, has macroglossia or disabling prognathism. Some surgeons hold the tracheostomy to be the 'gold standard' of treatment of OSAHS, as it conveniently bypasses the upper respiratory tract where the problem resides. However, this solution comes with its fair share of comorbidities, including loss of

natural speech to start with! The details of the various surgical techniques will not be discussed further in this thesis. The reader is referred to recent comprehensive reviews on the subject by Sher (2002) and Riley et al (2000). Likewise, bariatric surgery which may be extremely effective in the management of morbid obesity (and OSAHS concurrently) (Sugerman 2001; Scheuller and Weider 2001). The techniques involved have recently been reviewed by Gentileschi et al (2002) who conclude that randomised controlled trials comparing the various laparoscopic operations are strongly needed.

1.7.5 Pharmacotherapy

Drug therapy has been proposed as an alternative in the treatment of OSAHS. The imputed mechanisms include: reduction in the proportion of rapid eye movement sleep (REM), an increase in ventilatory drive and an increase in upper airway muscle tone during sleep. There is little evidence currently for any of the drugs that have been trialled in the management of OSAHS to be effective (Smith et al 2002). The most commonly described potential drugs for OSAHS treatment have been progestogens, shown to increase ventilatory drive (Cistulli et al 1994), acetazolamide and theophylline, both known to increase ventilatory drive. Tricyclic antidepressants and selective serotonin re-uptake inhibitors (SSRI's) reduce the proportion of REM sleep and theoretically the RDI (sleep disordered breathing is more common during REM). Clonidine has a similar action and has been proposed as a treatment for mild to moderate sleep apnoea (Hudgel 1995). Pathways involving serotonin and histamine have been shown to play an important role in modulating drive in the innervation of the pharyngeal musculature (for detailed discussion see Veasey 2002).

For this reason, ondansetron has also been trialled in the animal model of OSAHS, the English bulldog (Veasey et al 1999; 2001).

Treatment of residual sleepiness with a central nervous system stimulant, modafinil, in patients on CPAP has not shown to abolish sleepiness entirely (Kingshott et al 2001) and so this approach towards the management of this important component of OSAHS is also fraught with difficulty at present.

1.8 The OSAHS phenotype

Despite the increasing and already established recognition of OSAHS as an important syndrome with significant health implications for the individual as well as society, a precise characterisation of a particular phenotype is difficult.

In view of the multiplicity of conditions in which OSAHS may be manifest, there is no one specific human morphology that is typical such as is the case of e.g. Duchenne's muscular dystrophy or Marfan's syndrome. OSAHS remains a condition that must be classified on a physiological basis and on objective or subjective evaluation of sleepiness manifest as a daytime symptom. In view of this, the American Academy of Sleep Medicine Task Force (1999) recently defined OSAHS in the following way:

1. Diagnostic criteria. The individual must fulfil criterion A or B, plus criterion C.
 - A. Excessive daytime sleepiness that is not better explained by other factors;
 - B. Two or more of the following that are not better explained by other factors:
 - choking or gasping during sleep,
 - recurrent awakenings from sleep,
 - unrefreshing sleep,

- daytime fatigue,
 - impaired concentration; and/or
- C. Overnight monitoring demonstrated five or more obstructed breathing events per hour during sleep. These events may include any combination of obstructive apnoeas/hypopnoeas or respiratory effort related arousals, as defined below.

Obstructive apnoea/hypopnoea event

An event characterised by a transient reduction in, or complete cessation of, breathing. The event must fulfil criterion 1 or 2, plus criterion 3 of the following:

1. A clear decrease (>50%) from baseline in the amplitude of a valid measure of breathing during sleep. Baseline is defined as the mean amplitude of stable breathing and oxygenation in the two minutes preceding onset of the event (in individuals who have a stable breathing pattern during sleep) or the mean amplitude of the three largest breaths in the two minutes preceding onset of the event (in individuals without a stable breathing pattern).
2. A clear amplitude reduction of a validated measure of breathing during sleep that does not reach the above criterion but is associated with either an oxygen desaturation of $\geq 3\%$ or an arousal.
3. The event lasts 10 seconds or longer.

Respiratory effort-related arousal (RERA) event

A sequence of breaths characterised by increasing respiratory effort leading to an arousal from sleep, but which does not meet criteria for an apnoea or hypopnoea.

These events must fulfil both of the following criteria:

1. Pattern of progressively more negative oesophageal pressure, terminated by a sudden change in pressure to a less negative level and an arousal.

2. The event lasts 10 seconds or longer.

The American Academy of Sleep Medicine Task Force (1999) has also included definitions of severity of OSAHS, which is based on two components: severity of daytime sleepiness and of overnight monitoring of breathing. They suggest that the severity for the syndrome be based on the most severe component, having rated sleepiness and breathing events separately as below:

A. Sleepiness

1. Mild. Unwanted sleepiness or involuntary sleep episodes occur during activities that require little attention.
2. Moderate. Unwanted sleepiness or involuntary sleep episodes occur during activities that require some attention.
3. Severe. Unwanted sleepiness or involuntary sleep episodes occur during activities that require more active attention.

B. Sleep related obstructive breathing events.

1. Mild: 5 – 15 events per hour
2. Moderate: 15 – 30 events per hour
3. Severe: greater than 30 events per hour.

Although this constitutes a good general working definition of the disorder and can be applied satisfactorily in a research setting, it is pragmatic rather than soundly evidence based and does not take into account age-related or gender-related changes in sleepiness and sleep-disordered breathing. There are very few normative data for either in the population and the results obtained are highly dependent on the technology used to measure breathing during sleep or sleepiness. Table 1, 2, and 3

summarise the age-and gender-related frequencies of apnoeas and hypopnoeas from the most reliable and extensive studies available. The scoring definitions are listed under each Table in detail and appear to be equivalent. The study by Bixler et al (1998) may slightly overestimate the number of sleep disordered breathing events as they do not use arousals as part of their definition for a hypopnoea.

Table 1: Age and gender specific prevalence rates of the apnoea and hypopnoea index based on polysomnographic results for a sample of 1,050 men and 1,098 women from the Vitoria-Gasteiz region (Basque country, Spain)

| <u>Percentage of Subjects (95% confidence interval)</u> | | | | | |
|--|--------------|--------------|--------------|-------------|------------|
| AHI: | ≥5 | ≥10 | ≥15 | ≥20 | ≥30 |
| <u>Men</u> | | | | | |
| (age/yr) | | | | | |
| 30 – 39 | 9.0 (2 – 16) | 7.6 (0 – 15) | 2.7 (1-5) | 2.1 (0-4) | 2.1 (0-4) |
| 40 – 49 | 25.6 (14-37) | 18.2 (9-27) | 15.5 (7-24) | 10.1 (5-15) | 7.0 (3-11) |
| 50 – 59 | 27.9 (17-38) | 24.1 (15-34) | 19.4 (11-27) | 14.7 (8-21) | 11.4(6-17) |
| 60 – 70 | 52.1 (33-71) | 32.2 (17-48) | 24.2 (12-37) | 15.0 (8-22) | 8.6 (4-14) |
| <u>Women</u> | | | | | |
| (age/yr) | | | | | |
| 30 – 39 | 3.4 (0-7) | 1.7 (0-4) | 0.9 (0-2) | - | - |
| 40 – 49 | 14.5 (3-25) | 9.7 (0-19) | - | - | - |
| 50 – 59 | 35.0 (20-50) | 16.2 (5-27) | 8.6 (1-17) | 8.3 (0-16) | 4.3 (0-10) |
| 60 – 70 | 46.9 (31-63) | 25.6 (13-38) | 15.9 (6-26) | 13.0 (3-22) | 5.9 (0-13) |

(Based on Table 2, pg. 687 in Duran et al., 2001)

Technique for data collection: Overnight use of the portable recording system MESAM IV (Medizintechnik für Arzt und Patient, Munich, Germany). Polysomnography recorded using Alice 3 (Respironics Inc. Pittsburgh, OH).

Scoring: Manual scoring using conventional criteria. An abnormal breathing event was defined as complete cessation of airflow for ≥10 secs (apnoea) or a discernible 50% reduction in respiratory airflow accompanied by a decrease of ≥ 4% in SpO₂ and/or and EEG arousal (hypopnoea). Arousals were defined according to ASDA criteria (1992).

Table 2: Age and gender specific prevalence rates of the apnoea and hypopnoea index based on polysomnographic results for a sample of 352 men and 250 women from Wisconsin (United States of America)

| Percentage of Subjects (95% confidence interval) | | | |
|---|-----------------|----------------|---------------|
| AHI: | ≥5 | ≥10 | ≥15 |
| Men | | | |
| (age/yr) | | | |
| 30 – 39 | 17.0 (9.6 – 25) | 12.0 (5.4-19) | 6.2 (1.9-10) |
| 40 – 49 | 25.0 (18-32) | 18.0 (11-24) | 11.0 (6.7-16) |
| 50 – 60 | 31.0 (21-40) | 14.0 (7.5-20) | 9.1 (5.1-13) |
| Women | | | |
| (age/yr) | | | |
| 30 – 39 | 6.5 (1.4-11) | 4.9 (0.6-9.8) | 4.4 (1.1-7.3) |
| 40 – 49 | 8.7 (4.2-13) | 4.9 (1.7-8.1) | 3.7 (1.0-6.5) |
| 50 – 60 | 16.0 (5.2-26) | 5.9 (0.0-12.0) | 4.0 (0.0-10) |

(Based on Table 4., pg. 1230 in Young et al., 1993)

Technique for data collection: Overnight in-lab PSG recording in sound-attenuated, light-and temperature-controlled rooms using standard set-up and a 16-channel polygraph (Model 78d; Grass Instrument, Quincy, MA)

Scoring: Manual scoring using conventional criteria. An abnormal breathing event was defined as complete cessation of airflow for ≥10 secs (apnoea) or a discernible 50% reduction in respiratory airflow accompanied by a decrease of ≥ 4% in SpO₂.

Table 3: Prevalence rates of the apnoea and hypopnoea index by age based on polysomnographic results for a sample of 741 men from two counties in Southern Pennsylvania (United States of America)

| Percentage of Subjects (95% confidence interval) | | | | |
|---|----------|------------------|------------------|------------------|
| AHI: | n | ≥5 | ≥10 | ≥20 |
| AGE (yr) | | | | |
| 20-44 | 236 | 7.9 (5.0-12.1) | 3.2 (1.6-6.4) | 1.7 (0.6-4.4) |
| 45 – 64 | 430 | 19.7 (16.2-23.7) | 11.8 (9.1-15.3) | 23.9 (15.7-34.9) |
| 65-100 | 75 | 30.5 (21.1-41.7) | 23.9 (15.7-34.9) | 13.3 (7.3-23) |

(Based on Table 1., pg. 145 in Bixler et al., 1998)

Technique for data collection: Overnight in-lab PSG recording in sound-attenuated, light-and temperature-controlled rooms using standard set-up and a 16-channel polygraph (Model 78d; Grass Instrument, Quincy, MA)

Scoring: Manual scoring using conventional criteria. An abnormal breathing event was defined as complete cessation of airflow for ≥10 secs (apnoea) or a discernible 50% reduction in respiratory airflow accompanied by a decrease of ≥ 4% in SpO₂.

A similar dilemma exists with regard to measures of sleepiness (see 1.6.2 above). Probably the most widely used and best-validated instrument clinically is the Epworth Sleepiness Score designed by Johns in 1991 (see Appendix 1). This 8 point scale has been found useful internationally in populations as diverse as the Chinese (Chung 2000), the Spanish (Chiner et al 1999) and the Germans (Bloch et al 1999). Normative data for a general population are sparse. Nevertheless, there is sufficient evidence to suggest that an ESS in the range of 0 – 11 is within normal limits and an ESS of > 11 generally indicative of abnormal levels of daytime sleepiness irrespective of age (Parkes et al 1998; Manni et al 1999; Johns & Hocking 1997). Table 4 and 5 summarise the extant data on the ESS looking at normal populations by age group. The study by Carmelli et al (2001) looked at the heritability of the ESS in 818 monozygotic and 742 dizygotic male twin pairs and estimated it to be 38% (CI 95% 33 – 44%).

Table 4: Mean Epworth Sleepiness Score in a Normal or Control Population according to Age and Region

| N subjects | Age (yr) | ESS | Region | Reference |
|------------|-------------|-----------------|-------------|----------------------|
| | Mean (+SD) | Mean (+SD) | | |
| 30 | 75 \pm 6 | 6.93 \pm 3.54 | Australia | Crowley&Colrain 2000 |
| 30 | 20 \pm 3 | 7.23 \pm 2.96 | Australia | Crowley&Colrain 2000 |
| 188 | 49 \pm 16 | 4.5 \pm 3.3 | U.K. | Parkes et al 1998 |
| 70 | 47 \pm 13 | 10 \pm 5 | Spain | Chiner et al 1999 |
| 54 | 52 \pm 10 | 4.4 \pm 2.8 | Italy | Manni et al 1999 |
| 61 | 35 \pm 9 | 7.5 \pm 3 | Hong Kong | Chung 2000 |
| 1560 | 74 \pm 3 | 7.1 \pm 3.9 | U.S.A. | Carmelli et al 2001 |
| 72 | 22-59 | 4.6 \pm 2.8 | Australia | Johns & Hocking 1997 |
| 159 | 35 \pm 13 | 5.7 \pm 3 | Switzerland | Bloch et al 1999 |

Table 5: Mean Epworth Sleepiness Score in a Population of Drivers by Age

| Age Group (years) | Number of drivers* | ESS |
|--------------------------|---------------------------|------------|
| 17 – 24 | 582 | 5.7 |
| 25 – 34 | 648 | 6.1 |
| 35 – 44 | 735 | 6.0 |
| 45 – 54 | 841 | 6.6 |
| 55 – 64 | 850 | 6.5 |
| 65 and over | 905 | 6.2 |

* Number of drivers in whom ESS was recorded in general is ca 9% smaller than number quoted
(Based on Table 3., pg 457, Maycock 1997)

On the basis of the evidence presented above, the definitions for sleepiness, sleep-disordered breathing and OSAHS which will be applied throughout this thesis and which will define the sleep apnoea phenotype are as follows:

1. Sleepiness. This is defined as an ESS of ≥ 11 .
2. Sleep-disordered breathing. This will be defined as the frequency of apnoeas and hypopnoeas detected on overnight PSG in an age-specific context as follows:

Table 6: Classification of AHI according to Gender and Age

| Category | Age (years) | Apnoea/hypopnoea index/hour | |
|-----------------|--------------------|------------------------------------|-----------|
| Normal | 20 – 40 | M < 10 | F < 10 |
| | 40 – 60 | M < 15 | F < 10 |
| | > 60 | M < 20 | F < 15 |
| Indeterminate | 20 – 40 | M 10 – 15 | F 10 - 15 |
| | 40 – 60 | M 15 – 20 | F 10 - 15 |
| | > 60 | M 20 – 30 | F 15 - 20 |
| Abnormal | 20 – 40 | M > 15 | F > 15 |
| | 40 – 60 | M > 20 | F > 15 |
| | > 60 | M > 30 | F > 20 |

3. OSAHS. The obstructive sleep apnoea/hypopnoea **syndrome** will be defined if there is abnormal breathing during sleep **and** an abnormal level of daytime sleepiness as defined above. Those falling into the indeterminate category in respect to the AHI will also be classified as having OSAHS if they have an abnormal level of daytime sleepiness.

The **OSAHS phenotype** in this thesis will thus be clearly demarcated on the basis of criteria outlined above for the age-specific apnoea/hypopnoea index together with abnormal daytime somnolence. **Irregular breathing during sleep alone** will be defined on the basis of the AHI alone. **Sleepiness alone** will be defined on the basis of an abnormal ESS alone. Although this is a conservative definition of OSAHS overall, it allows for most individuals' data to be informative.

1.9 Concluding remarks

As outlined above, OSAHS is an important condition within our community with the potential of being a significant health burden. OSAHS is associated with a number of comorbidities and may contribute to the development of hypertension and other cardiovascular and cerebrovascular diseases. It is also a condition, which is a public health concern with respect to road safety and a small but important cause of further morbidity and mortality in this context. OSAHS is easily diagnosed and readily, although not always easily, treated.

The definitions for the phenotype of OSAHS to be used for further work presented in this thesis have been discussed in detail in section 1.8.

In Chapter 2, the familial nature of OSAHS and the potential candidate genes to be evaluated in this study will be described.

CHAPTER 2

CANDIDATE GENES IN OSAHS

2.0 Introductory Remarks

A number of studies have shown that OSAHS runs in families (discussed below) and that this can occur independently of obesity. Mechanisms contributing to the aetiology of OSAHS include genetically and environmentally induced changes in craniofacial dimensions, differential deposition of adipose tissue, abnormalities in upper airway control and differential susceptibility to sleepiness. All of these potential co-aetiologies have come under increasing scrutiny on a genetic level. With the completion of the Human Genome Project and the establishment of a single nucleotide polymorphism (SNP) gene map, enormous progress has been made in clarifying the genetic causes of phenotypic differences in the human population.

This chapter will focus on the hereditary aspects of OSAHS, discuss its polygenic nature and consider candidate genes in relation to its various aetiologies. The aims of this thesis are also outlined.

2.1 Is the Obstructive Sleep Apnoea/Hypopnoea Syndrome Hereditary?

Over a number of years, evidence has accumulated that there is a hereditary component to OSAHS. Redline and Tishler (2001) in a review on the genetic aspects of OSAHS have suggested that hereditary factors invoke 40% of the variance in the occurrence of OSAHS in the population, the rest attributable to obesity, upper airway control dysfunction and craniofacial components.

Initially, case reports were suggestive of a familial link for OSAHS. Strohl et al (1978) described 2 males and their father with severe hypersomnolence and obstructive sleep apnoea. A third, asymptomatic son was shown to have increased

upper airway resistance during sleep. The electromyographic recordings of the genioglossus muscle in this family showed loss of tonic activity when sleep apnoea occurred. The asymptomatic son showed loss of tonic activity in REM-sleep only. Two deaths were also present in the family, one in a four-month-old daughter of the asymptomatic son presumed to be secondary to SIDS and a brother (2nd generation of the family) who died aged 30 years in his sleep. The family members were hypersomnolent. Other case reports of a similar nature were published by Rostand (1978) who documented a man with OSAHS who had a son with OSAHS as well as a brother and Manon-Espaillet et al (1988) who described a family of sleep apnoeics who also suffered from anosmia, partial complex seizures and daltonism. This latter disorder was termed the 'sleep apnoea plus' syndrome.

The above case reports are not representative of the population at large, where 2 – 4% of middle-aged people have OSAHS. Douglas et al (1993) performed a prospective study of first-degree relatives of 20 consecutive non-obese patients with OSAHS. In studying a total of 40 relatives, they found that 10 of them had more than 15 apnoeas and hypopnoeas per hour of sleep and 8 had more than five 4% desaturations per hour. Teculescu et al (1994) suggested a familial basis for OSAHS. A study by Mathur and Douglas (1995) looked at, among other factors, cephalometry of relatives of patients with OSAHS compared to normal controls. The findings suggested that the relatives had more backset maxillae and mandibles than age and sex-matched controls.

Ferini-Strambi et al (1995) looked at snoring in 492 monozygotic and 284 dizygotic twins and found there was greater concordance for snoring among the former with obesity playing a more significant role in the latter.

Guilleminault et al (1995) conducted a mail survey of first-degree relatives of 157 patients with OSAHS and their friends who were unrelated to the patients and approximately the same age as the relatives. Despite limitation in method of this study, there was a suggestion that the relatives and index cases were distinguishable from their friends by snoring more and possessing slightly different craniofacial characteristics (numbers were 22 relatives vs. 6 friends).

Familial aggregation and segregation analysis of snoring and symptoms of OSAHS was performed by Holberg et al (2000) who suggested that for their sleep apnoea phenotype (applied to 584 pedigrees with 2019 cases enrolled in the Tucson Epidemiologic Study of Obstructive Airways disease) demonstrated Mendelian dominant or co-dominant transmission. However their analyses also suggested that a non-genetic model would fit the data equally well. They concluded by stating that their analysis showed that environmental factors contribute to the development of OSAHS and that maternal components may be more important than paternal ones. Ovchinsky et al (2002) who examined over 115 related caretakers of children with OSAHS suggested too that there may be a familial basis to the syndrome.

The results of family studies are summarised in Table 7 below.

Table 7: Prevalence of Sleep Disordered Breathing among First-degree Relatives of Proband with OSAHS (After: Table 1, pg 238, Redline et al 2002)

| Proband:Relative (n:n ratio) | Controls (n) | Phenotype | % relatives OSAHS | OR | Reference |
|---|-------------------------|--|------------------------------|-----------|--------------------------|
| 45:108 | 0 | AHI> 20 in-lab PSG | 33 | - | Pillar et al 1995 |
| 20:40 | 0 | AHI>15 in-lab PSG sleepy | 25 | - | Douglas et al 1995 |
| 0:51 | 51 | AHI > 15 in-lab PSG sleepy | 45 | 20 | Mathur & Douglas 1995 |
| 157:166 | 69 | AHI > 5 home PSG EDS >1 symptom | 84 | 46 | Guilleminault et al 1995 |
| | | AHI > 5 home PSG EDS abnormal hard palate | 68 | 11 | |
| 47:219 | 223 | AHI > age-specific threshold, home PSG | 21 | 2 | Redline et al 1995 |
| | | AHI >15 sleepiness | 13 | 5 | |

Buxbaum et al (2002) performed a segregation analysis on a sample of 177 Caucasian families and 125 African American families. With the use of very complex statistical techniques they demonstrated transmission of a putative candidate gene for OSAHS (typified solely by AHI) that was variable depending on whether

the mathematical model was age-or BMI-adjusted for the Caucasians and independent of BMI for the African Americans.

Unfortunately, this study is flawed on a number of counts. Not least are the lack of proper phenotyping and the use of AHI alone to characterise subjects. The OSAHS is a syndrome which therefore requires symptoms to be present. Thus, AHI alone is inadequate as a definition of the syndrome *per se*. Furthermore, AHI is variable within subjects on a nightly basis and subject to recording and scoring error and therefore, is not an immutable characteristic that substitutes for phenotype. There may be racial differences in the presentation of OSAHS. However, it is often taken for granted that the human species is divided into homogeneous groups or races among which biological differences are large (Barbujani et al 1997). Studies of allele frequencies **do not** support this view, as differences between members of the same population account for 85% of the total diversity. Differences among continents represent roughly 1/10 of human molecular diversity, which does not suggest that racial subdivision of our species reflects any major discontinuity in our genome (Barbujani et al 1997).

The postulated observation by Buxbaum et al (2001) is also not borne out by previous studies showing that BMI is just as important in the aetiology of OSAHS in African Americans as it is in American Caucasians (Redline et al 1997).

In summary, a number of studies over the last 25 years have suggested a familial component to OSAHS, which may influence the diverse aetiopathologies associated with its development including obesity, craniofacial structure and dysregulated upper airway function. What is clear from these studies is that OSAHS is not a monogenic disorder.

2.2 Craniofacial Morphology

The craniofacial complex is probably one of the most important heritable determinants of OSAHS development. The three components considered in this context are bone size; head shape and facial profile.

Retroposed maxillae and mandibles predispose to OSAHS (Douglas & Mathur 1995; Ferguson et al 1997). Such differences in jaw size can be inherited or acquired – for example, following nasal occlusion in childhood (Trask et al 1987; Linder-Aronson 1970; Harvold 1979, 1981). There are also a number of syndromes such as the Carpenter Syndrome and Apert's syndrome, which are associated with craniofacial anomalies leading to OSAHS (Gaultier & Guilleminault 2001). Here the genetic locus has been identified but the confounder remains the multitude of associated anomalies, which characterise these syndromes (they will not be discussed further). In order to discuss the genetic aspects of craniofacial characteristics, a brief overview of development is undertaken in Section 2.2.1 below.

2.2.1 Craniofacial Development

The facial primordia in humans appear around the primitive mouth (stomodeum) early in the fourth week of foetal development and are classified as follows:

1. The frontonasal prominence formed by the proliferation of mesenchyme ventral to the forebrain, constitutes the cranial boundary of the stomodeum.
2. The paired maxillary prominences of the first branchial arch form the lateral boundaries of the stomodeum.
3. The paired mandibular prominences of this arch form the caudal boundary of the stomodeum.

The mesoderm of the five facial primordia is continuous from one prominence to the other. There are no internal divisions corresponding to the grooves demarcating the prominences externally. Facial development occurs mainly between the fifth and eighth weeks. The mandible is the first part to form and results from the merging of the medial ends of the two mandibular prominences during the fourth week. Nasal placodes, bilateral oval-shaped thickenings of the surface ectoderm, develop by the end of the fourth week on each side of the lower part of the frontonasal prominence. Mesenchyme proliferates at the margin of each placode, giving rise to the medial and lateral nasal prominences. The nasal placodes come to lie in nasal pits.

The maxillary prominences enlarge and rapidly approximate each other as well as the medial nasal prominences. The nasolacrimal groove separates each lateral nasal prominence from its corresponding maxillary prominence. By the end of week five, the eyes have come slightly forward on the face and the external ear has begun to develop. Each maxillary prominence merges with the lateral nasal prominence by the end of week five and continuity is established between the side of the nose formed by the lateral nasal prominence and the upper cheek region formed by the maxillary prominence.

During the sixth and seventh weeks, the medial nasal prominences merge with each other and the maxillary prominences forming the intermaxillary segment which gives rise to a. the philtrum of the upper lip, b. the premaxillary part of the maxilla and associated gingiva and c. the primary palate.

The maxillary prominences merge with the mandibular prominences and form the lateral parts of the upper lip, the majority of the maxilla and the secondary palate.

The mandibular prominences give rise to the lower lip, the chin and lower cheek region. The various bony structures are also formed from these prominences.

The frontonasal prominence forms the forehead and the dorsum and the apex of the nose.

Development of the face is slow and results from changes in proportion and relative position of the facial components. During early foetal life, the nose is flattened and the mandible is underdeveloped. As the brain enlarges, creating a prominent forehead, the eyes move medially and external ears rise. The face at birth is small on account of rudimentary upper and lower jaws, unerupted teeth and the small size of the nasal cavities and maxillary sinuses.

An integral part of facial development is palatal genesis. The primary palate develops at the end of week five from the innermost part of the intermaxillary segment of the maxilla which comprises mesoderm. The primary palate becomes the premaxillary part of the maxilla, containing the incisor teeth. The secondary palate forms the hard and soft palates extending from the incisive foramen posteriorly. It develops from two horizontal mesodermal projections that extend from the internal aspects of the maxillary prominences, the lateral palatine processes. These latter processes project downward initially to flank the tongue on each side. With development of the jaws and neck, the tongue moves downwards and the lateral palatine processes elongate and move horizontally and superiorly to the tongue during the seventh week. The lateral palatine processes fuse with each other in the midline and also with the primary palate and the nasal septum commencing anteriorly in the 9th week with completion by the 12th week posteriorly in the region of the uvula. Bone develops in the premaxillary part of the maxilla which carries the incisor teeth, then extending

from the maxillae and palatine bones into the lateral palatine processes to form the hard palate. The posterior portions of the lateral palatine processes extending beyond the nasal septum remain as the soft palate and uvula.

Many genes are involved in the progressive development of the craniofacial complex. A hierarchy of control genes is activated in sequence, which specifies how the cells in a domain should develop. These controls are influenced by local feedback and intercommunication mechanisms between cells and tissues. The effect of other genes on contiguous tissue will also influence expression of the gene of interest with resultant effects on each other. This is an epigenetic phenomenon and may account for the variability seen in one particular tissue, which cannot be solely ascribed to a single gene polymorphism as would otherwise be postulated.

Thus, the craniofacial complex is comprised of a number of components which are interdependent in their growth patterns and which are so closely linked, that the growth and shape of one component will influence the rest. Generally, size influences shape and is probably the element that is most influenced by genes.

Genes identified through animal studies (mouse-mutants), human craniofacial syndromes and expression studies of signalling molecules during facial development have led to the identification of a variety of factors involved in the development of the jaw. Fig.3 summarises the putative genes involved in facial development and Table 8 summarises factors active in the developing face. For a more detailed discussion of this information, see Francis-West et al (1998); Mina (2001) and Thesleff (1998).

Fig 3: Processes involved in facial development (After: Fig.2, pg7, Francis-West et al 1998)

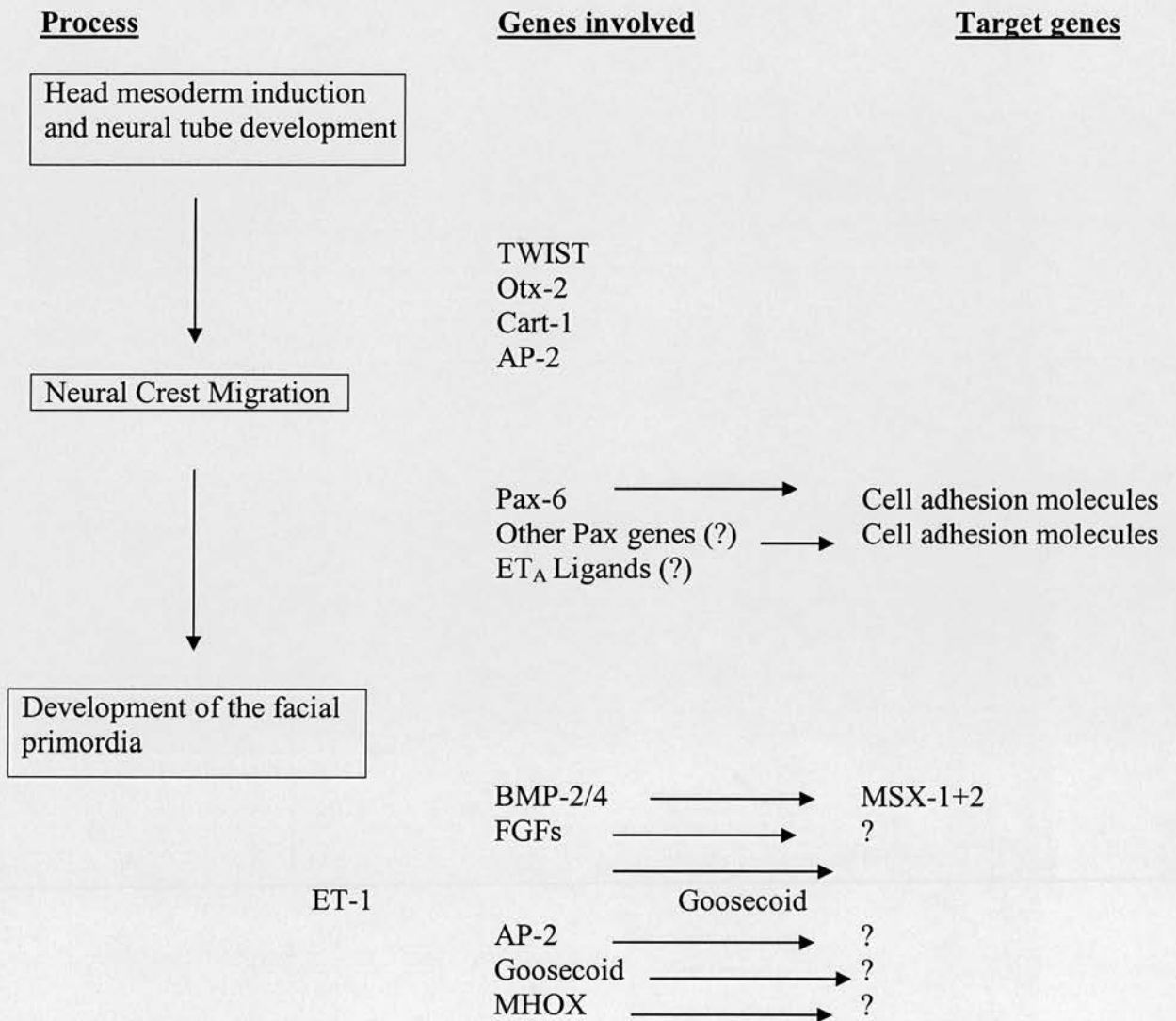


Table 8: Signalling and transcription factors involved in the developing face (not exhaustive) (After: Table 1, pg 6, Francis-West et al. 1998)

| | Signalling factor | Homeobox gene | Transcription Factors |
|-------------------------------|---|---|---|
| Migrating neural crest | Wnt-5a | En protein Dlx-1*, 2* Msx-1*‡, 2 ‡ Otx-2* Pax-3*‡, 7* | AP-2* Gli-2*, 3* twist *‡ |
| Facial Primordia | Activin β a* BMP – 2,4*,5*,7* EGF ET-1* FGF1,2,4,5,8,12† PDGF- α Jagged 1‡, 2* Shh*‡ TGF α TGF β 1,2*,3* Wnt-5a, 10a, 10b, 11 | Barx-1, 2 Dlx-1-6(1*,2*) En-2 GH-6 Gsc-1* MHOX* MSX-1*‡, 2‡ Otx-2* Pax-3*‡,7*,6*‡ Ptx-1 Reig‡ S8 HOX 11 Uncx-4.1 | AP-2* Gli-1,2*, 3* Pax-1* twist *‡ |

- * Mutation or loss of function by knockout studies in mice results in some facial abnormalities.
‡ Human syndrome associated with mutation of this gene.
† Several craniofacial syndromes are associated with the FGF receptor mutation.

The precise role played by each gene, signalling factor and transcriptional factor is as yet imperfectly understood. Known polymorphisms in genes regulating craniofacial development are linked to syndromes and generally inherited on an autosomal dominant basis and so are of little value in the context of the relatively mild abnormalities observed in the sleep apnoea population.

Of possibly greater interest is the regulation of facial growth and postnatal development of the maxilla and mandible as a potential future target for therapeutic intervention.

2.2.2 Postnatal Facial Growth

Growth of the craniofacial complex after delivery is dependent not only on genetic factors, but is also subject to a wide variety of environmental influences which may be related to the microenvironment of the face itself and its component structures but also to more global influences such as malnutrition.

Bone forms in two basic modes, endochondral and intramembranous and its growth is a mixture of two basic processes – deposition and resorption. Growth of the cranial vault is paced largely by growth of the enclosed brain. Basicranial growth is effected by a combination of synchondrosial elongation, sutural growth and cortical drift and remodelling. Nasomaxillary growth involves the sutures, endosteal and periosteal surfaces and alveolar processes. Alveolar remodelling influences height, width and length increases that correlate with tooth eruption. Mandibular growth occurs endochondrally with respect to length and also intramembranously with regard to width. Important determinants of growth and shape change are the areas where muscle and teeth are attached. The mandibular condyle is the chief determinant of growth in this region. Mandibular growth coincides roughly with periods of stature growth and may show coincident growth acceleration. There are important sexual differences in overall craniofacial growth with boys growing more, over a longer time and more likely to demonstrate growth spurts. Racial and ethnic differences in craniofacial structure are also well documented, but little is known about growth differences between groups of people of different descent.

The continued growth of the craniofacial skeleton through adulthood is an important concept that was initially best demonstrated by Behrents (1985). In performing an extensive follow-up of 163 dentate subjects in the Bolton-Brush study who had

ranged in age between 17 and 83 years he systematically documented the following observations (among others):

1. Craniofacial growth continues throughout adult life into old age
2. Significant sexual dimorphism exists with men being larger at all ages with more growth
3. Women demonstrated periods of increased growth beyond adolescence often associated with pregnancy
4. Mandibular orientation and occlusal relations were subject to growth and change throughout the life cycle.

Growth of the craniofacial complex can be affected by a number of environmental mechanisms. These include: deleterious orofacial muscle habits such as thumb-sucking and abnormal tongue posturing; nasopharyngeal disease and disturbed respiratory function which may produce mouth-breathing; oral/gingival tumours; dental caries with loss of teeth; loss of permanent teeth; malnutrition and endocrinopathy.

As a consequence of postnatal growth, head shape and facial profile assume certain characteristics that can be classified broadly as dolicephalic, mesocephalic and bradycephalic with ethnic associations towards the extremes. For instance, Caucasians are more likely to be dolicephalic and Asians more bradycephalic. There are three types of facial profile: retrognathic, orthognathic and prognathic (see Fig.4). The position of the cranial base (determined by the brain during embryogenesis) has a significant impact on the position of the maxillo-mandibular complex. In a dolicephalic head, the cranial base is flat. Consequently, the naso-maxillary complex

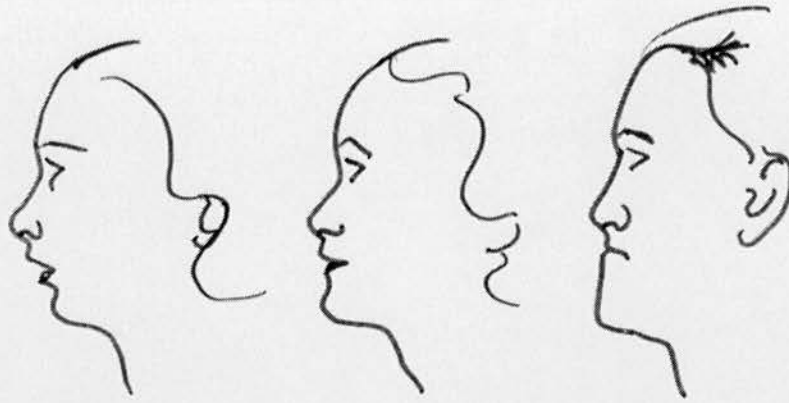


Fig 4: Facial Profiles (from left to right): retrognathic (skeletal class II); orthognathic (skeletal class I) and prognathic (skeletal class III)

is placed in a more protrusive position relative to the mandible and is lower than the mandibular condyle. There is therefore a tendency towards retrognathia. By contrast, in the bradycephalic head, the horizontal dimension of the middle cranial fossa is decreased with posterior placement of the maxilla. There is shortening of the horizontal length of the naso-maxillary complex and more forward placement of the entire mandible. This results in a prognathic profile. Head shape and size are determined genetically and it is likely that compensatory mechanisms that prevent the two extremes are also at work such as drift of maxillary and mandibular teeth and environmental factors including allergies, infections, otitis media, diet as discussed above. A partial failure in these compensatory mechanisms may be responsible for the observance of certain anatomic features noted more frequently in the OSAHS population, most notably lower facial height and overbite (retrusion of the mandible) (Pae and Ferguson 1999; Douglas and Mathur 1995) as well as bradycephaly (Cakirer et al 2001). Mandibular size and position seem to play the greatest role in determining facial alignment and predisposition to sleep related breathing disorders.

In summary, morpho-spatial disharmony of the craniomaxillary and mandibular complexes is dependent on genes that undergo gene-environmental interactions. The craniofacial skeleton continues to grow and develop throughout adult life and structural modifications can occur. Genes primarily involved in postnatal growth are discussed below.

2.2.3 Determinants of Skeletal Growth

Genes controlling final adult height and stature may also influence craniofacial growth. Studies using genome-wide linkage analysis have so far failed to confirm with certainty the potential positions of candidate genes for adult height. The first study in this area by Hirschhorn et al (2001) examined 4 different populations: 408 individuals from 58 families in Botnia, Finland; 753 individuals from 183 families in other parts of Finland; 746 individuals in 179 families in Southern Sweden and 420 individuals in 63 families from the Saguenay-Lac-St.-Jean region of Quebec. Linkage to stature was found on chromosomes 6, 7 and 13 in three of these ethnic groups, but not so convincingly in the Quebecois. These results were strengthened further by the study of Perola et al (2001) who found evidence of linkage to chromosome 7 and Wiltshire et al (2002) whose own data on a UK population suggested a locus on chromosome 3 to be strongly associated with height in their adult sample. These findings suggest that height is under the influence/control of multiple genes.

Studies using genome-wide linkage to look at mandibular structure and size have so far not been undertaken in humans. However, two groups have applied this technique in mice. Klingenberg et al (2001) utilised the method of geometrical morphometrics to quantify shape and size of the mouse mandible and were able to identify 12QTLs

(quantitative trait loci) for size, 25QTLs for shape and 5QTLs for left-right asymmetry in the F2 mice resulting from an inter-cross of Large (LG/J) and Small (SM/J) inbred mice from the Jackson Laboratory (Bar Harbor, ME). Using a different strain of mouse, the SMXA recombinant inbred mouse, Dohmoto et al (2002) found QTLs on chromosomes 10 and 11 influencing mandibular length.

Although genome-wide scans have the advantage of identifying genetic factors that do not require *a priori* knowledge of the underlying biology of a condition or risk alleles, there are significant drawbacks especially in the case of common diseases, or complex traits (see Section 2.9 below for discussion). At present it is more profitable to concentrate on SNPs in candidate genes identified as potentially important to the development of the craniofacial complex (discussed below). An integration of the two approaches would be ideal but is not feasible in terms of the current study.

Vitamin D Receptor

The vitamin D3 receptors (VDR) are intracellular polypeptides, 50 to 60 kD in size that bind 1, 25 (OH) 2D3 and interact with target-cell nuclei to effect a variety of biological actions. The human VDR gene is located on chromosome 12q12-q14, contains 11 exons and spans approximately 75 kb. The 5-prime end of the VDR gene is non-coding and includes exons 1A, 1B and 1C, whilst its translated product is encoded by exons 2 – 9. Polymorphisms in the VDR gene have been associated with a number of conditions, largely related to metabolism of vitamin D, e.g. vitamin-D resistant rickets, synthesis of osteocalcin, bone mineral density, osteoporosis, osteoarthritis, primary hyperparathyroidism and psoriasis. The importance of polymorphisms in VDR lies in its influence on the effectiveness of 1, 25 (OH) D3 in

regulating the differentiation or proliferation of osteoblastic, osteoclastic and chondrocytic lineage thereby affecting bone mineralization and linear bone growth.

The importance of the VDR gene in the development of the craniofacial complex can be inferred from a small number of studies examining the effects of vitamin D on mandibular condylar cartilage (Kyung et al 1992; Silberman et al 1987) and craniofacial chondrocytes (Takano-Yamamoto et al 1992) where it was found integral to the growth and differentiation process. *In vitro* effects of sex hormones/receptors on VDR gene function or modulation of vitamin D have not been investigated.

A number of studies have now looked at the influence of VDR gene polymorphisms on growth and height. Suarez et al (1997) studied the association between VDR genotype and growth in 589 healthy infants who were homogeneous for age, diet and vitamin D status. The *BsmI* polymorphism (825-bp in the intron separating exons 8 and 9) was significantly associated with differences in body size, which were found to be sex-specific. In a subsequent study, Suarez et al (1998) showed that the interaction between oestradiol receptor gene polymorphisms and the *BsmI* polymorphism in the VDR gene lead to significant variation in body growth during infancy, especially in boys.

A study by Lorentzon et al (2000) in 90 post-pubertal Caucasian males showed the *BsmI* polymorphism to be significantly related to final height (homozygotes being shorter) and to contribute to 8% of total variation in final height, taking environmental and parental influences into account.

Minamitani et al (1998) examined a different polymorphism in the VDR gene (exon 2 A (T/C) G substitution affecting a putative translation initiation site) in 90 healthy

female Japanese aged between 18 and 20 years, 159 Japanese 13 year olds and 24 children with constitutional short stature aged 6 – 10 years. The CC phenotype was found to be significantly associated with shorter stature in this heterogeneous population.

Beta-2-adrenergic receptor

The beta-2-adrenergic receptor (ADRB2) gene is located on chromosome 5q32-q34. The receptor is comprised of 413 amino acid residues encoding a protein containing 7 clusters of hydrophobic amino acids suggestive of membrane-spanning domains. The gene contains no introns in either the coding or untranslated sequences (Kobilka et al 1987). There are nine different point mutations in the β 2AR coding block. Four of these variants changed and encoded amino acid (Reihnsaus et al 1993; Liggett 1997). These latter four alter receptor function (Liggett 1997).

Apart from studies in asthma, the ADRB2 gene has been of interest in elucidating the mechanism of energy expenditure regulation and more specifically its role in obesity. In the context of growth, one study has been conducted examining the impact of ADRB2 polymorphisms on changes in weight and height in children. Matsuoka et al (2002) found that among 40 Japanese children of short stature treated with growth hormone, those with the Gly16Gly genotype remained shorter and more obese over a period of 4 years. These findings remain to be reproduced in other populations, but by inference would correspond to the observations in other studies looking at long-term weight gain where BMI (requiring both height and weight for the calculation) has remained higher in those with the Gly16Gly mutation (see e.g. Ellsworth et al 2002).

Growth Hormone

Growth hormone (GH) has a major role in the regulation of postnatal growth and it is generally accepted that it is the most important hormone in this respect (Ohlsson et al 1998). GH is a protein that contains 191 amino acids with 2 disulfide bonds and 4 α -helices. Its molecular mass is approximately 22,000 Da (Kopchick et al 2002). GH is located on chromosome 17q22-24. Growth hormone insensitivity which when complete is known as Laron dwarfism (Laron et al 1966) is characterised by small overall stature, classical craniofacial symmetrical reduction in size, constriction of the upper airway, obesity, elevated GH levels and insulin resistance. Recently the first report of a patient with Laron syndrome and OSAHS was published (Dagan et al 2001). GH is important in the regulation of carbohydrate and lipid metabolism (Nam and Marcus 2000) and is integral to the regulation of insulin at both a pre and post receptor level (Dominici and Turyn 2002 for review). In the obese adult and adolescent, GH levels are low (Attia et al) but growth continues, largely due to the effects of IGF-1 and low IGFBP-1. Recently it has been shown that the direct effects of leptin, sex hormones and insulin can directly activate the IGF system in growth plates and at bone growth centres (Philip et al 2002) independently of GH although GH can influence longitudinal bone growth directly without the mediation of IGF-1 (Ohlsson et al 1998). Current evidence available suggests that GH stimulates longitudinal bone growth directly by stimulating prechondrocytes in the growth plate followed by a clonal expansion caused both by the GH-induced local production of IGF-1 and the GH-induced increase in circulating levels of IGF-1. GH appears thus to be the major determinant for the stimulation of progenitor cells, although it is possible that IGF-1 might stimulate progenitor cells to some extent (see Ohlsson et al

1998 for detailed review). Thus, investigation into the other components of the growth hormone system may be equally productive in examining direct influences on bony development.

Insulin – like Growth Factor (IGF-1)

Growth promoting factors in serum were first identified in the 1950's (Salmon & Daughaday 1957) and their purification, demonstration of their biological properties and characterisation of their amino acid sequences revealed structural and functional similarity to insulin. Hence the term insulin-like growth factor (IGF) was introduced.

The IGF system comprises two ligands (IGF-1 and IGF-II), two receptors (IGF1R and IGF2R) and several IGF binding proteins. They have both paracrine and autocrine modes of action. Single genes encode IGFs: 12q22-q24.1 for IGF-1 and 11p15 for IGF-II. IGF-1 has 70 amino acid residues and 3 disulphide bridges and was first synthesised in 1983 using the solid-phase method (Li et al 1983). IGF-1 is continually synthesised and released as a single polypeptide chain primarily by the liver, but also by many other organs and tissues. IGFs stimulate glucose transport and metabolism as well as the differentiation of myoblasts, osteoblasts, neural cells, fibroblasts etc. Woods et al (1996) noted that there is no direct evidence that IGF1 has a prominent role in human foetal growth. Nevertheless, on the basis of experiments using IGF1 knockout mice, there is evidence that IGF1 is necessary for axonal growth and myelination with a high neonatal mortality in the knockouts.

IGF level and function is not only determined by the particular gene polymorphism, but also by GH level (major regulator), nutrition, adequate insulin secretion and thyroid function (Froesch et al 1985; Le Roith et al 1999). IGF gene expression is for the most part independent of GH in the foetus, but after birth, GH increasingly

begins to regulate expression and release of IGF-1 and is the primary regulator in the adult (Baker et al 1993; Ohlsson et al 1998).

Gene targeting strategies, which involve the alteration or knockout of critical regions of any gene, have been used to explore the contribution of single genes to the development of the entire organism. Two studies have looked in detail at the consequences of introducing mutations at the IGF-1 locus in mouse germ lines and constructing chimaeric animals.

Baker et al (1993) showed that IGF-1 (-/-) mutants grew at a slower rate and weighed 30% of the normal adult weight of their wild-type littermates. The IGF-1 null mutant mice were disproportionately and significantly smaller with a small head size and blunt nose. A more recent study by McAlarney & Rizos et al (2001) showed that IGF-1 null mutant mice had a 43 – 64% decrease in craniofacial size compared to wild-type and a non-allometric change of shape with significant changes in the facial and cranial areas. The mandible, although shorter, did not exhibit any shape changes. Further, Powell-Braxton et al (1993) showed that heterozygous IGF-1 null mutant mice were 10-20% smaller than wild-type litter mates – with a decrease in organ, muscle and bone mass.

Other evidence of IGF-1 influencing the growth of the mandibular condyle has come from the work of Maor et al (1993) in mice. Using immunofluorescence, they showed that the distribution of IGF-1 receptors in the chondroprogenitor and chondroblast cell layers of the mandibular condyle was parallel to that of IGF-1 production. They also showed intense staining with antibodies to IGF-1 in the mandibular condyle of 2-day-old mice. Immuno-inhibition of IGF-1 resulted in an almost complete inhibition of thymidine incorporation into DNA and marked

degenerative changes in the morphological appearance of the condyle. This supported the role of paracrine activity of endogenous GH-independent IGF-1 in early postnatal mandibular development. The importance of IGF-1 and its receptor in mandibular growth has also been demonstrated in experiments looking at the role of sex steroids on growth, e.g. testosterone (Maor et al 1999) which has been shown to promote growth through the mediation of IGF-1. The interdependence of GH and IGF-1 in stimulating chondrocyte growth has also been demonstrated many times e.g. by Smith et al (1989) who showed that GH alone had no effect on chondrocyte DNA and proteoglycan synthesis compared to IGF-1 alone and the two in combination resulted in the highest levels of adult chondrocyte extracellular matrix synthesis (see discussion above).

In summary, IGF-1 has been shown to be an important and independent regulator of maxillofacial and mandibular growth postnatally (see also McCarthy & Centrella 2001 for detailed discussion re: local IGF-1 expression and bone formation).

Deficiencies in IGF-1 may arise as a result of GH receptor/postreceptor abnormalities, IGF-1 receptor abnormalities or secondary to GH deficiency. Primary IGF-1 deficiency due to partial IGF-1 gene deletion is rare in humans and results in a syndrome characterised by short stature, sensorineural deafness and mental retardation (Woods et al 1996 and 2000; Camacho-Huebner et al 1999). Investigation into polymorphisms in the IGF-1 gene has been unhelpful in linking them to the phenotype of short stature, such as that of African pygmies (Bowcock and Sartorelli 1990) vs non-Pygmy black Africans. Direct sequencing of PCR-amplified DNA failed to demonstrate an alteration in the region upstream of the IGF-1 start site in Pygmies. A further study by Rasmussen et al (2000) considered the IGF-1 and IGF-

1R genes as candidates for low birth weight, insulin resistance and type II diabetes in a Danish population. They found no mutations predicting changes in the amino acid sequences of the IGF-1 or IGF-1R genes. Thus, at present it may be speculative to search for a particular gene polymorphism of IGF-1, which may be specifically linked to mandibular growth and maxillo-facial size. Since the effects of IGF-1 and GH are mediated by their receptors, it is integral to their understanding to examine these components involved in the growth pathway.

Insulin-like Growth Factor Receptor

The gene for IGF-1R is located at 15q25-q26. The complete primary structure of the receptor was first determined in 1986 by Ullrich et al and Abbott et al (1992) determined that the IGF-1R gene contains 21 exons and spans about 100kb. The gene sequence predicts a 1,367 amino acid receptor precursor with a 30-residue signal peptide, which is removed during translocation of the nascent polypeptide chain resulting in alpha and beta subunits. There is further cleavage after the removal of the signal peptide to generate the two subunits of the IGF-1R.

Gene function studies have demonstrated that mutation in the IGF-1R gene interferes with suppression of GH in cultured rat pituitary cells (Prager et al 1992). IGF-1R plays a pivotal role in transformation events in malignant tissues where it appears to function as an anti-apoptotic agent, thus enhancing cell survival (Kaaks and Lukanova 2001; Werner and Le Roith 1996; Maor et al 2000). Monozygosity for the IGF-1R results in severe abnormalities associated with intrauterine growth retardation, microcephaly, micrognathia, renal anomalies, lung hypoplasia and delayed growth and development Roback et al (1991). The study by Rasmussen et al (2000) looking at polymorphisms in the IGF1R gene has been discussed above.

One study so far has looked at hormonal control of condylar cartilage (Visnapuu et al 2001). In this study, the distribution of GH and IGF-1 receptors in the temporomandibular joint was investigated. GH receptors were detected in various components of the temporomandibular joint but not the fibrous articular surface or in the cartilage layers of the condyle where the IGF-1R was found. They concluded that early post-natal growth and development of the mandibular condylar cartilage seems to be IGF-1-dependent and not directly dependent on GH. Apart from this experimental evidence, no associations have been found for the remaining polymorphisms for the IGF-1R gene.

Growth Hormone Receptor (GHR)

GH binds the transmembrane receptor GHR that subsequently dimerizes and activates an intracellular signal transduction pathway leading to synthesis and secretion of IGF-1. After IGF-1 binds to the soluble IGF-1R in plasma, the resultant complex activates signal –transduction pathways resulting in the mitogenic and anabolic responses leading to growth. The gene for GHR is located on chromosome 5p13-p12. The gene has 9 exons encoding the receptor and several additional exons in the 5-prime untranslated region. The coding exons span at least 87kb (Godowski et al 1989). The molecular genetics of GHR has been chiefly concerned with mutations resulting in Laron dwarfism and idiopathic short stature (see Gastier et al 2000 and Woods et al 1997 for detailed discussion).

The functional role of GHR in the growth of the craniofacial unit has been discussed above in the context of IGF-1R. The function of all GHR variations is not characterised. A recent study by Yamaguchi et al (2001) aimed to quantitatively evaluate the relationship between craniofacial morphology and the Pro561Thr

(P56IT) variant in the GHR gene in a normal Japanese population. They showed that subjects without P56IT had a significantly greater mandibular ramus length as measured cephalometrically compared to those with P56IT. They postulated that the GHR gene P56IT variant may be associated with mandibular height growth and may be a genetic marker for it.

2.2.4 Summary of Craniofacial Factors

In summary, development of the craniofacial structures is complex and regulated by a large number of genes. Postnatal growth is also influenced by a large number of genetic and environmental processes which include regulation by the hormones GH, IGF-1 and their respective receptors. As yet, no specific variants in the genes of these hormones and their receptors have been linked to the development of the craniofacial unit in isolation from other somatic growth. However, a putative association of the P56IT GHR variant with mandibular size has been identified and deserves replication to establish the strength of the association in other populations.

2.3 Obesity

Obesity is the most commonly identified risk factor for OSAHS (Kushida et al 1997; Hoffstein & Szalai 1993). The mechanism whereby obesity may contribute to the development or expression of OSAHS appears to be due to reduction in nasopharyngeal calibre secondary to fat deposition or as a result of hypoventilation due to a decrease in chest wall compliance. Twin studies have shown that up to 70% of the variance in obesity within a population may be attributable to genetic factors (Stunkard et al 1990; Bodurtha et al 1990). Adoption studies, by contrast have

generated the lowest heritability estimates in the order of 30% with family studies showing a level of heritability intermediate between the latter and twin studies (Ravussin & Bouchard 2000). Bouchard et al (1997) included all series of relatives in the same analysis and concluded that the heritability estimate for BMI in large sample sizes was between 25% and 40%.

The susceptibility to becoming obese therefore seems likely to be determined significantly by genetic factors, but a favourable ‘obesogenic’ environment is necessary for phenotypic expression (Ravussin and Bouchard, 2000).

Obesity is defined in Table 9 below. Increasingly, it is becoming a global epidemic and is associated with a large number of comorbidities as well as having a strong negative impact on quality of life for the individual (Ravussin and Bouchard, 2000).

Table 9: WHO classification of weight in adults according to BMI (WHO 1997)

| Classification | BMI (kg/m²) | Risk of comorbidities* |
|-----------------------|-------------------------------|-------------------------------|
| Underweight | < 18.5 | Low – risk of other problems |
| Normal | 18.5 – 24.9 | Average |
| Overweight | 25 – 29.9 | Increased |
| Obese class I | 30.0 – 34.9 | Moderate |
| Obese class II | 35.0 – 39.9 | Severe |
| Obese class III | > 40.0 | Very severe |

**Increased risk of metabolic complications at waist circumference ≥ 94 cm in men and ≥ 80 cm in women. Substantially increased risk at waist circumference ≥ 102 cm in men and ≥ 88 cm in women (see National Institutes of Health guidelines 1998).*

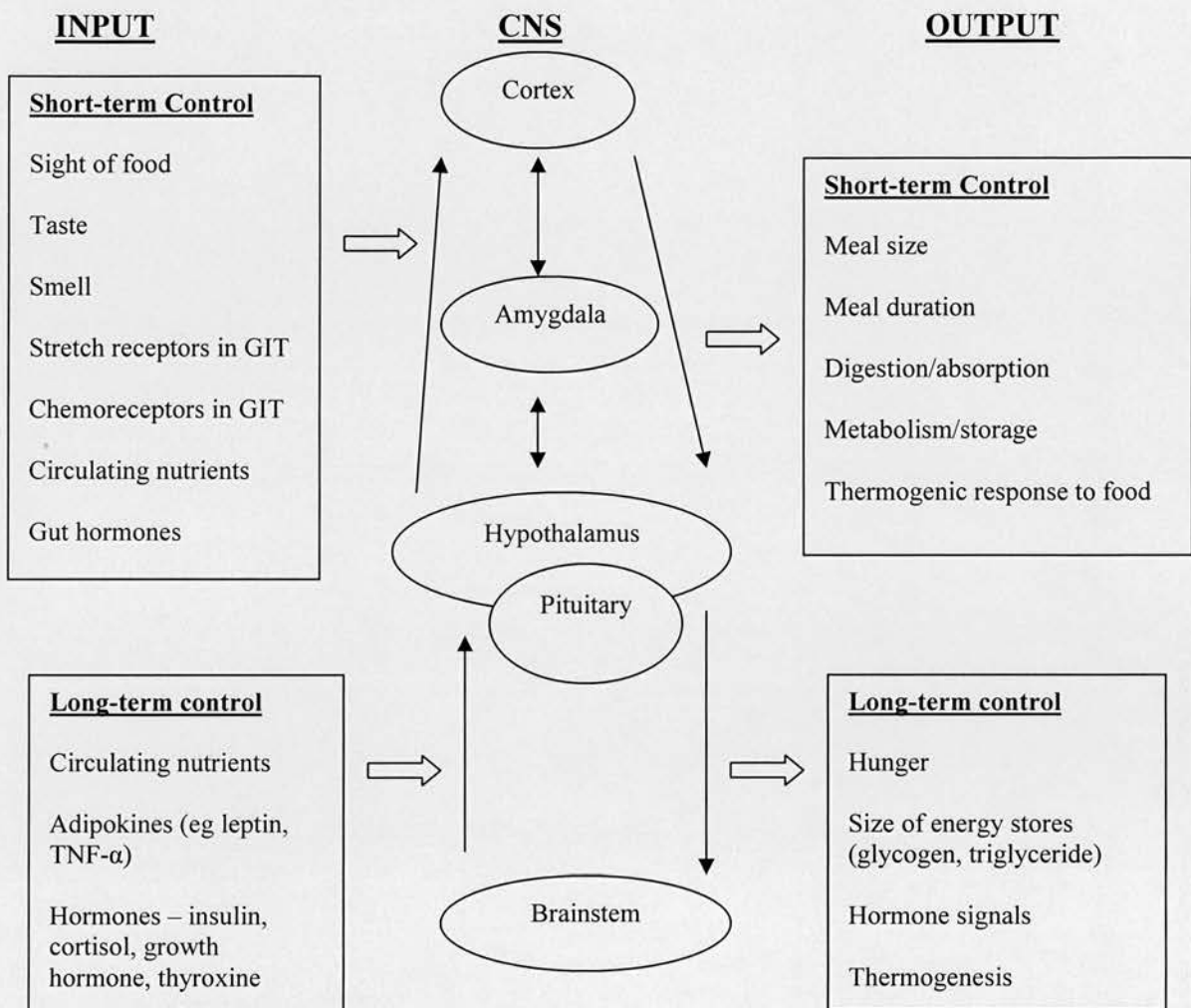
The regulation of energy expenditure comprises an extremely complex system with a large number of redundant pathways biased towards weight gain. Energy homeostasis is not only a function of lifestyle and environmental influences but has an important genetic component. Obesity develops when energy intake exceeds energy expenditure over time and there are a large number of short and long-term regulators of appetite and energy intake. The system is extremely complex and

outside the scope of this thesis. However, the major factors influencing energy balance are summarised in Fig 5 below and the chief neurotransmitters involved in energy expenditure are listed in Table 10.

Table 10: Neurotransmitters that influence energy expenditure and appetite (after Wilding 2002; *Diabetic Medicine* 19:621)

| <i>Neurotransmitters that increase intake</i> | <i>Neurotransmitters that decrease intake</i> |
|---|---|
| Agouti-related peptide | α - melanocyte-stimulating hormone |
| Neuropeptide Y | Bombesin/gastrin-releasing peptide |
| Melanin concentrating hormone | Calcitonin-gene related peptide |
| Orexin | Cholecystokinin |
| Galanin | Corticotrophin-releasing factor |
| Ghrelin | Glucagon |
| Nitric oxide | Glucagon-like peptide |
| Noradrenaline | Neurotensin |
| Opioids | Serotonin |

Fig 5: Short and long-term factors influencing energy homeostasis
(after Wilding 2002; *Diabetic Medicine* 19:621)



GIT = gastrointestinal tract

The search for obesity susceptibility genes has snowballed since the 1990s with numerous study designs being employed to identify genes of interest such as family linkage studies and association studies in affected vs. non-affected individuals (Chagnon et al 2000; Barsh et al 2000).

The information accumulated so far is so extensive, that it is currently published in updated form on an annual basis as *The Human Obesity Gene Map* (see Chagnon et al 2003 for most recent version) and is now available as a website

(<http://obesitygene.pbrc.edu>). This most current update (the ninth so far), incorporates published results on single-gene mutation obesity cases, Mendelian disorders exhibiting obesity as a feature, quantitative trait loci (QTLs) from human genome-wide scans and animal crossbreeding experiments as well as association and linkage studies with candidate genes and other markers. Transgenic and knockout murine models exhibiting obesity as a phenotype are included (n= 38 studies). Since October 2002, 33 Mendelian syndromes relevant to human obesity have been mapped to a genomic region and the causal or strong candidate genes have been identified for 23 syndromes. QTLs reported from animal models are 168 in number and there are 68 human QTLs for obesity candidate genes in targeted studies. Linkage peaks with candidate genes have been identified in targeted studies with seven genomic regions harbouring QTLs replicated in 2 to 5 studies. There are over 222 studies reporting positive associations with 71 candidate genes with 15 such replicated in at least 5 studies. Putative loci for obesity genes are present on all chromosomes except Y. In total, more than 300 genes, markers and chromosomal regions have been associated or linked with human obesity phenotypes.

Thus, candidate genes for obesity are numerous, but so far only a few single gene mutations causally related to obesity have been convincingly detected in a small number of people, often with other related conditions. These include the leptin receptor gene (Clement et al 1998), the leptin gene (Montague et al 1997; Strobel et al 1998), the pro-opiomelanocortin gene (Krude et al 1998), the prohomone convertase 1 gene (Jackson et al 1997) and the melanocortin MC₄ receptor gene (Cone 2000; Hinney et al 1999; Vaisse et al 1998; Yeo et al 1998). A recent meta-analytic review of the linkage association of the 3 leptin receptor gene

polymorphisms in a total of 3,263 individuals (> 74% Caucasian) showed no statistically significant association with waist circumference or body mass index at the $p = 0.05$ level (Heo et al 2002).

Other genes that have been sequenced and screened in the search for what predisposes to obesity include the agouti gene, the uncoupling proteins (UCP1 – 3), all the melanocortin receptor genes, the neuropeptide Y receptor 1 and 5 genes, TNF- α , peroxisome proliferation-activated receptor-gamma (PPAR- γ) and the β_3 -adrenoceptor genes among many others. As stated above, Mendelian disorders and a large number of linkage studies have also been conducted to date and are presented in the *Human Obesity Gene Map* (Chagnon 2003). It is important to note that none of the genetic associations reported so far has been proven to be the consequence of a mutation affecting the function or amount of a gene product. Many of the studies reporting single gene polymorphisms associations also need to be supported by cellular work identifying the functional consequences of the reported polymorphisms and it would be of greater clinical relevance if the environmental circumstances necessary for the full phenotypic consequence of these genes and their expression were identified.

In OSAHS, obesity constitutes a problem as it has been shown to contribute to the expression of the disease and to disease severity. From a purely clinical perspective, useful information when dealing with OSAHS patients would be knowledge regarding their propensity to lose or gain weight. This may affect the severity of OSAHS and determine choice of treatment modality. The question arises as to which patient will do well with calorie restriction, behaviour modification or potentially pharmacotherapy and which one won't?

There are far fewer studies on candidate genes associated with weight loss or weight gain in certain populations and they are summarised in Table 11 below. At present only a few of these studies have followed up the population under study for prolonged periods of time, but some of the work appears encouraging in terms of replication status in other studies. No work in this area has so far been carried out in the OSAHS population.

From the evidence available, the beta2-adrenoceptor gene (ADBR2) appears most promising. Catecholamines play a central role in energy expenditure both as hormones and as neurotransmitters. This regulation is in part effected by stimulating lipid mobilisation through lipolysis in adipocytes. ADBR2 is a major lipolytic receptor in human fat cells. As described in section 2.2.3 above, the gene for ADBR2 is found on chromosome 5q32-q34. ADRB2 has 6 different polymorphic forms comprising amino acid substitutions, the most common being gly16arg and gln27glu (Reihsaus et al 1993). All are in linkage disequilibrium. The most convincing animal study to date showing that the sympathetic nervous system is indeed the efferent arm of diet-induced thermogenesis is by Bachman et al (2002) who created mice that lacked ADBR1, 2 and 3. Beta-less mice fed a chow diet showed reduced metabolic rate and were slightly obese. On a high-fat diet, in contrast to wild-type mice, they became massively obese. Thus, beta-adrenergic receptors play a critical role in the body's defence against diet-induced obesity. Translating these results to humans, Corbalan et al (2002) showed that Spanish women with the glu27 ADRB2 polymorphism did not benefit equally from physical activity compared to noncarriers of the glu27 allele. They concluded that this could result from a blunting of the sympathetic nervous system activity response to exercise and thereby constitute a

Table 11: Positive association studies of candidate genes for weight loss or weight gain in humans

| Candidate gene | Allele | Population | Association | Reference |
|----------------------------|-------------------------------------|---|--|---------------------------|
| PPAR- γ 2 | Pro12Ala | 522 Finnish men and women | Weight loss with Ala12Ala | Lindi et al 2002 |
| 5-HT2C receptor | -759 C/T | 148 Caucasian women | Weight loss with -759T | Westberg et al 2002 |
| 5-HT1B receptor | G861C | 98 Caucasian women with bulimia nervosa | G/G – high BMI over lifetime | Levitan et al 2001 |
| COMT | Val/Met 158 | 51 Israeli anorexia nervosa women and 102 parents | Val/Val - 2 x risk of anorexia nervosa | Frisch et al 2001 |
| ADRB3 & IRS-1** | Trp64Arg & Gly972Arg | 310 German women | Decreased weight loss with Trp64Arg & Gly972Arg | Benecke et al 2000 |
| ApoA-4 | 360His | 186 Australians | Weight loss with ApoA-4 1/1 variant | Heilbronn et al 2000 |
| UCP# | 1 & 2 | 163 French | Weight loss with 2/2 | Fumeron et al 1996 |
| ADRB3 | Trp64Arg | 61 obese Japanese women with NIDDM [^] | Decreased weight loss with Trp64Arg | Sakane et al 1997 |
| 5-HTR2C | -759 T/C | 32 schizophrenic Chinese Han men | Less weight gain with - 759T on clozapine | Reynolds et al 2003 |
| LEPR ^{^^} | Lys109Arg Gln223Arg Lys656Asn | 536 Dutch men & women | Higher leptin & weight gain in men with Arg223 | Van Rossum et al 2003 |
| ACE*** | I/D | 959 Italian men | Weight gain with D/D | Strazzullo et al 2003 |
| Angiotensinogen | A-6G | 135 Spanish men & women | Weight gain with A/A | Chaves et al 2002 |
| Lipoprotein lipase HindIII | H1/H2 | 12 pairs Caucasian male twins | Weight gain with H2/H2 | Ukkola et al 2002 |
| ADRA2b | 12Glu9 | 210 Finnish NIDDM & non-NIDDM | Weight gain with Glu9/Glu9 | Sivenius et al 2001 |
| ADRB2 | Glu27Gly | 15 obese Spanish women | Lipolysis & fat oxidation blunted in Glu27/Glu27 | Macho-Azcarate et al 2002 |
| ADRB2 | Glu27Gly | 239 obese and non-obese Spanish men | Obesity with Glu27 in men | Corbalan et al 2002 |
| ADRB2 | Arg16Gly | 1151 African-American and Caucasian males and females | Weight gain in men with Gly16 | Ellsworth et al 2002 |
| ADBR2 | Glu27Gln | 12 pairs Caucasian male twins | Weight gain with Gln27Gln | Ukkola et al 2001 |
| ADRB2 | Gly16Arg & Gln27Glu | 419 men and 417 women - French | Weight gain with Arg16 & Gln27Gln in men | Meirhaeghe et al 2000 |

* Catechol-O-methyltransferase; ** Insulin receptor substrate 1; # Uncoupling protein; [^] NIDDM = Type II diabetes mellitus; ^{^^} Leptin receptor; *** Angiotensin-converting enzyme

physical activity-dependent factor for obesity risk. Studies along these lines have also shown similar results for ADBR2 gly27glu polymorphisms in men and women in other Caucasian populations (see Meirhaeghe et al 1999; Ukkola et al 2002; Macho-Azcarate et al 2002 a&b).

2.4 Sleepiness

Epidemiological studies have shown that sleepiness does not necessarily correlate with the severity of sleep apnoea and that there is a differential susceptibility to somnolence between individuals (Cluydts 2002; Moldofsky 1992). Therefore, mechanisms involved in sleep promotion need to be considered as part of the process aimed at elucidating the reasons for the observed differences and as a predisposition to the syndrome of OSAHS.

2.4.1 The Role of Cytokines

Sleep is regulated by neuronal and humoral mechanisms that are interdependent (Krueger et al 1999). Fig 6 below illustrates a multitude of factors that are currently thought integral to promoting sleep. To be considered a sleep regulatory substance, a substance must fulfil certain criteria (see Krueger et al 1999). For NREM sleep, sleep-regulating substances include IL-1, TNF- α , GHRH, PGD₂, adenosine and uridine (Kreuger and Obal 1997; Borbely and Tobler 1989). In REM sleep, the substances include prolactin and vasoactive intestinal peptide (Roky et al 1995). Although cytokines are pleiotropic and there is a great deal of redundancy within the cytokine network, much evidence has been accumulated about their integral role in the generation and promotion of sleep (Opp 2003).

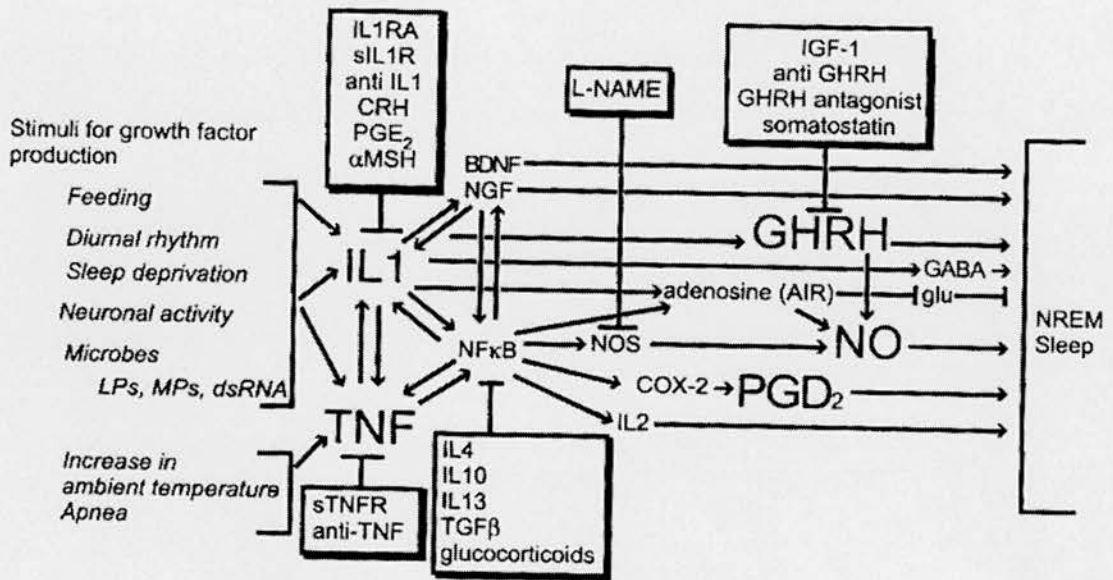


Fig 6: Factors involved in sleep promotion and regulation (After Krueger et al 1999; pg 57)

The mediation of a large number of neurohumoral factors by IL-1 and TNF- α appears to be central to the sleep activation pathway and will be discussed in detail.

Interleukin-1b belongs to the gene family that also includes IL-1a and IL-1 receptor antagonist. All three bind to two IL-1 receptors, the IL-1 type I and type II receptors.

The IL-1 type I receptor complexes with an accessory protein to form the unit for signal transduction, whereas the second type has no signalling role (Opp 2003). The genes for IL-1a and for IL-1b are found on chromosome 2q14. IL-1a is generally cell-associated whilst IL-1b is found in tissue fluids and plasma. Although IL-1a promotes sleep, most investigations into sleep regulation have used IL-1b. IL-1b induces SWS in every species examined so far, including rabbits, rats, cats, monkeys (Opp 2002) and anecdotally, humans (Dinarello 1991), whether administered

intravenously, intraperitoneally or intracranially. IL-1 receptors are located in CNS sleep pathways (Ban et al 1993; Cunningham et al 1993; Takao et al 1990), IL-1 containing neurones are found in the hypothalamus (Breder et al 1988) and IL-1 mRNA may be detected in many brain regions (Van Dam et al 1992; Schobitz et al 1994). IL-1 mRNA expression increases during sleep deprivation (Markiewicz et al 1996) and IL-1 is detected more frequently in plasma samples taken from humans during sleep than during wakefulness (Gudewill et al 1992). Plasma levels of IL-1 β peak in humans at sleep onset (Moldofsky et al 1986) and leukocyte cultures taken from blood samples during sleep produce more IL-1 than those taken during wakefulness (Hohagen et al 1993). Direct evidence for the involvement of IL-1 in sleep regulation comes from studies inhibiting binding of IL-1 to its receptor. This has been achieved either through IL-1 RA administration, IL-1 antibodies or a soluble IL-1 receptor fragment resulting in the reduction of spontaneous SWS in otherwise normal animals (Opp et al 1991; Opp et al 1992; Takahashi et al 1996). When experimental animals are pre-treated with anti-IL-1 or with an IL-1 receptor fragment, sleep deprivation-induced enhancement of SWS is either attenuated or blocked (Opp et al 1994; Opp et al 1994; Takahashi et al 1997).

TNF is also a pleiotropic cytokine that is expressed in normal brain. It exists in two forms – TNF- α , which is primarily produced by macrophages, and TNF- β , which is a product of lymphocytes. The genes for TNF- α and TNF- β map to the 6p23-q12 segment. Both proteins share about 30% amino acid sequence homology and have similar, though not identical modes of biological function (Opp 2002). TNF- β has been shown to promote sleep (Kapas et al 1992), but most sleep studies have used TNF- α . In the brain, TNF- α is produced by astrocytes, and TNF- α immunoreactive

neurones are located throughout the CNS (Breder et al 1993). There are two cell surface TNF receptors – TNF receptor 1 which maps to chromosome 12p13.2 and TNF receptor 2 whose gene is located on chromosome 1p36.3 – p36.2. Both mediate distinct actions (Schutze et al 1994), with TNF receptor 1 responsible for mediating the somnogenic effects of TNF- α (Fang et al 1997). The biological actions of IL-1 and TNF- α overlap and they induce each other's synthesis (Dinarello et al 1986; Philip and Epstein 1986). TNF- α induced SWS response is attenuated in animals pre-treated with a soluble IL-1 receptor and TNF antagonists inhibit somnogenic responses to IL-1 (Takahashi 1996). TNF- α induces SWS in a number of animal species (Opp 2002). TNF- α mRNA (Bredow et al 1997) and protein in brain and plasma fluctuate with circadian rhythm (Gudewill et al 1992; Darko et al 1995). In otherwise normal animals, the use of TNF- α antibodies (Takahashi 1995), binding proteins or soluble receptors or receptor fragments (Takahashi et al 1995) reduces SWS. Mice that lack TNF- α receptor 1 sleep less than wildtype mice do (Fang et al 1997) and pre-treatment of experimental animals with TNF- α antagonists attenuates SWS following periods of sleep deprivation (Takahashi et al 1996) or increases in ambient temperature (Takahashi et al 1997). TNF- α has also been associated with human obesity, although association studies are conflicting in their results (Romeo et al 2001; Herrmann et al 1998; Walston et al 1999; Pausova et al 2000). Other cytokines thought to induce sleep include IL-10, IL-6, interferon; IL-2, IL-4, GM-CSF and FGF (see Opp 2002). Interleukin-6 also has an increasingly important role in the genesis of somnolence (Opp 2002; Kreuger et al 1999; Spath-Schwalbe et al 1998; Redwine et al 2000).

Cytokine profiles that deviate from the normal circadian pattern may alter the normal expression of sleep-wake behaviour and it is possible that levels of sleep-inducing cytokines are increased during the daytime when they should be low in patients with OSAHS and thus may contribute to excessive daytime sleepiness. Two studies have addressed this hypothesis (Entzian et al 1996; Vgontzas et al 1997) and both suggest levels of these cytokines may be different in OSAHS compared to normal subjects. Entzian et al (1996) studied 10 patients with OSAHS compared to 10 normal controls. The patients with OSAHS were obese with an AHI of 48 ± 13 /hr. Blood samples were collected every 4 hours during the day (08:00 – 20:00) and 2-hourly whilst patients were asleep. Cytokine release was determined from whole blood cultures stimulated with LPS. Supernatant from the cultures was assayed for IL-1 β , IL-6, TNF- α and IFN- γ in addition to melatonin and cortisol. Circadian rhythmicity was evident in the production of all the substances. Concentrations and circadian profiles for IL-1 β , IL-6, IFN- γ , melatonin and cortisol were not statistically significantly different between cases and controls. The circadian rhythm of TNF- α production however, was markedly altered in sleep apnoea patients with peak concentrations in the afternoon - the time period during which concentrations in normal controls were at a minimum. Absolute IL-1 β concentrations were twice those obtained from controls although not statistically significantly different. The OSAHS group was then treated with CPAP for three months and the cytokine profiles re-examined. However, they were not normalised despite normalisation of sleep fragmentation, oxygen desaturation and arousal frequency. These findings were suggestive of a biological basis to excessive daytime somnolence not completely accounted for by sleep fragmentation.

Vgontzas et al (1997) measured morning plasma levels of TNF- α , IL-1 β and IL-6 in 12 patients with OSAHS, 11 patients with narcolepsy, 8 patients with idiopathic hypersomnia and 10 normal controls. Single blood samples were drawn between 0600 and 0700 hrs in the morning after completion of the overnight PSG. The cytokines were measured by ELISA. In the OSAHS patients, the TNF- α and IL-6 concentrations were significantly elevated compared to controls and TNF- α was significantly elevated in the narcoleptics compared to controls.

Both these studies are correlational and do not provide an explanation for why the cytokine profiles should be abnormal in OSAHS. Furthermore, cytokine concentrations were measured in the periphery rather than in the CNS and may not reflect the cytokine concentrations in the brain. However, the CNS does respond to peripheral events and there are many mechanisms whereby the brain may be signalled with subsequent effects on sleep (Dantzer 1994).

Differences among patients with OSAHS in terms of excessive daytime somnolence may in part be accounted for by differences in cytokine production, which in turn may be mediated by genetic polymorphisms. In narcolepsy, an examination for TNF- α polymorphisms in the promoter region of the gene has given conflicting results. Some investigators failed to show an association with narcolepsy in a Japanese population (Kato et al 1999) whilst others have demonstrated one (Hohjoh 1999, 2000, 2001). No studies looking at cytokine gene polymorphisms have been conducted thus far in patients with OSAHS.

2.4.2 Hypocretin (Orexin)

Animal studies and most recently human studies have identified that the neuropeptide hypocretin is integral to sleep pathways (see Mignot 2001 for

discussion). Most patients with narcolepsy have undetectable levels of hypocretin in the cerebrospinal fluid (Nishino et al 2000) and a marked decrease in hypocretin immunoreactivity and transcript levels in the perifornical hypothalamus (Peyron et al 2000; Thannickal et al 2000). A rare polymorphisms in the prepro-orexin gene has been associated with narcolepsy (Gencik et al 2001) but further population based studies comparing narcoleptic subjects with controls have shown no association of polymorphisms in the hypocretin gene nor in genes coding for its two receptors – *hcrt1* and *hcrt2* (Olafsdottir et al 2001; Hungs et al 2001). On the basis of these findings, the hypothesis has now been evolved that narcolepsy may be an autoimmune disorder associated with peripubertal destruction of hypocretin-containing cells (Mignot 2001).

The same pathways that cause sleepiness in narcolepsy may potentially be implicated in the induction of sleepiness in the normal population as well as in OSAHS.

However, the pathogenesis and clinical expression of the two disorders is so different that it is unlikely that a gene polymorphism in the hypocretin system is involved. Furthermore, hypocretin is involved also in the pathogenesis of cataplexy which does not occur in OSAHS.

2.5 The Upper Airway in OSAHS

2.5.1 Regulation of Upper Airway Tone

As discussed in Chapter 1, section 1.2, sleep-related reductions in pharyngeal muscle activity are thought integral to the pathogenesis of sleep-related breathing disorders.

A decrement in pharyngeal muscle activity leads to snoring and upper airway obstruction, which in turn leads to arousal from asleep. These arousals in turn

activate the pharyngeal muscles thereby restoring airway patency and more effective breathing.

Pharyngeal motor neurones share a number of common pre-motor inputs. Anatomical and electrophysiological evidence shows that some these pre-motor inputs exhibit sleep-state dependent activity and may be involved in the suppression of pharyngeal activity during sleep (Horner2000). Implicated are both inhibitory and disfacilitatory mechanisms (reduced excitation).

Most work in this area has been done studying the genioglossus muscle as it is considered to be the major upper airway dilator. The genioglossus is innervated by the hypoglossal nerve. The loss of activity of the genioglossus especially during REM-sleep (Sauerland and Harper 1976) is thought to contribute to the onset of airway narrowing and occlusion (Remmers et al 1978) but some controversy remains (Horner 2000).

Hypoglossal nerve output is subject to tonic or phasic pre-motor influences, which effect either slow time-varying or fast transient changes.

Respiratory pre-motor neurones also have important influences on hypoglossal motor neurones and these in turn may be subjected to the influence of sleep mechanisms (Orem 1994).

Reduced excitation of the hypoglossal nerve leading to sleep-related suppression of genioglossus activity has been demonstrated in a number of animal models (Woch et al 200; Yamuy et al 1999), especially in the instance of REM-sleep. The models used have involved the use of carbachol which itself is controversial (Horner 2000; Ursin 2002), but valuable insights have nevertheless been gained.

Fig. 7 below summarizes the current thinking in this area.

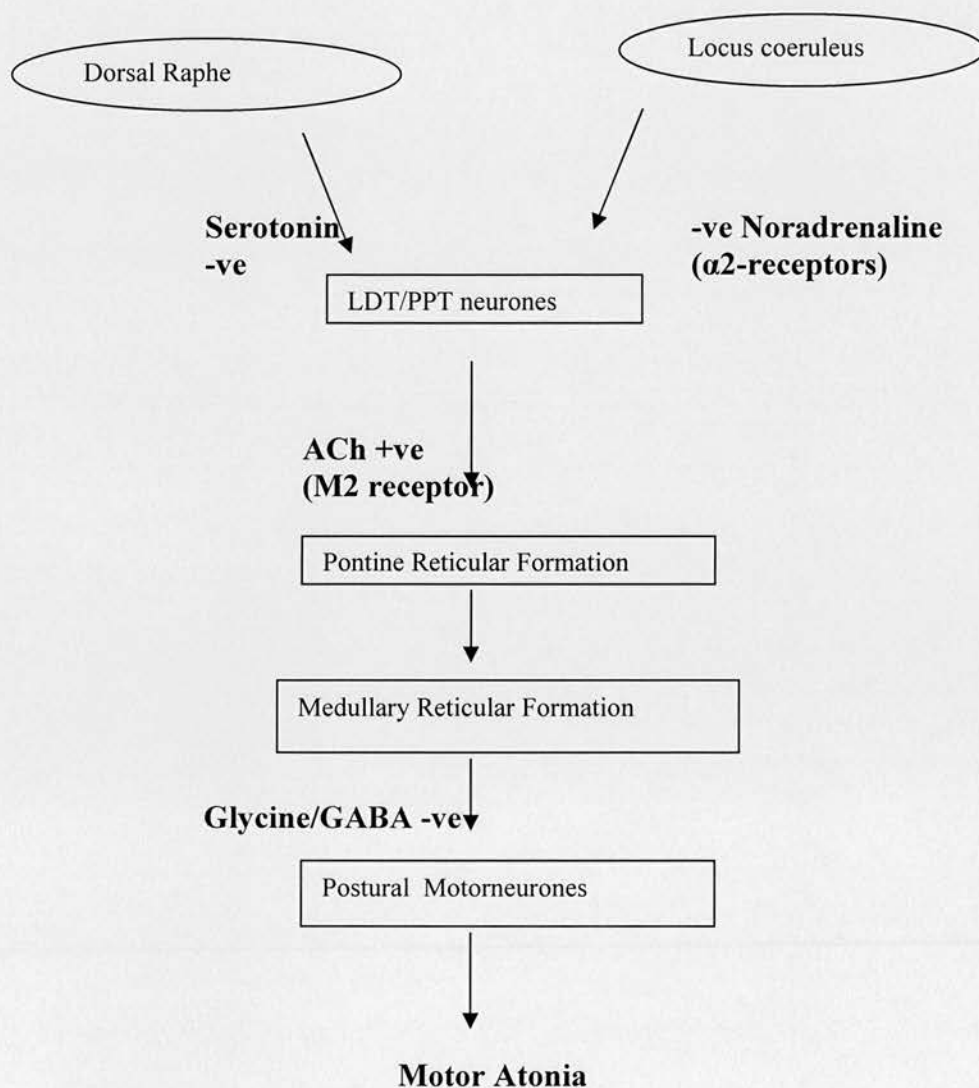


Fig 7: Diagram representing the neuronal circuitry thought to be involved in the pontine regulation of REM sleep and generation of postural motor atonia. (-ve = inhibition; +ve = excitation; M muscarinic)

Most evidence at present supports the hypothesis that non-REM sleep and especially REM sleep are associated with the withdrawal of tonic excitation of the hypoglossal motor neurones via reduced firing of predominantly serotonergic medullar raphe

neurons and less so by noradrenergic locus coeruleus neurones (Foote et al 1983; Parkis et al 1995).

However, depending on the animal model used, contradictory effects are seen when serotonin receptor antagonists are used.

One study using the normally respiring adult rat (Richmonds et al 1996) has shown that use of ritanserin (a 5HT_{2a}, 2c and 7 receptor antagonist) results in increased phrenic and hypoglossal activity whilst administration of the same drug in the English bulldog model of OSAHS causes decreased activity of upper airway dilator muscles with concurrent oxyhaemoglobin desaturation and collapse of the upper airway (Veasey et al 1996).

This may not be entirely a species-specific effect.

In a recent study, Nakano et al (2001) found that ritanserin did not alter ventilation in adult lean Zucker rat but did so in the obese Zucker rats in addition to reducing upper airway dilator activity, increasing collapsibility of the upper airway and increasing oxygen consumption at rest. These findings are similar to those found in the English bulldog model (Veasey et al 1996). This supports one hypothesis regarding serotonin receptor antagonists and their effect on the upper airway being state dependent. Upper airway collapse can occur in models where there is pre-existing compromise, which in turn may reflect 5-HT involvement in the neuronal adaptation mechanisms for narrowed upper airway. For example, long-term facilitation of respiratory motor output after exposure to intermittent hypoxia is 5-HT dependent (Bach et al 1996). Cervical dorsal rhizotomy may further augment the response to 5-HT antagonism (Kinkead et al 1998).

The pharmacology of 5-HT receptors is complex and at least 14 5-HT receptor subtypes are molecularly and pharmacologically distinguishable among mammals.

The 5-HT₃-receptor antagonist, ondansetron, produces very different effects in animal models of OSAHS in comparison to ritanserin. In the English bulldog, ondansetron reduces REM sleep disordered breathing events (Veasey et al) and in the normal rat increases respiratory drive by increasing hypoglossal activity and also reduces central sleep apnoeic events during REM sleep (Fenik et al 2000). This effect in turn is thought to be secondary to peripheral stimulation at the nodose ganglion, rather than directly originating in the nucleus of the hypoglossal nerve (Carley and Radulovacki 1999).

Molecular dissection techniques have most recently shown the 5HT_{2A} receptor to be the predominant receptor subtype in hypoglossal motor neurones (Fonseca et al 2001; Zhan et al 2002). Pharmacologic trials of 5-HT receptor agonists and antagonists support this receptor subtype as well as 5-HT_{2c} (found in much smaller quantities) as the predominant post-synaptic facilitator of hypoglossal motor neurones (McAll & Aghajanian 1980; Berger et al 1992; Kubin et al 1992; Al-Zubaidy et al 1996; Douse and White 1996; Bayliss et al 1997; Inoue et al 1999). A further study on the pharmacological activity of the serotonergic receptors in the hypoglossal nucleus (Fenik and Veasey 2003) showed that the receptor 2A antagonist MDL – 100, 907 dropped intrinsic hypoglossal nerve respiratory activity by 61% and significantly suppressed serotonin excitation of hypoglossal nerve activity in contrast to the 2C receptor.

Attempts have been made to alleviate obstructive apnoeas by neurochemical means in humans (Sunderram et al 1998; Slamowitz et al 1998) using selective serotonin

reuptake inhibitors (Hanzel et al 1991; Kraiczi et al 1999). The results have been mixed, with incomplete responses to the SSRIs despite demonstration of increased genioglossal activity as measured by EMG in the awake state. Part of this may reside in our currently incomplete knowledge of the exact mechanisms regulating pharyngeal motor control during sleep and the role of other upper airway muscle stabilizers and dilators (e.g. geniohyoid and myohyoid).

Nevertheless, in light of the current research in this area, it would be of potential therapeutic and scientific value to explore whether gene polymorphisms in the 5-HT_{2A} receptor may play a role in the modulation of serotonin metabolism potentially affecting upper airway responsiveness.

5HTR2A was first cloned and sequenced by Sparkes et al (1991) who showed it to be located on human chromosome 13q14-q21. Four polymorphisms have been identified – a silent T/C polymorphism at nucleotide 102; a Thr25Asn polymorphism; a -143861A polymorphism and the His452Tyr polymorphism. This latter SNP has functional consequences with the Tyr-allele being associated with smaller peak amplitude in intracellular calcium ion mobilization and different time course of calcium response (Erdmann et al 1996; Ozaki et al 1997). All polymorphisms are in linkage disequilibrium (Erdmann et al 1996). Because serotonin has been implicated in a large range of human disease including depression, obsessive-compulsive disorder, appetite control and schizophrenia, the vast majority of studies examining polymorphisms in the 5HTR2A gene have concentrated on its association with personality and mental illness. For example, Williams et al (1997) reported a meta-analysis of the association between 102T-C polymorphism of the 5HTR2A gene and schizophrenia backed up by Lohmueller et

al (2003). They concluded that the C allele is significantly associated with the disease (first reported by Inayama et al 1996).

No studies to date have examined the role of 5HTR2A polymorphisms in serotonin metabolism in the central nervous system with respect to motor function.

Another very important consideration with the 5HTR2A is the phenomenon of genomic imprinting. Genomic imprinting is an epigenetic process that causes non-allelic gene expression by silencing or activating one of the alleles of an autosomal gene, depending on the parent of origin (Tycko & Ashkenas 2000). Initially, the 5HTR2A gene was thought to be paternally imprinted and transcribed from the maternal allele only (Kato et al 1996). However, a study by Bunzel et al (1998) examined brain tissue in 18 patients and found that 4 samples showed mono-allelic expression, whilst the remaining 14 were bi-allelic for 5HTR2A expression. Thus, 5HTR2A demonstrates polymorphic functional imprinting. This is an important consideration in the context of disease-specific associations and pharmacogenetics.

In conclusion, there is some value in examining polymorphisms in the 5HTR2A receptor. It is important to bear in mind that other neurotransmitters are involved in the regulation of upper airway tone, so focussing on serotonin may lead to only a partial elucidation of the problem – however, it is the first step in considering pharmacological treatments for OSAHS.

2.5.2 Neuropathy in the Upper Airway – its role in OSAHS

OSAHS can progress in its severity and snoring can progress to OSAHS independently of weight gain (Pendlebury et al 1997; Lindberg et al 1999; Svanborg and Larsson 1993; Larsson et al 1991). This observation has led to the hypothesis

that a pharyngeal nerve lesion caused by the trauma of snoring (vibration and stretch) exists and is worsened by the hypoxia caused by hypopnoeas and apnoeas. (This has been demonstrated in peripheral nerves of hypoxaemic OSAHS patients by Mayer et al 1999). According to Friberg (1999), a pharyngeal afferent neuropathy may impair the reflex to the muscles for upper airway dilation and an efferent neuropathy may impair muscle strength. Table 12 summarises the major findings of studies that have demonstrated the pathophysiology and histology of this process.

Table 12: Pharyngeal nerve lesions in snorers and patients with OSAHS
(After: Table 1, pg 928, Friberg 1999)

| Number of subjects | | | Nerve type | Investigation | Tissue | Findings in snorers/ OSAHS | Reference |
|--------------------|---------|-------|------------|-------------------------|---------------------------|--|-----------------------|
| Controls | Snorers | OSAHS | | | | | |
| 4 | 4 | 4 | A + E | Histology | Uvula | Muscle atrophy Deg. Axons | Woodson et al 1991 |
| 7 | | 8 | E | Histology | Palato – pharygeus | Muscle atrophy | Edstrom et al 1992 |
| 15 | | 15 | A | Temperature thresholds | Soft palate | High threshold for warmth | Larsson et al 1992 |
| 11 | 11 | 10 | A | Immunohisto-chemistry | Soft palate | Abnormal nerve endings | Friberg et al 1997 |
| 13 | 11 | 24 | A | Laser doppler flowmetry | Soft palate | Abnormal vascular reactions | Friberg et al 1998 |
| 10 | 11 | 10 | E | Histology | Palato – pharygeus | Progressive neuropathy | 1998 Friberg et al |
| 15 | 12 | 38 | A | Sensation Vibration | Soft palate | Reduced sensation Vibration | Kimoff et al 2001 |
| 7 | 34 | ? | ? | Histology | Soft palate | Inflammation Fibrosis | Berger et al 2002 |
| 5 | 11 | | A+E | Histology | Palato-pharygeus Uvula | Denervation Degeneration Increased connective tissue | Lindman & Stal 2002 |

E = efferent nerves; A = afferent nerves; Deg. = degenerating

Neuropathy of afferent nerves is characterised by axonal degeneration or segmental demyelination. Reinnervation generally occurs within 1-2 weeks with regrowth of neurofilament immunoreactive nerve fibres in close connection with blood vessels

(Hermansson et al 1987). Degeneration of efferent (motor) nerves leads to lack of electro-mechanical activity in muscle fibres. Denervated muscle fibres can be reinnervated from the destroyed axon or from sprouting from nearby intact axons. This process may result in 'type grouping' with blocks of muscle fibres of a single histochemical type. Within this block there will be atrophied as well as hypertrophied muscle fibres. This may explain the histological findings on tissues taken from the oropharynx of patients with heavy snoring or OSAHS.

The success of peripheral nerve regeneration is highly dependent on its microenvironment. Axotomy or crush injury leads to degeneration of the distal nerve stump referred to as Wallerian degeneration (WD). Schwann cells respond to this axonal loss by extrusion of their myelin sheaths, down-regulation of myelin genes, dedifferentiation and proliferation. Molecular changes in the distal stump include up-regulation of neurotrophins, neural cell adhesion molecules, cytokines and other soluble factors and their receptors (Stoll and Muller 1999; Kury et al 2001). One of the more important factors involved in neural regeneration is brain derived neurotrophic factor (BDNF) (Zhang et al 2000) and nitric oxide has also been shown to be a key player in successful regeneration (Zochodne 2000; Levy et al 2001). Studies simulating deprivation of BDNF during regeneration have shown impairment in myelination of regenerating axons (Zhang et al 2000). The BDNF gene is located on chromosome 11p13. Although initially regarded to be responsible for neurone proliferation, differentiation and survival through its uptake at nerve terminals and retrograde transport to the cell body, it has now been shown that it is also transported anterogradely and released upon neurone depolarisation. BDNF has been shown to potentiate intracellular signals and action potentials in central neurones and recently

has been considered important in the regulation of dopamine. For this reason, a number of studies have examined gene polymorphisms in BDNF in Parkinson's disease (Momose et al 2002), bipolar disorder (Sklar et al 2002), addictive behaviour (Uhl et al 2001), schizophrenia (Virgos et al 2001; Krebs et al 2000; Wassink et al 1999) and Alzheimer disease (Kunugi et al 2001). Only one study has examined BDNF gene polymorphisms in peripheral neuropathy (Davar et al 1996). This study focussed on kindred with hereditary sensory neuropathy II (an autosomal recessive disorder characterised by loss of peripheral sensory modalities) but found no segregation of polymorphic alleles at loci for p75NGFR, TRKA, TRKB or BDNF.

A further consideration in snoring is the trauma of vibration. This has long been recognised as a cause of vibration white finger in those who work in heavy industry necessitating the long-term use of low-frequency (< 500Hz) hand-held vibrating tools (Liapina 2002; Noel 2000). The result is a decrease in sensitivity to vibration and temperature. The histological lesion comprises muscle hypertrophy, demyelinating neuropathy and axonal regeneration without myelin sheaths (Takeuchi et al 1986). The vascular disorder has now been classified as digital organic microangiopathy; a digital vasospastic phenomenon and arterial thrombosis (Noel 2000). The vasospastic phenomenon has been recognised since 1990 as the consequence of an imbalance between endothelin-1 and calcitonin-gene-related peptide (CGRP) (Noel 2000). A further important observation has been the deficiency of immunoreactive CGRP nerve fibres in the cutaneous microvasculature of patients with primary and secondary white finger with the distribution and quantity of other types of nerve fibres not significantly altered (Goldsmith et al 1994). The study by Kimoff et al (2001) alluded to in Table 12 not only

demonstrated a selective reduction in the detection of mechanical stimuli in the upper airway of snorers and patients with OSAHS, but also showed a significant, but not complete reversal of this neuropathy on use of CPAP. The fact that the deficiencies were only partially reversible is suggestive of either permanent neuronal injury secondary to snoring (i.e. an epiphenomenon) or may be due to increased susceptibility to damage due to underlying genetic factors governing sensory function. In the latter instance, it may be of interest to examine polymorphisms in BDNF and other neurotrophic factors as well as CRGP in a population with OSAHS. At present however, it remains unresolved as to whether the neuropathy of OSAHS and snoring is a by-product or a primary aetiological factor in disease expression.

2.5.3 Disorders of Connective Tissue

Connective tissue can have a role in OSAHS pathogenesis through increased upper airway collapsibility. Marfan's syndrome (classically a defect in the fibrillin gene) is the best characterised of the disorders showing marked increases in upper airway collapsibility (Cistulli & Sullivan 1995). One study has also suggested that sleep disordered breathing is increased in patients with Ehlers-Danlos syndrome, although the frequency of other sleep-related disorders was also high, e.g. periodic limb movements (Verbraecken et al 2001).

Both disorders are well characterised at a molecular level and will not be discussed further as they represent only a tiny minority of patients with OSAHS.

Polymorphisms in the elastin and collagen (specifically collagen type 1, alpha1 and 2) genes have been investigated in the context of disorders such as cutis laxa, Ehlers-Danlos syndrome, atypical Marfan's syndrome, supraaortic stenosis, the Williams-Beuren syndrome, osteogenesis imperfecta and osteoporosis. One study so

far has examined the Ser422Gly elastin gene polymorphism in human carotid arteries in respect of aging and distensibility (Hanon et al 2001). It is at present unknown, whether this work can be extended to examine soft tissue structures, which likewise are affected by the ageing process. There is also limited information regarding polymorphisms in collagen and elastin genes in the general population, which might go towards explaining the spectrum of upper airway elasticity contributing to a 'floppier' upper airway.

2.6 Control of Ventilation

The existence of genetic diseases such as the congenital central hypoventilation syndrome (Shea 1997; Gozal 1998) points to specific genetic mechanisms involved in the control of breathing. Genetic influences may play a role in determining the wide variability in the magnitude of response to hypoxia and hypercapnia in the adult human (Hirshman et al 1975; Kawakami et al 1985 and 1984; Collins et al 1978; Lahiri et al 1976). Studies in adult monozygotic twins have shown concordance in responses to hypoxia, but not consistently to hypercapnia (Kobayashi et al 1993; Arkinstall et al 1974). Thomas et al (1993) showed a high degree of heritability of peripheral chemoreceptor response to hypoxia and hyperoxia in monozygotic twins during infancy compared with dizygotic twins exposed to similar environmental conditions.

Familial studies examining ventilatory responses have suggested that healthy family members (Kawakami et al 1982, 1985; Mountain et al 1978) share reduction in ventilatory response to hypoxia with the index case. Examination of the ventilatory drive in OSAHS patients and their healthy relatives as well as healthy unrelated

controls has been undertaken by a number of investigators with conflicting results. A summary of these studies is presented in Table 13 below.

Table 13: Ventilatory control abnormalities in patients with SDB, family members and controls

| Number of subjects | | | HVR | HCVR | HCVR + loading | Reference |
|--------------------|--------|----------|------------------------------------|---------------------------------|---|-----------------------|
| SDB | Family | Controls | | | | |
| 10 | 12 | 9 | SDB and family lower than controls | No difference across groups | Respiratory impedance greater in family c.f. controls | Redline et al 1997 |
| 8 | | 8 | | | Decreased VE in SDB | Rajagopal et al 1984 |
| 13** | 32 | | No difference between relatives | No difference between relatives | | Javaheri et al 1992 |
| 16* | 26 | | | | | |
| 35 | | 17 | No difference between groups | Lower response in SDB | | Gold et al 1993 |
| 16 | | 12 | Greater increase VE in SDB | No difference between groups | | Narkiewicz et al 1999 |
| ‡20 | | 19 | No difference between groups | No difference between groups | | Marcus et al 1994 |
| 37 | | 14 | No difference between groups | No difference between groups | | Radwan et al 2000 |

Patients with SDB are normocapnoeic unless otherwise indicated; **Hypercapnoeic patients; * Normocapnoeic patients; ‡ Children (mean age 8 years)

Based on the information in Table 13, it is difficult to conclude that there is one single abnormality in ventilation in SDB. This makes search for a candidate gene currently untenable. Knowledge with regard to neural control of breathing in vertebrates is still in its infancy – see Fortin et al (2000) – and strategies defined in invertebrates are being applied in the investigation of the vertebrate brainstem. Table 14 summarises gene deletion models and their effects on ventilatory responses in transgenic mice. These potentially play their most important role during early embryonic development and for a brief and transient period only, e.g. Hox and Krox-20 (Lumsden and Krumlauf 1996).

Table 14: Gene deletion models in transgenic mice and effects on ventilatory response to chemical challenges (After Table 1, pg 283, Gaultier and Guilleminault 2001)

| Genes | -/- | +/- | Baseline breathing pattern | | HVR | HCVR |
|-------------------|-----|-----|----------------------------|------------------|-----|------|
| | | | Apnoeas | Respiratory Rate | | |
| Krox-20 | + | + | | Low | NS | |
| Tst-1/Oct-6/SCIP+ | | | | Low | NS | NS |
| Rnx | + | + | | High | | |
| Ret | + | | | | NS | D |
| Ret | | + | | | NS | D |
| Mash-1 | | + | | | D | D |
| Mash-1 (adults) | | + | | | NS | NS |
| ET-1 | + | | | | D | D |
| Et-1 (adults) | | + | | | D | D |
| ETA | + | | | | D | D |
| ECE-1 | | + | | | D | NS |
| ECE-1 (adults) | | + | | | NS | NS |
| BDNF | + | + | | Low | D | NS |
| PDGF-b (adults) | | + | | | I‡ | |
| NEP (adults) | + | | | | I | |
| NOS (adults) | + | | | | I | NS |
| Leptin (adults) | | | | | | D |

-/- = homozygous; +/- = heterozygous; NS = not significantly different to wildtype animals; D = decreased; I = increased; all mice new-born unless otherwise stated; ‡ increased in late phase of HVR

A number of studies have shown that gene expression in the cardiorespiratory sites of the brainstem during development and in later life may be modified by responses to hypoxia in the context of environmental constraints (Larnicol et al 1994; Belegu et al 1999; Dumas et al 1996; Bianchi et al 1995). Hypoxia inducible factors (HIF) such as HIF-1a (Wang et al 1995; Gassman and Wenger 1997) is upregulated in the mammalian brainstem in response to a hypoxic stress compatible with normal respiratory physiology (Pascual et al 2001). The role of HIF in control of respiratory rhythms is currently under investigation. The role of neurotrophins during development of the brainstem has also been found integral to establishment of functional respiratory control (Fortin et al 2000). The best studied of these is BDNF. BDNF, acting through the TrkB receptor tyrosine kinase has been implicated in the

chemoafferent control of breathing. In BDNF $-/-$ mice, ventilatory responses to hyperoxia and hypoxia are depressed but sensitivity to hypercapnia is intact (Erickson et al 1996). Respiratory frequency and tidal ventilation per body mass are smaller than normal and lead to chronic hypoventilation and death within 3 weeks of birth (Erickson et al 1996). There is a hypoplasia of cranial sensory ganglia with an intact brainstem anatomy (Conover et al 1995). Neuronal growth factors are thought to regulate survival by modulating genetically programmed cell death and thereby quantitatively match neuronal populations to the size of their target field (Oppenheim 1991). A sub-population of dopaminergic chemo-afferent neurones that controls the respiratory network is selectively reduced by 50% in mice lacking BDNF (Erickson et al 1996). Balkowiec and Katz (1998) have also shown that BDNF is integral to the normal development of the central respiratory rhythm, including its stabilisation by modifying the size of specific neuronal populations within the respiratory network (Balkowiec and Katz 1997) in these transgenic mice. The molecular biology of BDNF is discussed in 2.5 above.

The other major issue is whether respiratory control is implicated in the pathogenesis of OSAHS. Although there are abnormalities of respiratory control in OSAHS (Ayappa et al 2002; Garcia-Rio et al 2002; Asyali et al 2002) , these reverse with CPAP (Moura et al 2001; Lin et al 1994; Verbraecken et al 1995; Verbraecken et al 2000). Thus the changes may be secondary rather than causative. This makes searching for a gene cause of OSAHS relating to respiratory control likely to be low yield.

2.7 Apolipoprotein E – a role in OSAHS?

A recent study by Kadotani et al (2001) looked at APOE e4 allele frequencies in a population with sleep disordered breathing. The hypothesis was that complex interactions among sleep disordered breathing, brain pathology (such as early onset Alzheimer's disease) and cardiovascular disorders may occur with increased frequency in subjects heterozygous or homozygous for APOE e4.

Apolipoprotein E (**APOE = gene; ApoE = protein**) occurs in all lipoproteins and its major role is thought to be the conversion of LDL proteins to IDLs (Davignon et al 1988). It is a 299 amino-acid polypeptide residue found at position q13.2 on chromosome 19. The 3 major isoforms of human apo E (apoE2; apoE3 and apoE4) are coded for by 3 alleles (epsilon 2, 3, 4). The difference among the isoforms is found at two sites – residue 112 (site A) and residue 158 (site B). At site A/B apoE2 contains cysteine/cysteine; apoE3 contains cysteine/arginine and apoE4 contains arginine/arginine (Weisgraber et al 1981; Rall et al 1982). ApoE3 is the most commonly occurring isoform (wildtype) in human populations. Four different mutations giving a band at the E2 position with isoelectric focusing have been described: E2 (arg158-to-cys), E2 (lys146-to-gln), E2 (arg145-to-cys) and E2-Christchurch (arg136-to-ser). E2 (arg158-to-cys) is the most common of the four.

There is now a large body of data on the APOE allelic variants and their gene frequencies in various populations. Corbo and Scacchi (1999), Gerdes et al (1992 and 1996), Schiele et al (2000) and Roychoudhury and Nei (1988) have most extensively documented these. As stated above, the APOE3 allele is the most frequent in human populations the world over and especially so in populations with a long-established agricultural economy such as those in the Mediterranean basin

where the frequency of the allele is 0.849 – 0.898. The APOE4 allele frequency remains higher in populations such as the Pygmies (0,407), Aborigines of Malaysia (0.240) and Australia (0.260), Papuans (0.368), some Native Americans (0,280) and Lapps (0.310). The current theory links the occurrence of this allele with a population where foraging still exists or where food supplies tend to be scarce and sporadically available within traditional lifestyles. The APOE2 frequency fluctuates with no apparent trend (0.145 – 0.02) and is absent in Native Americans. Corbo and Scacchi (1999) have suggested on the basis of these observations that APOE4 may be a ‘thrifty allele’ and that the exposure of the allele to the environmental conditions of a Western diet and longer lifespan may have rendered it a susceptibility allele for atherosclerosis and Alzheimer disease. The absence of the association of APOE4 with either disorder in sub-Saharan Africans and presence of the association in African Americans is viewed as supportive of this hypothesis. Table 15 below shows frequencies for the 3 allelic variants in a European population.

Table 15: Distribution of APO E allelic frequencies among a European population (*After: Table 1, pg 303, Corbo and Scacchi 1999*)

| Population | n | APOE2 | APOE3 | APOE4 |
|-------------------|----------|--------------|--------------|--------------|
| Lapps | 70 | 0.050 | 0.640 | 0.310 |
| Swedes | 279 | 0.119 | 0.675 | 0.206 |
| Danes | 466 | 0.085 | 0.741 | 0.174 |
| Finns | 2245 | 0.044 | 0.748 | 0.208 |
| Dutch | 2318 | 0.085 | 0.752 | 0.163 |
| Belgians | 760 | 0.072 | 0.765 | 0.163 |
| Icelanders | 185 | 0.068 | 0.767 | 0.165 |
| UK | 734 | 0.089 | 0.767 | 0.144 |
| French | 1228 | 0.108 | 0.771 | 0.121 |
| Germans | 2031 | 0.077 | 0.778 | 0.145 |
| Norwegians | 395 | 0.087 | 0.781 | 0.132 |
| Tyroleans | 469 | 0.090 | 0.789 | 0.117 |
| Hungarians | 202 | 0.064 | 0.807 | 0.129 |
| Swiss | 173 | 0.072 | 0.821 | 0.107 |
| Poles | 137 | 0.055 | 0.839 | 0.106 |
| Italians | 2000 | 0.060 | 0.849 | 0.091 |
| Spaniards | 1286 | 0.052 | 0.856 | 0.091 |
| Turks | 8453 | 0.061 | 0.860 | 0.079 |
| Greeks | 551 | 0.054 | 0.878 | 0.068 |
| Sardinians | 280 | 0.050 | 0.898 | 0.052 |

In disorders of lipid metabolism and cardiovascular disease, the E2 isoform appears to play the greatest role as it has been shown to bind remnants to hepatic lipoprotein receptors defectively (Schneider et al 1981; Rall et al 1982) and to delay their clearance from plasma (Gregg et al 1981). Expression of the defect is however modulated by other factors such as obesity; diabetes and age (see Hazzard et al 1981 and Goldstein 1983 for more detailed discussion). There is some suggestion from epidemiological studies that ApoE4 is associated with greater risk of atherosclerosis (Bockxmeer and Mamotte 1992; Stengard et al 1995; Stengard et al 1996; Lehtimaki et al 1990; Tiret et al 1994; Luc et al 1994), but this has not been a consistent finding (Payne et al 1992; O'Malley and Illingworth 1992; De Knijff et al 1992) and some authors postulate that environmental influences have a major impact on expression of coronary artery disease in conjunction with the APOE4 allele (Corbo et al 1999).

ApoE4 has also been associated with Alzheimer disease as well as a number of neurological disorders, including Creutzfeldt-Jakob disease, Down's syndrome, schizophrenia and vascular dementia. The reason for the intense research in this area is based on the observation that ApoE is found in senile plaques, congophilic angiopathy and neurofibrillary tangles of Alzheimer's disease (AD). When Strittmatter et al (1993) compared the binding of synthetic amyloid beta peptide to purified ApoE4 and ApoE3, both bound to it in the oxidised form. However, binding to ApoE4 was observed in minutes whereas binding to ApoE3 occurred within hours. The conclusion drawn was that isoform-specific differences in ApoE binding or oxidation might be involved in the pathogenesis of the lesions of AD. Further to the numerous studies linking ApoE4 with the presence of AD, early onset AD and preferential development of AD in postmenopausal women, are studies suggesting

that the E2 allele may confer protection against AD (Talbot et al 1994; Corder et al 1994). In these latter studies, although 65% of AD is attributable to the presence of E4 alleles, risk of AD is lowest in subjects with the E2/E3 genotype with an additional 23% of AD attributable to the absence of an E2 allele. A large study by Myers et al (1996) which examined the association of apolipoprotein E4 with AD and other dementias in 1,030 elderly individuals in the Framingham cohort study did find an increased risk of these conditions in patients either homozygous or heterozygous for E4. However, most apoE4 carriers did not develop dementia and about half of the patients with AD did not have the E4 allele. Bennett et al (1995) examined APOE genotypes in families with a positive history and those with a negative history of AD and found that the allele distribution of at-risk but unaffected sibs for E4 was in excess and this did not differ from that of affected sibs. They found no evidence for linkage between the APOE4 locus and AD and speculated that it may modify preclinical progression at most.

As outlined above, E4 is neither necessary nor sufficient for the expression of AD which is also affected by environmental or other genetic factors.

With respect to sleep disordered breathing, 3 studies have been published, with a fourth paper looking at APOE4 in relation to chronic daytime somnolence.

The first by Saareleinen et al (1998) examined allelic variants in APOE on the basis of its role as an 'injury-response' macromolecule in peripheral nerves and neuromuscular junctions (Akaaboune et al 1994). The primary hypothesis was to examine the importance of APOE genotype in OSA in relation to pharyngeal dilator muscle patency and also increased risk of cardiovascular disease. 291 patients with OSA (AHI > 5 events/hour) with no neurological or cerebrovascular disease were

examined in association with 728 controls from a random sample of the Finnish population used in previous studies without any polysomnography. The cases and controls were age matched (mean ages 53.3 and 53.7 years respectively). The mean AHI was 38 (5 – 106) in cases. There was no significant difference in distribution of either APOE alleles or genotypes between cases and controls as shown in Table 16 below.

Table 16: Distribution of APOE genotypes and alleles in OSA and Controls (After Table 1, pg 148, Saareleinen et al 1998)

| | OSA (n=291) | Controls (n=728) | p-value |
|-------------------------|--------------------|-------------------------|----------------|
| Genotype | | | |
| E2/E2 | 0 (0%) | 0 (0%) | - |
| E2/E3 | 25 (8.6%) | 70 (9.6%) | NS |
| E2/E4 | 4 (1.4%) | 18 (2.5%) | NS |
| E3/E3 | 166 (57%) | 408 (56%) | NS |
| E4/E3 | 79 (27.1%) | 206 (28.3%) | NS |
| E4/E4 | 17 (5.8%) | 26 (3.6%) | NS |
| Allele frequency | | | |
| E2 | 5% | 6% | NS |
| E3 | 74.9% | 75% | NS |
| E4 | 20.1% | 19.0% | NS |

The major criticism of this study resides in the absence of data regarding sleep disordered breathing in the control group and the absence of stratification of the cases by degree of SDB.

The second study to be published in this area by Kadotani et al (2001) looked exclusively at the presence or absence of apolipoprotein E4 alleles in a group of patients who had all undergone polysomnography (up to three times per patient) and were classified by the authors as all having sleep apnoea according to their criteria (AHI > 5). No control group was used and subjects were grouped as either APOE4 positive (n=222) or APOE4 negative (n=569). The two groups did not differ significantly in terms of mean age (49 years), percentage of males in each group, ethnicity, BMI or presence of hypertension. Sleep architecture was likewise not

significantly different between the two groups. There was a significantly higher mean AHI in the E4 positive group (6.5SE0.6 vs. 4.8SE0.3) and there was a higher percentage of patients with AHI >15 in the E4 positive group (12% vs. 7%). However, the distribution of the AHI in the groups was non-parametric and the median values between groups were not significantly different – 1.3 (0 – 121) in the E4 negative group vs. 2 (0 – 81) in the E4 positive group. Additionally, rather than averaging the AHI for an individual who had had more than one sleep study, results for all sleep studies ever undertaken in both groups were reported as if for individual patients, i.e. 960 in the E4 negative group (n=569) vs. 384 (n=222). The authors went on to suggest that increased SDB in E4 positive patients might be responsible for the observed association of recalling napping behaviour and subsequent development of AD (Asada et al 2000). However, they may have simply demonstrated a statistical association.

The third study to report findings in this area by Foley et al (2001) looked at 718 Japanese-American men in Hawaii aged between 79 to 97 years. Only 18% of the sample had the E4 allele. Moderate to severe SDB (AHI > 15) was present in 42% of this sample of men and adjustment for age, BMI, smoking and use of antihypertensive medication did not reveal an association between E4 and an AHI > 15. The authors postulated that the difference between their results compared to those of Kadotani et al (2001) may have been attributable to the high false-positive rate of a genetic association study, the differences in the populations in terms of age, sex, ethnicity and BMI as well as SDB and the fact that risk factors for chronic disease often have weaker relationships in older persons. This latter bias may stem from

selective survival, misclassification and changes in biological relationships associated with increased age.

The most recently published study has examined APOE4 homozygosity in the context of memory and chronic daytime somnolence (Caselli et al 2002). In this study, 42 patients homozygous for E4 were matched to 42 patients heterozygous for E4 and 42 non-carriers of the E4 allele. None of the patients underwent a sleep study. The mean ESS was not high in any of the three groups – 6.4 (sd 3.8) in the E4 homozygotes, 8.2 (sd 3.8) in the heterozygotes and 6.8 (sd 4.5) in the nonE4 carriers ($p= 0.85$, one-way ANOVA). All groups were matched in terms of age (mean age 55years), gender, BMI and obesity. However, analyses were confined to E4 homozygotes vs. nonE4 carriers and no comparison was made in a 3-way test across the groups for the multitude of neuropsychological tests that were carried out. Although the authors concluded that chronic daytime somnolence was associated with a decline in verbal memory in cognitively normal APOE E4 homozygotes in comparison to non-carriers of E4, the study displayed a large number of flaws in method, definitions and statistical analysis for any definitive conclusions to be drawn.

In summary, Apo E is associated with the presence of atherosclerosis and Alzheimer's disease and potentially neuropathy. The association with sleep apnoea is potentially even more complex. No controls without SDB have been examined in the positive studies. Secondly, OSAHS is associated with a number of comorbidities that have independently been shown to associate with increased frequency of the E4 allele, such as atherosclerosis, and coronary artery disease irrespective of the presence of SDB. Thirdly, even if the E4 allele were found with increased frequency

in an OSAHS population, this would not constitute causation and indeed, there appears to be no biologically plausible mechanism currently under consideration that would allow for a hypothesis to be generated in this regard.

2.8 Hypertension in OSAHS

As discussed in Chapter 1, section 1.5, OSAHS is an independent risk factor for diurnal hypertension (Stradling et al 2000, Faccenda et al 2000; Pepperell et al 2002) and has now been implicated as a risk factor for first stroke, recurrent stroke and post-stroke mortality (Dyken et al 1996). The Wisconsin Sleep Cohort Study (Peppard et al 2000), which prospectively investigated the association between OSA and the development of hypertension at four years of follow-up showed that sleep disordered breathing present at initial evaluation was accompanied by a substantially increased risk of future hypertension. Subjects with an apnoea/hypopnoea index (AHI) of > 15 events per hour had a risk ratio for developing new hypertension over a 4 year period that was threefold compared to those without any apnoea.

There are a number of potential mechanisms that may mediate the vasculopathy that contributes to the development of hypertension. In OSA, repetitive episodes of airway occlusion resulting in hypoxaemia, hypercapnia and changes in intrathoracic pressure elicit chemoreflex activation with consequent increases in sympathetic vasoconstrictor traffic to peripheral blood vessels (Somers et al 1995). This results in increases in blood pressure and elevated levels of circulating catecholamines. Whilst sympathetic activation is one mechanism contributing to diurnal hypertension, the pressor effect of vasoactive peptides such as endothelin may sustain the elevated blood pressure (Faller 1999). Lastly, endothelial dysfunction may contribute to

vasculopathy in OSA and is a characteristic of hypertension (Panza et al 1990) and atherosclerosis (Celermajer et al 1992). Theoretically, it may result from the repetitive hypoxaemia and pressor surges evident in patients with OSA.

The treatment of OSA with continuous positive airway pressure (CPAP) has been shown to attenuate the above effects (Narkiewicz et al 1999) and to result in the reduction of blood pressure in both hypertensive (Stradling et al 2000, Faccenda et al 2000) and normotensive patients with OSA (Faccenda et al 2000; Pepperell et al 2002). This effect has been most marked in those with a high number of oxygen desaturations during sleep associated with apnoeas and hypopnoeas.

Recent studies have shown abnormal vascular responses in patients with OSAHS which are present even in the absence of overt cardiovascular disease, and are similar to those in patients with essential hypertension (Kraiczi et al 2001; Kraiczi et al 2000). OSAHS may be associated with increased vasoconstrictor sensitivity (Kraiczi et al 2000), impaired endothelium dependent vasodilatation to acetylcholine (Kraiczi et al 2000) and bradykinin (Kato et al 2000; Duchna et al 2000), and decreased alpha- and beta 2-adrenergic vascular responses (Grote et al 2000). Nocturnal hypoxaemia was associated with decreased endothelium-dependent vasodilatation in a recent correlational study (Kraiczi et al 2001). Methods employed in these studies have differed between groups and so it is difficult to compare results between patient populations and to determine reliably the reproducibility of the reported findings.

The endothelium plays a vital role in the control of blood flow, coagulation, fibrinolysis and inflammation. Consequently, the maintenance and regulation of tissue perfusion critically depends upon the integrity of endothelial function and the release of potent endothelium-derived factors. In a small study, Kato and colleagues

(2000) demonstrated impaired endothelium-dependent vasodilatation to acetylcholine but unchanged responses to sodium nitroprusside and verapamil in the forearm circulation of 8 patients with OSAHS. This suggests that OSAHS is an endothelial dysfunctional state.

Endothelium-dependent vasomotion may not be representative of other aspects of endothelial function. The effects of intermittent nocturnal hypoxaemia resulting in hypertension are thought to at least partly involve the release of vasoactive peptides. These determine the response of the vascular endothelial and/or smooth muscle cells to the hypoxic stimulus resulting in remodelling, fibrosis and angiogenesis. The key peptides involved include vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) (Faller 1999). Levels of expression and activity of these factors may be determined by genotype.

VEGF levels have been found to be elevated in patients with OSAHS (Imagawa et al 2001, Schulz et al 2002, Gozal et al 2002) and the levels decrease with treatment with CPAP (Lavie et al 2002).

The result of studies examining circulating levels of ET-1 in patients with OSAHS with and without hypertension have been contradictory (Grimpen et al 2000; Phillips et al 1996; Saareleinen et al 1997; Kanagy et al 2001; Phillips and Somers 2000) and each study has displayed limitations in method which may have affected results. No studies are available to date looking at the gene polymorphisms of vasoactive peptides in the context of OSAHS. One study examining angiotensin converting enzyme inhibitor (ACE-I) gene polymorphisms has been reported in the literature in subjects with and without OSA and hypertension (Zhang et al 2000).

The importance of looking at gene polymorphisms in OSAHS with respect to hypertension is largely related to the reversibility of abnormal vascular responses with treatment. The distribution of polymorphisms potentiating hypertension may or may not be similar to that in patients with essential hypertension as they share much comorbidity. However, it is of secondary importance to this work, which is focussed largely on the causes of OSAHS rather than its consequences.

2.9 Methods of Genetic Studies

Genetic polymorphisms occur throughout the human genome approximately once every 500-1000 base pairs (bp). A genetic polymorphism is defined as a variation in the DNA sequence that occurs at least once in every 100 copies (Vogel & Motulsky 1996). Apart from the genes on the sex chromosomes, each individual will have two copies of every gene so the variation needs to occur at least once in every 50 individuals to be classified as a polymorphism. Rarer sequence variants are usually referred to as isolated mutations. The normal DNA sequence for a particular gene is called the wild-type allele and the rarer sequence the variant allele. Different polymorphisms that occur close together are often linked and within a single chromosome, this arrangement is known as a haplotype. When haplotypes are found together more frequently than would be expected by chance, they are said to be in linkage disequilibrium. The most common type of genetic polymorphism is the single nucleotide polymorphism where one base is replaced by another. Sometimes insertions of additional sequences or deletions can occur. SNPs can be silent or functional. SNPs are useful markers in the investigation of susceptibility in polygenic diseases. The Human Genome Project has made possible a SNP map which is a high-

density map of 200,000 – 300, 000 SNPs and a database that contains 1.8 million SNPs accessible via the internet (<http://snp.cshl.org/>).

Two general approaches in study design have been used in linking human disease with a genetic component (Table17).

Table 17: Study designs for the identification of disease genes
(after Daly & Day 2001; *Br J Clin Pharmacol* 52: 490)

| |
|--|
| <p><u>Linkage Studies</u></p> <ol style="list-style-type: none">1. Used mainly in the study of single gene disorders2. Utilize genetic markers situated throughout the genome which are not necessarily functionally significant in terms of phenotypic effect3. Use extended families (rarely in study cases and controls)4. Linkage of genotype for a genetic marker to disease may be unique to the particular family |
| <p><u>Association Studies</u></p> <ol style="list-style-type: none">1. Used to identify genes involved in polygenic disorders.2. Determine genotype for polymorphism (may be functionally significant) in candidate gene of biological relevance to disease3. Use cases and appropriately matched controls (unrelated usually)4. Association of a genotype or phenotype with disease is a statistical finding not necessarily reflecting genetic linkage |

In the last 20 years, genes implicated in Mendelian diseases have been identified by using genetic linkage and positional cloning methods. These methods have allowed for the identification of relative risk genes but have not been so successful in identifying genes involved in polygenic disorders. The failure of these methods (linkage studies) in complex diseases arises from three main features inherent to them:

1. Complex diseases vary in severity of symptoms and are generally of late onset which means parents of the index case are not usually available for family studies and often makes definition of an appropriate phenotype difficult. Furthermore, a number of epigenetic phenomena, such as parental

imprinting, disease fluctuation and sex- effects (see Petronis 2001) can also alter phenotype.

2. Complex diseases may vary in their aetiological mechanisms which might involve various biological pathways.
3. Complex diseases are more likely to be polygenic with each gene having a small overall contribution and relative risk for disease expression.

Therefore, association studies using candidate-gene approaches are more likely to be more effective tools than linkage studies in the investigation of complex traits. By utilizing the case-control method of study design, a candidate-gene study has the advantage of both the increased statistical efficiency of association analysis of a complex disease (Risch & Merikangas 1996; Tabor et al 2002) and the biological understanding of the phenotype, tissues, genes and proteins that are likely to be involved. Association studies may provide adequate power to detect relative risks as low as 1.5 which is generally not possible in family studies (Risch 2000) and by looking at functionally significant polymorphisms, concerns about linkage disequilibrium and adequacy of SNP markers to detect association are not relevant (Weiss & Terwilliger 2000). Segregation and linkage analysis studies have difficulties with non-Mendelian disorders. Segregation analysis is very sensitive to biases and linkage analysis requires the specification of a precise genetic model which includes disease gene frequency and information on the penetrance of each genotype (Gambaro et al 2000).

However, in order to be effective, a number of relevant issues in the design of case-control studies must be addressed including choice of candidate gene and SNP; recruitment methods; control matching and number of study subjects

(especially with respect to power) – see Little et al (2002) for exhaustive discussion on design adequacy and checklist on gene-disease association studies and Daly & Day (2000). It is also important to consider the confounding factors inherent in case-control studies using the candidate-gene approach which include:

1. Population stratification – this can be minimised by using internal family control subjects (Hassan et al 2001; Silverman & Palmer 2000)
2. Phenotypic heterogeneity
3. Linkage disequilibrium of the SNP under study with other causal SNPs
4. No biological role for the gene/protein studied; SNP studied has no functional effect even though the protein is involved
5. Degree of generalisability to other populations
6. Lack of replication of results in subsequent studies or studies undertaken in different populations (due to false-positive, false-negative studies or true variability among different populations).

Largely related to questions regarding causality (does the candidate gene cause or contribute to the disease process?), these issues are less of a concern if there is biological plausibility in the association and the association is strong. Furthermore, Lohmueller et al (2003) and Tabor et al (2002) have argued that consistent replication in different populations is strong evidence of causality but that lack of replication does not necessarily imply lack of causality. A large number of SNPs studied may have real but modest effects on common disease risk but the studies are underpowered leading to false-negative reports (Lohmueller 2003).

Despite these limitations, the association study remains the most cost-effective, practical and reliable method of examining for candidate genes in complex diseases such as OSAHS.

2.10 AIMS OF THE THESIS

The aims of this thesis were to conduct a case-control study examining the association of 5 SNPs in 5 candidate genes with OSAHS. On the basis of evidence presented above, OSAHS is considered to be a complex disease.

The genes of interest included:

- 1. Tumour necrosis factor α (SNP: - 308 A/G).** The most widely studied polymorphism within the TNF- α gene involves the G/A substitution at position -308 (relative to transcription start site of (+1)) in the promoter region. It was first described by Wilson et al in 1992. Guanine at position -308 is considered to be the wildtype variant (TNF1) whilst the adenine at this position is considered the minor allele (TNF2). The functional relevance of the TNF2 polymorphism *in vivo* remains in contention. The postulated functional effect would appear to be an effect on transcription of the TNF- α gene. Two approaches have been used to investigate the effects of the TNF2 polymorphisms: 1. investigation of the influence of polymorphism on transcriptional activity of reporter genes expressed in various cell lines; 2. investigation of the expression of TNF2 by cells derived from individuals with varying TNF- α promoter genotypes.

Each approach has its advantages and disadvantages (see Allen 1999 for detailed discussion). In Table 18 and Table 19 below, the results of studies on the TNF- α G/A polymorphisms are summarised according to the investigative approach used.

Table 18: Findings from studies of the relationship between -308 genotype and TNF- α production by stimulated peripheral blood mononuclear cells (after Allen 1999, pg 1022)

| Authors | N for G/A: G:G | Ratio G/A: G:G* | P – value | Cell stimulant used |
|---------------------|----------------|-----------------|-----------|---------------------------------------|
| Bouma et al 1996 | 13: 26 | 1.3 x | < 0.05 | 1 μ /ml each anti-CD3 & anti-CD28 |
| Danis et al 1994 | 6: 29 | 1.4 x | NS | 5 ng/ml IFN- γ |
| Huizinga et al 1997 | 6: 67 | 1.2 x | NS | 10 or 1000 ng/ml LPS |
| Pociot et al 1993 | ? | 1.2.x | 0.08 | 250pg/ml LPS |
| Louis et al 1998 | 16: 41 | ~ 1.8 x | < 0.05 | 1 or 100 ng/ml LPS |
| Mycko et al 1998 | ? | 0.95 x | NS | 10 μ g/ml LPS |

* Genotype at position -308. Ratio of TNF- α production from cells of heterozygotes compared to cells of homozygotes

Table 19: Study results using reporter gene constructs to investigate the transcriptional effects of the -308 G/A polymorphism (after Allen 1999, pg 1023)

| Author | 5' segment used | 3'UTR segment | Cell line | Origin of line | Effect of SNP |
|------------------------|-----------------|----------------|--------------|----------------|---------------|
| Braun et al 1996 | -627 to +164 | Nil | BHK | Hamster | Positive A>G |
| Kroeger & Abraham 1996 | -993 to +110 | Nil | Jurkat U937 | Human | Negative |
| Kroeger & Abraham 1996 | -993 to +110 | +1957 to +2792 | Jurkat U937 | Human | Positive A>G |
| Wilson et al 1997 | -585 to +106 | Nil | Raji | Human | Positive A>G |
| Brinkman et al 1996 | -619 to +108 | +1951 to +3013 | Raji, Jurkat | Human | Negative |
| Stuber et al 1996 | -1173 to +130 | Nil | ANA-1 | Murine | Negative |
| Uglieroro et al 1998 | -977 to +93 | Nil | A20, Ar-5 | Murine | Negative |

The conflicting results clearly arise largely from problems with statistical power in the studies looking at TNF- α production in cells from individuals with different genotypes (Table 18). A further confounder may lie in the possibility that TNF- α is in linkage disequilibrium with the MHC and that the -308 allele is specifically in

linkage disequilibrium with HLA-DR3 (Wilson et al 1993). This latter association results in a relatively high TNF- α production (Jacob et al 1990). However, Bouma et al (1996) showed that secretion of TNF- α was elevated in -308G/A individuals irrespective of whether they were DR3+ or DR3-. The high TNF- α production associated with the HLA-DR3 allele may be due to its close association with the -308 A TNF- α promoter allele.

Studies using the reporter gene approach also show conflicting results (Table 19). The one variable that may be contributing to this may be the segment of the promoter used. A sequence as short as 6 – 8 bp could bind a transcription factor and have repressor or activator activity which could be significant (whereby DNA structure is modified). Despite the variation in results, studies that do report a difference report the same type of difference. Taken together, the two approaches to studying the effects of an SNP on gene function, suggest that there may be a small but significant effect of the -308 A allele increasing the levels of TNF- α transcription. As Allen (1999) points out, the degree to which an increase or decrease in transcriptional activity is required to be biologically relevant is still unknown. It is possible that even a 5% difference in levels of locally produced TNF- α could alter the kinetics of an ongoing immune response at a crucial time. Kaijzel et al (1998) showed that TNF- α increases its own production by a positive feedback mechanism (Amiot et al 1997; Philip and Epstein 1986). Thus, a small initial increase in TNF- α production may be magnified during an *in vivo* immune response and therefore have a significant effect on the outcome of that response. In this respect, promoter polymorphisms may not need to exert a large effect on transcription in order to influence the immune response.

The TNF- α -308 A polymorphism has been associated with an increased risk of cerebral malaria (McGuire et al 1994), obesity (Hermann et al 1998) and a diverse range of inflammatory conditions including asthma (Moffat & Cookson 1997), usual interstitial pneumonia (Whyte et al 2000; Pantelidis et al 2001; Riha et al 2004) and chronic lymphocytic leukaemia (Demeter et al 1997). The number of conditions with which the -308 SNP is associated or in which it has been tested is so diverse and so numerous, that it has prompted the establishment of a website that regularly updates the information and which can be found at:

<http://www.bris.ac.uk/Depts/PathAndMicro/services/GAI/cytokine4.htm>

It is also important to bear in mind that the TNF- α gene is located within the highly polymorphic major histocompatibility complex (MHC) region on chromosome 6p21.3. The TNF gene cluster contains many polymorphisms including microsatellites and single nucleotide polymorphisms. Many of these polymorphisms, including TNF- α -308 have been found to be in linkage disequilibrium with HLA class I and II alleles, including lymphotoxin- α (TNF-b – one of five microsatellites within the TNF locus). The association of TNF- α SNPs with various conditions may therefore be due to the direct influence of the SNP in question and/or due to linkage disequilibrium with other polymorphisms within the TNF- α gene or the HLA system. The reasons that the TNF- α gene may be important in the pathogenesis of OSAHS are as follows:

1. TNF- α levels are raised in OSAHS. To date, four studies have demonstrated this in subjects with OSAHS (Vgontzas et al (1997; 2000), Entzian et al (1996) and Liu et al (2000)) and can be summarised as follows:

- a. TNF- α is elevated in OSAHS independently of obesity

b. TNF- α may play a role in daytime sleepiness experienced by the obese even in the absence of OSAHS.

2. TNF- α may promote sleepiness. This is discussed in Section 2.4.1.

3. TNF- α may be associated with hypertension (Pausova et al 2000).

4. TNF- α may be associated with obesity. A number of studies have shown an increased association of the TNF- α -308 A allele with obesity in various ethnic populations including Pima Indians (Norman et al 1995), French and Northern Irish (Hermann et al 1998), French Canadians with differential body fat deposition in line with gender differences (Pausova et al 2000) and Germans (Brand et al 2001). See also Das (2001) for detailed discussion.

5. TNF- α is implicated in bone growth and remodelling. Gerstenfeld et al (2001) have shown that TNF- α plays a crucial role in promoting postnatal bone repair through the induction of osteoprogenitor cell recruitment or osteogenic cell activation in the context of intramembranous bone formation. Suda et al (2001) have demonstrated that TNF- α plays an important role in pathological bone resorption due to inflammation and Cheng et al (2003) showed TNF- α to be integral to osteoclastogenesis.

Thus, TNF- α may play a significant role in the pathogenesis and expression of OSAHS.

2. Apolipoprotein E (Alleles: E2, E3, E4). As discussed in section 2.7 above, the APOE gene products are intimately associated with the regulation and binding of lipoprotein receptors. The product of the 3 APOE alleles differs in such properties as its affinity for binding to apoE (protein) and low density lipoprotein

(LDL) receptors and its affinity for lipoprotein particles (Davignon et al 1988). *In vitro* observations have associated APOE4 (gene) with higher levels of total and LDL cholesterol and apoB, thereby acting as a risk factor for atherogenesis. APOE 4 (gene) carriers show lower apoE levels (protein) compared to the other alleles (Davignon et al 1988). The largest amount of APOE mRNA is found in the liver with the second largest concentration in the brain. Brain apoE is synthesized locally (as shown in liver transplant recipients) and is the major apolipoprotein in the cerebrospinal fluid. It has been implicated in lipid transport and cholesterol redistribution following peripheral and CNS injury and it is possible that it acts as a neurotrophic factor, antioxidant and mediator of immune responses. ApoE4 is found in senile plaques, congophilic angiopathy and neurofibrillary tangles of Alzheimer's disease. When Strittmatter et al (1993) compared the binding of synthetic amyloid beta peptide to purified ApoE4 and ApoE3, both bound to it in the oxidised form. However, binding to ApoE4 was observed in minutes whereas binding to ApoE3 occurred within hours. It appears that the ApoE4 allele gives rise to structural changes that affect binding kinetics and transport. In the case of OSAHS, these observations may be of relevance in respect of changes in the brain related to chronic intermittent hypoxaemia (e.g. study by Davies et al 2001 which showed a higher number of white matter lesions of unknown significance using MRI in patients with OSAHS compared to perfectly matched controls) or the development of atherogenic complications. Two studies have suggested that the APOE4 allele may be more frequently associated with OSAHS than expected by chance alone (section 2.7).

3. Serotonin receptor 2A (SNP: T102C).

The 5HT_{2A} receptor was one of the first serotonin receptors to be cloned (Saltzman et al 1991; Chen et al 1992; Stam et al 1992). 5HTR_{2A} is a member of the G-protein linked receptor superfamily and thereby shares a common mechanism for signal transduction with other HT₂ receptors which when activated stimulate phosphoinositide (Pi) hydrolysis. A number of SNPs have now been identified in the coding and regulatory regions (see Table 20).

Table 20: DNA sequence variants identified in the human 5HTR_{2A} gene

| Name | Location (nucleotide position) | Sequence Change | Region of Gene | Function? |
|-----------|--------------------------------|-----------------|---------------------|-------------------------------|
| Thr25Asn | +74 | C → A | Coding (N-terminus) | Unknown |
| 102T/C | +102 | T → C | Coding | Silent |
| 516C/T | +516 | C → T | Coding | Silent |
| His452Tyr | +1354 | C → T | Coding (C-terminus) | Ca ⁺⁺ mobilization |
| -1438 G/A | -1438 | G → A | Promoter | Promoter activity |

Inayama et al (1994) were the first to report an association with the silent mutation in the coding region T102C with schizophrenia. The mutation does not alter the expression or structure of the protein and this suggests that a functional receptor variant of 5HTR_{2A} may be in linkage disequilibrium with T102C. This prompted Erdmann et al (1996) and Ozaki et al (1996) to search for two other relatively abundant amino acid substitutions in the 5HTR_{2A} receptor which were also located in the coding region. These were identified as C+74A (Thr25Asn) and C+1354T (His452Tyr) and were found to be located close to the N- and C-terminals respectively of the 5HTR_{2A} molecule. At that stage no functional role for the protein could be assigned, but further studies using radioligand binding in human platelets demonstrated an effect on Ca⁺⁺ mobilization.

Ozaki et al (1997) looked at the influence of the His452Tyr SNP on cellular 5HT functions using 5HT_{2A} binding and 5HT-stimulated Ca⁺⁺ response in platelets

derived from clinically matched subjects (patients with seasonal affective disorder and normal controls). The His452Tyr heterozygotes showed significantly smaller amplitude of intracellular Ca^{++} mobilization after stimulation with two different doses of 5HT compared to the His452His homozygotes. There was also no apparent effect of increasing concentrations of serotonin to augment calcium mobilization in the heterozygotes compared to a clear dose-response relationship seen in the His452His homozygotes. However, the T102C SNP was not found to be in linkage disequilibrium with these two potentially functional SNPs discussed above and continued to independently associate with the schizoaffective disease state (Erdmann et al 1996; Spurlock et al 1998). Spurlock et al (1998) and Nothen and Propping (1998 - unpublished) identified an A-G polymorphism at -1438 in the promoter region of the 5HTR2A which was in complete linkage disequilibrium with the silent T102C SNP, also confirmed by Ohara et al (1999) and Basile et al (2001). Two clinical studies examining the 5HTR2A -1438G/A promoter polymorphism showed an association with increased food and alcohol intake in those with the G allele (Aubert et al 2000) and a higher BMI, waist to hip ratio and abdominal sagittal diameter in homozygotes for the G allele compared to homozygotes for the A allele (Rosmond et al 2002).

Functional analysis of A-1438G using luciferase assay demonstrated significant basal promoter activity in serotonin expressing HeLa cells by both the A and G variants. Comparison of the A and G variants however, showed no significant differences in basal activity or when promoter activity was induced by cAMP and protein kinase C-dependent mechanisms (Spurlock et al 1998). Though these are disappointing findings, the 5HTR2A gene is greater than 20kbp in length and thus, it is possible

that other areas that affect expression of the gene may vary and have not as yet been identified.

The T102C SNP in the 5HTR2A gene, despite being silent, has been chosen in the context of OSAHS to examine for associations in relation to obesity, hypertension and potential responsiveness to selective serotonin re-uptake inhibitors. The serotonin 2A receptor has been identified as the primary receptor on motor neurones involved in the function of the hypoglossal nerve and is thereby instrumental to the regulation of upper airway tone.

The size of the 5HTR2A gene which continues to be sequenced suggests that the silent SNP T102C studied is in linkage disequilibrium not only with the A-1438G SNP but potentially with other, as yet unidentified polymorphisms which may affect function of the gene.

4. Beta-2 adrenoceptor (SNP: C+79G).

The structure of ADRB2 is similar to other G protein coupled receptors. The amino terminal end is localized extracellularly, has seven transmembrane spanning domains and an intracellular carboxyl-terminus.

The C+79G SNP is one of nine polymorphisms in the coding block of ADRB2. C+79G is one of four SNPs that change and encode amino acid and is found in the amino terminus of the receptor. The change is designated Gln27 → Glu. Initial studies have focussed on the association of the various SNPs in the ADRB2 with asthma and response to β_2 -agonist treatment.

The consequences of the alterations in receptor function have been assessed by using recombinant expression systems where each SNP is mimicked using oligonucleotide-directed site-specific mutagenesis of the wild-type ADRB2 cDNA. These constructs

are inserted into mammalian expression vectors and transfected into Chinese hamster fibroblasts that normally do not express ADRB2. Permanent cell lines are then established and receptor function is studied (Green et al 1993; Green et al 1994).

Findings for the Glu27 variant showed that agonist binding and coupling to adenylyl cyclase remained intact, but that agonist downregulation was absent.

Studies by Green et al (1993 & 1994) in human bronchial smooth muscle cells obtained by rapid autopsy in disease-free individuals showed about 25% downregulation in receptor expression in the Glu27 variant cells. This was much less however, than for some of the other SNPs, e.g. the Arg16 – Gly SNP. Liggett (1997) summarises at length the associations of the various SNPs with asthma in a number of populations. The results of these studies have not been consistent in their findings (Martinez et al 1997; Tan et al 1997; Israel 2000). It has now been demonstrated that haplotype for a total of 13 SNPs in the upstream and coding sequences of the ADRB2 appear to be a better predictor of agonist response with 5 different haplotypes relatively common among Caucasians (Drysdale 2000). This is an example of how determination of haplotype may be more useful than a single SNP in predicting treatment responses in a diseased population.

All the SNPs located in the ADRB2 are in tight linkage disequilibrium. It should also be noted that the ADRB2 gene is localized to chromosome 5q32-34 very close to several cytokine genes. It is thus possible that the individual SNPs of the ADRB2 may be markers for the action of cytokines in various pathological processes.

As discussed above in sections 2.2.2 and 2.3, ADRB2 mediates response to catecholamines, is therefore involved in the energy pathways of the body especially

in regard to lipolysis and has also been implicated in the growth process and the development of hypertension.

In this thesis it was postulated that in OSAHS, the ADBR2 SNP Glu27Gln may show an association with obesity (as a result of poor energy expenditure) and possibly independently associate with hypertension. Knowledge of a differential response to exercise according to genotype may help predict those who respond to certain lifestyle and behavioural modifications, thereby enhancing their clinical management.

5. Growth Hormone Receptor (SNP: + 561 T/G).

As discussed previously in section 2.2.2, the GHR gene consists of 9 coding exons, spanning at least 87kb of chromosome 5. Exon 2 encodes the signal peptide; exons 3 – 7 the extracellular domain, exon 8 the transmembrane domain and exon 9 and part of exon 10 the intracellular domain (Godowski et al 1989). The majority of mutations in the GHR gene are located in the portion involving exons 3 – 7 which also codifies for the GH binding protein (GHBP) generated by limited proteolysis of the GHR (Leung et al 1987). This accounts for the majority of patients with growth hormone insensitivity having lower levels of GHBP (Daughaday & Trivedi 1987).

Over 30 different missense, nonsense, frameshift and splice mutations of the GHR gene have been reported, which also include the P561T polymorphism which occurs in exon 10. This SNP results in an amino acid substitution Pro561 → Thr but its functionality *in vitro* has not been assessed. Goddard et al 1997 reported this SNP to occur at a frequency of 2% in 102 control subjects in their large study of GHR polymorphisms. Chujo et al (1996) identified the SNP in a Japanese child with Noonan syndrome who showed a blunted IGF-1 response to an acute injection of

growth hormone. Forty-one men and 55 women aged 20 – 80 years were recruited from the population and tested for presence of the SNP. Fourteen of the 96 (15%) were heterozygous for the P561T mutation but it was not associated with any differences in body height. Yamaguchi et al (2001) aimed to quantitatively evaluate the relationship between craniofacial morphology and the Pro561Thr (P561T) variant in the GHR gene in 80 subjects taken from the Japanese population. They showed that subjects without the P561T allele had a significantly greater mandibular ramus length as measured cephalometrically compared to those with P561T. They postulated that the GHR gene P561T variant may be associated with mandibular height growth and may be a genetic marker for it.

There have been no further functional studies in the area of GHR SNPs in terms of examining binding or signalling properties *in vitro*. Only 3 larger studies have examined the prevalence of various mutations in the GHR in terms of *in vivo* responses to GH and GHBP levels (see Sjoberg et al 2001; Goddard et al 1997; Sanchez et al 1998).

The +561G/T SNP in the GHR has been chosen to act as a marker for possible disease associations as it may be in linkage disequilibrium with potentially more functional polymorphisms. GHR SNPs have been associated with postnatal bone and soft tissue growth and with obesity. The GHR P561T polymorphism (within 10kb of the +561 T/G SNP in this study) has recently and uniquely been associated with mandibular size in a group of Japanese subjects (Yamaguchi 2001).

2.11 Summary

In this chapter, the hereditary aspects of OSAHS have been discussed. It is a complex, polygenic disease with a number of aetiologies interacting to produce a single phenotype. The aims of the thesis are to investigate the possible roles of 5 candidate genes in the aetiology of OSAHS. The methods used in this study are presented in Chapter 3.

CHAPTER 3

METHOD

This chapter outlines the rationale, tests and techniques used in this thesis to examine the variables of sleepiness and sleep disordered breathing, the craniofacial complex, and the presence of genetic polymorphisms in the study population. The statistical methods employed in the subsequent analyses of the results are also discussed.

3.0 Introduction

A case-control study based on the recruitment of sib-pairs commenced in January 1997 with completion in December 2002 and data analysis in February 2003.

In order to augment the recruited numbers, data from another study conducted at the Scottish Sleep Centre were included in the final analysis (Vennelle et al 2003) as were 192 random anonymous UK blood donors to act as additional controls (<http://www.ecacc.org.uk>).

The study was approved by the Lothian Research Ethics Committee, the Wellcome Trust Clinical Research Facility Scientific Advisory Committee and had Research and Development approval from the Lothian University Hospitals NHS Trust. All participants in the study signed a written informed consent (copies Appendix 2 & 3). Copies of the consent form and an information letter were also sent to each participating subject's general practitioner (Appendix 3). All subjects with abnormal sleep study results were notified by letter, as was their general practitioner.

3.1.1 Recruitment of subjects

Between 1997 and 2000, 296 consecutive patients attending the sleep laboratory were chosen as they had symptoms of OSAHS, a body mass index less than 30kg/m² and an AHI \geq 15. Recruitment was undertaken directly in the Sleep Centre by approaching

patients and by a mail-out based on a register of all patients attending for studies previously and who fulfilled criteria. Index cases in the mail-out lived within a 50-mile radius of Edinburgh. Each index patient was sent a letter requesting participation in the study (Appendix 2). The letter requested that the index cases contact their siblings. As we sought a sleep apnoea enriched relative group, siblings who were willing to participate were asked to complete and return a short questionnaire about their sleep, sleepiness and snoring (Appendix 2). The siblings who were sleepest or snored were then asked to attend the Scottish Sleep Centre for polysomnography, cephalometry and to have blood taken. If the index case had only one other sibling, he/she was requested to participate regardless of their sleepiness or snoring status.

Response rates for this phase of the study were:

165 index cases agreed to participate in the study (165/296)

97 index cases had siblings who were willing to participate

21 siblings defaulted despite originally agreeing to participate in the study

76 sib-pairs completed the study

8 pairs had inadequate or missing blood samples.

This left a total of 68 complete sib-pairs.

The second phase of recruitment for the study commenced in 2001 and was completed in 2002. The inclusion criteria for the index cases were changed to a BMI $\geq 30\text{kg/m}^2$ with an AHI ≥ 15 and symptoms of OSAHS. The reason for the shift in recruitment criteria was to augment the number of obese cases with OSAHS. Index cases were taken from the register of patients on CPAP at the Scottish Sleep Centre who lived within a 50-mile radius of Edinburgh. Potential index cases were also approached during sleep clinics and at two annual meetings of the Scottish Association for Sleep Apnoea.

An invitation to participate in the study was given or mailed out (Appendix 4 for study protocol and letters) and followed up by a telephone call. A second letter was sent to participants who did not respond. 261 index cases were approached of whom 147 could be reached by telephone or responded to the letter.

58 of 147 index patients participated (4 had died, 10 declined outright, the remainder because they had no sibs or their siblings were unavailable; 7 index patients defaulted after originally agreeing to participate).

38 siblings participated (3 index cases had 2 siblings each participating)

35 sib-pairs completed the study.

The overall participation rates for the study were thus:

162 of 557 index subjects contacted had siblings and were willing to participate in the study (29% response rate); 103 sib-pairs (209 subjects in total) completed the study with blood samples available (18%). The 557 subjects approached represent 0.01% of the total Scottish population (~ 5 million).

The lowish participation rate overall is largely attributable to the availability of siblings who could travel easily to the Scottish Sleep Centre in Edinburgh. There has been widespread migration from Scotland in the last 50 years, which is reflected in the Census data over that period (General Register Office for Scotland 2002).

Younger subjects with few or no sibs reflected the societal trend for smaller families in the last 30 years and in the older patient groups (now in their 50s and 60s) where larger families were more common, siblings had passed away, emigrated or were more likely to have a chronic illness or increased frailty which precluded long-distance travel.

3.1.2 Data collection

All subjects had height; weight and neck circumferences measured and were asked to fill out a standard sleep questionnaire that has been extensively validated in the Scottish Sleep Centre (see Appendix 5 for copy of questionnaire).

Height was measured without shoes and socks in centimetres using a stadiometer. Weight was measured in light clothing without shoes on an electronic scale in kilograms. Neck circumference was measured with the subject seated with a soft dressmaker's tape measure at the level of the thyroid cartilage (in centimetres).

All siblings had overnight polysomnography (see section 3.3 below). Index patients had either overnight PSG or a home study (see section 3.3 below). All subjects participating in the study had cephalometry (see section 3.2 below) and 20 mls of blood collected (see section 3.4.1 below). Blood pressure was measured on one occasion using a mercury sphygmomanometer.

All data were recorded manually and then transferred onto an electronic spreadsheet.

Where required, travelling expenses for participating siblings were paid.

3.1.3 Additional Subjects without a diagnosis of OSAHS

In order to augment the number of subjects without a diagnosis of OSAHS, we utilised bloods obtained from a previously conducted study at the Scottish National Sleep Centre (Vennelle et al 2003) aimed at examining the polysomnographic and cephalometric parameters in parents of SIDS victims. Although a number of papers have been published suggesting that there is a possible increase in the prevalence of OSAHS in families of SIDS victims (Oren et al 1987; Tishler et al 1996; Mathur and Douglas 1994; Pinholster 1995), there is no proof of causality, or indeed anything more than a tenuous link between OSAHS and SIDS despite the use of sophisticated

statistical analysis or conducting a population-based study (Gislason et al 2002). Use of DNA and sleep study data obtained in this study, represents a valid study group from the same Scottish population. The method of case selection was as follows: SIDS victims were identified by the Departments of Paediatric Pathology in the Royal Hospital for Sick Children in Edinburgh and Yorkhill Hospital, Glasgow. In all cases, expert medical and legal staff, following a combination of inquiry and a detailed post-mortem examination, had excluded known causes of sudden infant death syndromes. The parents of 265 consecutive SIDS cases were then contacted and invited to take part in this study, all approaches being made through the family practitioner on whose list the SIDS victim had been at the time of death. The parents of 57 of the SIDS victims could not be traced by the family practitioner and the family practitioners indicated that the parents of 12 victims should not be contacted for emotional reasons. The parent(s) of 171 of the 196 SIDS cases were located. 119 pairs of parents declined to participate in the study and 18 pairs agreed to take part but did not attend for their sleep studies. 55 parents of 34 SIDS victims participated. Control subjects for this study were obtained from family practice registers and were matched with the parents studied on a one to one basis for gender, age within 5 years, height to within 5 cm and weight to within 5 kg. Each was approached by an independent research guardian and asked to participate in an unspecified medical research study. 132 possible subjects were approached before 55 suitable controls agreed to participate. The Local Medical Ethical Advisory Committees approved the study and all parents and controls gave written informed consent (see appendix for copy of consent form and information sheet). Consent included the collection of 20mls of venous blood for future DNA analysis.

All participants in the study completed the standard clinical sleep symptoms questionnaire (Appendix 5) and underwent overnight polysomnography in the Sleep Centre as discussed below (section 3.3). Prior to the sleep study, all subjects had their height and weight measured as detailed above and all were asked to have lateral cephalometry performed to determine upper airway dimension and facial bone structure (section 3.2).

Of the subjects recruited, 34 complete data sets (blood, cephalometry and sleep study) were available for inclusion in this study – comprising 24 SIDS parents and 10 controls.

3.1.4 Phenotyping

Phenotype for OSAHS was defined using Table 6 in Chapter 1. Each subject's AHI was first scored as either definitely abnormal (1), indeterminate (2) or definitely normal (0) on the basis of gender and age. Each subject's ESS was then scored as either sleepy = 1 ($ESS \geq 11$) or not sleepy ($ESS < 11$) = 0.

OSAHS was then classified as being definitely present, indeterminate or definitely absent as represented in Table 21 below.

Table 21: Presence or absence of OSAHS phenotype using scores derived for SDB and sleepiness

| SCORE FOR SDB | SCORE FOR SLEEPINESS | OSAHS PHENOTYPE |
|---------------|----------------------|-----------------|
| 1 | 1 | OSAHS |
| 1 | 0 | INDETERMINATE |
| 2 | 1 | OSAHS |
| 2 | 0 | NO OSAHS |
| 0 | 1 | NO OSAHS |
| 0 | 0 | NO OSAHS |

3.1.5 Blood donors

As a second set of controls, DNA from UK Caucasian human random control DNA panels (Product No. HRC-1 96 array and No. HRC-2 96 array) produced by ECACC[®] (European Collection of Cell Cultures; <http://www.ecacc.org.uk>) and distributed by Sigma[®] was used. The 96 array panels are intended as an aid to determining the characteristics of DNA from apparently normal, randomly selected subjects as a basis for comparison with DNA from subjects that have been selected for particular phenotypic characteristics. The arrays can be used directly in automated gene analysis systems.

Human random control (HRC) DNA is extracted from lymphoblastoid cell lines derived by Epstein Barr Virus (EBV) transformation of peripheral blood lymphocytes from fresh, single donor blood samples. The donors selected for this study were all UK Caucasian and were characterised (incompletely) by gender and age at venesection. All donors had given written informed consent for their blood to be used for research purposes.

3.2 Cephalometry

3.2.1 Technique of cephalometry

Hofrath in Germany and Broadbent in the USA first introduced radiographic cephalometry in 1934 (Moyers 1988). The original purpose of cephalometry was for research on growth patterns in the craniofacial complex. Apart from its utility in following treatment progress in the sphere of orthodontics, cephalometry is useful in the evaluation of dentofacial proportions and in the clarification of the anatomic basis for malocclusion. The goal of cephalometry is therefore to establish the horizontal and vertical relationships between the functional components of the face – the cranium

and cranial base, the skeletal maxilla (the portions of the maxilla that would remain if there were no teeth or alveolar processes), the skeletal mandible (similarly defined), the maxillary dentition and alveolar process, and the mandibular dentition and alveolar process.

There are two basic approaches in cephalometry. The first approach pioneered by Down (see Moyers 1988) and followed by most workers in this field is the use of selected linear and angular measurements to establish appropriate comparisons between the functional components of the face. The other approach is to express the normative data graphically rather than as a series of measurements and to compare the patients' dentofacial form directly with the graphic reference (template). Any differences can be observed without making measurements.

In our study, we recorded the measurements and angles on each subject's radiograph and focussed on the bony components of the craniofacial complex rather than on the dentition in an effort to establish craniofacial characteristics that might be associated with OSAHS. Because 50% of the sample was on CPAP, soft tissue measurements were not analysed in this study (Mortimore et al 1996; Schwab et al 1996). Neck angles were also not analysed.

3.2.2 Method of measurement and recording of results

Radiology

Cephalometric roentgenograms were carried out in the Department of Radiology at the Royal Infirmary Edinburgh using the Orthoceph ® 10 (Type No.: D 3198; Siemens AG, UB Med). Subjects were seated and facing at 90° to the X-ray beam (see Fig 8), 152.4cm (60 inches) from the target of the x-ray tube with their left side toward the film. The ear olives were placed into the external auditory meati and the

nasal root supported and aligned with the nose support to prevent lateral head tilt or rotation. The distance of the median plane to the film was 18cm. The subjects were directed to face forward holding their heads in a natural position. They were asked to close their mouths with their normal resting occlusion and their lips together to allow the tongue to relax onto the floor of the mouth and not to swallow during the exposure. The peak kilo voltage was adjusted to optimise the contrast of both hard and soft tissues. Generally, for an average built subject, the voltage and exposure times were kV 71, mAS 15, 0.3 seconds and for those more obese - kV 77, mAS14, 0.4 seconds. Magnification was 1:1.1 for all films. Radiographs were taken with the subject exhaling slowly from a deep breath by radiographers familiar with the procedure. The films were taken using a 24X30 fast speed cassette with Agfa© Ortho H film and Curix® Standard screens (Agfa Ltd).

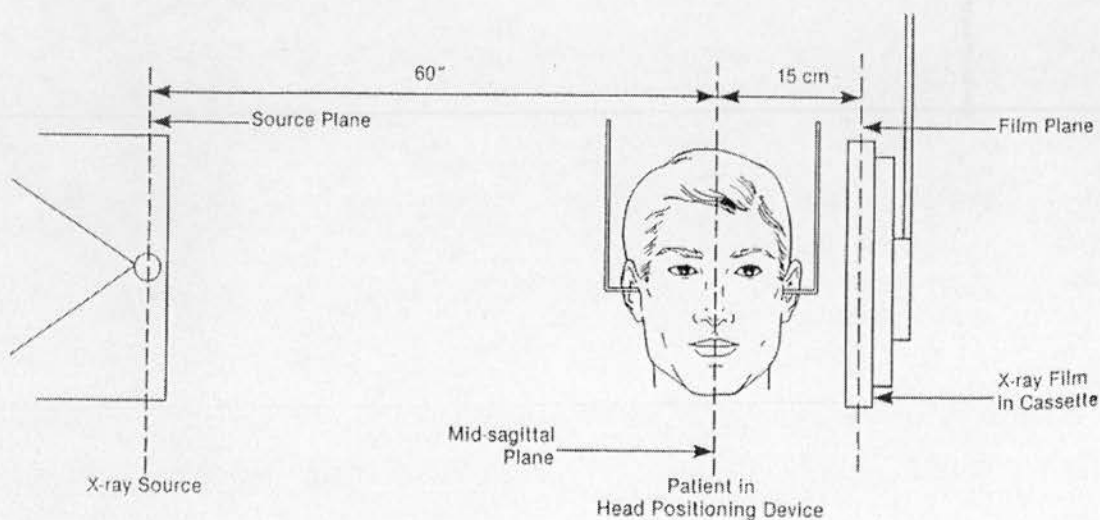


Fig 8: Technique of performing cephalometry

Measurement

In this study the reference work for all points and planes used was by Rakosi (1982).

All radiographs were secured with tape on a light box. Acetate tracing paper was placed on the radiograph and the points, lines and angles traced by hand using a fine tip permanent pen (0.5mm) and measured using a ruler (millimetre markings) and protractor. All measurements taken were recorded manually on a data collection sheet separately for each subject and converted to life size.

Cephalometric landmarks are represented in Fig 9 and planes in Fig 10. Table 22 shows the measurement abbreviations with their definitions.

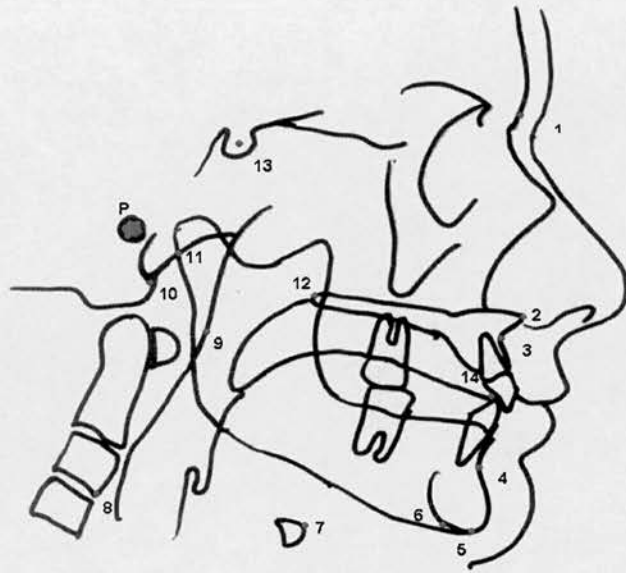


Fig 9 Cephalometric Points of Reference

(1 =Na; 2 =ANS; 3 =point A; 4 =point B; 5 = Gn; 6 = RtGn ; 7 = H ; 8 = C3 ; 9 = Post. Phar. wall ; 10 = Ba ; 11 = Ar ; 12 = PNS; 13 = Sella; 14 = tip of tongue; P = porion)

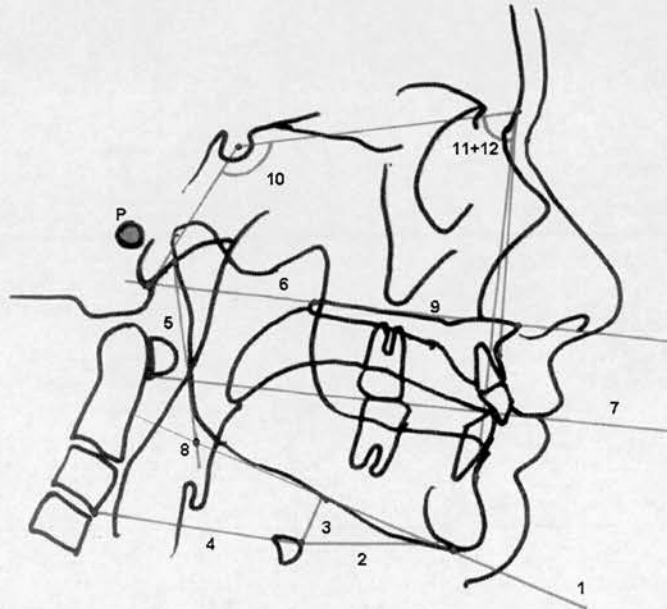


Fig 10 Cephalometric Planes and Angles

(1 = Go-Gn; 2 = H-RtGn; 3 = H-MP; 4 = C3-H; 5 = Go-Ar; 6 = PNS-Ba; 7 = Int max length; 8 = Go; 9 = ANS-PNS; 10 = SN-Ba; 11 = SNA; 12 = SNB; P = Porion)

Table 22: Abbreviation of Cephalometric Distances and Angles

| ABBREVIATION | DEFINITION |
|---------------------|---|
| Go-Gn | Distance from gonion to gnathion |
| Go-Ar | Distance from gonion to articulare – the intersection of a line along the posterior border of the mandibular process and the temporal bone |
| ANS-PNS | Distance between anterior nasal spine and posterior nasal spine |
| PNS-Ba | Horizontal distance from posterior nasal spine to basion |
| H-MP | Distance from the anterior-superior point of the body of the hyoid bone perpendicular to mandibular plane (a plane constructed from gnathion to gonion) |
| C3-H | The distance between the inferior and anterior tip of the C3 vertebra and hyoid |
| H-Rtgn | Distance from hyoid to retrognathion (most infero-posterior point of the mandibular symphysis) |
| ANSFOP | Anterior maxillary length, vertical line between functional occlusal plane and anterior nasal spine |
| GnFOP | Anterior mandibular length, vertical line between gnathion and functional occlusal plane |
| Int max length | Internal maxillary length – linear distance measured along the facial occlusive plane from the intersection with tongue tip anteriorly to where it intersects posterior pharyngeal wall |
| Post max height | Post maxillary height - perpendicular distance between ANS-PNS plane to Gn-Go plane which passes through the point where functional occlusal plane intersects the posterior pharyngeal wall |
| SNA | Angle between lines from sella to nasion and nasion to point A (subspinale) |
| SNB | Angle between lines from sella to nasion and nasion to B (submentale) |
| NS-Ba | Angles between lines from nasion to sella and sella to basion, cranial base angle or cranial base flexure |
| ANB | Angle difference between SNA and SNB which indicates skeletal class (1 – orthognathic; 2 – retrognathic; 3 - prognathic) |

3.2.3 Reproducibility

All studies were scored blind to index/control status and sleep study result and a research assistant performed the blinding.

Two measurers scored all studies (MV, RR) and had 3 calibration sessions together prior to performing their number of measurements independently.

No facilities were available for digitisation.

Although cephalometric measurements are made in accordance with strict criteria, there is a subjective element in performing and measuring radiographic tracings. This is due to variability in the quality of the radiographs in terms of exposure (film density and sharpness), rotation in the cephalostat, imprecise anatomic definitions of landmarks, degree of osteoporosis in the subject, presence or absence of teeth and oedema in soft tissue in the upper airway as well as in the experience of the measurer.

Therefore, inter-rater and intra-rater reliability was evaluated by reanalysing a randomly selected number of studies as follows: 10 radiographs originally traced by Operator 1 (MV) retraced by Operator 2 (RR); 10 radiographs originally traced by Operator 2 (RR) retraced by RR. Operator 1 (MV) also retraced 10 cephalograms that she had originally scored. All reassessments were done blinded to previous outcome and at least 2 weeks apart.

Results for inter- and intra-rater reproducibility for four measurements and 3 angles: is shown in Table 23. The correlation coefficient (using Spearman's rank correlation) for both inter- and intra-rater scores was high. Additionally, the mean differences between the initial (measurement 1) and subsequent measurements (measurement 2) demonstrate high reproducibility.

Table 23: Inter and Intra-rater Reproducibility for Cephalometric Measurements

| Cephalometric Variable | Score 1 Mean (sd) | Score 2 Mean (sd) | Typical Error | Spearman's ρ | p-value |
|--|-------------------|-------------------|---------------|-------------------|----------|
| Inter-rater Reliability (n = 20) | | | | | |
| GoGn | 81 \pm 5 | 80 \pm 3 | \pm 2.6 | 0.8 | 0.004 |
| GoAr | 60 \pm 6 | 58 \pm 7 | \pm 2.5 | 0.95 | < 0.0001 |
| ANSFOP | 32 \pm 3 | 32 \pm 4 | \pm 1.9 | 0.8 | 0.006 |
| GNFOP | 47 \pm 3 | 47 \pm 4 | \pm 3.5 | 0.95 | < 0.0001 |
| SNA | 81 \pm 4 | 79 \pm 3 | \pm 2 | 0.8 | 0.005 |
| SNB | 78 \pm 4 | 78 \pm 4 | \pm 2 | 0.93 | < 0.0001 |
| NSBA | 132 \pm 6 | 131 \pm 5 | \pm 2.5 | 0.8 | 0.004 |
| Intra-rater Reliability (MV) (n = 10) | | | | | |
| GoGn | 81 \pm 5 | 80 \pm 3 | \pm 1.6 | 0.8 | 0.004 |
| GoAr | 60.4 \pm 6 | 58 \pm 7 | \pm 2.3 | 0.95 | < 0.0001 |
| ANSFOP | 32.5 \pm 3 | 32 \pm 4 | \pm 1.5 | 0.8 | <0.0001 |
| GNFOP | 47 \pm 3 | 47 \pm 3 | \pm 0.8 | 0.95 | <0.0001 |
| SNA | 81 \pm 4 | 79 \pm 3 | \pm 2 | 0.8 | 0.005 |
| SNB | 78 \pm 4 | 78 \pm 4 | \pm 1 | 0.94 | <0.0001 |
| NSBA | 132 \pm 6 | 131 \pm 5 | \pm 3 | 0.82 | 0.004 |
| Intra-rater Reliability (RR) (n = 10) | | | | | |
| GoGn | 80 \pm 4 | 82 \pm 7 | \pm 2.3 | 0.93 | < 0.0001 |
| GoAr | 59 \pm 8 | 59 \pm 9 | \pm 1.7 | 0.93 | < 0.0001 |
| ANSFOP | 33 \pm 3 | 33 \pm 3 | \pm 1.1 | 0.87 | 0.001 |
| GNFOP | 43 \pm 3 | 44 \pm 2 | \pm 1.5 | 0.75 | 0.012 |
| SNA | 83 \pm 6 | 82 \pm 5 | \pm 1 | 0.97 | < 0.0001 |
| SNB | 80 \pm 4 | 79 \pm 4 | \pm 1 | 0.9 | 0.001 |
| NSBA | 126 \pm 7 | 127 \pm 7 | \pm 0.8 | 0.93 | < 0.0001 |

All measurements in mm except for SNA, SNB and NSBa measured in degrees

The cephalograms for the subjects included from the SIDS study were all scored by one operator (MV) who was blinded to case status.

Random method error in the series of radiographs was assessed using Dahlberg's method (Dahlberg 1940) and ranged from 0.46 to 1.6 mm and 0.035 – 0.64 degrees.

Systematic error, which is revealed when applying a t-test at the 10% level (Houston, 1983), was also assessed. Houston's coefficient of reliability was calculated for all repeated measurements and ranged from 86 – 95%. No systematic errors were detected.

3.3 Polysomnography

3.3.1 Technique of measurement and measurement parameters recorded

Home – based Sleep Studies

Home-based sleep study monitoring was used to obtain a diagnosis of OSAHS in 7% (n=16/228) of the cases.

This was performed using the limited sleep study system Edentrace (EdenTec Model 3711 Digital Recorder, Nellcor, Eden Prairie, Minnesota, USA) that has been extensively validated against polysomnography (Parra et al 1997; Whittle et al 1997).

This device records oronasal airflow using a thermocouple, chest wall movement by electrical impedance, heart rate using an electrocardiogram, and finger pulse oximetry. There is no EEG recorded.

Patients are instructed in the application of the system by the sleep technician on the afternoon of their study and then sleep overnight at home. The equipment is returned the next morning. Patients are requested to keep a note of the time they retire in the evening and the time at which they rise. This time in bed is then assumed to represent roughly the total sleep time.

All information recorded during the night is stored electronically. This is subsequently downloaded, printed out as a trace on paper and scored manually with frequencies of respiratory events calculated per hour of recording.

Apnoea is defined as the cessation of airflow for ≥ 10 seconds and hypopnoea as a reduction of $\geq 50\%$ in chest wall movement for ≥ 10 seconds. An apnoea-hypopnoea index thus obtained is considered significant for sleep disordered breathing if it measures greater than 30 events per hour.

In-lab Polysomnography

Two hundred and nine subjects (92%) underwent in-lab polysomnography. Studies were performed in soundproofed and electrically screened bedrooms at the Edinburgh Sleep Centre (hospital-based). (The three remaining subjects are discussed below).

Sleep was recorded using electroencephalography (EEG), electro-oculography (EOG) and electromyography (EMG) using bipolar signals from silver chloride surface electrodes. EEG was recorded using the 10/20-electrode placement system (Cooper et al 1980) from 2 scalp sites – Cz/Pz. Frontal EEG was also recorded using ‘mixed’ channels comprising EEG/EOG signals (Cz/Fp1, Cz/Fp2). The EOG was recorded from electrodes placed at sites on the outer canthus of the eye and Fp1 and Fp2. This allows both horizontal and lateral eye movements to be monitored. Submental EMG was recorded using 2 electrodes placed on the belly of the genioglossus. A grounding electrode was placed at Fpz. Electrodes placed on the right and left anterior tibialis recorded leg EMG. All signals were filtered using a high and low bandpass in order to reduce artefact in addition to 50Hz notch filtering. The aim was to achieve electrode impedances of less than 5 kOhms.

3.3.2 Sleep staging and scoring of respiratory events

With the exception of oxygen saturation, all sleep studies were scored manually using standard in-lab definitions (Gould et al 1988; Cheshire et al 1992).

Sleep was staged in 30-second epochs using the criteria developed by Rechtschaffen and Kales (1968). Sleep stages were classified from 1 – 4, REM sleep, a wake and movement time and are defined below.

Awake (Fig. 11): the EEG comprises alpha activity (8 – 11 Hz (cycles per second)) and /or low voltage, mixed frequency activity (beta waves greater than 16 Hz) for more than 50% of the epoch. Eye blinks on the EOG and high EMG activity may be present.

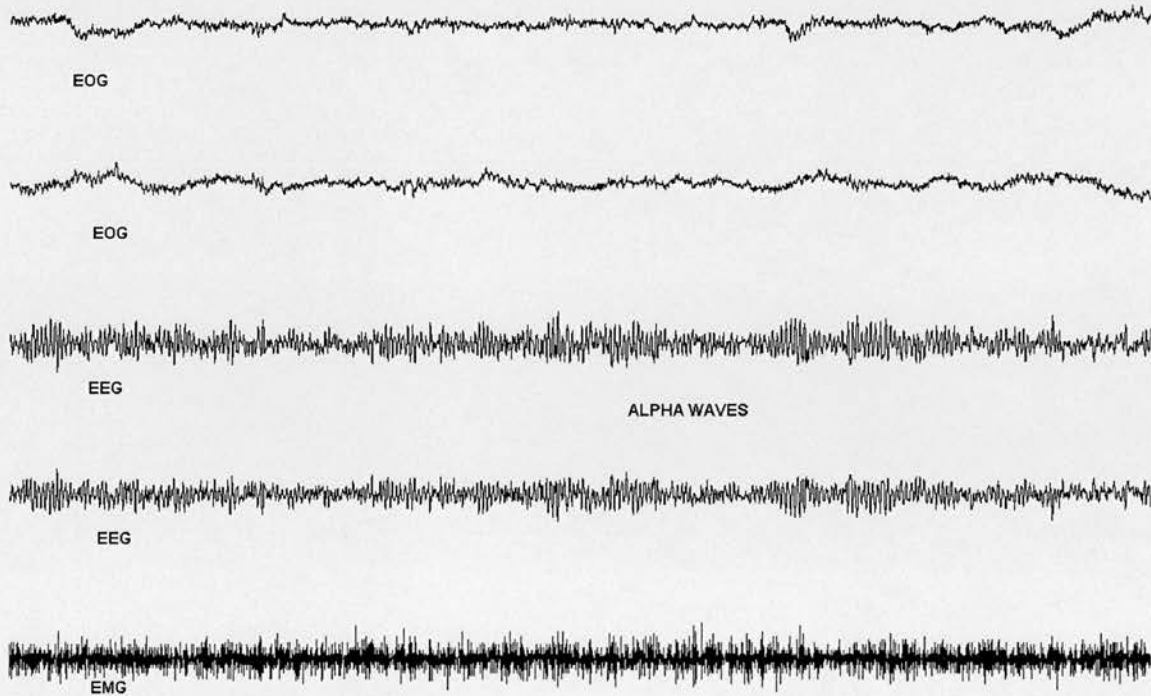


Fig 11: Example of Awake EEG

Stage 1: This is a transient sleep stage comprising relatively low voltage, mixed frequency EEG. More than 50% of the epoch should consist of theta EEG frequencies (4 – 7Hz). There is an absolute absence of clearly defined K complexes and sleep spindles. Slow eye movements may be present (approximately 7 seconds in

duration) – rapid eye movements are absent. Tonic EMG levels are below those of relaxed wakefulness.

Stage 2 (Fig12): is defined by the presence of sleep spindles and/or K complexes and the absence of sufficient high amplitude, slow activity to define the presence of stages 3 and 4. 50% of the epoch must comprise EEG frequencies in the theta range (4-7Hz). Sleep spindles must be minimum 0.5 seconds (up to 2 seconds) duration and comprise sigma frequencies (12 – 15 Hz). K complexes are EEG waveforms with a well-delineated negative sharp wave, which is immediately followed by a positive component lasting more than 0.5 seconds.

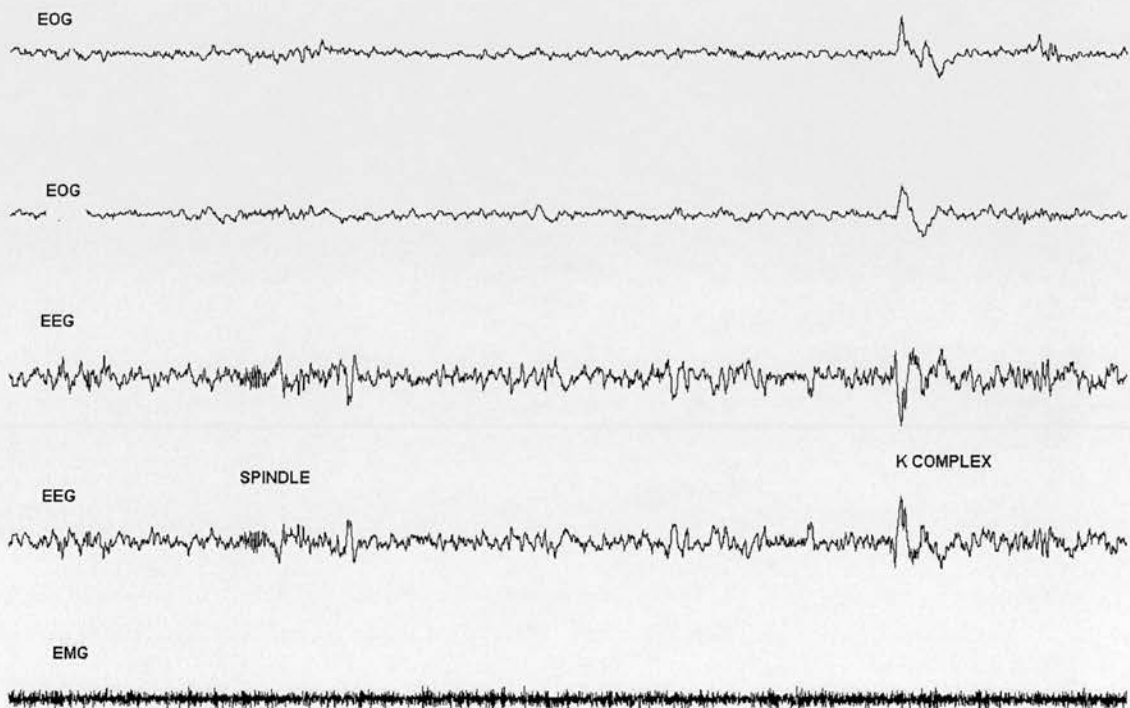


Fig 12: Example of Stage 2 Sleep

Stage 3: At least 20% but not more than 50% of the sleep epoch consists of delta waves (0 – 3Hz) not less than 75 uV in amplitude. Sleep spindles may or may not be present.

Stage 4 (Fig 13): The EEG record shows more than 50% of the epoch comprising waves of 0 - 3 Hz which have amplitudes greater than 75 uV. Sleep spindles may or may not be present.

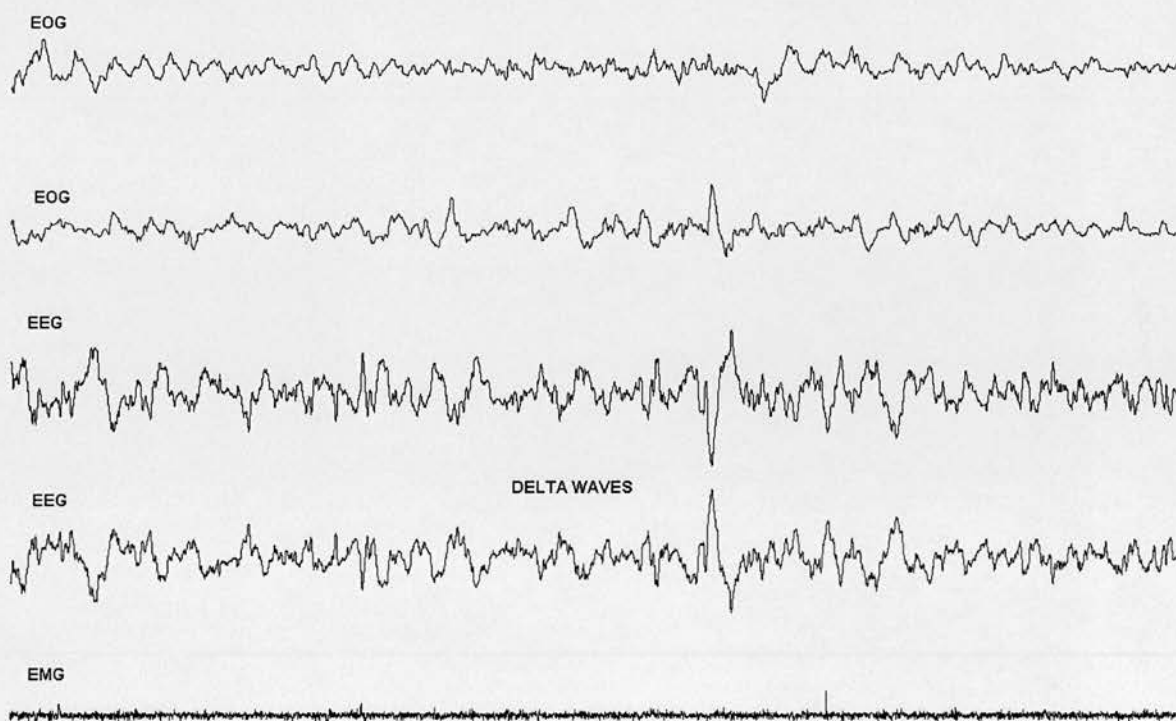


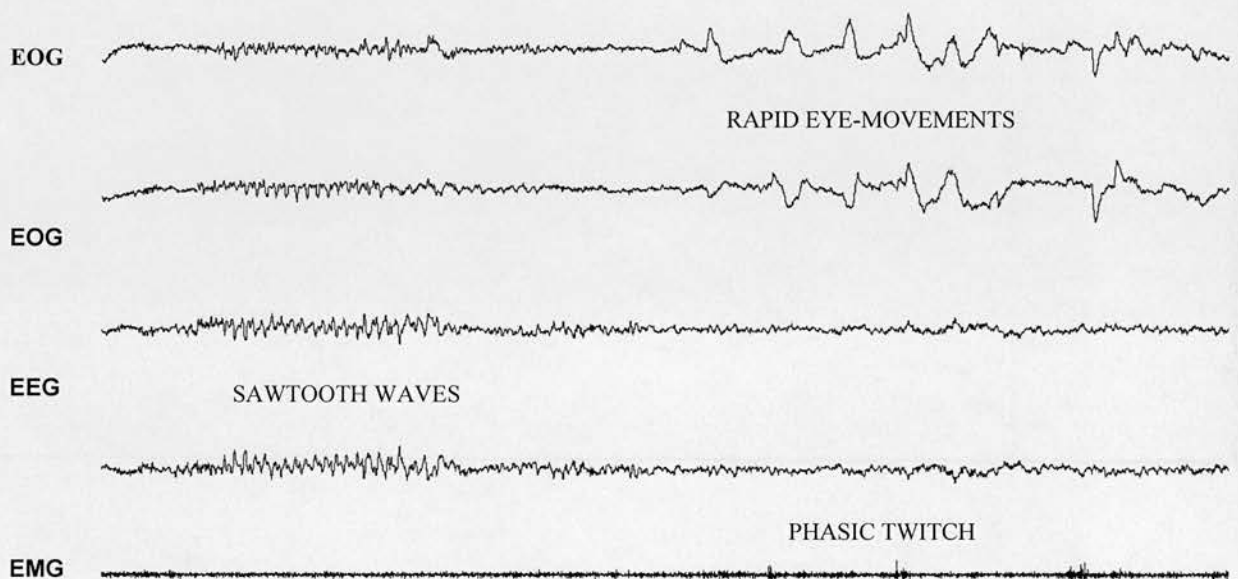
Fig 13: Example of Stage 4 Sleep

Stage REM (Fig 14): This stage is defined by the appearance, concomitantly, of relatively low voltage, mixed frequency EEG activity and episodic rapid eye movements. The EEG pattern resembles that of Stage 1. However, the vertex sharp waves are not prominent. Distinct ‘saw-tooth’ waves are often present in vertex and frontal regions in conjunction with bursts of REM. Alpha waves are prominent and the frequency is generally 1- 2 Hz slower than during wakefulness. There is an

absolute absence of sleep spindles and K complexes. REM should not be scored in the presence of relatively elevated tonic mental-submental EMG activity although phasic twitches do occur. In epochs where there are no rapid eye movements but the remainder of the record has the appearance of REM sleep, REM sleep should be scored provided 3 minutes has not passed since the last rapid eye movement. REM sleep always follows stage 2 or 4 sleep.

Rechtschaffen and Kales (1968) discuss more complex aspects of scoring REM sleep.

Fig 14: Example of REM Sleep



Movement time is a term used when more than 50% of a sleep epoch consists of movement artefact and the underlying stage cannot be determined from any EEG or EOG channel.

Normal young adults with good sleep hygiene will generally have a sleep architecture comprising less than 5% of awake time; 2-5% of stage 1, 45-50% stage

2, 3 – 8% stage 3, 10-20% stage 4 and 20 – 25% stage REM sleep (Carskadon and Dement 1989).

Patients with severe sleep disordered breathing often have disrupted sleep alternating between Stage 2 and awake. This presents a potential scoring difficulty according to the Rechtschaffen and Kales (1968) criteria that suggest Stage 1 always precedes Stage 2 after an awake period. In such cases, I followed this laboratory's policy, which is to score these epochs as stage 2 if they meet criteria.

Respiratory Events

These events are classified as central, mixed and obstructive apnoeas and hypopnoeas.

Central events are defined as the complete cessation of respiratory movement and flow for 15 seconds (some authors use 10 seconds)

Obstructive apnoea is defined as the complete cessation of flow for a minimum of 10 seconds but with continued respiratory effort throughout the apnoea.

Mixed apnoea is defined as the complete cessation of flow for a minimum of 10 seconds with respiratory effort initially absent at the beginning, but returning midway through the apnoea.

Hypopnoea (Fig 15) is defined as a minimum 50% reduction in thoracoabdominal movement for a minimum of 10 seconds.

Definitions of hypopnoea differ between laboratories. Some use a 50% reduction in flow as the guideline and others require an additional minimum 4% oxygen desaturation. The above definition has been validated in a series of patients with clinical features of sleep apnoea but who do not have apnoeas (Gould et al 1988) and is accepted as the current definition by the American Academy of Sleep Medicine (1999). The total number of apnoeas and hypopnoeas is calculated and divided by total sleep time to give the apnoea and hypopnoea index per hour of sleep (AHI).

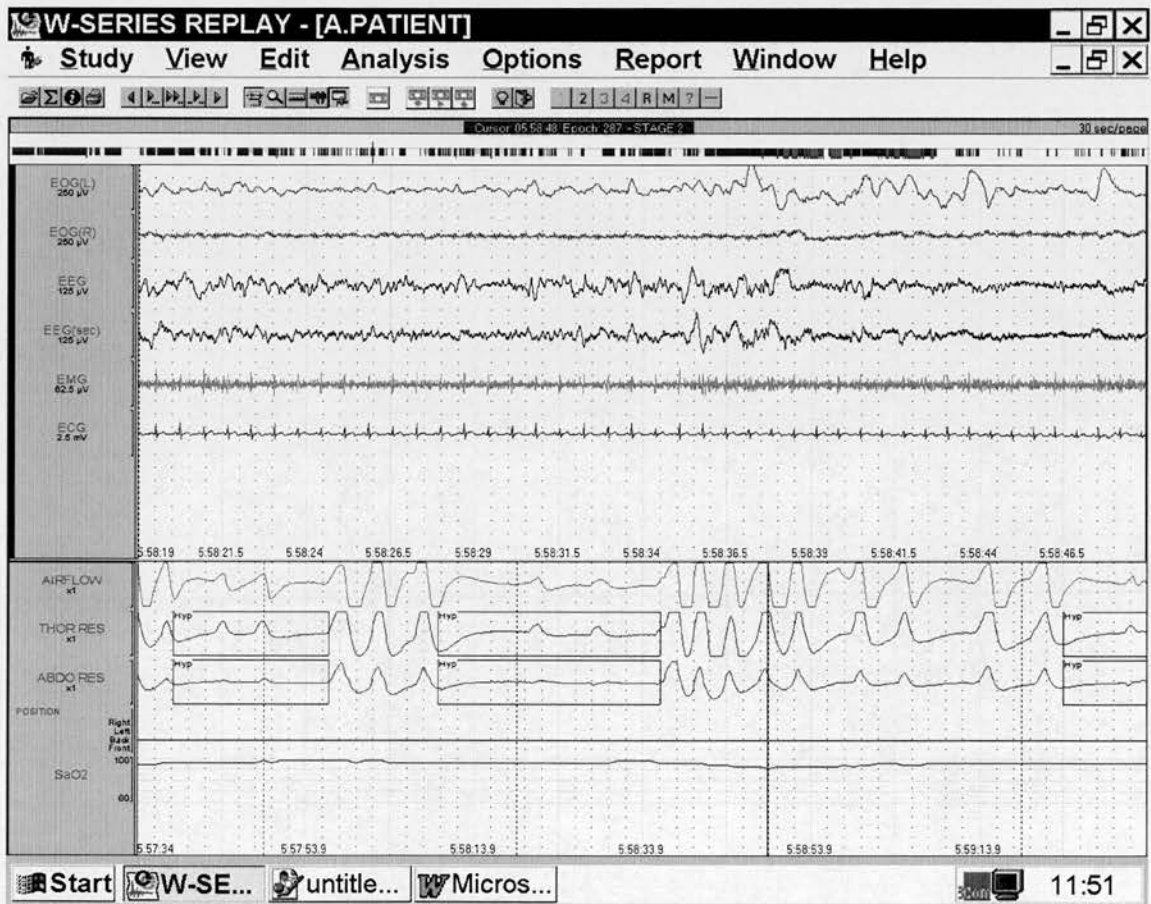


Fig 15: Example of a Hypopnoea on the Compumedics™ W-Series® Split Screen

Hypoxaemia

The frequency of oxygen saturation dips during sleep (SpO₂) of 2, 3 and 4 % from the running peak, along with minimum overnight saturation were analysed using an automatic desaturation detection algorithm (Compumedics S, Australia).

Oxygen saturation whilst awake (SaO₂) was measured manually from the oximetry channel prior to sleep onset on the overnight polysomnography where relevant.

Arousals were not scored as part of this work.

3.3.3 Reproducibility

All studies were scored blind to index and control status.

A research assistant (MV) not involved in the study performed the blinding.

Three different operators scored studies over the period 1997 – 2002 (PB, NMcA, RR). Although sleep stages and variability in breathing is scored according to strict criteria as outlined above, there is a subjective element in marking such events. This is due to variability in the quality of overnight recordings, unpredictability in the occurrence of respiratory events, arousals and sleep patterns found in the study population compared with a textbook pattern as well as in the experience of the scorer.

Therefore, inter-rater and intra-rater reliability was evaluated by reanalysing a randomly selected number of studies as follows:

9 studies originally scored by Operator 1 (PB) rescored by Operator 3 (RR)

9 studies originally scored by Operator 2 (NMcA) rescored by Operator 3 (RR)

12 studies originally scored by Operator 3 (RR) re-scored by RR (blinded to previous outcome) after a period of 3 months.

Results for inter- and intra-rater reproducibility for four criteria: AHI, REM-time, TST and sleep efficiency is shown in Table 24. The correlation coefficient (using Spearman's rank correlation) for both inter- and intra-rater scores was high. Additionally, the mean differences between the initial (score 1) and subsequent score (score 2) for operator 3 (RR) demonstrate high reproducibility.

Table 24: Scoring reproducibility for AHI, REM-sleep time, TST and Sleep Efficiency

| Nocturnal Variable | Score 1 Mean (sd) | Score 2 Mean (sd) | Typical Error | Spearman's ρ | p-value |
|---|-------------------|-------------------|---------------|-------------------|----------|
| Inter-rater Reliability (n = 18) | | | | | |
| AHI (per hour slept) | 18 (24) | 18 (24) | ± 2 | 0.91 | < 0.0001 |
| REM-sleep (mins) | 69 (24) | 76 (29) | ± 11 | 0.92 | < 0.0001 |
| TST (mins) | 336 (50) | 339 (60) | ± 25 | 0.79 | < 0.0001 |
| Sleep Efficiency (%) | 75 (10) | 75 (13) | ± 4 | 0.808 | < 0.0001 |
| Intra-rater Reliability (n = 12) | | | | | |
| AHI (per hour slept) | 21 (17) | 21 (18) | ± 2 | 0.91 | < 0.0001 |
| REM-sleep (mins) | 86 (15) | 86 (15) | ± 6 | 0.83 | 0.001 |
| TST (mins) | 391 (53) | 390 (55) | ± 4 | 0.94 | < 0.0001 |
| Sleep Efficiency (%) | 84 (11) | 84 (11) | ± 0.9 | 0.97 | < 0.0001 |

Finally, one index case had OSAHS diagnosed initially on overnight oximetry and two index cases were diagnosed on the basis of an AHI detected by a nocturnal trial of Autoset T®. These patients were not initially studied at the Scottish Sleep Centre in Edinburgh, but the diagnosis was considered sufficiently reliable to allow for inclusion in the study as both patients had all the hallmarks of OSAHS, had been commenced on CPAP therapy and were continuing on treatment.

3.4 DNA Extraction

3.4.1 Blood collection and storage

Venesection was performed on all subjects in the study at the initial interview.

20 mls of blood was collected after venepuncture at the cubital fossa using a 23 gauge needle and vacuum collection system directly into 2 x 10ml (or 4x 5ml) EDTA (ethylenediamine tetraacetic acid) tubes. Blood tubes were labelled with the name and date of birth of each subject, or alternatively they were labelled with the subject's study number.

Blood collected on subjects between 1997 and 2000 was subsequently packaged and sent directly by first class post to Oxford where it was stored and DNA extraction was performed (see section 3.4.2 below and Appendix 6).

Blood collected in 2001 – 2002 was stored in a -80°C freezer within hours of venesection in the Edinburgh Wilkie Laboratory, from which it was taken for processing.

Blood collected from subjects involved in the SIDS study was centrifuged and the red cells and plasma decanted and stored separately in Eppendorf™ tubes in a -80°C freezer. For DNA extraction purposes, the red cell and plasma portions were reconstituted at room temperature.

Prior to DNA extraction and archiving at the Wellcome Trust Research Facility, Edinburgh, all samples were labelled by code only so that no personal identification was possible at the laboratory during processing.

3.4.2 Method of DNA extraction

Two methods of DNA extraction were employed using two different commercially available kits.

Blood samples collected between 1997 and 2000 (n=138) were processed at the DNA Extraction Core Laboratory in the Wellcome Trust Centre for Human Genetics, Oxford using the Wizard[®] R Genomic DNA Purification Kit (Part # TM050; Promega[™]; <http://www.promega.com>). DNA was extracted from frozen 10ml sample volumes of blood. The procedure was based on cell lysis, isolation of white blood cells, nucleic lysis, protein precipitation and DNA extraction followed by an ethanol wash. DNA was rehydrated using a rehydration solution and incubated at 65°C for one hour (see Appendix for protocol). DNA was then stored frozen at –20°C until required.

The DNA samples were transported to the Wellcome Trust Clinical Research Facility Genetics Core Laboratory in May 2002 and subsequently stored in a –80°C freezer until analysed.

Working stocks of DNA were prepared at 50ng/ul. Measurement of mean purity using a spectrophotometer (at 260/280 Å) gave a result of 1.6 – 1.7 for all samples.

Blood samples collected in 2001 – 2002 (n= 73) and blood samples from the SIDS study (n = 17) were processed directly at the Edinburgh Wellcome Trust Clinical Research Facility Genetics Core laboratory using the Nucleon Extraction and Purification Protocol (Product Code: Nucleon BACC3 RPN 8512 for extraction of DNA from 10 ml of whole blood; © Amersham International plc).

The procedure involved cell lysis, deproteinisation, DNA extraction; DNA precipitation; DNA washing and DNA resuspension (see Appendix 6 for protocol).

Blood was taken out of storage and allowed to defrost at room temperature. Before the extraction procedure was commenced, 100µl of blood of each sample was spotted onto a Whatman FTA card, numbered and dated. The cards were stored at room

temperature in sealed bags containing silica desiccant. This procedure is part of the quality control protocol of the laboratory to enable a check DNA at the start and end of the extraction process, but was not required in this study.

Once extracted, the DNA in solution was placed on a rotary wheel and allowed to re-dissolve (one week) prior to storage at -2° to -8°C in the refrigerator (working stock) or -40°C (master stocks).

Working stock was prepared as described above and the concentration of DNA was measured using the spectrophotometer as above.

Blood Donor Panels

The DNA in the two 96-DNA array panels was received extracted and purified by ECACC. Their process yields high molecular weight material as verified by agarose gel electrophoresis. The composition of each 96-sample array is completely defined and standardised by ECACC so that each product lot will be identical. The identity of each product lot is checked using STR (short tandem repeat) DNA profiling.

3.5 Genotyping

Two different techniques were used to obtain data on genotype. For Apolipoprotein E, Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) was used and the remainder of the gene polymorphisms in the study (ADRB2; TNF- α ; GHR; 5HT2A) were determined using the TaqMan-based allelic discrimination assay (see 3.5.2 below). At present, an assay is not available for Apolipoprotein E using the latter technique.

Except where noted, staff of the Genetics Core did all following procedures. Twenty-five percent of DNA extractions as above were performed by me (RR) as well as the PCR-RFLP under the supervision of AC.

3.5.1 Polymerase Chain Reaction – Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

A genotyping procedure developed by Ossendorf and Prellwitz (2000) was used to examine the APOE alleles in the study sample. The technique represents a substantial improvement on previous PCR-RFLP techniques for APOE, which used only the *HhaI* restriction endonuclease site resulting in small fragments (19-91 base pairs) with subsequent poor resolution on agarose gel and other technical difficulties. This has in the past led to a high rate of misclassification (Lahoz et al 1996).

The new technique uses the primers E2mut and E3 to amplify a 318 bp region of the APOE gene that contains a variable number of restriction sites depending on the allele (E2, E3 or E4) and uses two enzymes for the digestion process: *HaeII* and *AfIII*.

The two primers used in the amplification process were:

1. Upstream primer **E2mut** (forward primer):

5' ACT GAC CCC GGT GGC GGA GGA GAC GCG TGC

(the underlined sequence differs from the genomic sequence, which creates the *AfIII* recognition site in the amplified fragment)

2. Downstream primer **E3** (reverse primer):

5' TGT TCC ACC AGG GGC CCC AGG CGC TCG CGG

The actual sequence of the PCR product is shown in Fig 16.

Fig 16: 318 base pair APOE sequence amplified during PCR-RFLP

```

actgacccccggtggcgaggagacgcgtgcacggctgtccaaggagctgcagacggcgagggc
ccggctgggcgcgacatggaggacgtgtgcggccgcctggtgcagtaccgcgggcgaggt
gcaggccatgctcggccagagcaccgaggagctgcgggtgcgcctcgctcccacctgcg
caagctgcgtaagcggctcctccgcgatcccgatgacctgcagaagcgcctggcagtgta
ccaggccggggcccgcgagggcgccgagcgcggcctcagcgccatccgcgagcgcctggg
gccctggtggaaca
    
```

* The two variable positions are highlighted – the first is the cleavage site for *AflIII* and the second for *HaeII*

Digestion of the PCR product by *HaeII* and *AflIII* results in fragments of variable size depending on the allele. Table 25 shows the RFLP pattern allowing for allele and genotype identification.

Table 25: PCR-RFLP pattern for APOE using *HaeII* and *AflIII*

| Genotype | RFLP (base pairs) according to enzyme | |
|----------|---------------------------------------|--------------|
| | <i>AflIII</i> | <i>HaeII</i> |
| E2 E2 | 231 | 267 |
| E3 E3 | 231 | 232 |
| E4 E4 | 295 | 232 |
| E2 E3 | 231 | 232 AND 267 |
| E2 E4 | 231 AND 295 | 232 AND 267 |
| E3 E4 | 231 AND 295 | 232 |

Preparation of the PCR Reaction Mixture

96 well plates were used for the PCR. Each well contained 50 µl of mixture comprising 5 µl of 1ηg/ul of DNA/H2O added to 45 µl of master mix.

The recipe for the master mix was as follows:

5 µl of 10x PCR buffer (15mM MgCl₂)

10 µl of 5 x Qiagen™ solution (Q)

1.25 µl of 10µM E2 mut f primer (Genosys™; 41.1nmol+41.1 nmol TE (Tris-EDTA) = 1µM solution)

1.25 µl of 10µM E3 r primer (Genosys™; 30.2 nmol+30.2 µmolTE = 1µM solution)

1 μ l 10mM dNTPs (deoxynucleotide mix; SigmaTM D-7295 0.2mls Lot 60K7029; 10mM solution)

0.3 μ l Taq polymerase (QiagenTM)

26.2 μ l H₂O

Each 96 well plate was prepared with at least two wells of 50 μ l H₂O to act as negative controls. Reaction mixtures were incubated at 94°C for 3 minutes and subjected to 40 cycles of amplification on the Peltier Thermal Cycler (PTC-225 DNA tetrad PCR machine®; MJ ResearchTM) as follows: 94°C 30 seconds; 67°C 30 seconds; 72°C 30 seconds; 72°C for 10 minutes and then were left to hold at 15°C overnight.

Restriction digests were prepared using 10 μ l of PCR product in each well to which was added 0.5 ml of *HaeII* (10U) and 1.2 μ l of 10xBSA (Biolabs New England B900IS) for stabilisation of the enzyme or 2 μ l of *AflIII* (10U). This mixture was then incubated at 37°C overnight on the Tetrad 37°C Programme. The products of digestion were prepared for gel electrophoresis as follows: 1 μ l Blue juice (loading dye) added to 5 μ l of digest mixture were placed in each well. Controls used were H₂O with Master Mix that had undergone PCR and one well with uncut PCR product. A 100 bp ladder (InvitrogenTM Cat.No. 15628-019; 1 μ g/ml) was included in each row on the 4% low melting point agarose gel to facilitate the sizing of the RFLP patterns. The gel was prepared in advance using 15g agarose mixed with 375 ml of 0.5 x TBE (Tris-borate EDTA) and 7 drops of ethidium bromide. Four combs with 26 wells each were set in the horizontal electrophoresis unit (HU-25; SCIE-PLASTM Ltd) and the gel was poured at room temperature and stored overnight in a cold room

covered with 0.5 x TBE. The digest products were run at 250V for a maximum of 3 hours.

RFLP patterns on the gel were read under UV light using a Syngene™ camera (Gene genius bioimaging system®). An example of the results obtained is shown in Fig.18 in Chapter 4. The allele and genotype were read and scored by two independent investigators (AC and RR). Data were entered onto a spreadsheet manually and then transferred to an electronic spreadsheet.

3.5.2. Allelic Discrimination Analysis using TaqMan®

The TaqMan system is a method of following PCR in real time, which can be adapted for the analysis of DNA sequence polymorphisms (Livak 1995). In this study single nucleotide polymorphisms in the genes TNF- α , GHR, ADRB2 and 5-HT2A receptor were determined using this technique. The basic chemistry of the technique is illustrated below in Fig.17.

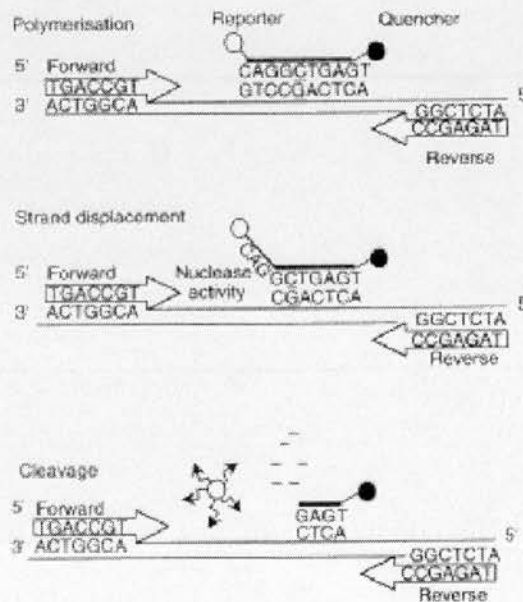


Fig 17: Chemistry of the TaqMan-based Allelic Discrimination Analysis (explained below)

DNA was arranged in several 96 well plates and transferred to 2 x 384 well plates for the assay. DNA quantity in each well was 10ng in 1µl volume.

Two probes are used in this system to detect the bi-allelic SNP of interest. Each probe is specific to an allelic variant and labelled with a different reporter fluorophore (6-carboxy-fluorescein (FAMTM) or (VICTM)). The probe consists of an oligonucleotide, complementary to the sequence of interest, located directly over the single nucleotide polymorphism. It is labelled at the 5' end with reporter and the 3' end with the quencher dye tetramethylrhodamine. The proximity of the quencher to the reporter dye reduces the observed fluorescence signal if the probe is intact. This also ensures that the 3' end of the probe is blocked and cannot act as a primer. During PCR, as the forward primer extends, the target specific probe is degraded by the 5'-3' exonuclease activity of the DNA Taq polymerase. With each round of amplification, there is an increase in the intensity of fluorescence related to the accumulation of PCR product.

The presence of two probes, each labelled with a different fluor allows for the detection of both alleles in a single tube. Because the probes are included in the PCR, genotypes are determined without any post-PCR processing - a feature unavailable with most genotyping methods (Landegren et al 1998).

Fluorescence is then measured directly in the reaction well by the ABI PRISM 7900 HT Sequence Detector TM(Applied Biosystems®).

A series of controls are also added to the plates on which the samples are analysed which contain no DNA template. Thus fluorescence levels are due solely to uncleaved probe. At the endpoint of PCR, a laser collects a fluorescence spectrum (a linear combination of the fluorescence emissions from the component dyes) and

algorithms within the software extract the contribution of each component dye to the observed spectrum to produce a scatter diagram. On this are plotted the allele-specific components of each reaction (see Fig.19 & 20 in Chapter 4).

The statistical analysis is performed automatically. Normalised fluorescence values (R_n), defined as the amount of fluorescence from each reporter dye divided by that from the reference dye, are imported into a statistical software package that is part of the 7900HT® genotyping platform. An increase in signal of either FAM™ or VIC™ indicates homozygosity for that particular allele and an increase in both signals indicates heterozygosity. Confidence limits of 99.7% must be met for a genotype to be determined.

Alleles were read and classified on the plots by two readers independently who were both blind to subject status (SB and AF).

Assay Design

Assay-on-Demand® and Assay-by-Design® (ABI™) were used to design probes and primers. All are designed to work under standard conditions.

The precise sequences of the unlabelled amplification primers and labelled allele-specific probes (published in part for Assay-on-Demand® only) used in this study are copyrighted to the company. Table 26 below shows the SNPs for which primers and probes were available.

Table 26: SNPs for which probes and primers were designed

| Gene | SNP position | Type of SNP | Base Change | Type of Assay |
|---------------|--------------|--|-------------|-----------------|
| ADBR2 | + 79 | Functional amino-acid substitution in coding block | C:G | Assay by Design |
| TNF- α | -308 | Functional promoter polymorphism | G:A | Assay by Design |
| 5HTR2A | 102 | Silent mutation | T:C | Assay on Demand |
| GHR | 561 | Missense mutation in cytoplasmic domain | C:T | Assay on Demand |

Each 25µl of PCR contained 10ng of genomic DNA, 900nM primers, 250nM probes and 12.5µL of TaqMan Universal PCR master mix (ABI), which contains buffer, uracil-N-glycosylase, deoxyribonucleotides, uridine, passive reference dye (ROX) and TaqGold DNA polymerase and MGB groove binders

The solution was pipetted into each well of a 96 or 384 well plate using an automated liquid handling robot.

Amplification was performed using the Peltier Thermal Cycler (PTC-225 DNA tetrad PCR machine®; MJ Research™). Fluorescence in each well was measured before and after PCR using an ABI 7900 HT® machine (ABI™) as described above.

3.6 Data Collection and Statistical Analysis

All data collected in the course of this study was entered into a spreadsheet using the statistical programme SPSS (release 10.0 for Windows, Chicago, Illinois).

For categorical variables, the chi-squared test was utilised to examine differences between groups.

For continuous variables, comparisons between groups were made using the unpaired and paired Student's t-test and ANOVA if data were parametric and the Mann-Whitney U test and Kruskal-Wallis test for non-parametric distribution. The Wilcoxon Rank sum test was used for paired data that were non-parametrically distributed.

Tests of correlation were carried out using either Pearson or Spearman's rank tests depending on whether the variables were parametrically or non-parametrically distributed respectively. Typical error was calculated for the standard deviation for the difference in score between two measures, divided by root 2.

Binary logistic regression was used to assess the association of variables. This test also allows for forward conditional regression analysis to be carried out examining the significance of the interaction between covariates and their effect on the dependent variable.

The significance of frequency differences of OSAHS between genotype discordant sibling pairs was estimated using one sibling pair from each family by McNemar's test of symmetry.

Power calculations were performed using the formulae and methods described by Machin et al 1997 for case-control studies comparing two proportions and using the odds ratio. The Stanford Power Calculator for case-control studies was also utilised to check results (<http://calculators.stat.ucla.edu/powercalc/>). This programme is based on Xlisp Stat by Jason Bond, based on FORTRAN examples of *stplan* by Barry Brown et al. (bwb@odin.mdacc.tmc.edu) using CGI forms (UCLA Dept. of Statistics, California, USA). Power was checked at the $\alpha = 0.05$ level.

Hardy-Weinberg (H-W) equilibrium was checked using the formula $1 = p^2 + 2pq + q^2$ for allelic distribution in the population and the frequencies of minor and major alleles were compared with published frequencies. Hardy-Weinberg equilibrium indicates that the genotype frequencies can be determined directly from the allele frequencies. Failure to demonstrate H-W equilibrium may result from genotyping errors, inbreeding, genetic drift, mutation or population substructure (Silverman & Palmer 2000). H-W equilibrium can be assessed with a goodness-of-fit chi square test for biallelic markers and for markers with multiple alleles, using a Markov Chain Monte Carlo method (Guo & Thompson 1992). Significant deviations from the expected proportions of homozygote and heterozygote classes in a population of case

subjects may be caused by association with the disease allele. Lack of consistency with H-W equilibrium among controls should prompt investigation for potential confounders, including genotyping errors and population stratification.

P-values were corrected for multiple comparisons by the method of Holm (Holm 1979; Aickin and Gensler 1996). This method maintains the experiment-wise error rate at $\alpha = 0.05$ and does not assume independence among the multiple tests. The advantage in this method lies in fixing the type 1 error at α and thereby decreasing the type 2-error rate, unlike the Bonferroni method which is arguably too conservative and may result in too many false negatives (see Perneger 1998 for discussion). Furthermore, comparisons shown to be different by the Bonferroni test will also be different using the Holm adjustment.

Significance was defined at an experiment-wise error of $\alpha \leq 0.05$, two-sided. The Holm's adjustment procedure for each set of comparisons involved first ordering the p-values from smallest to largest and assigning a rank number (i) of 1 through to n . Starting with the smallest p-value, each p_i value was compared with its critical value $\alpha/(n-i + 1)$. If the p-value was less than or equal to its critical value, the paired comparison was considered statistically significant at an experiment-wise α of 0.05.

In this thesis the p-value of each test looking at allelic/genotype distribution between and within groups is presented with the critical α -value in brackets at which each p-value is either accepted or rejected. All p-values in this thesis are for two-tailed tests. Results are considered significant at the $\alpha = 0.05$ level.

CHAPTER 4

RESULTS

4.0 Characteristics of the Study Population

The characteristics of the index cases and their siblings as well as subjects recruited for the SIDS study are presented below. Results for the subjects recruited for the SIDS study are not included in the sibling analyses.

The total number of subjects was 228. All subjects were Caucasian.

DNA samples for 17 (34%) of the SIDS subjects and 8 (3%) of the original sibling study cohort were technically unsatisfactory or inadequate, and they were thus excluded from the study. All values are expressed as mean +/- standard deviation (\pm sd), except for gender, which is expressed in whole numbers as a ratio. The p-value for the ESS and AHI are not represented as they were the defining values for the groups.

Table 27: Characteristics of the Study Population (n=228)

| Variable | No OSAHS (n =83) | Indeterminate OSAHS (n = 35) | Definite OSAHS (n = 110) | p-value* |
|-----------------------------|---------------------|-------------------------------------|--------------------------------|-----------|
| Sex ratio (M:F) | 39:44 | 24:11 | 87:23 | < 0.0001¶ |
| Age (years) | 48 \pm 11 | 51 \pm 9 | 52 \pm 9 | 0.017 |
| BMI (kg/m ²) | 27 \pm 5 | 30 \pm 7 | 30 \pm 6 | < 0.0001¶ |
| Neck circumference (cm) | 37 \pm 4 | 40 \pm 4 | 41 \pm 4 | < 0.0001¶ |
| SBP (mmHg) | 129 \pm 17 | 135 \pm 17 | 137 \pm 18 | 0.002¶ |
| DBP (mmHg) | 82 \pm 12 | 82 \pm 10 | 83 \pm 12 | 0.75 |
| ESS (n/24) | 8 \pm 5 | 6 \pm 3 | 15 \pm 4 | |
| AHI (events/hr) | 9 \pm 5 | 37 \pm 18 | 42 \pm 23 | |
| Sleep efficiency** | 78 \pm 12 | 73 \pm 13 | 72 \pm 15 | 0.023 |
| REM time (mins) | 72 \pm 27 | 68 \pm 27 | 65 \pm 30 | 0.1 |
| NREM time (mins) | 282 \pm 51 | 277 \pm 50 | 274 \pm 61 | 0.44 |
| SaO ₂ (%) awake | 97 \pm 2 | 96 \pm 2 | 96 \pm 2 | 0.01 |
| Lowest SpO ₂ (%) | 91 \pm 6 | 86 \pm 8 | 83 \pm 10 | < 0.0001¶ |

* For comparison of no OSAHS vs definite OSAHS (χ^2 -test for sex and Mann-Whitney test for continuous variables)

** Sleep Efficiency as a percentage of the total time spent asleep divided by the time of the study

¶ Significant using Holm correction for α for 24 comparisons (Table 27 and 28)

Snoring was reported in 58 (70%) subjects without OSAHS, 33 (94%) in the indeterminate category and all 110 (100%) subjects with definite OSAHS. This reflects largely the selection criteria applied when recruiting the siblings of index cases. Other data relevant to the population are presented in Table 28 below.

Table 28: Characteristics of the Study Population (n=228)

| Variable | No OSAHS (n =83) | Indeterminate OSAHS (n = 35) | Definite OSAHS (n = 110) | p-value* |
|-----------------------|------------------------|-------------------------------------|--------------------------------|-----------|
| Snoring | 58 | 33 | 110 | |
| Witnessed apnoeas | 13 | 20 | 71 | |
| Asthma | 2 | 2 | 11 | 0.044 |
| Bronchitis | 5 | 3 | 11 | 0.432 |
| Hayfever | 9 | 9 | 16 | 0.45 |
| Fractured nose | 4 | 2 | 10 | 0.4 |
| Current smoking | 21 | 7 | 22 | 0.38 |
| Alcohol (units) | 9(IQR 5 – 22) n= 49 | 7(IQR 3 – 20) n = 24 | 10(IQR 5-15) n=74 | 0.5 |
| Diabetes mellitus | 0 | 1 | 5 | 0.07 |
| Myocardial infarct | 0 | 2 | 11 | 0.003¶ |
| Hypertension | 3 | 6 | 30 | < 0.0001¶ |

* For comparison of no OSAHS vs. definite OSAHS (χ^2 -test; Fisher's exact test; Mann-Whitney test for continuous variables)

¶ Significant using Holm correction for α for 24 comparisons (Table 27 and 28)

Subjects classified as indeterminate for OSAHS were not included in subsequent genetic analyses or in cephalometric analyses.

4.1 Cephalometry

Cephalograms were available for 224 of 228 subjects: 148 men and 76 women.

All measurements are corrected for the magnification factor (1:1.1) and represent actual dimensions in millimetres. The angles SNA, SNB and NSBA are recorded in degrees.

As stated above, all subjects indeterminate for OSAHS were excluded from the analyses: 24 men and 26 women.

Of the remaining 148 men, 19 (13%) were edentulous and were discarded from subsequent cephalometric analyses. Of the 76 women, 15 (20%) were edentulous and were likewise not included in analyses presented below. Forward conditional regression analysis comparing the dentate and edentulous populations including BMI, age and sex in the analysis showed that the edentulous had a significantly more backset maxilla (more acute SNA) ($p=0.009$) and shorter anterior maxillary height ($p< 0.0001$). Furthermore, location of the occlusal plane is difficult to determine with any degree of accuracy in those without teeth and affects other measurements that are dependent on it such as posterior maxillary height, internal maxillary length and anterior mandibular height.

Owing to the sexual dimorphism inherent in the skull, results for males and females are presented separately.

In the male group, 8 subjects were non-snorers and in the female group, there were 3 non-snorers. These 11 subjects in total are represented in Table 29 but not included in subsequent analyses or regression analyses. Table 29 below shows the bony measurements and angles for male and female snorers with and without OSAHS as well as the non-snorers.

Table 29: Bony measurements and angles for dentate non-snoring and snoring males and females with and without OSAHS.

| Variable* | OSAHS | Men | Women |
|-----------|-------------|------------------|------------------|
| | | (n=109) | (n=56) |
| | | <u>Range</u> | <u>Range</u> |
| GoGn | Yes | 75±5 (65 – 87) | 70±4 (64 – 77) |
| | No | 77±5 (66 – 86) | 71±5 (63 – 84) |
| | Non-snorers | 78±5 (68 – 85) | 70±8 (62 – 78) |
| GoAr | Yes | 56±7 (44 – 73) | 46±6 (35 – 58) |
| | No | 57±5 (47 – 65) | 46±5 (34 – 60) |
| | Non-snorers | 56±4 (48 – 62) | 49±7 (41 – 56) |
| ANSPNS | Yes | 50±4 (42 – 61) | 48±4 (40 – 54) |
| | No | 51±5 (44 – 60) | 48±4 (40 – 54) |
| | Non-snorers | 52.5±3 (48 – 56) | 46±0.9 (46 – 47) |
| PNSBA | Yes | 44±4 (36 – 55) | 42±4 (34 – 50) |
| | No | 44±3 (37 – 49) | 41±2 (36 – 45) |
| | Non-snorers | 48±5 (42 – 57) | 44±4 (39 – 46) |
| HMP | Yes | 23±6 (6.4 – 36) | 20± 6 (5.5 – 35) |
| | No | 19±6 (11 – 30) | 17±4 (11 – 24) |
| | Non-snorers | 19±5 (11 – 27) | 16±7 (9 – 23) |
| C3H | Yes | 41±5 (32 – 53) | 36±6 (26 – 47) |
| | No | 40±6 (31 – 57) | 33±4 (29 – 42) |
| | Non-snorers | 41±3.6 (37-47) | 32±2 (30 – 33) |
| HRGN | Yes | 41±6.5 (25 – 56) | 40±7 (31 – 54) |
| | No | 40±4 (30 – 47) | 37±7 (26 – 52) |
| | Non-snorers | 40±4 (35.5-47) | 36±2 (33 – 37) |
| ANSFOP | Yes | 30±4 (19 – 41) | 29±3 (21 – 34) |
| | No | 29±3 (24 – 34) | 28±3 (22 – 33) |
| | Non-snorers | 29±5 (21 –36) | 28±1 (26 – 28) |
| GNFOP | Yes | 44±4 (26 – 54) | 41±3 (38 – 48) |
| | No | 44±4 (37 – 52) | 38±3 (33 – 43) |
| | Non-snorers | 43±1.8 (41 – 46) | 38±5 (34 – 43) |
| IntMax | Yes | 77±6 (64 – 89) | 69±6 (60 – 83) |
| | No | 77±4 (69 – 84) | 71±5 (57 – 79) |
| | Non-snorers | 73±6 (66 – 81) | 69±3 (69 – 70) |

Table 29 continued

| | | | |
|---------------|-------------|-------------------|------------------|
| PMAXHT | Yes | 41±7 (26 – 58) | 35±6 (18 – 45) |
| | No | 40±6 (26 – 52) | 34±7 (23 – 53) |
| | Non-snorers | 39±7 (32 – 52) | 30±7 (23 – 35) |
| SNA | Yes | 82±4 (72 – 95) | 79±3 (72 – 84) |
| | No | 82±5 (72 – 88) | 82±5 (74 – 91) |
| | Non-snorers | 82±4 (78 – 91) | 80±1 (79 – 81) |
| SNB | Yes | 79±4 (70 – 87) | 75±2 (72 – 80) |
| | No | 80±4 (71 – 87) | 77±6 (66 – 86) |
| | Non-snorers | 79±3 (76 – 85) | 76±5 (70 – 80) |
| NSBA | Yes | 129±6 (116 – 144) | 131±5(121 – 138) |
| | No | 128±6 (120 – 142) | 129±4(122 – 136) |
| | Non-snorers | 129±6 (119 – 136) | 132±4(130 – 136) |

(Men: Yes n = 74; No n = 27; Non-snorers n = 8)

(Women: Yes n = 16; No n = 37; Non-snorers n = 3)

* Except for the angles SNA, SNB and NSBA, which are measured in degrees, all other measurements are in millimetres.

Abbreviations: *GoGn* = gonion to gnathion distance; *GoAr* = gonion to articulare distance; *ANSPNS* = anterior nasal to posterior nasal spine distance; *PNSBA* = posterior nasal spine to basion distance; *HMP* = hyoid to mandibular plan distance; *C3H* = cervical vertebra 3 to hyoid distance; *HRGN* = hyoid to retrognathion distance; *ANSFOP* = anterior nasal spine to occlusal plan distance; *GNFOP* = gnathion to occlusal plane distance; *IntMax* = internal maxillary length; *PMAXHT* = posterior maxillary height; *SNA* = sella-nasion-point A angle (maxillary angle); *SNB* = sella-nasion-point B angle (mandibular angle); *NSBA* = nasion-sella-basion angle (cranial base angle).

Since age and BMI can affect craniofacial growth and structure, these two variables were included in all regression analyses.

Forward conditional logistic regression for all bony measurements and angles showed that the distance of the hyoid bone to the mandibular plane was the only significant factor associated with a diagnosis of OSAHS compared to snoring alone in the male group ($p = 0.01$).

In the female group, the same analysis revealed that OSAHS was significantly associated with a higher BMI ($p = 0.002$), a greater distance of the hyoid bone from the mandibular plane ($p = 0.038$) and greater anterior mandibular length ($p = 0.001$)

compared to snoring alone. When conditional logistic regression analysis was undertaken looking at anterior lower facial height (anterior mandibular and anterior maxillary height (GNFOP +ANSFOP)) in relation to OSAHS vs. snoring alone, the association of a longer lower face with OSAHS was significant ($p=0.016$; OR 1.26; CI95% 1 – 1.5) and explained 27.6% of the observed variance. When forward logistic conditional regression analysis was performed using all variables as above, but substituting GNFOP and ANSFOP with lower facial height alone, only higher BMI and longer lower facial dimensions were significantly associated with OSAHS, explaining 49.6% of the variance ($p = 0.015$ and $p = 0.013$) respectively.

A comparison by forward conditional regression analysis was made between male and female craniofacial dimensions, controlling for age, BMI and a diagnosis of OSAHS. Not surprisingly, all measurements were significantly shorter in the female group. The females had significantly more backset mandibles as judged by SNB compared to men ($p < 0.0001$). However, there was no difference in the SNA or NSBA between the sexes.

When subjects with OSAHS alone were examined in relation to the effect of obesity on craniofacial dimensions (Table 30), the male subjects with a BMI $\geq 30\text{kg/m}^2$ had significantly greater posterior maxillary height, mandibular ramus height and hard palate length compared to the non-obese. In women the most significantly different dimensions were the distance of the hyoid bone from C3 in snorers and the cranial base angle in the group with OSAHS.

Table 30: Significant Comparisons in Cephalometric Variables between Obese and Non-Obese Men and Women with and without OSAHS

| Variable** | BMI < 30 kg/m ² | BMI ≥ 30 kg/m ² | p-value |
|-----------------------------------|----------------------------|----------------------------|-----------|
| Male Non-apnoeic snorers | | | |
| C3H2 | 38±4 (n=21) | 46±6 (n=6) | 0.002* |
| Male OSAHS | | | |
| ANSPNS | 49±4 (n=52) | 53±4 (n=22) | < 0.0001* |
| GoAr | 54±6 | 60±6 | < 0.0001* |
| PmaxHt | 39±7 | 45±7 | 0.003* |
| C3H2 | 40±4 | 43±5 | 0.005 |
| NSBA | 127±5 | 131±7 | 0.013 |
| Female Non-apnoeic snorers | | | |
| C3H2 | 32±3 (n=16) | 38±4 (n=4) | 0.007 |
| Female OSAHS | | | |
| NSBA | 128±4 (n=7) | 133±4 (n=8) | 0.027 |

* Significant using Holm correction for α for 14 comparisons

**Except for the angles SNA, SNB and NSBA, which are measured in degrees, all other measurements are in millimetres.

In order to explore determinants of OSAHS in siblings, 22 pairs of dentate male sibs, **discordant** for the diagnosis of OSAHS were analysed. Results showing a trend towards association with OSAHS in the male sibs with smaller mandible and longer distance of the hyoid from the mandibular plane are presented in Table 31 below.

Table 31: Significant Comparisons in Cephalometric Variables between Male Sibs **discordant** for a Diagnosis of OSAHS

| Variable** | Sibs with OSAHS (n = 22) | Sibs without OSAHS (n = 22) | p-value* |
|--------------------------|--------------------------|-----------------------------|--------------------------|
| Age (years) | 50±9 | 51±9 | 0.54 |
| BMI (kg/m ²) | 28±4 | 28±4 | 0.39 |
| GoGn | 82±5 | 86±5 | 0.024 |
| GoAr | 59±6 | 63±6 | 0.031 |
| HMP | 26±6 | 21±6 | 0.013 ($\alpha=0.003$) |

* No significance using Holm correction for α for 15 comparisons of bony measurements

**Except for the angles SNA, SNB and NSBA, which are measured in degrees, all other measurements are in millimetres.

Forward conditional regression analysis controlling for age and BMI showed that only a shorter mandibular corpus and lower hyoid in relation to the mandibular plane

were significantly associated with a diagnosis of OSAHS in this group ($p = 0.013$ and $p = 0.006$ respectively).

The effect of ageing on the craniofacial complex was examined by dividing the dentate men and women respectively into two groups: one group over 51 years and the other group less than 51 years. (This age cut-off was chosen on the basis of studies which have shown evident morphological changes in the craniofacial complex occurring around the age of 50 years – see Doual 1997; Behrents 1985; Macho 1986). Forward conditional logistic regression analysis in the male group showed only GoAr (mandibular ramus height) to be significantly greater in those ≥ 51 years ($p = 0.01$). There were no significant differences in any bony measurements or angles in the female group.

Skeletal Class

Jaw profile is classified as orthognathic (skeletal Class I), retrognathic (skeletal Class II) or prognathic (skeletal Class III). Classification is based on the difference between the angles SNA and SNB which is the derived value ANB. Not surprisingly, female subjects were significantly more likely to have skeletal class II profiles owing to their backset mandibles compared to males (see Table 32).

Table 32: Distribution of Skeletal Classes between Male and Female Dentate Subjects

| | CLASS I | CLASS II | CLASS III |
|-----------------------|----------------|-----------------|------------------|
| FEMALES (n=61) | 16 | 40 | 5 |
| MALES (n=129) | 60 | 51 | 18 |

p=0.004; χ^2 -test; 2 x 3 contingency table

A comparison of the female subjects with and without OSAHS showed no difference in skeletal class distribution (χ^2 -test; $p = 0.8$; 2x3 contingency table). Similarly, there

was no difference in profile overall between obese and non-obese women (χ^2 -test; $p = 0.3$; 2x3 contingency table) regardless of OSAHS diagnosis, nor between women aged 51 years and over compared to the younger age group (χ^2 -test; $p = 0.6$; 2x3 contingency table). Results were similar for men. There was no significant difference in profile distribution between men with and without a diagnosis of OSAHS (χ^2 -test; $p = 0.95$; 2x3 contingency table); the obese vs. the non-obese (χ^2 -test; $p = 0.3$, 2x3 contingency table) and those aged 51 years and over compared to those who were younger (χ^2 -test; $p = 0.23$, 2x3 contingency table).

4.2 Blood Donor Controls

As stated previously, information on sex was provided for all anonymous blood donors and age for half of them. Of the 192 donors whose DNA was utilised in this study, 50% (n=96) were male ($p = 1$, χ^2 - test). Mean age and age range according to sex is shown in Table 33 below.

Table 33: Mean Age and Age Range of Anonymous Blood Donors

| | Males (n = 37) | Females (n = 51) | p-value* |
|------------------------------|-----------------------|-------------------------|-----------------|
| Mean age + sd (years) | 39 ± 9 | 38 ± 7 | 0.36 |
| Age range (years) | 24 - 61 | 24 - 54 | |

* Student's t-test, equal variances assumed

The sex distribution between the blood donor controls and the 228 other study subjects was significantly different ($p=0.003$, χ^2 -test) as was the mean age ($p < 0.001$).

4.3 APOLIPOPROTEIN E

As discussed in Chapter 3.5.1, the method used for examination of APO E carriage in this population was PCR-RFLP. An example of the gels obtained in the process is

presented in Fig 18 below. Seventy-eight samples of DNA were analysed. However, 5 were discarded due to difficulty in reading the gel accurately by either investigator independently.

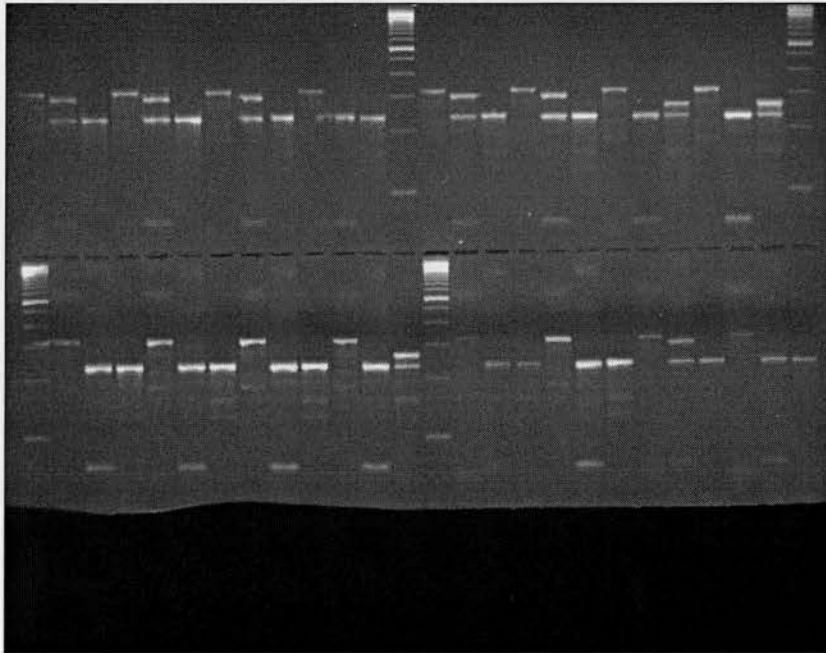


Fig 18: Gel showing RFLP patterns for APOE using *Haell* and *AflIII* restriction enzymes

Complete results for 73 subjects were available and the characteristics of the study population including the Apolipoprotein E genotype and allelic frequencies are presented in Table 34 below.

Table 34: Characteristics of the Study Population for Apolipoprotein E Genotype and Allelic Frequencies

| Variable | No OSAHS (n =33) | Indeterminate OSAHS (n =8) | Definite OSAHS (n =32) | p-value* |
|-----------------------------|----------------------|-----------------------------------|-------------------------------|-----------|
| Sex ratio (M:F) | 16:17 | 6:2 | 23:9 | 0.04 |
| Age (years) | 51 _± 9 | 47 _± 9 | 52 _± 10 | 0.9 |
| BMI (kg/m ²) | 27 _± 4 | 28 _± 2 | 28 _± 3 | 0.58 |
| Neck circumference (cm) | 37 _± 4 | 43 _± 4 | 40 _± 3 | 0.003¶ |
| SBP (mmHg) | 133 _± 16 | 140 _± 20 | 136 _± 15 | 0.24 |
| DBP (mmHg) | 86 _± 9 | 78 _± 8 | 80 _± 12 | 0.94 |
| ESS (n/24) | 7 _± 4 | 6 _± 3 | 14 _± 3 | < 0.0001¶ |
| AHI (events/hr) | 16 _± 11 | 43 _± 22 | 42 _± 19 | < 0.0001¶ |
| Sleep Efficiency | 78 _± 11 | 73 _± 7 | 71 _± 14 | 0.027 |
| TST (mins) | 367 _± 61 | 339 _± 30 | 332 _± 65 | 0.009 |
| REM time (mins) | 68 _± 23 | 59 _± 16 | 64 _± 27 | 0.45 |
| NREM time (mins) | 302 _± 47 | 280 _± 23 | 269 _± 46 | 0.003¶ |
| SaO ₂ (%) awake | 97 _± 1.5 | 97 _± 2 | 96 _± 2 | 0.24 |
| Lowest SpO ₂ (%) | 90 _± 6 | 89 _± 2 | 85 _± 8 | 0.001¶ |
| Genotype | N = 33 | N = 8 | N = 32 | |
| E3/E3 | 14 | 6 | 14 | 0.23 |
| E2/E3 | 5 | 0 | 7 | |
| E3/E4 | 12 | 2 | 8 | |
| E2/E2 | 0 | 0 | 3 | |
| E2/E4 | 2 | 0 | 0 | |
| Allele | N = 66 | N = 16 | N = 64 | |
| E2 | 7 | 0 | 13 | 0.13 |
| E3 | 45 | 14 | 43 | |
| E4 | 14 | 2 | 8 | |

* For comparison of subjects with no OSAHS vs those with definite OSAHS (χ^2 -test for sex and Mann-Whitney test for continuous variables)

** Sleep Efficiency is calculated as a percentage of the total time spent asleep divided by the time of the study

¶Significant using Holm correction for α for 14 comparisons

There was no significant difference among groups in the distribution of either genotype or alleles.

In order to retest the hypotheses put forward by Kadotani et al (2001) (see Chapter 2.6) the group was divided into those with an AHI greater than or less than fifteen (Table 35).

Table 35: Characteristics of the Study Population divided by AHI of greater than or less than 15/hr

| Variable | AHI <15 (n = 23) | AHI ≥ 15 (n = 50) | p-value* |
|-----------------------------|---------------------|----------------------|-----------|
| Sex ratio (M:F) | 8:15 | 37:13 | 0.001¶ |
| Age (years) | 48+8 | 52+9 | 0.13 |
| BMI (kg/m ²) | 28+4 | 27+3 | 0.56 |
| Neck circumference (cm) | 37+4 | 40+3 | 0.001¶ |
| SBP (mmHg) | 132+17 | 136+16 | 0.337 |
| DBP (mmHg) | 87+10 | 81+10 | 0.024 |
| ESS (n/24) | 8+4 | 11+5 | 0.046 |
| AHI (events/hr) | 11+3 | 39+19 | |
| TST (mins) | 378+56 | 334+60 | 0.005¶ |
| Sleep Efficiency** | 79+11 | 72+13 | 0.03 |
| REM time (mins) | 72+20 | 62+25 | 0.071 |
| NREM time (mins) | 306+45 | 275+45 | 0.009 |
| SaO ₂ (%) awake | 97+1 | 96+2 | 0.017 |
| Lowest SpO ₂ (%) | 93+2 | 86+7 | < 0.0001¶ |
| Genotype | N = 23 | N = 50 | |
| E3/E3 | 12 | 22 | 0.33 |
| E2/E3 | 2 | 10 | |
| E3/E4 | 9 | 13 | |
| E2/E2 | 0 | 3 | |
| E2/E4 | 0 | 2 | |
| Allele | N = 46 | N = 100 | |
| E2 | 2 | 18 | 0.080 |
| E3 | 35 | 67 | |
| E4 | 9 | 15 | |

* For comparison of subjects with no OSAHS vs. those with definite OSAHS (χ^2 -test for sex and Mann-Whitney test for continuous variables)

** Sleep Efficiency is calculated as a percentage of the total time spent asleep divided by the time of the study

¶Significant using Holm correction for α for 14 comparisons

In order to examine whether those carrying the APOE4 allele differed in any way to those without it, a further analysis was performed, the results of which are presented in Table 36.

Table 36: Study Subjects according to Apolipoprotein E4 carriage

| Variable | ApoE4 -ve (n = 49) | ApoE4 +ve (n = 24) | p-value* |
|-----------------------------|-----------------------|-----------------------|----------|
| Sex ratio (M:F) | 33:16 | 12:12 | 0.15 |
| Age (years) | 51 _± 9 | 51 _± 8 | 0.98 |
| BMI (kg/m ²) | 27 _± 3 | 27 _± 4 | 0.97 |
| Neck circumference (cm) | 39 _± 4 | 38 _± 4 | 0.54 |
| SBP (mmHg) | 135 _± 16 | 133 _± 16 | 0.73 |
| DBP (mmHg) | 83 _± 10 | 85 _± 12 | 0.52 |
| ESS (n/24) | 10 _± 5 | 9 _± 5 | 0.29 |
| AHI (events/hr) | 32 _± 21 | 26 _± 19 | 0.26 |
| TST (mins) | 345 _± 56 | 357 _± 73 | 0.45 |
| Sleep Efficiency | 74 _± 12 | 76 _± 14 | 0.6 |
| REM time (mins) | 64 _± 24 | 68 _± 25 | 0.53 |
| NREM time (mins) | 281 _± 45 | 294 _± 51 | 0.3 |
| SaO ₂ (%) awake | 97 _± 2 | 97 _± 1.5 | 0.07 |
| Lowest SpO ₂ (%) | 88 _± 7 | 88 _± 8 | 0.83 |

* For comparison of subjects with no OSAHS vs. those with definite OSAHS (χ^2 -test for sex and Student's t-test for continuous variables)

Finally, the distribution of alleles in the study group was compared to published frequencies for the UK population (Table 37). There was no statistically significant difference between the two groups ($p=0.3$, χ^2 -test, 2x3 contingency table).

Table 37: Allelic Frequencies in the Study Population vs. a UK population

| | Study group (n=73) | UK population (n=734)* |
|----|--------------------|------------------------|
| E2 | 13% | 9% |
| E3 | 71% | 77% |
| E4 | 16% | 14% |

* From: Corbo and Scacchi (1999)

No cephalometric comparisons were made in this group, as it was not considered biologically relevant.

Based on odds ratios, the power for detecting a difference in the distribution of alleles between the groups with or without OSAHS was greater than 80%. However, the number needed to show a difference between E4 carriage alone based on AHI at

a power of at least 80% (proportional differences) would have been 469 subjects in each group.

4.4 RESULTS FOR TaqMan ANALYSES

The technique is described fully in Chapter 3.5.2 and was used for examination of SNPs in the genes for TNF- α , GHR, ADRB and the 5HTR2A. The graphical analysis of the alleles is shown in Fig 19 and 20 below. The plot for the serotonin receptor gene- 2a polymorphism demonstrates the clarity of results for an ABI-designed primer for commercial use whilst the plot for the ADRB polymorphism was custom-designed for the study and showed a high rate of drop-out due to failure of the primer as well as difficulty of delineating the polymorphism category by the plot reader.

The results for TNF- α and GHR are not shown, as the plots were similar to those for the 5HTR2A.

Results for each gene will be discussed in turn below. For all significance levels, tables were constructed to establish rank and thereby the Holm-corrected α -level at which the obtained p-value was either rejected or accepted.

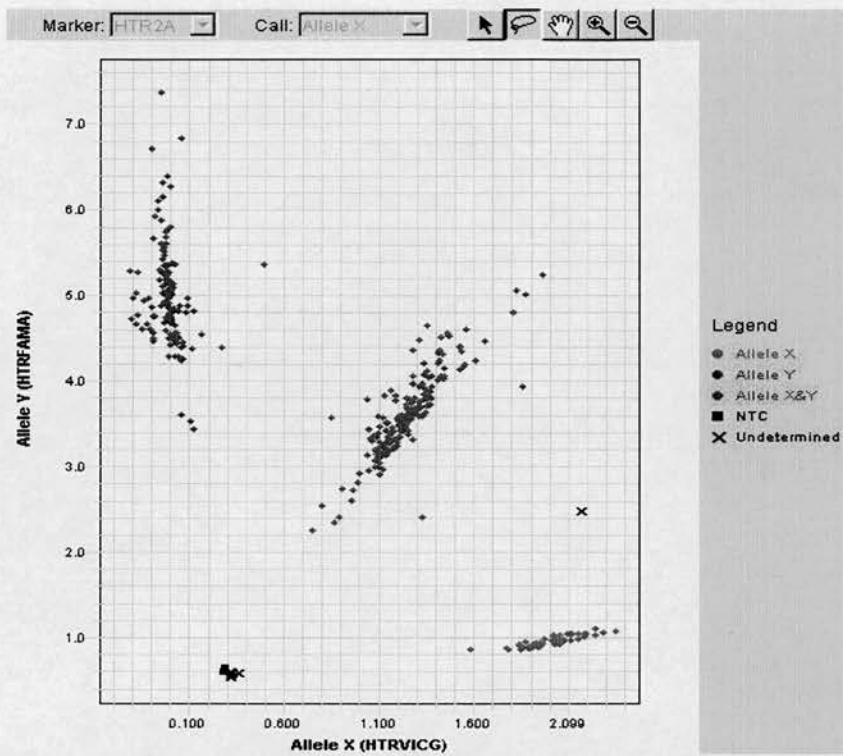


Fig19: Plot of the 5HTR2A promoter polymorphism C/T after TaqMan analysis

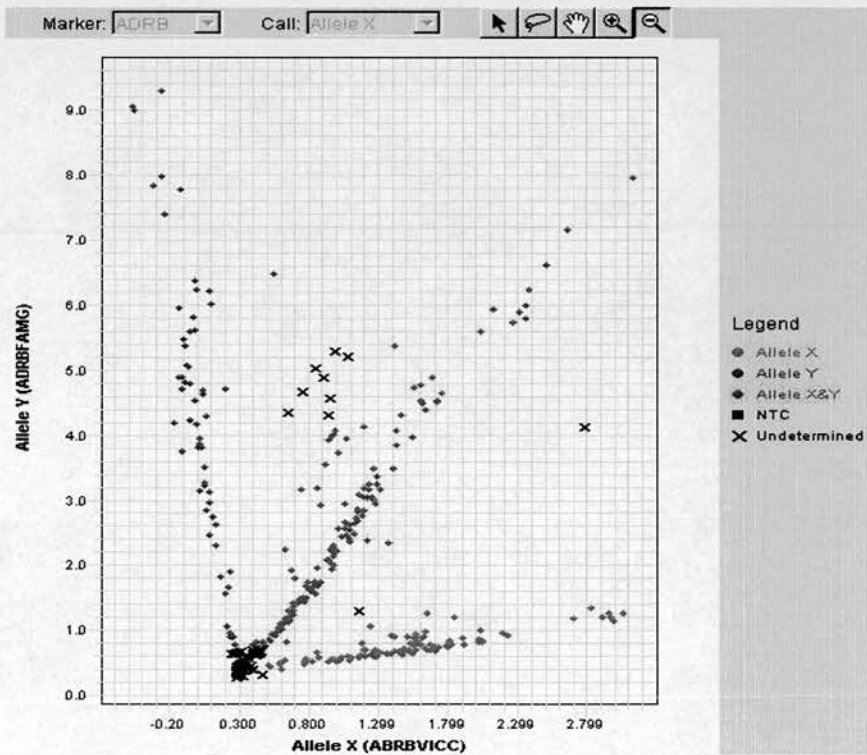


Fig 20: Plot of the ADRB2 polymorphism C+79G after TaqMan analysis

4.5 Tumour Necrosis Factor- Alpha

Genotyping results examining the -308 (A/G) SNP for TNF- α were available for 190 UK blood donor controls (99%) and 216 of the 228 study subjects (95%). Of the blood donor controls, 95 (50%) were male and the mean age was 38 ± 8 years.

Population characteristics for the other 216 study subjects did not differ from those presented in Table 27 above. The sex distribution between the blood donor controls and the other study subjects was significantly different ($p=0.003$, χ^2 -test) as was the mean age ($p < 0.001$).

The distribution of allelic frequencies and genotypes was significantly different between subjects with a definite diagnosis of OSAHS and all subjects without a diagnosis of OSAHS (Table 38). Allele distribution in the control group was in Hardy-Weinberg equilibrium.

Binary logistic regression analysis showed a significant association for the TNF- α (-308A) allele carriage with male sex (OR 1.6; CI95% 1.03 – 2.34; $p=0.036$), older age (OR 1.04; CI95% 1.01 – 1.06; $p=0.046$) and a definite diagnosis of OSAHS (OR 1.82; CI95% 1.15 – 2.89; $p = 0.01$). These observations explained 1.5%, 4.6% and 2.4% of the variance in total. Forward conditional logistic regression showed that when these three variables were entered into the equation, only age remained significantly associated with the TNF- α (-308A) allele (OR 1.04; CI95% 1.014 – 1.06; $p=0.001$).

Logistic regression analysis showed no association of the (-308) A allele with AHI, ESS, BMI, weight, height, neck circumference or blood pressure. Cephalometric variable were not included in the analysis, as this was considered biologically untenable as a hypothesis despite the implication of TNF- α in bone growth.

Table 38: Allelic and Genotype Frequencies for TNF- α (-308) A/G Polymorphism in Subjects with definite OSAHS vs. those without.

| Genotype | No OSAHS (n= 269) | Definite OSAHS (n= 103) | p-value* |
|----------|-------------------|-------------------------|----------|
| G/G | 175 (65%) | 52 (50%) | 0.034 |
| A/G | 83 (32%) | 44 (43%) | (0.025) |
| A/A | 11 (4%) | 7 (7%) | |
| Alleles | N = 538 | N = 206 | |
| G | 433 (81%) | 148 (72%) | 0.011 |
| A | 105 (19%) | 58 (28%) | (0.0125) |

* χ^2 -test; Holmed α -value in brackets (multiple comparisons for data on polymorphisms in ADRB, GHR, TNF- α and 5-HT-2a)

Twenty-one pairs of sibs **discordant** for carriage of the A allele (carriage of the A allele vs. no carriage of the A allele) were discordant for the phenotype of OSAHS ($p = 0.001$). All siblings who carried the A allele had OSAHS compared to none of the non-carriers.

Power for detecting a difference in -308 A allele carriage in the entire group ($n = 372$) was 67% based on comparison of proportions. To achieve a power of 80% to detect a difference in allele carriage would have required $n = 322$ in each group.

4.6 GROWTH HORMONE RECEPTOR

Genotyping results examining the +561 (T/G) SNP for the GHR were available for 192 UK blood donor controls (100%) and 218 of the 228 study subjects (96%). Characteristics of the subjects did not differ significantly from those outlined above (4.0).

The sex distribution between the blood donor controls and the other study subjects was significantly different ($p=0.003$, χ^2 -test) as was the mean age ($p < 0.001$).

The distribution of allelic frequencies and genotypes was significantly different between subjects with a definite diagnosis of OSAHS and all subjects without a diagnosis of OSAHS (Tables 39). Allele distribution in the control group was in Hardy-Weinberg equilibrium.

Table 39: Allelic and Genotype Frequencies for +561 (T/G) GHR Polymorphism in Subjects with definite OSAHS vs. those without.

| Genotype | No OSAHS (n= 272) | Definite OSAHS (n= 102) | p-value* |
|----------|-------------------|-------------------------|----------|
| T/T | 84 (31%) | 23 (23%) | 0.016 |
| T/G | 139 (51%) | 47 (46%) | (0.0125) |
| G/G | 49 (18%) | 32 (31%) | |
| Alleles | N = 544 | N = 204 | |
| T | 307 (56%) | 94 (46%) | 0.011 |
| G | 237 (44%) | 110 (54%) | (0.0167) |

* χ^2 -test; Holmed α in brackets (multiple comparisons for data on polymorphisms in ADRB, GHR, TNF- α and 5-HT-2a)

Binary logistic regression showed a significant association for carriage of the genotype G/G with OSAHS ($p = 0.008$; OR 2.4; CI 95% 1.3 – 4.5). This explained 5% of the variance.

There was no significant association with any other variables except for BMI.

Those with a BMI greater than or equal to 30kg/m^2 were significantly more likely to have the G/G genotype ($p = 0.004$; OR 4 CI 95% 1.5 – 8.5; 10% variance). The association of BMI and OSAHS with the G/G genotype was examined using forward conditional logistic regression. Only a BMI greater than 30kg/m^2 was found to be significantly associated with G/G ($p = 0.026$), explaining 3% of the variance in the association. The G/G genotype is therefore not independently associated with the OSAHS phenotype.

Carriage of the G allele alone was significantly associated with BMI ($p = 0.009$, χ^2 -test, 2x2 contingency table) as shown in Table 40 below.

None of the bony measurements or angles examined in the craniofacial complex was associated with the G allele. Posterior maxillary height alone showed a trend towards being larger in the group carrying the G allele ($p = 0.057$).

Table 40: Carriage of the +561 G GHR allele in subjects with a BMI greater than or equal to 30 kg/m²

| G-carriage | BMI<30 (n=151) | BMI > 30 (n = 64) | p-value* |
|------------|----------------|-------------------|----------|
| ABSENT | 50 (33%) | 10 (16%) | 0.009 |
| PRESENT | 101 (67%) | 54 (84%) | |

* χ^2 – test, 2 x 2 contingency table

McNemar test for sibs discordant for the G/G genotype did not associate significantly with a particular phenotype.

The power of detecting a difference in G and T allele carriage in the study group ($n = 374$) was 47% at $\alpha = 0.05$. For power to be 80% would have required a total $n = 768$. However, power was 93% to detect a difference between subjects with the G/G genotype compared to those without it.

4.7 SEROTONIN RECEPTOR- 2A GENE

Genotyping results examining the promoter region polymorphism C/T for the serotonin 2A receptor gene were available for 191 UK blood donor controls (99%) and 218 of the 228 study subjects (96%). Population characteristics did not differ from those presented in 4.0 above. The distribution of allelic frequencies and genotypes was not significantly different between subjects with a definite diagnosis

of OSAHS and all subjects without a diagnosis of OSAHS (Tables 41). Allele distribution in the control group was in Hardy-Weinberg equilibrium.

Table 41: Allelic and Genotype Frequencies for 5HTR2A promoter polymorphism C/T Subjects with definite OSAHS vs. those without.

| Genotype | No OSAHS (n= 272) | Definite OSAHS (n= 103) | p-value* |
|----------|-------------------|-------------------------|----------|
| C/C | 45 (17%) | 12 (12%) | 0.397 |
| C/T | 138 (51%) | 59 (57%) | (0.05) |
| T/T | 89 (33%) | 32 (31%) | |
| Alleles | N = 544 | N = 206 | |
| C | 223 (41%) | 83 (40%) | 0.862 |
| T | 321 (59%) | 123 (60%) | (0.05) |

* χ^2 -test; Holmed α in brackets (multiple comparisons for data on polymorphisms in ADRB, GHR, TNF- α and 5-HT-2a)

There were no significant associations with any other variables including BMI, age, sex or blood pressure.

Sibs discordant for genotype were not significantly different in phenotype. Power to detect a difference in the total population (n = 390) between C and T allele carriage was 5% at the $\alpha = 0.05$ level. To achieve a power of 80% would have required 1,377 subjects as cases and the same number for controls.

4.8 Beta- 2 Adrenoceptor

Genotyping results examining the C+79G (Gln27Glu) polymorphisms in the ADRB-2 gene were available for 177 (92%) UK blood donor controls and 165 of the 228 study subjects (72%). Despite the smaller numbers overall, the population characteristics (shown in Table 42 below) did not differ significantly from those presented above in section 4.0. The p-value for the ESS and AHI are not represented, as they were the defining values for the groups.

Table 42: Characteristics of the Study Population with and without definite OSAHS (n=138)

| Variable | No OSAHS (n=65) | Definite OSAHS (n=73) | p-value* |
|--------------------------|-----------------|-----------------------|-----------|
| Sex ratio (M:F) | 30:35 | 57:16 | < 0.0001¶ |
| Age (years) | 48±11 | 52±9 | 0.04 |
| BMI (kg/m ²) | 27±4 | 31±6 | < 0.0001¶ |
| Neck circum. (cm) | 37±6 | 41±7 | < 0.0001¶ |
| SBP (mmHg) | 128±17 | 139±19 | 0.003 |
| DBP (mmHg) | 81±12 | 83±12 | 0.3 |
| ESS (n/24) | 7±5 | 15±4 | |
| AHI (events/hr) | 10±5 | 42±23 | |

* For comparison of subjects with no OSAHS vs. those with definite OSAHS (χ^2 -test for sex and Mann-Whitney test for continuous variables)

¶ Significant using Holm correction for α for 8 comparisons

The UK blood donor controls comprised 38 men and 57 women and had a mean age of 38±8 years.

The distribution of genotypes but not allelic frequencies was significantly different between subjects with a definite diagnosis of OSAHS and all subjects without a diagnosis of OSAHS (Tables 43). Allelic distribution in the control group was in Hardy-Weinberg equilibrium.

Table 43: Allelic and Genotype Frequencies for G+79C ADBR-2 Polymorphism in Subjects with definite OSAHS vs. those without.

| Genotype | No OSAHS (n= 242) | Definite OSAHS (n= 73) | p-value* |
|----------|-------------------|------------------------|----------|
| G/G | 80 (33%) | 23 (32%) | 0.023 |
| G/C | 123 (51%) | 28 (38%) | (0.0167) |
| C/C | 39 (16%) | 22 (30%) | |
| Alleles | N = 484 | N = 146 | |
| G | 283 (59%) | 74 (51%) | 0.096 |
| C | 201 (41%) | 72 (49%) | (0.05) |

* χ^2 -test; Holmed α -value in brackets (multiple comparisons for data on polymorphisms in ADRB, GHR, TNF- α and 5-HT-2a)

There were no significant associations of the C allele with any other variables including, BMI, blood pressure, age or sex. However, there was a significant difference in the distribution of the CC genotype (Gln27Gln) between those with and without OSAHS (Table 44). Binary logistic regression showed that the association of the C/C genotype with OSAHS was significant ($p = 0.009$; OR 2.25; CI 95% 1.23 – 4.16). There was no association of the C/C genotype with any variables save for height in men. Men with the C/C genotype were significantly taller than men without it (1.79 ± 6 m vs 1.76 ± 6 m; $p = 0.009$).

Table 44: Distribution of the C+79G ADRB-2 Polymorphism in Subjects with definite OSAHS vs. those without.

| C/C Genotype | No OSAHS (n= 242) | Definite OSAHS (n= 73) | p-value* |
|--------------|-------------------|------------------------|----------|
| Present | 39 (16%) | 22 (30%) | 0.008 |
| Absent | 203 (84%) | 51 (70%) | (0.0167) |

* χ^2 -test; Holmed α - value in brackets (multiple comparisons for data on polymorphisms in ADRB, GHR, TNF- α and 5-HT-2a)

Comparison of siblings discordant for the C/C genotype showed no association with phenotype. Power to detect a difference between C/C genotype in the population (total n = 315) was 81% at the $\alpha = 0.05$ level.

4.9 Conclusion

In this chapter, the focus has been on a detailed presentation of results arising from the study. The next chapter will be devoted to the discussion of the results; potential shortcomings of the study and future directions for work in this area.

CHAPTER 5

5.0 INTRODUCTION

This chapter comprises a detailed discussion of results presented in Chapter 4. The main findings are summarised briefly below and discussed in turn. Not surprisingly, study subjects with OSAHS differed significantly from those without it in all but a few minor characteristics. There were significant differences in the cephalometric measurements between apnoeics and snorers. In the genetic analyses, the APOE ϵ 4 allele was not associated significantly with a diagnosis of OSAHS nor was it significantly more common in those with an $AHI \geq 15$. The SNPs examined for each of the 4 other candidate genes in this study (TNF- α , GHR, 5HTR2A, ADRB-2) showed variable degrees of association with the OSAHS phenotype (Table 45).

Table 45: Summary of p-values (in bold when statistically significant) for allelic distribution, minor allele carriage and genotype for subjects with and without OSAHS for the genes TNF- α , GHR, 5HTR2A and ADRB-2.

| Gene | Allele/Genotype | p-value | Rank | Holm - α |
|------------------------------|-----------------|----------------|------|-----------------|
| Allelic Distribution | | | | |
| TNF- α | A/G | 0.01080 | 1 | 0.0125 |
| GHR | G/T | 0.0114 | 2 | 0.0167 |
| ADRB-2 | G/C | 0.096 | 3 | 0.025 |
| 5-HT-2a | C/T | 0.862 | 4 | 0.05 |
| Minor Allele Carriage | | | | |
| TNF- α | A | 0.010 | 1 | 0.0125 |
| GHR | G | 0.112 | 2 | 0.0167 |
| 5-HT-2a | C | 0.239 | 3 | 0.025 |
| ADRB-2 | C | 0.804 | 4 | 0.05 |
| Minor Genotype | | | | |
| GHR | G/G | 0.006 | 1 | 0.0125 |
| ADRB-2 | C/C | 0.008 | 2 | 0.0167 |
| TNF- α | A/A | 0.276 | 3 | 0.025 |
| 5-HT-2a | C/C | 0.76 | 4 | 0.05 |
| Genotype | | | | |
| GHR | G/G; G/T; T/T | 0.016 | 1 | 0.0125 |
| ADRB-2 | C/C; G/C; G/G | 0.023 | 2 | 0.0167 |
| TNF- α | A/G; A/A; G/G | 0.034 | 3 | 0.025 |
| 5-HT-2a | C/T; C/C; T/T | 0.397 | 4 | 0.05 |

All allelic frequencies in the control group were in Hardy-Weinberg equilibrium ($p > 0.05$). The TNF- α (-308A) allele showed significant association with OSAHS phenotype.

5.1 CHARACTERISTICS OF THE STUDY POPULATION

5.1.1. Subjects with and without OSAHS

The differences between subjects with and without sleep apnoea were marked and not surprising. The most significant changes were for the sex ratio, with more men than women in the OSAHS group. This is what would be expected from the epidemiology with OSAHS twice as common in men (see Chapter 1, section 1.1). In addition, those in the OSAHS group had a higher systolic blood pressure in keeping with the association of OSAHS with raised blood pressure (see Chapter 1, section 1.5).

Examining for genetic associations in siblings reduced problems inherent to population stratification in some of the analyses. The population studied was Caucasian. Those who were recruited were largely drawn from Edinburgh and the immediate environs, with one sibling pair from Glasgow, 2 sibling pairs from Fife and 5 sibling pairs from Aberdeenshire. This population is generally representative of the Caucasian population in the UK. According to the 2001 Population Report Scotland based on Scotland's Census 2001 (General Register Office for Scotland 2002), of just over 5 million people living in Scotland, 98% were white, 1% were of Indian origin, 0.3% were Chinese and 0.7% came from other ethnic groups. The vast majority of people were also born in Scotland (87.1%) with 0.3% being born in Wales, 1.1% in Ireland, 0.9% in other EU countries and 2.5% elsewhere. According

to the latest figures from the census, there is significant mobility within the UK itself with 11.6% of people moving into Scotland, 0.6% of whom move from outwith the UK. There has been a 2% decline in the Scottish population from 1981 – 2001. Much of this is attributable to large flows in migration. It is estimated that annually, there are flows of about 50,000 people each way with the rest of the UK and 20,000 each way with the rest of the world. Within the age group of this study (majority in their 40's and 50's), migration and movement within and outwith the country is much more limited as evidenced by the ageing of the population.

Thus, the study population is broadly comparable in ethnic terms to a random assortment of UK Caucasian blood donors. This makes it unlikely that inter-ethnic differences could have a major effect on allelic distribution. The fact that the SNPs in the genes *TNF- α* ; *ADRB-2*, *5-HTR2A* and *GHR* were in Hardy-Weinberg equilibrium in the control group (mixture of UK DNA and Scottish recruits) mitigates against population stratification. The *TNF- α* alleles were also not significantly differently distributed in this group compared to samples from South-East England and Newcastle (Reynard et al 2000). Short tandem repeat (STR) profiling, used in forensic work, has shown no practical differences in the Caucasian population across regions of England and Scotland (Lee et al 1996; Evett et al 1996). The majority of studies performed in Scotland using genetic association in SNPs have used a West of Scotland population (e.g. Eskdale et al 2000, Kinane et al 1999, McGarry et al 1999, Gallagher et al 1997 to name a few). This population is more homogeneous than that found in the South-East (Lothian (Edinburgh)) and has less fluctuation and migration to introduce genetic diversity (General Register Office for Scotland 2001). In addition the fact that Edinburgh has had very significant

immigration from other parts of the UK would minimise any potential differences between our patient population and the UK Caucasian blood donors.

5.1.2. Phenotyping

One of the critical issues regarding the population under study is accurate phenotyping. The factors used to determine phenotype were AHI and Epworth score. The limitations of using AHI include whether it is the best index of OSAHS, reproducibility, difficulty in extrapolating results between different centres due to differing sensors and definitions used, and variation with age and gender (see Chapter 1, section 1.0 and 1.8).

While there is not universal agreement on its precise definition, AHI remains the more widely adopted measure for classifying the presence and severity of OSAHS. Hypopnoea definitions remain disputable and may vary widely from centre to centre (see for example Manser et al 2002). However the definition we have developed (Gould et al 1988) and used is widely accepted including by the American Academy of Sleep Medicine (1999).

In order to minimise the gender and age-related differences inherent in classifying OSAHS, population-based studies documenting the range of AHI in specific age categories according to sex were utilised to develop a classification scheme. The populations used were Caucasian to reflect the race of the study population.

However, the AHI for an individual is also subject to the technique employed in obtaining it and analysing it and may vary from night to night. The degree of variation is not consistent. Most studies however, show there are no differences in the mean AHI in a patient group on the first or subsequent nights (Bittencourt et al

2001; Aber et al 1989; Bliwise et al 1991; Chediak et al 1996; Dealberto et al 1994; Dean and Chaudhary 1992; Mosko et al 1988; Rebuffat et al 1994). However, a proportion of subjects in all of these studies changed their classification of OSAHS severity from the first to the second nights. This generally occurred in subjects with milder forms of OSAHS. This issue was addressed in a study by Le Bon et al (2000), which showed that the gain in detection of OSAHS with an additional night of PSG if the first one was negative was 15 – 25%. The value of performing a second night when diagnosing OSAHS if it was suspected to be mild, was considered less efficient but potentially helpful by Mendelson (1994).

We performed a single overnight study in all patients (whether using home-based monitoring or in-lab PSG). The virtue in classifying patients as having OSAHS by combining both a score for the degree of sleep disordered breathing and a measure of sleepiness is that those who may be slightly underestimated in the degree of SDB on a one night study, will nevertheless be appropriately classified. Furthermore, the existence of an indeterminate score further reduces misclassification by making a wide distinction between those with definite sleep apnoea and those definitely without it

All study participants were investigated in the same laboratory, using the same standard techniques for setting up, recording and scoring events. There was a high intra-and inter-rater reproducibility for analysing results which were performed blind to subject status.

Further issues arise with the measurement of sleepiness. Although the ESS is one of the most widely used and easily administered tests to ascertain a level of somnolence, it always remains subjective. Olson et al (1997) addressed this issue by

comparing scores on the ESS, Symptom Checklist 90 and MSLT in patients at a sleep disorders clinic with diagnoses including OSAHS and narcolepsy. They found that the ESS was a poor predictor of results on the most objective of the tests, the MSLT and was influenced by psychological factors. However, Johns – the originator of the ESS (1991; 1992; 1993; 1994) and Chervin et al (1997) found mild to moderate negative correlations in ESS with reduced sleep latency on the MSLT. At present, though neither an ideal nor complete measure of sleepiness, the ESS does allow for a slightly more realistic appraisal of somnolence in daily life situations, which other more objective tests may not. It is by far the simplest, most practical and cheapest of the tools available for general clinical and research use and has been validated in countless studies. Furthermore sleepiness is the major presenting symptom of OSAHS (Whyte et al 1989) and the ESS is the best validated and most widely used measure of subjective sleepiness (see Chapter 1, section 1.7). As long as its limitations are borne in mind, then it is also very useful in evaluating the diagnosis of OSAHS.

5.2 CEPHALOMETRY

The present study is the first to compare bony landmarks and angles in male and female apnoeic and non-apnoeic snorers separately and to examine the differences between male siblings discordant for a diagnosis of OSAHS. The findings are discussed under separate headings below.

There are not many papers in the cephalometric literature dealing with OSAHS that fulfil all the criteria for reproducible methods. The technique for performing cephalometry is not described in detail in many papers (e.g. European methods for

taking the radiograph differ from those in America, the UK and Australia), error measurements are either omitted or inadequately described, and magnification of the radiograph is generally omitted as is the method for identifying landmarks and performing tracings. Furthermore, many papers are flawed in the selection criteria of cases and controls, often fail to include correction for multiple comparisons and show variation in cut-off points selected to distinguish controls from snorers from subjects with OSAHS - all of which makes them difficult to compare to each other (see Miles et al 1996 for discussion – over 30 papers can be identified). In addition, despite the obvious differences between men and women anatomically, many papers pool results for measurements without correction for sex, thereby invalidating their data (e.g. Prachartam et al 1992). For further discussion of these problems see Miles et al (1996), who reviewed all papers published up to 1995. For these reasons, many papers that are quoted frequently in the OSAHS and cephalometry literature are not included in the subsequent discussion.

5.2.1 Cephalometric Findings in Apnoeic vs. Non-apnoeic Snorers

In this study, there were few differences between males with OSAHS and simple snorers with respect to bony landmarks or angles. Significantly, the hyoid bone was lower in relation to the mandibular plane in those with OSAHS ($p = 001$).

This finding is comparable to that of Battagel et al (2000) who studied 115 Caucasian UK dentate male subjects, 45 of whom had OSAHS, 46 of whom were snorers and 24 of whom were controls. There was no difference between the OSAHS and snoring groups in terms of dentoskeletal measurements apart from the hyoid bone which was significantly more inferiorly positioned in the OSAHS group.

These findings are in agreement with previous studies by Andersson and Brattstrom (1991), Maltais et al (1991), Zucconi et al (1992) and Frohberg et al (1995). Furthermore, an extrapolation of the work done on subjects originally recruited for the Bolton-Brush longitudinal study in the USA on orthodontic and craniofacial parameters by Nelson et al (2003) showed that male snorers, compared to non-snorers had a more inferiorly positioned hyoid bone throughout life. The importance of this observation derives from the observation that the tongue mass is more concentrated in the hypopharyngeal region with a low-set hyoid. This hyoid position may therefore be a poor prognostic indicator for successful use of mandibular advancement splints [Lyberg et al (1989) and Mayer and Meier-Ewart (1995)].

There was no difference in bony angles between the OSAHS and snoring groups. Neither mandible nor maxilla was more retroposed in one group compared to the other, nor was there any difference in the cranial base angle.

These findings are supported by Battagel et al (2000), Jamieson et al (1986), Battagel and l'Estrange (1996), Steinberg and Fraser (1995) and Robertson (2002) who looked specifically at the cranial base differences between snorers and OSAHS patients. The implication of a wider NSBa is the placement of the craniofacial complex closer to the cervical spine, thus contributing to the reduction in airway space.

Studies addressing specifically the differences and similarities between female apnoeics and simple snorers are not available and the small numbers reported in the literature are either subsumed by the larger male group or not discussed separately.

In this study, women with OSAHS had a lower set hyoid bone in relation to the mandibular plane than non-apnoeic female snorers ($p = 0.038$). These results and

their implications mirror those for the male group. Furthermore, women with OSAHS had a significantly greater lower facial height (mirrored in the greater anterior mandibular length) compared to snorers. This was not present in the male group. Reasons for this difference could be accounted for by small sample size (false positive association), environmental/behavioural influences on the development of the face (eg lifelong mouth breathing, nasal obstruction) beyond the scope of this study and possible dental influences such as missing molar teeth etc (although all subjects in this analysis were dentate). These findings have been reported previously in male groups with OSAHS (Lowe et al 1986, 1996; Bacon et al 1990; Tsuchiya et al 1992; Pae & Ferguson 1996). In a recent study, Kawashima et al (2002) found that children with 25 – 75% of their tonsils visible and who had sleep disordered breathing had a greater anterior lower facial height and a short nasal floor compared to controls.

5.2.2 The Associations of Obesity and Cephalometric findings in OSAHS

In the last decade, increasing attention has been focussed on the different morphological characteristics of the obese and non-obese craniofacial skeleton.

Male subjects with OSAHS in this study who were obese (BMI \geq 30kg/m²) had significantly longer mandibular rami, hard palate lengths and posterior maxillary heights compared to the non-obese. There was also a trend toward a longer mandibular corpus, greater distance of the hyoid from C3 anteriorly and a more obtuse cranial base angle (but these were not significant when corrected for multiple comparisons). In the female group, the only significant difference between the obese

and non-obese subjects with OSAHS was in a more obtuse cranial base angle. However, the numbers of women with OSAHS were small ($n = 15$).

In the male population studied, the findings for the posterior maxillary height and C3 to hyoid distance are similar to those in the Battagel et al (2000) study and Tangugsorn et al (2000). A study by Paoli et al (2001) also showed differences between 46 obese apnoeics ($BMI > 30\text{kg/m}^2$) and 39 non-obese apnoeics which comprised greater back-setting of the mandible (more acute SNB angle), longer anterior cranial base measurement and greater mandibular ramus length in the obese group. However, no corrections were made for multiple comparisons (31 in total), which when I performed them using the Holm method, left only the hypopharyngeal space and hyoid to chin distance significantly greater in the obese group.

The study by Ferguson et al (1995) which divided 161 men with OSAHS into 3 groups on the basis of neck circumference (correlated with BMI), showed that the larger the neck circumference, the longer the maxilla and the mandible. Neck circumference was also inversely proportional to the skeletal class (as measured by ANB) and related to the SNB angle (thereby, retrognathia). The overall results of the study suggested that bony abnormalities were of greater importance in the pathogenesis of OSAHS in the less obese compared to the obese where soft tissue parameters such as enlarged tongue and retropharyngeal tissue thickness played a potentially more significant role. However, the division of the data set by neck circumference rather than BMI and the use of Pearson's correlation rather than statistical techniques incorporating correction for multiple comparisons make this study difficult to compare directly with other reported findings.

Why should there be a difference in bony measurements between the obese and non-obese subjects with OSAHS?

One recent study in obese adolescents (male and female) showed that they had significantly larger craniofacial parameters and were more prognathic than their age and sex-matched controls (Ohm et al 2002). Interestingly, the authors postulated that growth of the craniofacial complex in the obese population may be largely regulated by increased levels of circulating IgF-1 acting directly at the level of the bone and independently of GH. Growth hormone and IGF-1 normally increase during adolescence. Fasting insulin levels are increased in obese adolescents (and adults) compared to lean ones and this hyperinsulinaemia chronically suppresses levels of IGF-binding protein – 1. Low levels of IGFBP-1 may thus lead to an increase in the bioavailability of free IGF-1, resulting in low circulating GH and total IGF-1 (Attia et al 1998).

This is in contrast to acromegaly, which may manifest similar changes in bony structures to obesity, most notably prognathism, but is driven largely by excess GH although elevated levels of IGF-1 can also exist (Dostalova et al 2001).

However, the changes in acromegaly are very gross (see for example Dostalova et al 2001) and more subtle differences are seen in the obese patient with OSAHS. The number of patients in this study with prognathism (Class III skeletal change) was very small (18 men and 5 women). The vast majority of men were orthognathic. These findings compare with those of Ferguson et al (1995) who reported neck circumference to be inversely related to angle ANB ($r^2 = 0.03$; $p < 0.05$).

It is possible that there may be a small direct effect of GH and/or IGF-1 on bone growth (and concurrently on soft tissue growth), which may be modulated by the

patient's genotype for receptor or binding proteins. Alternatively, soft tissue growth may be primary, and the alterations in the bony skeleton an adaptive compensatory response, e.g. more obtuse cranial base angle, larger posterior maxillary height and nasopharyngeal space to accommodate increased soft tissue bulk and allow for adequate gas exchange.

5.2.3 The Effects of Age on Cephalometric findings in OSAHS

The effects of age were limited to an increase in the mandibular corpus height in male study subjects over the age of 51 years compared to those who were younger. No differences were observed between the younger and older female age groups. It is important when considering the craniofacial morphology in patients with OSAHS to bear in mind changes that occur with age regardless of pathology and which may be contributors to sleep disordered breathing as a result of this. Behrents (1985) was the first to show systematically that the craniofacial complex continued to grow in adult life using subjects from the Bolton-Brush study. Using subjects from the same cohort, Nelson et al (2003) showed that the HMP distance increased significantly with age regardless of snoring status (mandibular height was not measured). This finding is consistent with two other studies by West et al (1999) and Maltais (1991). A further study by Kawashima (2002) demonstrated that in male preschool children with sleep-disordered breathing, the hyoid bone position was situated more anteriorly, and they had a greater anterior lower facial height compared to girls. The descending position of the hyoid with age is thought to be the result of the tongue's enlargement in relation to the intermaxillary space – a trend pronounced in males (Cohen and Vig, 1976; Kollias and Krogstad 1999).

In the Nelson et al study (2003) there was no significant change with age in the bony angular measurements of NSBa, SNA and middle cranial fossa alignment from puberty through to adulthood. A study by Doual et al (1997) investigating the influence of ageing on craniofacial and cervical morphology in 84 men and 102 women (ages range 21 – 101 years) showed that morphological changes in the craniofacial complex during adulthood became pronounced at around fifty years in both sexes. They suggested that bone structures of membranous origin underwent more significant modification compared with structures of endochondral origin. Similar findings were shown by Macho (1986).

5.2.4 The Effects of Gender on Cephalometric findings in OSAHS

Differences in the craniofacial complex with gender are well recognised (Behrents 1985; Ursi et al 1993; Kollias and Krogstad 1999; Ingerslev and Solow 1975; Buschang et al 1983) so it is difficult to understand why numerous studies looking at OSAHS have lumped results for males and females together (e.g. Prachartam et al 1992; Douglas et al 1995). Females are smaller at all ages, grow less and have significantly shorter mandibles than males. Furthermore, mandibular plane rotations in a forward direction are seen in males with posterior rotations in females accompanied by compensatory changes in the dentition. At any age, therefore, women have significantly more backset mandibles than men on average. This was demonstrated in the current study population, even when allowance was made for BMI, age and a diagnosis of OSAHS. Interestingly, there was no significant difference overall between men and women in the SNA or NSBA angles.

5.2.5 Genetic Factors affecting the Craniofacial Complex in OSAHS

In an attempt to examine whether there are certain heritable craniofacial features that make sleep disordered breathing more likely, the bony measurements and angles for male sibling pairs discordant and concordant for OSAHS were examined. (There were insufficient female sibling pairs for adequate power for such an analysis).

There was no significant difference in any of the bony measurements or angles between brothers **concordant** for the diagnosis of OSAHS. However, in the 22 pairs of brothers **discordant** for OSAHS (equivalent in age, BMI and neck circumference), those with the diagnosis had a trend towards a shorter mandibular corpus and mandibular ramus and a lower-swung hyoid in relation to the mandibular plane. Those without OSAHS, however, were still all snorers, thus limiting the likelihood of finding cephalometric differences.

These results suggest that though siblings share the same characteristics that predispose to snoring and sleep disordered breathing in relation to the rest of the population, additional morphological characteristics are necessary to cause OSAHS independently of obesity.

The observation that the hyoid is lower in sibs with OSAHS is especially interesting in light of studies that have shown that children between 7 and 14 who snored had significantly lower hyoid bones than did non-snoring children (Kulnis et al 2000). Apnoeic children had also been found to have a longer HMP distances (Shintani et al 1992; Kawashima 2002). Nelson et al (2003) in their longitudinal study of snoring vs. non-snoring subjects found that snorers had significantly lower-set hyoids throughout life – from pre-puberty right through to adulthood although the HMP distance increased throughout life in all subjects. This finding is confirmed for the

age between 22 and 42 years especially in the male population by Kollias and Krogstad (1999). It is possible that the low-slung hyoid, related to tongue bulk, is present from childhood and may predispose toward snoring and apnoea in later life. Numerous studies examining the adult OSAHS population have shown that the hyoid is low-slung in this condition compared to either non-snorers or snoring controls (Battagel et al 2000, Tangugsorn et al 2000; Andersson and Brattstrom 1991, Maltais et al 1991, Zucconi et al 1992 and Frohberg et al 1995).

The regulation of mandibular size, especially in postnatal growth, has been discussed in Chapter 2. Sibs with smaller mandibles may have inherited the less functional alleles of the GH, GHR, IGF-1 receptor and the various binding protein genes quite apart from the other regulators of bone growth from sex hormones to Vitamin D.

In respect of whether genetic factors or environment are most important in the development of the craniofacial complex, several studies have been published looking at the heritability of the craniofacial complex (Ramsey et al 1992; Byard et al 1984; Saunders et al 1980; Byard et al 1985; Byard et al 1988; Sorin et al 1991; King et al 1993). All of these studies demonstrate that a proportion of facial characteristics are the result of a polygenic mode of inheritance, with a certain proportion subject to environmental influences or other disease conditions e.g. perennial allergic rhinitis which may alter growth of the maxillo-mandibular complex in those who are predisposed to its development (Trask et al 1987); nasal blockage in early life (Yamada et al 1997).

There have been no extensive studies exploring craniofacial similarities or differences between siblings with and without OSAHS to date.

There have also been no studies comparing controls to subjects with OSAHS and their relatives. One study examined 51 non-obese relatives (25 males) of patients with OSAHS and compared them to age, BMI and sex-matched controls recruited from a GP register (Mathur and Douglas 1991). Unfortunately, only 36 pairs of study subjects had available cephalograms. The major finding was of a backset mandible and maxilla in the relatives compared to normal population controls. The authors suggested that this could possibly indicate a heritable craniofacial structure predisposing to OSAHS. Unfortunately no mention was made of whether the population was dentate or not. The angles reported, especially for the SNA were high in relation to other reported means in the literature for a Caucasian population (e.g. Battagel et al 2000) which may be related to the small sample size. Regardless of these points it was the first study to show that there are certain craniofacial features that may divide the general population into those likely to develop snoring and OSAHS and those that do not regardless of obesity.

5.2.6 Other considerations

An important consideration in all cephalometric studies is whether the subjects are dentate or not. In the current study, the edentulous population had a significantly more backset maxilla and a shorter maxillary height. Whether or not this may independently contribute to a diagnosis of OSAHS has never been examined. A few studies are available that have examined the effect of denture wearing over time. Tallgren and Solow (1984) showed that over a period of 15 years, a change in hyoid position occurred with denture wearing due to changes in the mandibular and cervical inclination and craniocervical angulation. Karkazis et al (1997), in line with

previous studies, demonstrated significant reduction in anterior mandibular height, which was especially dramatic in women within two years of starting dentures. There are only three reports in the literature with respect to edentulism and sleep apnoea, two concentrate on the production of prostheses for the edentulous (Meyer and Knudson 1990; Robertson 1998) with the third being a case report on the worsening of OSAHS with complete edentulism (Bucca et al 1999). Interestingly, in the latter report, a striking decrease in the anteroposterior oropharyngeal wall distance on supine cephalometry was shown when the patient had his dentures out. A study of a further six men (Bucca et al 1999) showed that if they slept without dentures, there was a marked decrease in the retropharyngeal space, an increase in the AHI and a decrease in both mean and lowest arterial oxygen desaturation.

Another consideration in cephalometry is the role of ethnicity in the population under study. In this population, all subjects were Caucasian and came from the Scottish population which has been subject to genetic influx over the centuries (Picts, Celts, Romans, Anglo-Saxons and others). A number of studies have explored racial differences in cephalometric variables (Owens et al 2002; Moate and Darendeliler 2002; Liu et al 2000), some in relation to OSAHS (Will et al 1995). It is important not to include those of different race in the same analyses. However, Richardson (1980) in examining Caucasians of different ethnic groups (e.g., Swedish whites vs. American whites) stated that quantifiable differences between such ethnic (rather than racial) groups are small and there is an enormous degree of overlap. In such instances, the greatest differences are to be found in the alveolar and dental areas of the craniofacial skeleton.

5.2.7 Limitations of the cephalometric Study

The major limitation of this study, as for all cephalometric studies, lies in the examination of a three-dimensional structure using a two-dimensional technique.

However, the method is sufficiently powerful to allow for the recognition of trends and changes in the craniofacial skeleton in a way that makes it comparable to other studies in similar populations. Furthermore, its relative simplicity, ease of access, low radiation exposure and low cost as well as its routine use in the practice of prosthodontics and orthodontics makes it a more practical tool in the investigation of the craniofacial complex than more sophisticated techniques.

The other limitation of the study comprised the lack of soft tissue measurements in the study group. These were not performed owing to changes in soft tissue parameters that are common in patients that use CPAP (Mortimore et al 1996; Schwab et al 1996). Approximately 50% of the study group were already on CPAP, which would invalidate the measurements and make comparison between groups difficult.

Lastly, the study would have been further enhanced if there were age and sex-matched controls from the normal population. However, this was not the original study design and the population examined allowed for accurate assessment of heritable factors in craniofacial structure and confirmed some of the previous findings in purely apnoeic and non-apnoeic snoring populations from published studies.

5.3 GENOTYPING

5.3.1 DNA EXTRACTION

DNA extraction using both techniques documented is standard. The only problem arose with storage method of the bloods collected in the SIDS study making 50% of them unusable. On initial collection, the bloods were spun and the plasma and cell fractions were stored separately. Recombination of the two components did not yield sufficient sample volume in 50% of the group. However, the DNA quality in the samples that could be extracted was adequate to allow for analysis.

5.3.2 PCR-RFLP

There were no significant problems with the technique used for genotyping APOE alleles. Small alterations were made from the published technique by Ossendorf and Prellwitz (2000), which are documented in the Method section (Chapter 3, section 3.5.1). Of the 78 patients genotyped, 5 results could not be clearly read by either scorer, independently and so were discarded.

5.3.3 TAQMAN- BASED ALLELIC DISCRIMINATION

The technique itself was not problematic. Primers designed by ABI systems that were used in the study for GHR and 5HTR2a had minimal dropout on the plates. Likewise, the primer designed for the TNF- α -308 (A/G) reacted consistently with the DNA samples. The most problematic primer to construct was that for the C+79G ADRB polymorphisms, which though passing quality control checks at ABI failed to bind with the DNA in 38 % of the total sample. At present, the reason for this failure

has not been fully elucidated but may have a connection to GC-rich regions within ADRB2 which can also interfere with specificity in annealing in PCR-RFLP. Approximately 15% of all SNPs that go through the assay-by-design (ABI systems™) service will experience this problem. A slightly different technique has been reported in the literature as posing problems with inaccuracies in base calling with relation to ADRB2 (Humma et al 2000). Reassuringly, alleles in the control population were in Hardy-Weinberg equilibrium and did not differ significantly from published frequencies for a UK population mitigating against genotyping error in the sample used.

TaqMan based allelic discrimination testing has an estimated error-rate of <1 in 2000 genotypes (Ranade et al 2001). This level of accuracy is achieved by ensuring uniform buffer conditions by making up a large batch of master-mix and utilising a robot in the pipetting procedure to eliminate small errors caused by hand. Even a small error can cause large differences in post-PCR fluorescence values. To correct for pipetting errors, fluorescence values were measured prior to PCR. Theoretically, all wells prior to PCR should have equal amounts of fluorescence from either the reporter or reference dyes. However, the amounts tend to vary in practice and can cause predictable variation in post-PCR fluorescence values. Mean fluorescence values prior to PCR for each reporter dye are calculated for each plate. If the mean value of a plate is significantly different from the others, as judged by a non-parametric Wilcoxon signed-rank test, then post-PCR values for that particular plate are adjusted accordingly.

Although reading of the data is not automated in the system used in this study, visual examination of the plots by two researchers blind to subject status ensured independent verification of point allocation, and with a high degree of correlation.

The single greatest limitation of the technique is the fact that it is biallelic (ie can only assay for one SNP at a time). (However, the whole technique of SNP genotyping is biallelic, as opposed to microsatellite markers or short tandem repeats that have multiple alleles). In Taqman based allelic discrimination analyses, there are also problems with probe construction, especially if GC-rich areas in the gene are encountered in the area of the SNP, which is the case with APOE and also with ADRB2. This may affect accuracy and reliability, and in such instances, PCR-RFLP or DNA-sequencing are currently still the best available methods. (They were not conducted in our study due to time and budgetary constraints).

5.4 APOLIPOPROTEIN E

A third of the 73-strong population assayed for APOE was indeterminate for OSAHS. Not surprisingly, there were significant differences between those with and without a definite diagnosis of OSAHS in terms of the sex distribution (more men had OSHAS), neck circumference and lowest nocturnal desaturation. However, importantly, there were no significant differences between age, BMI and blood pressure, both systolic and diastolic, at the time of the study. This is an important consideration in view of the associations of ApoE with increased risk of vascular disease and lipid metabolism.

Results for the genotype and allelic distributions of the APOE alleles, E2, E3 and E4 show no significant association with a diagnosis of OSAHS in the population

studied. Reclassification of the group of 73 subjects according to an AHI of greater than or equal to or less than 15 likewise failed to show a difference in genotype or allelic frequency between the two groups.

A further hypothesis examined in this group was whether those carrying the APOE4 allele differed in any way compared to those who didn't. There was no difference in any of the characteristics including AHI, sleep variables, nocturnal desaturation or blood pressure. The same hypothesis tested previously by Mignot et al (2000) suggested that those with the E4 allele had a greater AHI (see Chapter 2). The results obtained in this study support the findings of Kadotani et al (2000) and Saaraleinen et al (1998) that there are no differences in APOE alleles in OSAHS.

It is important to stress that the distribution of alleles in this study did not differ significantly from that of a white UK random population (Corbo and Scacchi 1999). Furthermore, the study was adequately powered to detect a true difference in allelic frequency between the groups to 90% at the $\alpha = 0.05$ level.

To detect the difference in APOE4 carriage alone in association with either OSAHS or an AHI of 15 or greater would require 469 subjects in each group. To date no study has presented genotyping results in this number with regard to ApoE in relation to sleep.

Is there a link between sleep-disordered breathing or OSAHS and APOE4?

On the basis of previous results and the current study, it seems unlikely.

As previously discussed in Chapter 2, it appears biologically implausible that carriage of the E4 allele should be associated with sleep disordered breathing directly, and might only indirectly influence it. As a thrifty allele it may contribute to obesity. However, even if this were the case, differences in the allelic frequencies

should be – and were not - detected between the obese and non-obese group in this study and others. The presence of ApoE4 in various conditions ranging from Alzheimer's disease to neuropathy is suggestive of association rather than of direct causation. Its contribution to the pathobiology of these conditions is also disputed. It is possible that it may act as a marker for another disease locus or gene as yet unidentified and this may go some way toward explaining the variability of associations from disease to disease and population to population.

Many attempts have been made to link sleep disordered breathing with cognitive decline in aging (see Bliwise 2002 for discussion) but the link remains tenuous at best. Of greater interest is the possible role for ApoE4 as a marker of vulnerability for, or the presence of, cerebrovascular disease. The role of ApoE4 in the central nervous system is complex, but some data suggest that ApoE expression is up-regulated in response to cerebral ischaemia. Furthermore, a few studies have shown ApoE4 subtype to be associated with poorer recovery from cardiac by-pass surgery, haemorrhagic stroke and head injury.

Although one study has associated a significantly higher number of infarcts on brain MRI in a group of men with OSAHS perfectly matched with normal controls (Davies et al 2001), other studies that have been equally carefully conducted have shown no increase in SDB in an older population with transient ischaemic attacks compared to healthy age and sex-matched controls (McArdle et al. 2003). Other studies examining the risk of stroke in association with sleep-disordered breathing, though flawed in method, suggest a link (Bassetti and Aldrich 1999).

However, in the light of results presented in this study and previous studies, there is currently no evidence for a direct association of ApoE4 with OSAHS. Pursuing a

possible link may not be a fruitful path forward at present and we may be better served by focussing on genetic aspects of hypertension in OSAHS and the effects of nocturnal hypoxaemia on vascular endothelial function at a molecular level.

5.5 Tumour Necrosis Factor Alpha

TNF- α is a potent inflammatory cytokine. The main source of TNF- α is activated mononuclear leukocytes but it is widely secreted by a variety of other immune and non-immune cell types including fibroblasts, smooth muscle cells, astrocytes and neurones. Elevation of TNF- α occurs in many infectious, inflammatory, autoimmune and neoplastic diseases. An exhaustive list is documented and updated at the following website:

<http://www.bris.ac.uk/Depts/PathAndMicro/services/GAI/cytokine4.htm>

A functional TNF- α gene polymorphism consisting of a guanine (G) to adenine (A) substitution exists at position -308 in the promoter region. Compared to the wildtype, the -308A variant results in greater increase in TNF- α production *in vitro* and *in vivo* (see e.g. Louis et al 1998; Bouma et al 1996; Huang et al 1999; Kroeger et al 2000; Maurer et al 1999).

In this study, the TNF- α -308 A allele was significantly more common in subjects with a diagnosis of OSAHS. Carriage of the A allele was significantly associated with the OSAHS phenotype in allele discordant sib pairs, suggesting TNF- α may have a disease-promoting role in OSAHS. The TNF- α -308 allele was also significantly more common in the older age group (discussed below). The -308 A allele was also associated with male sex. This last finding is most likely due to the higher number of males in the OSAHS group and therefore to be spurious as there is

no rationale for expecting such an association. Both differences disappear when age and sex are controlled for in regression analysis.

OSAHS is an inflammatory condition

To date, four studies have demonstrated hypercytokinaemia in subjects with OSAHS. Vgontzas et al (1997; 2000), Entzian et al (1996) and Liu et al (2000) the results of which can be summarised as follows:

1. TNF- α and IL-6 are elevated in OSAHS independently of obesity
2. BMI positively correlates with TNF- α and IL-6 levels. This suggests that these two cytokines may play a role in daytime sleepiness experienced by the obese in the absence of OSAHS.

C-reactive protein (C-RP) levels have been found to be elevated in untreated sleep apnoeics without any known comorbidities (Shamsuzzaman et al 2002). C-RP production is enhanced in the liver by IL-6 and is a more stable marker of inflammation over a 24-hour period than cytokines. An elevated C-RP level has been shown to be a strong predictor of cardiovascular disease and is present secondary to the hypoxaemia of high altitude together with elevated levels of IL-6. C-RP has also been found to be elevated in obesity secondary to increased expression of IL-6 in adipose tissue (Crichton et al 1996; Mohamed-Ali et al 1997; Bastard et al 1999). A higher BMI has been demonstrated in association with higher C-RP concentrations in young adults aged 17 – 39 years, confirming a state of low-grade systemic inflammation in overweight and obese persons (Visser et al 1999).

C-RP, IL-6 and TNF- α appear to produce their harmful effects by inducing endothelial dysfunction (Das 2001). TNF- α damages endothelial cells, causes apoptosis of endothelial cells and triggers procoagulant activity and fibrin deposition.

TNF- α also enhances the production of reactive oxygen species including inducible nitric oxide (NO) and decreases myocardial contractility in a dose-dependent fashion (Das 1990, 1993, 1999).

In the overweight and obese, serum levels of TNF- α , serum-soluble TNF receptor p55 and serum-soluble TNF receptor p75 have been found to be significantly higher than in lean subjects (Tsukui et al 2000; Zahorska-Markiewicz et al 2000).

An increased association of the TNF- α -308 A allele with obesity in various ethnic populations has also been shown, including Pima Indians (Norman et al 1995), French and Northern Irish (Hermann et al 1998), French Canadians with differential body fat deposition in line with gender differences (Pausova et al 2000) and Germans (Brand et al 2001).

In the present study, although no association was found between obesity and the higher TNF- α secreting genotype, the BMI overall was relatively increased - subjects without OSAHS had a BMI of approximately 27 compared to those with OSAHS who had a BMI of about 30. Our negative findings support those of Walston et al (1999) who examined 424 non-diabetic patients of mixed race at the John Hopkins Weight management centre in the USA and of Romeo et al (2001) who studied 194 Caucasian patients at an obesity clinic in Rome and found no association of obesity with the TNF- α -308A allele.

The role of TNF- α in hypertension

Elevated TNF- α levels have been identified as a risk factor for recurrent myocardial infarction and cardiovascular death and are associated with an increased risk of stroke (Elkind et al 2002). TNF- α levels have also been correlated with ankle-brachial index (Bruunsgaard et al 1999) and other measures of atherosclerosis

(Elneihoum et al 1997) and most recently, atherosclerotic carotid plaques (Elkind et al 2002). TNF- α levels are elevated in non-diabetic hypertensive subjects compared to normal controls (Demirbas et al 2002). Samad et al (1999) showed that TNF- α is a key factor in obesity-linked elevation of plasminogen activator inhibitor 1 in mice. Because TNF- α is chronically elevated in adipose tissue in obesity and contributes to insulin resistance and elevated TGF- β , there is a potential that this cascade of events may lead to elevated plasminogen activator, tissue factor and possibly the activation of other haemostatic genes that may promote cardiovascular risk (Samad et al 1999; Das 2002).

Population studies searching for an association between TNF- α gene polymorphisms and hypertension have been few and contradictory. Pausova et al (2000) in a French-Canadian group of patients showed an association of the TNF- α -308 SNP with higher blood pressure in relation to obesity, whilst results for over 641 patients and 710 controls in the Franco-Irish study by Hermann et al (1998) were negative.

There was no association of TNF- α genotype with raised systolic or diastolic blood pressure within the study group in this thesis.

TNF- α is involved in the generation of sleepiness

TNF- α is known to induce Slow Wave Sleep (SWS) in several species (Kapas and Krueger 1992; Shoham et al 1987) and TNF- α mRNA and protein expression in the brain exhibits circadian activity (Floyd and Krueger 1997; Bredow et al 1997). Takahashi et al (1995) showed that the use of TNF- α antibodies, binding proteins, soluble receptors or receptor fragments reduce SWS in otherwise normal rats and rabbits. Fang et al (1997) showed that mice lacking the 55kDa TNF receptor slept less than normal control strains.

Studies in human subjects have shown that plasma cytokine levels vary with the sleep-wake cycle. Darko et al (1995) showed that plasma levels of TNF- α varied in phase with EEG slow-wave amplitudes. Other studies have shown that TNF- α levels peak during sleep (Gudewill et al 1992; Entzian et al 1996; Vgontzas et al 1999) in young adults. The peak occurs close to the offset of sleep – a rhythm not present in the older adult.

The only studies to examine TNF- α polymorphisms in the field of sleep so far have been in relation to narcolepsy (Kato et al 1999; Hohjoh et al 2001a & 2001b; Hojoh 2000). However, the results have been contradictory and have also focussed on the TNF- α (-857 T/C) promoter polymorphisms as well as the TNF receptor gene (-196R). No correlation was made with the degree of sleepiness experienced by subjects.

In this study, the TNF- α genotype in the population did not correlate with sleepiness as measured by the ESS. However, this is a relatively crude measure influenced by psychological factors which are absent on more objective tests of sleepiness such as the MSLT or MWT (Olson et al 1998). The fact that the degree of sleep disordered breathing did not independently associate with TNF- α A allele carriage, but a diagnosis of definite OSAHS did (combination of SDB and ESS), suggests that there may be a potential role for genotype influencing sleepiness.

TNF- α is associated with aging as a risk factor for OSAHS

There is a growing body of evidence that aging is an inflammatory condition. Enhanced *in vitro* production of IL-6, TNF- α and IL-1b by mononuclear cells after mitogen stimulation have been found in the older age group (Born et al 1995; Fagiolo et al 1993; Grau et al 2001) and population studies have shown that the

magnitude in increase in the concentration of IL-6 is a reliable marker for functional disability and a predictor of disability and mortality in the elderly (Ferrucci et al 1999; Harris et al 1999).

Genotype may play a significant role in aging. One study (Bonafe et al 2001) of 700 individuals between 60 and 110 years of age showed a positive correlation of IL-6 levels with age. The IL-6 promoter polymorphism (-174 G/G) that predisposes toward high levels of this cytokine's production decreased in frequency with age. However, this effect was seen only in the male group – an observation that has been made for other pro- and anti-inflammatory cytokine gene polymorphisms in various disease conditions such as sepsis (Schroder et al 2000; Lio et al 2002a; 2002b).

The TNF- α -308 SNP has been investigated in only one group of elderly subjects to date – 250 Finnish nonagenarians (198 women) who all came from the same town with a population of 182,000 and were compared to 400 healthy blood donors (18 – 60 years) from the town and the surrounding district. There was no statistically significant difference between the two groups in genotype, but there was a likelihood of significant consanguinity and controls were not stratified by decade or sex. Furthermore, it is possible that by comparing healthy nonagenarians with healthy young blood donors, that an element of bias was introduced whereby a proportion of the population with comorbidity who were unable to donate blood or who had deceased earlier than their same-aged counterparts may have had higher carriage of TNF2 (speculative).

In this thesis, carriage of the -308 A allele was less common in the (younger) UK blood donor population than in the older Scottish population studied. The rates of this allele were 18% in the donors and 24% in the older Scottish controls ($p = 0.1$, χ^2 -

test) (compared with 27% in the OSAHS patients ($p = 0.5$ vs Scottish controls)). Such a small and non-significant difference in -308 A allele carriage between blood donors and Scottish controls suggests that it is not age alone which is responsible for increased TNF2 carriage in those with OSAHS. Furthermore, any such difference would be unlikely to account for the difference in carriage of the -308 A allele by OSAHS phenotype in allele discordant sib pairs. Thus, there appears to be a genuine association of this allele with OSAHS, apart from its association with older age. This is borne out by a significant association of the A allele with OSAHS even when age and sex are controlled for.

This study has several limitations. As a case-control study, it can only demonstrate an association of the -308 A allele with OSAHS and not causality. However, carriage of the A allele was significantly associated with the OSAHS phenotype in allele discordant sib pairs and this presents the strongest evidence that TNF- α may have a disease-promoting role in OSAHS. There may also have been residual confounding by other conditions affecting the prevalence of the -308 A allele in the OSAHS population. All subjects were asked to report associated medical conditions by questionnaire on enrolment into the study. The prevalence of conditions including bronchitis, diabetes mellitus and known ischemic heart disease in the OSAHS population, though higher than in the non-OSAHS population was low – 10%, 5% and 10% respectively.

Furthermore, the distribution of the G and A alleles between the Scottish OSAHS and Scottish non-apnoeic controls was non-significant ($p = 0.5$) as was the difference between the latter group and the UK blood donors ($p=0.1$). All allelic and genotype frequencies for the control group as a whole were in Hardy-Weinberg equilibrium

according to published information (see e.g. Allen 1999; Reynard et al 2000), mitigating against genotyping errors or problems with population stratification. Allelic frequencies, when compared to published UK data for Caucasians for TNF- α were also not significantly different excluding bias based on ethnicity (Reynard et al 2000). The power of the study was also adequate to determine a difference in allelic frequency (two-group continuity corrected X^2 -test of equal proportions showed power of 99% at $\alpha = 0.05$ level). Although the sleep disordered breathing status of the control group was unknown, this would tend to dilute the reported observation rather than strengthen it.

Finally, it should be borne in mind that TNF- α -308 has been found to be in linkage disequilibrium with HLA class I and II alleles, the class III region which encodes several components of the complement system and the MHC class IV cluster which includes lymphotoxin- α (one of five microsatellites within the TNF locus) and lymphotoxin- β (Ruuls and Sedgwick 1999). The association of TNF- α -308 A with OSAHS may therefore be due to the direct influence of the SNP in question and/or due to linkage disequilibrium with other polymorphisms within the TNF- α gene or other genes within the HLA system.

In conclusion, this is the first known study to examine the TNF- α -308 (A/G) SNP in subjects with OSAHS, lending support to the argument that OSAHS is a disease associated with inflammation.

5.6 GROWTH HORMONE RECEPTOR

The SNP +561 T/G for GHR has not been widely investigated. It is a variant of the gene which lies close to the P561T SNP (within ~ 10 kb) in the cytoplasmic domain (exon 10) of the GHR, was identified by Chujo et al (1996) and Goddard et al in

1997 and was the only one associated with increased mandibular height in a Japanese population (Yamaguchi et al 2000). The +561G/T SNP in the GHR was chosen in this study to act as a marker for possible disease associations as it may be in linkage disequilibrium with more functional polymorphisms.

In this study, the GHR +561 G allele was more commonly found in the OSAHS subjects, as was the G/G genotype. Of greater importance, was the independent association of the G allele and G/G genotype with a BMI \geq 30kg/m² in the study population. There was no association with craniofacial bony measurements in the whole population or when analysed separately in either men or women. Likewise, there was no association with height within the study population. This latter finding supports a previous report from Japan, which likewise did not find an association with a GHR variant in the normal population (Chujo et al 1996) with height. Other studies have been restricted to examining for GH and GHR variants in children and adults with short stature (Goddard et al 1997; Goddard et al 1995 etc).

The absence of an association of the +561G/T GHR SNP with bony measurements in the craniofacial complex is in contrast to the findings of Yamaguchi et al (2000). However their study found a link with a slightly differently located SNP within the cytoplasmic domain and with one bony measurement only. This finding in 100 members of the general population may have been by chance alone. Furthermore, there is an ethnic difference between the populations studied and Yamaguchi et al were not searching for an association with OSAHS.

The highly significant association of the G allele with obesity is of interest in view of the major role of GH in the regulation of postnatal growth. Growth hormone insensitivity which when complete is known as Laron dwarfism (Laron et al 1966) is

characterised by small overall stature, classical craniofacial symmetrical reduction in size, constriction of the upper airway, obesity, elevated GH levels and insulin resistance. Recently the first report of a patient with Laron syndrome and OSAHS was published (Dagan et al 2001). GH is important in the regulation of carbohydrate and lipid metabolism (Nam and Marcus 2000) and is integral to the regulation of insulin at both a pre and post receptor level (Dominici and Turyn 2002 for review). In the obese adult and adolescent, GH levels are low (Attia et al) but growth continues, largely due to the effects of IGF-1 and low IGFBP-1. Recently it has been shown that the direct effects of leptin, sex hormones and insulin can directly activate the IGF system in growth plates and at bone growth centres (Philip et al 2002) independently of GH.

With regard to sleep, it is known that GH levels influence the amount of SWS that an individual has. There is a linear relationship between the amount of SWS and increased GH secretion (Van Cauter and Copinschi 2000). During ageing, SW sleep and GH secretion decrease exponentially and with the same chronology (Van Cauter et al 2000; Murck et al 1999). There is also a sex-dependent effect on GH secretion and its effects on sleep (Steiger 2002).

One study has examined GH secretion and peripheral sensitivity to its effects in the obese and patients with OSAHS (Gianotti et al 2002). A group of 13 adult males with OSAHS, weight -matched patients with simple obesity without sleep-disordered breathing and 10 normal lean males were studied with regard to their response to: 1. GH response to GHRH and arginine and 2. IGF-1 and IGFBP-3 responses to a very low dose of recombinant human GH treatment for 4 days. The GH response to the first intervention in OSAHS was lower than the response of the

obese group, which was lower than the lean group. Further, the IGF-1 rise in response to recombinant GH in OSAHS was significantly lower than in the obese group. The findings suggested that OSAHS is characterised by a concomitant impairment of GH secretion and sensitivity. It would not be unreasonable to consider that GH and GHR interactions may play some part in explaining these observations.

In fact Savage et al (2001) writing in the context of research into short stature, stated that the effects of mild growth hormone insensitivity due to an abnormal GHR which may also affect metabolism, specifically insulin resistance, IGF-1 levels and IGFBP levels and increase levels of obesity (Savage et al 2001) remained unknown and as yet unresearched in the general population.

However, it is also possible that the hypoxaemia associated with OSAHS may contribute to the reduction in circulating GH and peripheral sensitivity. It has been shown that the reduction of the nocturnal secretion of GH in overweight OSAHS patients is reversed by CPAP prior to a significant change in body weight (Grunstein et al 1989; Saini et al 1993; Cooper et al 1995).

The degree to which an aberrant allele affects function depends on where the polymorphism occurs in the gene. GH binding of the GHR induces dimerisation of the GHR and activation of the tyrosine kinase JAK2 (Frank 2001). This leads to tyrosine phosphorylation of the JAK2 itself and GHR and activation of a variety of signalling molecules, including signal transducer and activator of transcription (STAT) members, mitogen activated protein (MAP) kinase, phosphatidylinositol 3-phosphate kinase etc (Carter et al 1996; Frank 2001).

Depending on which part of the receptor the SNP is situated and whether it results in an amino acid substitution, which can affect configuration or not will determine its

effect. If the latter occurs, then the somatogenic effects of GH will be curtailed or blunted resulting in short stature or reduced growth (as demonstrated in STAT5b knockout mice – Udy et al 1997), whereas if the receptor just shows reduced but not entirely defective binding, then the effects may be more subtle and manifest as abnormal lipid and carbohydrate metabolism.

In addition to the effects of the GHR polymorphisms on the somatogenic effects of GH, there is new research in the area of GHR distribution in the CNS. In the last 10 years or so, evidence has accumulated that GH may exert profound effects on the CNS. Replacement therapy in the GH deficient adults and children has revealed beneficial effects on memory, mental alertness, motivation and working capacity as well as ameliorating behavioural problems (Nyberg 2000).

Specific binding sites in the CNS have been identified including the choroid plexus, hippocampus, hypothalamus and spinal cord. The density of GHR in the various brain regions has been found to decline with age and the receptors differ somewhat in size compared to their peripheral counterparts (Nyberg 2000).

Although many factors and pathways predispose to and are involved in the psychological and mental dysfunction associated with OSAHS, it is also possible that one of the pathways may involve growth hormone which reduces in amount with age.

All allelic and genotype frequencies for the control group were in Hardy-Weinberg equilibrium according to published information (Applied Biosystems™; <http://abassays.celera.com> – minor allele frequency in Caucasians = 0.46), mitigating against genotyping errors or problems with population stratification. Allelic frequencies, when compared to published data for Caucasians were also not

significantly different excluding bias based on ethnicity. Although the SDB status of the control group was unknown, this would tend to dilute the reported observation rather than strengthen it.

At present, the implications of a GHR SNP on CNS function and metabolic derangement in the context of OSAHS are largely speculative. However, the association of the +561 G/T GHR polymorphism with obesity should be investigated further.

5.7 SEROTONIN RECEPTOR- 2A

The silent polymorphism T102C for the 5HT_{2A} receptor (5HTR_{2A}) showed no differences in allelic association between subjects with definite OSAHS and controls without it.

Inayama et al (1994) were the first to report an association with the silent mutation in the coding region T102C in the 5HTR_{2A} in association with schizophrenia. The mutation does not alter the expression or structure of the protein and this suggests that a functional receptor variant of 5HTR_{2A} may be in linkage disequilibrium with T102C. Spurlock et al (1998) and Nothen and Propping (1998) identified an A-G polymorphism at -1438 in the promoter region of the 5HTR_{2A} which was in complete linkage disequilibrium with the silent T102C SNP, also confirmed by Ohara et al (1999) and Basile et al (2001). Functional analysis of A-1438G using luciferase assay demonstrated significant basal promoter activity in serotonin expressing HeLa cells by both the A and G variants. Comparison of the A and G variants however, showed no significant differences in basal activity or when promoter activity was induced by cAMP and protein kinase C-dependent mechanisms (Spurlock et al 1998). The size of the 5HTR_{2A} gene (20kbp) which

continues to be sequenced suggests that the silent SNP T102C studied is in linkage disequilibrium not only with the A-1438G SNP but potentially with other, as yet unidentified polymorphisms which may affect function of the gene.

The 5HT2A receptor has most recently been shown to be the predominant receptor subtype in hypoglossal motor neurones (Zhan et al 2002), which are intimately involved in the regulation of the major upper airway dilator during sleep, the genioglossus muscle. Pharmacologic trials of 5-HT receptor agonists and antagonists support this receptor subtype as well as 5-HT2c (found in much smaller quantities) as the predominant post-synaptic facilitator of hypoglossal motor neurones (McAll & Aghajanian 1980; Berger et al 1992; Kubin et al 1992; Al-Zubaidy et al 1996; Douse and White 1996; Bayliss et al 1997; Inoue et al 1999). A further study on the pharmacological activity of the serotonergic receptors in the hypoglossal nucleus (Fenik and Veasey 2003) showed that the receptor 2A antagonist MDL – 100, 907 dropped intrinsic hypoglossal nerve respiratory activity by 61% and significantly suppressed serotonin excitation of hypoglossal nerve activity in contrast to the 2C receptor.

Whether the receptor subtype is modulated by and contributes exclusively to sleep apnoea remains the subject of ongoing research at present. Certainly, the lack of a distinct genotype in the OSAHS population for the 5-HT2a receptor would be consistent with the observed variable responses to SSRIs that have been observed in published trials (Jagadeeshan et al 2000; Berry et al 1999; Kraiczi et al 1999). However, such a finding is not unexpected, as there has been much contradictory literature in the psychiatric field. Genes encoding for the 5-HT transporter and 5HT2A receptor have long been proposed as candidates for possible involvement in

mood disorders. The T102C polymorphism was identified (Erdmann et al 1996) and investigated for a possible association with schizophrenia (Inayama et al 1996), mood disorders (Zhang et al 1997) or response to treatment (Arranz et al 1995). However, the results in favour of an association could not be replicated (Frisch et al 1999; Tsai et al 1999) in other populations. A study by Minov et al (2001) examined 2 polymorphisms of the 5HT-2A receptor (T102C and His452Tyr) in a German population with unipolar depression (173 patients; 121 controls) as well as polymorphisms in the promoter region of the serotonin transporter gene (5-HTTLPR). His group found no significant association with depression in any of the gene polymorphisms studied with depression. The most interesting finding was a small but significant improvement in treatment response in patients (n=86) who carried the C-allele of the 5-HT2A-receptor polymorphism ($p = 0.023$). However, the patients were on various drug regimens (SSRI, TCA and ECT combinations) so the results cannot be generalized. So far, this remains the only study reporting an effect of the 5HT2A T102C receptor polymorphism on treatment outcomes in relation to SSRIs.

SSRIs may not be the ideal pharmacological option in OSAHS as they are not entirely selective for 5-HT in microdialysis experiments (Pozzi et al 1999) and may inhibit dopamine function in the midbrain (Kapur and Remington 1996). Furthermore, SSRIs have been associated with a decreased response to serotonin-2 (Glennon and Dukat 1995) so other more selective agents may be required.

Another very important consideration, which cannot be controlled for in rat and other animal experiments or indeed human experiments in relation to treatment responses, is the impact of genetic imprinting. With imprinted genes, despite biparental

inheritance resulting in 2 copies of the same gene, only one is expressed – the second copy is ‘silent’ or imprinted. The expressed copy is governed molecularly by a gamete-specific tag resulting in exclusive or predominant expression from the maternal or paternal copy (monoallelic expression). Functional imprinting can behave as a polymorphic trait - some but not all individuals express only the allele from a given parent. The 5-HT_{2a}-receptor gene was found to be paternally imprinted and transcribed from the maternal allele only by Kato et al 1996. However, a study by Bunzel et al (1998) examined brain tissue in 18 patients to assess the imprinting status of 5HT-2a receptor in the CNS. They found that 4 of 18 samples showed monoallelic expression, whilst the remaining 14 were bi-allelic for 5HTR2a expression with no difference between imprinting status according to brain area. Thus, 5HTR2A demonstrates polymorphic functional imprinting. This has essential consequences for the evaluation of genetic association studies and studies where responses to medications are analysed. Taking genomic imprinting into consideration in an extended family study (using trios) no association of bipolar disorder with 5HTR2A was shown in a recent study (Murphy et al 2001).

Other associations that have been investigated in relation to the 5HTR2A have been obesity and hypertension. Weight loss has been more studied in the 5HTR2C receptor in association with anorexia nervosa in girls (Westberg et al 2002) and Type II diabetes in association with obesity (Yuan et al 2000). Studies for the 5HTR2A receptor have yielded conflicting data (Collier et al 1997; Ziegler et al 1999) in this area. Whether obese patients with OSAHS may have a greater propensity to lose weight depending on genotype may become clinically relevant in the future and may play a role in what constitutes good medical advice. Two studies examining the

5HTR2A -1438G/A promoter polymorphism which is in absolute linkage disequilibrium with the T102C polymorphism have shown an association with increased food and alcohol intake in those with the G allele (Aubert et al 2000) and a higher BMI, waist to hip ratio and abdominal sagittal diameter in homozygotes for the G allele compared to homozygotes for the A allele (Rosmond et al 2002).

There was no difference in distribution of alleles or genotypes in the 5HTR2A T102C polymorphisms in relation to obesity in our study group. Neither was there an association with systolic or diastolic blood pressure, which has been shown specifically for the T102C polymorphism in a study by Liolitsa et al (2001) in elderly females with essential hypertension.

All allelic and genotype frequencies for the control group were in Hardy-Weinberg equilibrium according to published information (Applied Biosystems™; <http://abassays.celera.com>), mitigating against genotyping errors or problems with population stratification. Allelic frequencies, when compared to published data for Caucasians were also not significantly different excluding bias based on ethnicity (e.g. Williams et al 1997). Although the SDB status of the control group was unknown, this would tend to dilute the reported observation rather than strengthen it.

Power to detect a difference between carriage of the C and T alleles in association with OSAHS was very low. However, this does not preclude the *possibility* of a modest genetic effect of the C allele on development of OSAHS. Lohmueller et al (2003) in reviewing a meta-analysis of HTR2A associations with schizophrenia commented that with a relative risk of 1.07 for a positive association, a replication study would require 6,900 case-control pairs to achieve 80% power at $p < 0.05$. This is much larger than a typical association study with only hundreds of individuals. With

an OR of 1.03 for OSAHS in association with the C allele, replication of this study would require over 3,000 individuals.

Bearing this in mind and in contrast to the potential problems of treatment response studies with 5HTR2A, more promising results have been obtained with the serotonin transporter (5-HTT). This molecule is the initial site of action of SSRIs. A number of polymorphisms have been identified, but the two most important ones are located in the promoter region and the second intron of the gene (VNTR) (see Serretti et al 2002 for detailed discussion). The promoter region polymorphism has shown the most promising results in terms of reproducibility in a number of populations when treatment has been instituted. Probably the best study to date by Rausch et al 2002, examined SSRI dose-response relationship to 5-HTT kinetics with significant associations of the long promoter allele with placebo and drug response in comparison to the short allele carriers.

Bearing in mind the considerable problems inherent in association and treatment studies that polymorphisms in the 5HTR2A gene pose as well as the lack of replicated results to date, it might be more fruitful to concentrate on the serotonin transporter gene if pharmacological treatments for OSAHS are to be developed and implemented successfully.

5.8 BETA-2 ADRENERGIC RECEPTOR

The ADRB2 C+79G SNP results in a change in amino acid structure (Gln27Glu) which significantly alters receptor function (Liggett 1997). This SNP has been associated with obesity, changes in lipolysis, oxidative metabolism and energy expenditure, growth and hypertension. The other functional polymorphisms within the ADB2 receptor are in tight linkage disequilibrium. It should also be noted that the

ADBR2 gene is localized to chromosome 5q32-34 very close to several cytokine genes. It is thus possible that the individual SNPs of the ADRB2 may be markers for the action of cytokines in various pathological processes.

In our population, we failed to show a significant difference in the distribution of alleles for the C+79G ADRB2 gene polymorphism between subjects with OSAHS and controls. Genotype did differ however, and this was largely attributable to the significantly increased prevalence of the C/C genotype (Gln27Gln) in subjects with OSAHS. This distribution was significant in both male and female subsets of the population and was not related to obesity or hypertension. In fact the only difference was found for height in men with those with the C/C genotype being significantly taller than those without it ($p = 0.009$).

The potential importance of the ADRB2 C+79G SNP lies in its association with the regulation of energy expenditure and growth and its link with obesity and hypertension.

ADRB2 is the dominant lipolytic receptor in white human adipose tissue (Yang and McElligot 1989) and treatment of obese animals with selective beta2-agonists promotes a marked redistribution of body composition with a decrease in the fat mass and increase in the muscle mass (Yang and McElligot 1989). A recent study correlating these findings in 1152 French men and women showed no significant difference in the distribution of genotype in the population (Meirhaeghe et al 1999). However, they also assessed individuals on the basis of their BMI and the amount of regular physical activity that they undertook. Men with the Gln27Gln polymorphism were significantly more likely to be obese than those without it. However, the degree of obesity was counteracted by regular physical exercise. The authors concluded that

those individuals with the polymorphism were capable of modifying their BMI through exercise and thus, genotyping could prove useful in targeting prevention such as physical activity in those were most likely to benefit.

A study by Hellstrom et al (1999) also examined for the association of polymorphisms in the ADRB2 in relation to gender. They found that in 138 Swedish men, the Glu27 allele was significantly decreased when they were obese (BMI > 27kg/m²), whereas this allele frequency was increased in the obese group of 109 women. They concluded that different genetic factors contribute to obesity in males and females. Previous work by this group (Large et al 1997) had shown that female homozygotes for the Glu 27 allele (G/G) were significantly more obese and had 50% larger fat cells than controls and a fivefold increase in ADRB2-agonist affinity (using terbutaline). Studies attempting to replicate these findings in other populations have shown no such clear associations in either men (Bengtsson et al 2001; Kortner et al 1999 – German population; Echwald et al 1998 – Danish population; Hayakawa et al 2000 – Japanese population) or women (Bengtsson et al 2001; Oberkofler et al 2000 – Austrian population; Kortner et al 1999 – German population). A more detailed study looking at cardiovascular risk factors (including blood pressure, cholesterol levels, triglyceride levels etc) in a group of 284 Swedish men over the age of 51 years, showed the Glu27Glu genotype to be associated with elevated leptin and triglyceride levels only (Rosmond et al 2000). Bengtsson et al (2001) showed a weak association of Gln27 with hypertension, but more importantly, demonstrated this allele to be in linkage disequilibrium with the Cys19 allele of the 5'LC-Arg19Cys polymorphism which has much stronger associations with hypertension, obesity and insulin resistance.

The lack of an association between ADRB2 receptor gene polymorphisms (including Gln27Glu) with hypertension has been further demonstrated by Hermann et al (2002) who examined 1885 subjects with and without systolic hypertension in France, Scotland and Northern Ireland. This supports other similar findings by Xie et al (2000) whose population comprised black and white Americans. An association of the Gln27Glu genotype has also been found with diabetes. Carlsson et al (2001) showed that Type II diabetics carried the Gln27 polymorphism more frequently and that siblings of these subjects with the Gln27 allele had significantly more insulin resistance. They concluded that Glu27 was protective.

What does emerge from this body of contradictory evidence is that the ADRB2 gene may be important in function and change rather than being directly associated with a particular level of obesity or hypertension. Apart from the study by Meirhaeghe et al (1999) quoted above, 2 studies by Ukkola et al have shown that polymorphisms in the ADRB2 gene associated with codon 27 have significant impact on differential energy expenditure (Ukkola et al 2001) and gene-gene interaction with alpha-2 and beta-3 adrenergic receptor genes (Ukkola et al 2000). A third study by Macho-Azcarate et al (2002) demonstrated that obese women with the Gln27Gln genotype were better exercise responders.

All allelic and genotype frequencies for the control group were in Hardy-Weinberg equilibrium mitigating against genotyping errors or problems with population stratification. Allelic frequencies, when compared to published UK data for Caucasians for ADRB2 were also not significantly different excluding bias based on ethnicity (see e.g. Liggett et al 1997). Although the SDB status of the control group was unknown, this would tend to dilute the reported observation rather than

strengthen it. Power to detect a difference in phenotype with the C/C genotype was 81% at the $p < 0.05$ level.

The association of the C/C genotype of the ADRB2 with OSAHS may be significant in terms of distribution of body fat particularly as it has been shown to be in strong linkage disequilibrium with the Arg16Cys polymorphism (Oppert et al 1995), energy expenditure and insulin resistance. These factors may play an important role in the development of cardiovascular morbidity in OSAHS by affecting vascular response to hypoxaemia (Garovic et al 2002). There does not appear to be a link with blood pressure of the Gln27Glu SNP in the population studied. Future studies examining the role of ADRB2 in OSAHS, obesity or hypertension may need to take functional aspects into consideration in order to be meaningful.

5.9 Concluding Remarks

In this section the results for the study presented in Chapter 4 have been discussed in detail. The next section will summarise the findings of this thesis, highlighting the most pertinent of these, and discuss potential research directions for the future.

CHAPTER 6

6.1 CONCLUSIONS

This thesis explores genetic aspects and associations with OSAHS.

From the results of the study the following conclusions can be drawn:

1. OSAHS is not just a sporadic, but also a familial condition. The degree of environmental influence on its development is currently unknown but is almost certainly considerable including effects on obesity and face structure.
2. The OSAHS phenotype must take age and gender related changes in AHI into account to try and achieve greater accuracy. The OSAHS phenotype identified in this study is the product of many different changes at a single point in time. Whether a definite OSAHS phenotype exists and remains static throughout life and in relation to the effects of BMI and craniofacial change is unknown at present.
3. Cephalometry is a simple and well-validated technique for examining the craniofacial complex. In males, a smaller mandible and a lower-set hyoid bone are the most important characteristics distinguishing siblings with from sibs without OSAHS, independently of BMI and age. The influence of environment on this structural difference is unknown at present.
4. Male and female apnoeics have lower-set hyoid bones than non-apnoeic snorers.
5. Age, gender and BMI play a small but significant role in the continued growth of the craniofacial complex in adulthood and may affect changes associated with OSAHS. The impact of BMI on craniofacial parameters

raises questions as to whether it is the chicken or the egg in terms of determining the development of OSAHS.

6. Edentulism is a confounder in cephalometric studies in OSAHS and can significantly affect bony measurements and angles. This is an environmental effect on the craniofacial structure, which may independently predispose to or worsen OSAHS.
7. Apolipoprotein E alleles are not significantly differently distributed in a population with OSAHS compared to a general population from the same ethnic group. There is no evidence for an increased AHI in subjects with the E4 allele.
8. There is a significant association of the TNF- α -308 A allele carriage with OSAHS and it is likely to be a 'disease-associated gene'. Carriers of the allele may have increased generalised inflammation associated with OSAHS. There is also an association of the allele with ageing which may contribute to the development or worsening of OSAHS.
9. The GHR +561 G allele carriage is significantly related to morbid obesity. There is no association of this allele with bony measurements or height in the OSAHS population.
10. There was no difference in frequencies of the 5HTR2A T/C SNP between the cases and controls. A polymorphism in the 5HRT2A gene or one that is in strong linkage disequilibrium with it may not be directly responsible for mediating the observed differences in upper airway collapsibility in OSAHS.

11. ADRB2 Gln27Gln homozygosity is significantly more common in OSAHS.

There is a *potential* association with changes in energy expenditure, obesity, insulin resistance and hypertension.

OSAHS appears to be a polygenic disorder with a complex phenotype and this lends it unsuitable to genome-wide scans at the present time. The most common (and informative) study design in complex disease using genome scans to find areas of linkage is in affected sib pairs. However, the number of sib pairs required to lend sufficient power to the study would require an unrealistically large effort and would be economically and logistically unfeasible for a single research centre to attain (see e.g. Gu et al (1996) for discussion of power in sib pair studies for linkage analysis).

Despite these considerations, Palmer et al (2003b) have published the first study to perform a 9cM genome scan for OSAHS and obesity in 66 European American pedigrees comprising 349 subjects. OSAHS was phenotyped on the basis of AHI alone. The pedigrees were chosen on the basis of either an affected individual with overnight, in-home measurement of breathing using a portable monitor (Edentec®) or a proband who was a neighbourhood control individual. DNA was pooled and multipoint variance-component linkage analysis was performed for the OSAHS-associated quantitative phenotypes of AHI and BMI. The analysis identified candidate regions on chromosomes 1p (LOD score 1.39), 2p (LOD score 1.64), 12p (LOD score 1.43) and 19p (LOD score 1.40) for linkage with AHI. BMI was linked to the following regions: chromosome 2p (LOD 3.08), 7p (LOD score 2.53) and 12p (LOD score 3.41). Further statistical modelling indicated that evidence for linkage to AHI was removed after adjustment for BMI, excepting regions on chromosomes 2p (adjusted LOD score 1.33) and 19p (adjusted LOD score 1.45). When the inverse

was done (i.e. when BMI linkages were adjusted for AHI) the LOD scores were roughly halved. Palmer et al (2003b) concluded on the basis of these results that the interrelationship between OSAHS and obesity in Caucasians might be partially explained by a common causal pathway involving one or more genes regulating both AHI and BMI. Interestingly, the strongest association in this study, the chromosome 2p region contains the ADRB2 receptor. Furthermore, the 12q region contains the vitamin D receptor and IGF-1 and the 19q region contains APOE.

In spite of this, there are a number of potential limitations to this study. Firstly, as in the study by Buxbaum et al (2002) (see Chapter 2.1), AHI alone cannot be used to phenotype OSAHS. Secondly, the statistical method used is not classical for family studies - when families are used to examine for a disease-specific association, the simpler the statistical test the more real the result. Risch and Teng (1998) have discussed at length the use of the Transmission Disequilibrium Test and the SDT as the most informative statistic. A summary of statistical methods used in family-based studies is discussed by Kruglyak & Lander (1995), Holmans (2001), Whittemore & Tu (2000), Hassan et al (2002). Furthermore, in many respects, the above study by Palmer et al (2003b) became a case control study comparing families positive for the diagnosis of OSAHS with control families living in the same area without it. Theoretically, this should enhance the study, but the size of the sample remained modest and would have limited the power of the genome screen to detect linkage and increased the possibility of Type I errors (Terwilliger and Goring 2000). Further, the study should have pointed out how it had fulfilled the criteria of Lander and Kruglyak (1995) for genome-wide scans and which Palmer as co-author on a previous paper reviewing 101 genome-wide scans in humans (Altmüller et al 2001)

advocated. LOD scores of less than 2 are not considered to demonstrate significant linkage under most circumstances, especially in the context of modest power of the sample size, lack of a reported significance threshold for the sample and the use of quantitative traits. The latter possibly demonstrate intra-individual fluctuations over time. In addition, difficulties in the consideration of covariate adjustment for quantitative intermediate phenotypes may bias the phenotypes, introducing noise into the linkage analysis. Perhaps it is premature to utilise genome-wide scans in OSAHS at present and it is less likely to be successful as the phenotype is complex, at present difficult to define over more than a single point in time. Like all complex diseases, OSAHS also appears to be polygenic.

As Silverman and Palmer (2000) have pointed out, a significant limitation of linkage analysis is the difficulty of fine mapping the location of the gene influencing a complex disorder. There are usually insufficient meioses within 1 to 2 megabases of the disease gene to detect recombination events. With the effects of phenocopies and genetic heterogeneity in complex disease, critical recombination events may not be identified with certainty.

Choosing candidate genes for OSAHS in case-control studies remains difficult because a large number of disparate co-aetiologies need to be considered. These include obesity, craniofacial structure, upper airway control and sleepiness. Each of these aetiologies in turn is regulated by a wide variety of genes and mechanisms that are not necessarily related yet may interact and influence each other. Furthermore, the role of epigenetics is becoming increasingly important consideration and emphasizes the potential importance of influences that are difficult to measure with accuracy. Above all, this study highlights that there is still much to be done in our

search for the relevant genetic factors that will lead us to a greater understanding of this complex disease.

6.2 FUTURE DIRECTIONS

This study has answered few questions, but raised many more. Each broad area will be discussed in turn.

6.2.1 Phenotyping

Further investigation should be undertaken into whether the OSAHS phenotype remains static throughout life, or whether it changes with time and under different environmental conditions. At present, we are limited to studying the phenotype at a single point in time – when it calls itself to clinical attention, generally in middle age. Longitudinal studies should be undertaken, firstly identifying those who have OSAHS in childhood and following them through and secondly, continuing to follow those with sleep disordered breathing identified in adult life. There may be large differences in underlying genotype, for instance, between those progressing into old age with asymptomatic SDB, compared to those who develop symptoms and require treatment. We may discover that we are dealing with a range of diseases that manifest as a single phenotype at a particular point in time in the individual's life rather than a single disorder. Further study is needed to determine the best variables to be used to define phenotype and the age and gender related cut-offs to be used.

6.2.2 Craniofacial Development

Cephalometry continues to be a cheap, effective and universally applicable tool for identifying discrepancies in growth and craniofacial structure.

Further work should be undertaken in examining siblings in relation to age and sex-matched controls who are unrelated and extending this to the use of technologies such as MRI and CT scanning. Once again, longitudinal studies using unique data sets like the Bolton-Brush and Fels studies would be greatly enhanced by improved phenotyping of individuals with OSAHS by conducting PSG and other studies.

Further studies should be conducted in looking at the differences in diagnosis and treatment response in the dentate vs. the edentulous. This has been overlooked completely in sleep apnoea research.

6.2.3 Obesity

Obesity is a highly complex disorder and the large number of redundant energy pathways makes true candidate gene association studies difficult. On the basis of current results, functional studies exploring the role of the ADRB2 polymorphisms on weight loss and energy expenditure as well as the existence of insulin resistance and body fat distribution in OSAHS (Mortimore et al 1998) may lead to clinically meaningful and potentially useful results. The same applies to GHR SNP studies in obesity, which should be combined with functional endocrinological investigations.

6.2.3 The Upper Airway

Further work needs to be done on defining the role of other transmitter-mediated pathways in the regulation of upper airway tone during sleep.

It is too simplistic by far to accept that the hypoglossal nerve alone is the only significant regulator of this and by implication the serotonin receptor.

However, it remains without doubt the most important mechanism that we are aware of at present. Of greater importance than the serotonin receptor in mediating different pharmacological responses may be the serotonin transporter. In functional studies looking at the treatment of depression, the serotonin transporter polymorphism has had functional correlates with differing clinical response. One fruitful avenue in the area of OSAHS would be to examine for differences in allele distribution in the promoter polymorphism of 5HTT and its correlation to functional outcomes in a double blind, placebo controlled treatment trial of SSRI.

6.2.4 Cytokines, Sleep and Aging

The role of cytokine gene polymorphisms in the regulation of sleep appears to be inextricably linked to ageing. Further studies need to be conducted examining the role of IL-6 and IL-1 gene polymorphisms in sleepiness, OSAHS and ageing. Work on the TNF- α SNP identified to be of importance in this study should include more objective testing of sleepiness in the human population coupled with measurement of responses to treatment. Furthermore, changes in upper airway could be potentially different in the population with -308A carriage. The study needs to be replicated in other populations and also in populations of different ages with symptomatic and asymptomatic SDB.

6.3 Summary

OSAHS is a complex disorder and future work attempting to unravel its genetic basis may be better served by utilising a combination of linkage analysis with association studies on SNPs in the areas of interest. At present, much remains to be done in elucidating the precise role of genes involved in the regulation of craniofacial growth as well as upper airway control. Once the basic biochemical and physiological processes have been completely understood, then the role of various SNPs and their impact on the process will be rapidly resolved.

For the time being though, it seems that OSAHS, this common, polygenic disease will continue to be a challenge and at many levels a mystery for a long time to come.

The origin of all science is in the desire to know causes; and the origin of all false science and imposture is in the desire to accept false causes rather than none; or, which is the same thing, in the unwillingness to acknowledge our own ignorance.

‘Burke and the Edinburgh Phrenologists’ – *The Atlas*, February 15, 1829

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Appendix 1:

Epworth Sleepiness Questionnaire

The Epworth Sleepiness Scale

Sex (Male = M / Female = F)

Date:

Age (years)

How likely are you to doze off or fall asleep in the situations described in the box below, in contrast to feeling just tired?

This refers to your usual way of life in recent times.

Even if you haven't done some of these things recently, try to work out how they would have affected you.

Use the following scale to choose the most appropriate number for each situation:

- 0 = Would never doze
- 1 = Slight chance of dozing
- 2 = Moderate chance of dozing
- 3 = High chance of dozing


| Situation | Chance of Dozing |
|---|------------------|
| Sitting and reading | |
| Watching TV | |
| Sitting, inactive in a public place (eg a theatre or a meeting) | |
| As a passenger in a car for 1 hour without a break | |
| Lying down to rest in the afternoon when circumstances permit | |
| Sitting and talking to someone | |
| Sitting quietly after a lunch without alcohol | |
| In a car, while stopped for a few minutes in the traffic | |

Thank you for your co-operation

Appendix 2:

Enrolment Questionnaire

ton Place
urgh EH3 9YV
hone 0131 536 1000
131 536 1001



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CHIEF EXECUTIVE

John J Owens BSc, OMS, MBA

0131 536 2355 (Secretary) - 0131 536 2356 (Appointments) - 0131 536 2636 (Staff Nurses)
0131 536 2362
Director: Professor N J Douglas

Cons. Physician: Dr T W Mackay

NJD.MO.

As you may know, we have been doing some research which shows that sleep apnoea tends to run in families. To allow us to understand the mechanism of this and, hopefully to allow us to develop drug treatments for sleep apnoea, we wish to try to identify the genes which determine whether you develop sleep apnoea. I write to ask you whether you will be able to help us with this research.

We require to get the help of 150 of our thinner patients with sleep apnoea - and you come into this category. For each patient, we also require the help of one brother or sister. I would, therefore, be grateful if you would do three things to help us. Firstly, would you please fill in the enclosed form (1) indicating the number of brothers and sisters that you have. Secondly, I would be grateful if you would send one copy of the enclosed questionnaire to each of them and ask them to return the questionnaire to YOU. If you need more copies of the questionnaire, please phone Maggie on the above number. Thirdly, I would be grateful if you would then return all the completed questionnaires to us, along with Form 1, as soon as possible - a stamped addressed envelope is enclosed.

The reason that we are sending out the questionnaires is so that we can select the most appropriate brother or sister to help with our studies and ask them if they would agree to participate. For your information, your brother or sister would be asked to help us by spending a single night in the Sleep Laboratory and also by having one small blood test taken and - where agreeable - a single x-ray of the head. We will also ask you to have the simple blood test at your next clinic visit or by arrangement.

I have very much hope that you will be able to help us with this important research. Please do not hesitate to contact me if you require any further information.

Yours sincerely

Neil J Douglas MD FRCPE
Professor of Respiratory & Sleep Medicine

Enc:



+ Telephone Numbers

FORM 1

PATIENT'S NAME

NAMES OF BROTHERS

.....

.....

.....

NAMES OF SISTERS

.....

.....

.....

on Place
rgh EH3 9YW
one 0131 536 1000
31 536 1001



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CHIEF EXECUTIVE

John J Owens BSc, DMS, MBA

NJD/ED

20th June 1997

Telephone Numbers - Sleep Laboratory

0131-536-2355 (Secretary)

0131-536-2356 (Appointments)

0131-536-2636 (Staff Nurses)

Director: Professor N J Douglas

Consultant Physician: Dr T W Mackay

Dear

SLEEP APNOEA RESEARCH

I recently wrote to you asking for your help in a research project on the genetics of sleep apnoea and I enclose a copy of that letter. As best I can tell, I have not yet received a reply from you and write to ask if you would be kind enough to help us in this project. I am sure you realise that advances in the management of the condition from which you suffer are only achieved with a great deal of effort and co-operation and I very much hope that you will be able to assist with this important area of research.

Please do not hesitate to contact me if you wish any further information.

With kind regards,

Yours sincerely,

NEIL J DOUGLAS MD FRCPE

Professor of Respiratory & Sleep Medicine

ENC.



INFORMATION SHEET FOR PATIENTS INVOLVED IN THE STUDY OF GENETIC LINKAGE IN SLEEP APNOEA

1) Purpose of the study

Previous studies that we have carried out have shown that the sleep apnoea/hypopnoea syndrome runs in families. We are now trying to identify why it runs in families and specifically whether there is a particular gene involved. Identification of a gene might allow us to develop new therapies for this condition from which you suffer. This is a very long term objective and it is unlikely that this study will benefit you in the short term.

2) What you will need to do:-

We will ask you to list your brothers and sisters and pass on to them the enclosed Questionnaire and return the completed Questionnaires to the Sleep Laboratory. This is in order to allow us to select the brother or sister who is most likely also to have the sleep apnoea/hypopnoea syndrome. Clearly, this study is also voluntary on their part as well and we will enclose Information Sheets for them too.

a) We will require to take one blood sample from you at a mutually convenient time. This sample will allow us to identify whether sleep apnoea is associated with a particular gene. Excess blood will be destroyed after the testing has been done.

b) We stress that you are under no obligation to participate in the study. If you require further information, please do not hesitate to contact Dr Brander at 0131-536-2355. If you wish to take independent advice about the study, this can be obtained from Dr Tom Mackay, via the same telephone number. He is not involved in the study and can provide unbiased advice about your participation and the overall value of the study. All data obtained in the study will be confidential. We are, however, required to notify your general practitioner about your involvement in this project.

On completion of the study, we will inform all participants about the results via a meeting of the Scottish Association for Sleep Apnoea Meetings and Newsletter.

Appendix 3:

Sibling Enrolment Questionnaire



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CHIEF EXECUTIVE

John J Owens BSc, DMS, MBA

Lauriston Place

Edinburgh Sleep Laboratory

Tel: 0131 536 2355 (Secretary)

0131 536 1001

Fax: 0131 536 2362

Director: Professor N J Douglas

Consultant Physician: Dr T MacKay

Dear

A study of your breathing during sleep will be carried out in the Sleep Laboratory, Ward 48, Royal Infirmary, Lauriston Place, Edinburgh. Enclosed is a map of the Royal Infirmary. If you are coming by car there is earmarked parking available for overnight sleep study subjects and this is shown on the map. Please display the enclosed permit in your car. Car entrance is by Chalmers Street only. Pedestrian access is available from Lauriston Place as well.

You should report to the Sleep Laboratory at 9 pm. The Sleep Laboratory is part of Ward 48 and is on the top floor of the building indicated. There is a lift you may use. Once you get to the top floor, please ring the bell at the Sleep Laboratory door when the night sister will come to welcome you. I (or Dr Nigel McArdle) will be there as well. Please note that there will be no staff in the Sleep Laboratory between 5 pm and 8.30 pm (or on Sundays before 8.30 pm).

You should bring with you to your sleep study your normal night attire and wash things. You will be given a single room to sleep in. In the morning, you will be provided with breakfast. After taking the single blood sample and the x-ray, you will be free to leave at about 10 am. For local (Scottish) subjects the travel expenses will be paid later by the University of Edinburgh. For that purpose, we would need your National Insurance Number. I will inform you later about the results of your sleep study. If there is something to be concerned about, we would like to inform your GP as well. For that purpose, I would need the name and address of your GP.

With many thanks

Looking forward to seeing you

Yours sincerely

Dr Pirkko E. Brander, MD, postdoctoral fellow



INFORMATION SHEET FOR RELATIVES INVOLVED IN THE STUDY OF GENETIC LINKAGE IN SLEEP APNOEA

1) Purpose of the study

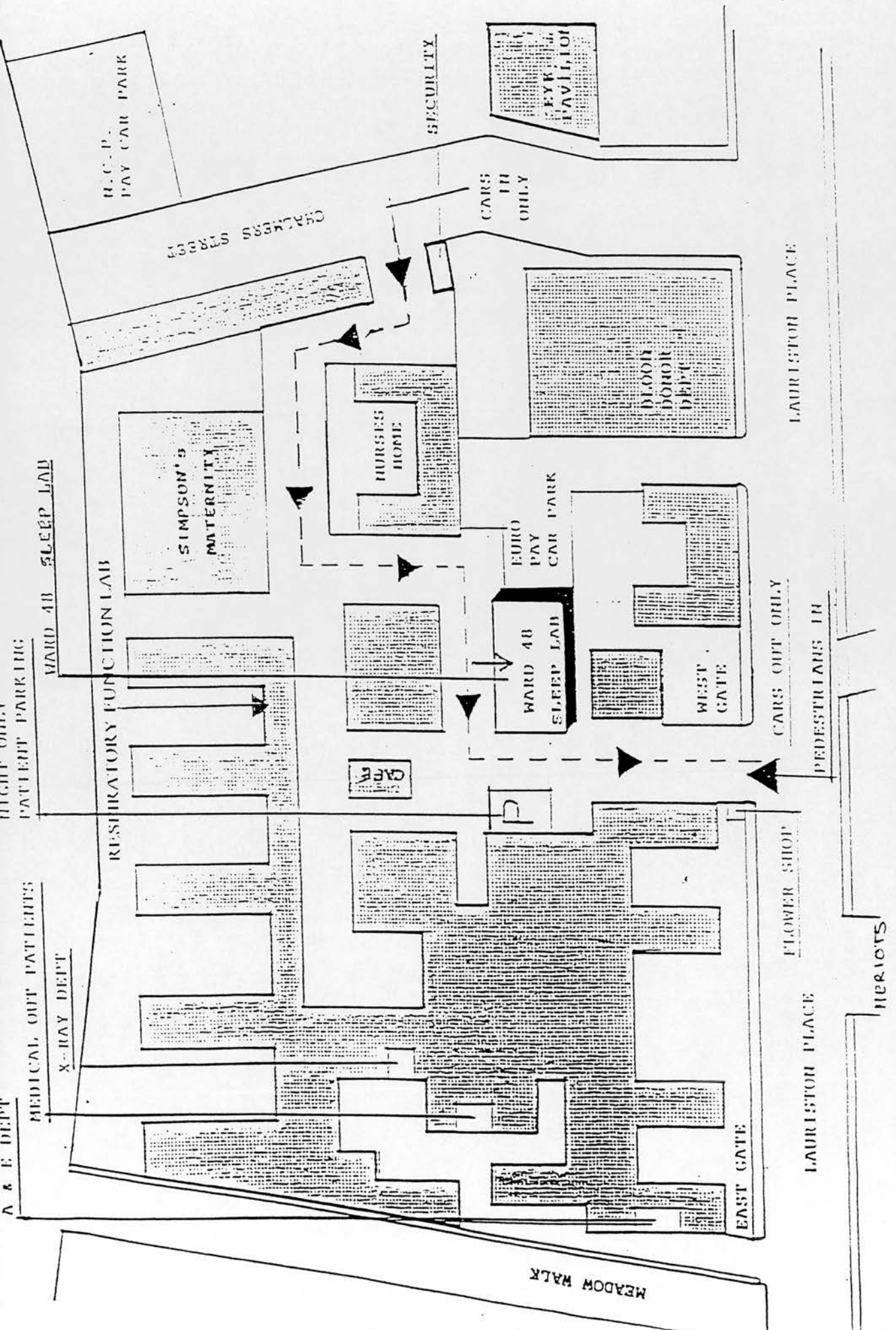
Previous studies that we have carried out have shown that the sleep apnoea/hypopnoea syndrome runs in families. We are now trying to identify why it runs in families and specifically whether there is a particular gene involved. Identification of a gene might allow us to develop new therapies for this condition.

2) What you will need to do

As you know, you have a brother/sister (delete as appropriate) with this condition and they will have passed on to you this information and the enclosed questionnaire. We would be grateful if you would complete the Questionnaire and return it to your brother/sister who will send it back to the Sleep Laboratory. This is in order to allow us to select the brother or sister who is most likely to have some irregular breathing at night. This study is entirely voluntary on your part. The study will involve you in:-

- a) Completing the Questionnaire: If selected, we will ask you to attend the Sleep Laboratory for one night for a sleep study which will be similar to the study which your brother or big sister had when sleep apnoea was diagnosed. During this night, we will record your breathing pattern and sleep pattern by taping wires on to your body. None of this is painful and there are no needles involved. You will be required to attend the Sleep Laboratory about 9 p.m. at night and will be given breakfast at about 7.30 a.m.
- b) Either the next morning or at a mutually convenient time, you will be asked to attend the X-Ray Department at the Royal Infirmary for a single head x-ray. This is to allow us to assess the structure of your facial bones which are an important factor in the development of sleep apnoea. The x-ray dose involved in this is low and about the equivalent of a chest x-ray. If you are female, the head x-ray will only be performed if you are post-menopausal, have previously had a hysterectomy, have had a surgical sterilisation more than 2 years previously or have a negative pregnancy test.
- c) In the morning after the sleep study or at some other mutually convenient time, we would take a single blood sample from you. This sample will allow us to identify whether sleep apnoea is associated with a particular gene. Excess blood will be destroyed after the testing has been done.
- d) We stress that you are under no obligation to participate in the study. If you require further information, please do not hesitate to contact Dr Brander at 0131-536-2355. If you wish to take independent advice about the study, this can be obtained from Dr Tom Mackay, via the same telephone number. He is not involved in the study and can provide unbiased advice about your participation and the overall value of the study. All data obtained in the study will be confidential. We are, however, required to notify your general practitioner about your involvement in this project.

On completion of the study, we will inform all participants about the results via a meeting of the Scottish Association for Sleep Apnoea Meetings and Newsletter or a letter to you.



ston Place
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WITH COMPLIMENTS

PATIENT ATTENDING FOR SLEEP STUDY

(Please display this on your dashboard)

/DMO/7/94
EEP1.CH3

Support Services Office
June 1994



LOTHIAN RESEARCH ETHICS COMMITTEE

STANDARD CONSENT FORM

TITLE OF THE PROPOSED RESEARCH:

Genetic Linkage in Sleep Apnoea.

NAME OF INVESTIGATOR:

Professor NJ Douglas.

ADDRESS:

Respiratory Medicine Unit, Department of Medicine, RIE.

TELEPHONE:

536-3252

FURTHER INFORMATION IS AVAILABLE FROM: (A person who is not involved in the study)

Dr TW Mackay.

LIST ANY DRUGS TO BE GIVEN IN THE STUDY EXPLAINING THEIR ACTION:

None.

LIST ANY PROCEDURES REQUIRED IN ADDITION TO THE STANDARD PROCEDURES:

Sleep study; Head x-ray; Blood sample.

- I agree to participate in this study.
- I have read this consent form and Patient/Subject Information Sheet and had the opportunity to ask questions about them.
- I agree for notice to be sent to my General Practitioner about my participation in this study.

- I agree to the provision of any clinically significant information to my General Practitioner.
- I understand that I am under no obligation to take part in this study and that a decision not to participate will not alter the treatment that I would normally receive.
- I understand that I have the right to withdraw from this study at any stage and that to do so will not affect my treatment.
- I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

Signature of Patient/Subject

Name of Patient/Subject:

Signature of Investigator:

Date:

Four copies to be made

Top copy to be retained by Investigator

Second copy to be retained by patient/subject

Third copy to be sent to patient's/subject's General Practitioner

An additional copy to be filed in any relevant hospital case notes

PERSONAL INFORMATION:

Name:..... Date of Birth:..... Tel No:.....

Address:

1. How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired? This refers to your usual way of life in recent times. Even if you have not done some of these things recently, try to work out how they would have affected you. Use the following scale to choose the **most appropriate** number for each situation:

Scale

- 0 = would **never** doze
- 1 = **slight** chance of dozing
- 2 = **moderate** chance of dozing
- 3 = **high** chance of dozing

| <u>Situation</u> | <u>Chance of dozing (use scale)</u> |
|---|-------------------------------------|
| Sitting and reading | _____ |
| Watching TV | _____ |
| Sitting inactive in a public place(e.g. a theatre or a meeting) | _____ |
| As a passenger in a car for an hour without a break | _____ |
| Lying down to rest in the afternoon when circumstances permit | _____ |
| Sitting and talking to someone | _____ |
| Sitting quietly after a lunch without alcohol | _____ |
| In a car, when stopped for a few minutes in the traffic | _____ |
| TOTAL | _____ |

- 2. Do you snore during sleep? Yes/No
If yes: Do you snore on your back only/on back and side/in all positions?
- 3. Has your bed partner ever noticed that you stop breathing when asleep?
Yes/No
- 4. In the morning do you feel that your nights sleep was refreshing /satisfactory?
always/ 4-6 nights per week/ 1-3 nights per week/ never

| |
|---|
| DATA COLLECTION SHEET |
| Name |
| Sex |
| DOB |
| Age |
| Index/Relative |
| Height |
| Weight |
| BMI |
| Neck circumference |
| SBP |
| DBP |
| POLYSOMNOGRAPHY RESULTS |
| |
| Sleep latency, minutes |
| REM sleep latency, minutes |
| Sleep period time, minutes |
| Time awake during sleep period, minutes |
| Total sleep time, minutes |
| NREM sleep, minutes |
| REM sleep, minutes |
| Stages 1+2, minutes |
| Stages 3+4, minutes |
| Sleep efficiency, % |
| Arousal index |
| AHI |
| SaO2 awake % |
| Lowest SpO2 % |
| 4% desaturations sleep |

| SLEEP QUESTIONNAIRE RESULTS | SCORE |
|--|-------|
| ESS | |
| Current smoker (current=1; other 0) | |
| Alcohol consumption (units/week) | |
| Question 4 (1-4; waking d night) | |
| Question 6 (snoring; yes=1; no=0) | |
| Question 6a (score 1-4; how long soring) | |
| Question 6b (score 1-3; how often snoring) | |
| Question 6c (score 1-3; postion) | |
| Question 7 (score 1-4; toilet at night) | |
| Question 8 (score 1-4; refreshing sleep) | |
| Question 10 (score 1-4; choking attacks) | |
| Question 11 (witnessed apnoeas; 0=no, yes=1) | |
| Question 12 (1-4; sleeping against will) | |
| Question 14 (driving accident yes=1; no=0) | |
| Asthma (yes=1; no=0) | |
| Bronchitis | |
| Emphysema | |
| Diabetes | |
| Heart attacks | |
| High blood pressure | |
| Ankle swelling | |
| Tonsillitis | |
| Hay fever | |
| Broken nose | |
| Nerve problems | |
| Nose operations | |
| Throat operations | |
| Drugs | |

| |
|--|
| CEPHALOMETRY MEASUREMENTS |
| Gonion-Gnathion |
| Gonion-articulare |
| ANS-PNS |
| PNS-Basion (horizontal) |
| H-MP |
| C3 (ant.inf.)-H |
| H-Retrognathion |
| H-PhW (parallel to B-Go) |
| PAS (on a line from B to Go) |
| UP-PhW (parallel to B-Go) |
| UT-PhW (parallel to B-Go) |
| PNS-PhW (parallel to B-Go) |
| UL (PNS-UT) |
| UW |
| Ant. Maxillary length (ANS-FOP) |
| Ant. Mandibular length (Gn-FOP) |
| Intern. Maxillary length (on FOP.Tt-PhW) |
| Post. Maxillary height |
| SNA |
| SNE |
| NS-Basion |
| C2C4SN |
| C2tang-SN |
| Neck angle (C2-C4) |

Appendix 4:

Enrolment Questionnaire and Letter



Dear

As you may know, the Sleep Centre at the Royal Infirmary of Edinburgh is doing research that shows that sleep apnoea runs in families. We are trying to identify the genes that may determine whether or not you develop sleep apnoea.

We would greatly appreciate your help with our research and also that of any of your brothers or sisters that might be willing to participate.

All we require of you is a simple blood test and to enquire with your brothers and sisters whether they would like to help out with the research.

We would then send a questionnaire to each of your brothers and sisters to help us determine which one would best be able to help out on the grounds of snoring and tiredness.

Your brother or sister would then be asked to come in for an overnight study to check their breathing just like your initial study, to have a small blood test and to have a head X-ray.

I shall call you within a week of receipt of this letter to ask you whether you would be able to help us with this very important research.

If you require any further information, please do not hesitate to contact either one of us.

Thank you.

Dr Renata L Riha BMedSc FRACP
Clinical Research Fellow

Prof. Neil J Douglas MD FRCPE
Professor of Respiratory and Sleep Medicine
Director Sleep Laboratory

Encl. Information sheet



**INFORMATION SHEET FOR PATIENTS INVOLVED IN THE
STUDY OF GENETIC LINKAGE IN SLEEP APNOEA**

1) Purpose of the study

Previous studies that we have carried out have shown that the sleep apnoea/hypopnoea syndrome runs in families. We are now trying to identify why it runs in families and specifically whether there is a particular gene involved. Identification of a gene might allow us to develop new therapies for this condition from which you suffer. This is a very long term objective and it is unlikely that this study will benefit you in the short term.

2) What you will need to do

We will ask you to list your brothers and sisters. We will then contact your brothers and sisters by letter explaining the study and asking them to fill out a questionnaire. We will provide a stamped, self-addressed envelope for your brothers and sisters to return the questionnaire to the Sleep Laboratory. This is in order to allow us to select the brother or sister who is most likely also to have the sleep apnoea/hypopnoea syndrome. Clearly, this study is also voluntary on their part as well and we will enclose Information Sheets for them too.

- a) We will require to take one blood sample from you at a mutually convenient time. This sample will allow us to identify whether sleep apnoea is associated with a particular gene. Excess blood will be destroyed after the testing has been done.
- b) We stress that you are under no obligation to participate in the study. If you require further information, please do not hesitate to contact Dr Riha on 0131-536-2360. If you wish to take independent advice about the study, this can be obtained from Dr Tom Mackay via 0131-536-2355. He is not involved in the study and can provide unbiased advice about your participation and the overall value of the study. All data obtained in the study will be confidential. We are however, required to notify your general practitioner about your involvement in this project.

On completion of the study, we will inform all participants about the results via a meeting of the Scottish Association for Sleep Apnoea Meetings and Newsletter or a letter to you.

INFORMATION SHEET FOR RELATIVES INVOLVED IN THE STUDY OF GENETIC LINKAGE IN SLEEP APNOEA

1) Purpose of the study

Previous studies that we have carried out have shown that the sleep apnoea/hypopnoea syndrome runs in families. We are now trying to identify why it runs in families and specifically whether there is a particular gene involved. Identification of a gene might allow us to develop new therapies for this condition.

2) What you will need to do

As you know, you have a brother/sister (delete as appropriate) with this condition and they will have passed on to you this information and the enclosed questionnaire. We would be grateful if you would complete the Questionnaire and return it ~~to the Sleep Laboratory~~ to the Sleep Laboratory. This is in order to allow us to select the brother or sister who is most likely to have some irregular breathing at night. This study is entirely voluntary on your part. The study will involve you in:-

- a) Completing the Questionnaire: If selected, we will ask you to attend the Sleep Laboratory for one night for a sleep study which will be similar to the study which your brother or big sister had when sleep apnoea was diagnosed. During this night, we will record your breathing pattern and sleep pattern by taping wires on to your body. None of this is painful and there are no needles involved. You will be required to attend the Sleep Laboratory about 9 p.m. at night and will be given breakfast at about 7.30 a.m.
- b) Either the next morning or at a mutually convenient time, you will be asked to attend the X-Ray Department at the Royal Infirmary for a single head x-ray. This is to allow us to assess the structure of your facial bones which are an important factor in the development of sleep apnoea. The x-ray dose involved in this is low and about the equivalent of a chest x-ray. If you are female, the head x-ray will only be performed if you are post-menopausal, have previously had a hysterectomy, have had a surgical sterilisation more than 2 years previously or have a negative pregnancy test.
- c) In the morning after the sleep study or at some other mutually convenient time, we would take a single blood sample from you. This sample will allow us to identify whether sleep apnoea is associated with a particular gene. Excess blood will be destroyed after the testing has been done.
- d) We stress that you are under no obligation to participate in the study. If you require further information, please do not hesitate to contact Dr Brander at 0131-536-2355. If you wish to take independent advice about the study, this can be obtained from Dr Tom Mackay, via the same telephone number. He is not involved in the study and can provide unbiased advice about your participation and the overall value of the study. All data obtained in the study will be confidential. We are, however, required to notify your general practitioner about your involvement in this project.

On completion of the study, we will inform all participants about the results via a meeting of the Scottish Association for Sleep Apnoea Meetings and Newsletter or a letter to you.

PERSONAL INFORMATION

Name:..... Date of Birth:..... Tel No:.....

Address:.....

Brother or Sister of patient named:.....

Weight:..... Height:.....

1. How likely are you to doze off or fall asleep, in the following situations, in contrast to feeling just tired? This refers to your usual way of life in recent times. Even if you have not done some of these things recently, try to work out how they would have affected you. Use the following scale to choose the most appropriate number for each situation.

- 0 = would never doze
- 1 = slight chance of dozing
- 2 = moderate chance of dozing
- 3 = high chance of dozing

| Situation | Chance of Dozing |
|--|------------------|
| Sitting and reading | |
| Watching TV | |
| Sitting inactive in a public place (e.g. a theatre or a meeting) | |
| As a passenger in a car for an hour without a break | |
| Lying down to rest in the afternoon when circumstances permit | |
| Sitting and talking to someone | |
| Sitting quietly after a lunch without alcohol | |
| In a car, while stopped for a few minutes in traffic | |
| <u>TOTAL</u> | <u>.....</u> |

2. Do you snore during sleep? Yes/no _____

If yes: Do you snore on your back only/on back and side/ in all positions

3. Has your bed-partner/ roommate ever noticed that you stop breathing when asleep?

Yes/no

4. In the morning do you feel that your night's sleep was refreshing/satisfactory?

Always/ 4-6 nights per week/ 1-3 nights per week/ never



Sleep Laboratory Tel: 0131 536 2355 (Secretary) Fax: 0131 536 2362
Director: Professor N J Douglas Consultant Physician: Dr T MacKay

Dear

A study of your breathing during sleep will be carried out in the Sleep Laboratory, Ward 48, Royal Infirmary, Lauriston Place, Edinburgh. Enclosed is a map of the Royal Infirmary. If you are coming by car there is earmarked parking available for overnight sleep study subjects and this is shown on the map. Please display the enclosed permit in your car. Car entrance is by Chalmers Street only. Pedestrian access is available from Lauriston Place as well.

You should report to the Sleep Laboratory at 9 pm on

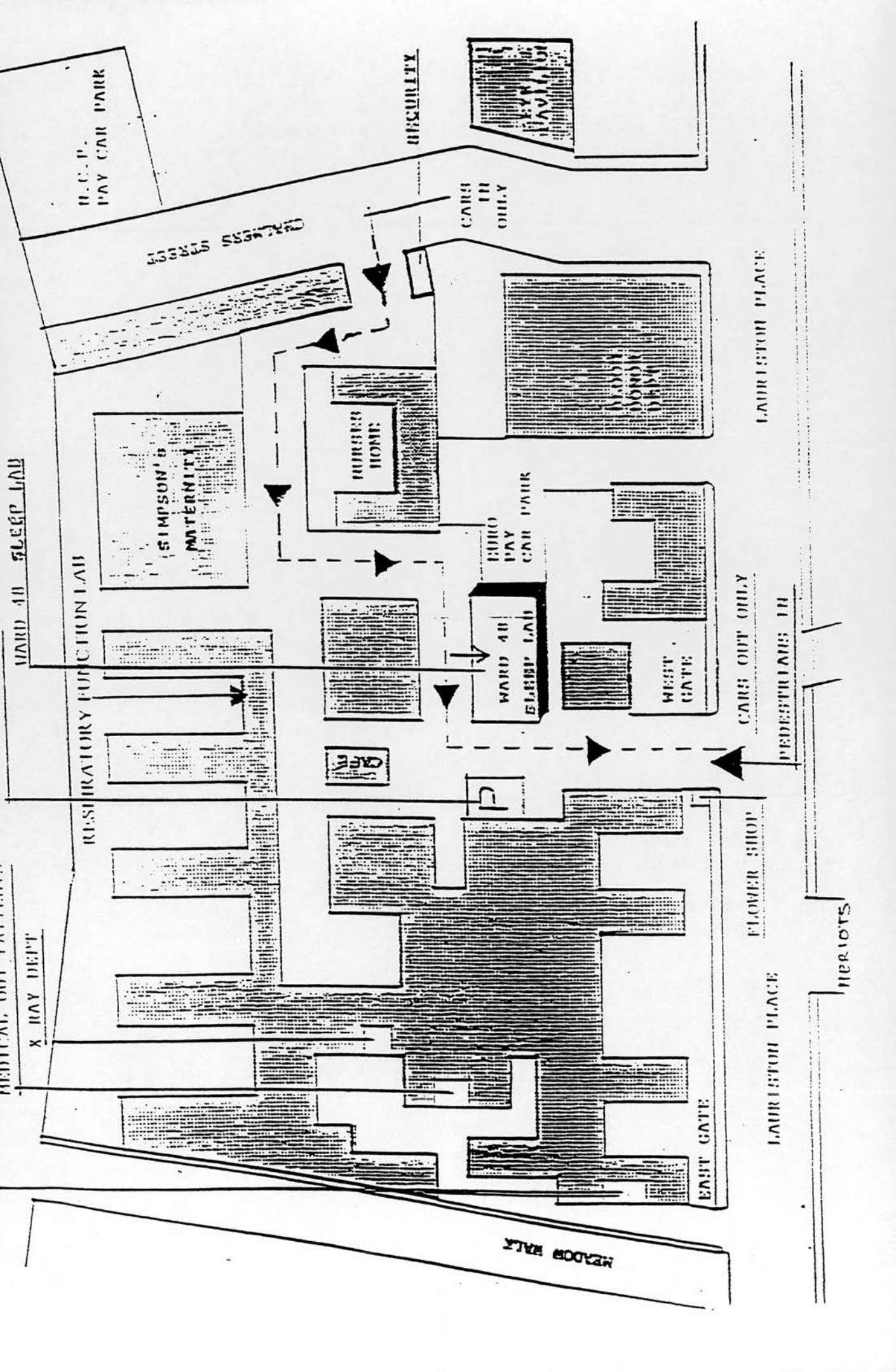
The Sleep Laboratory is part of Ward 48 and is on the top floor of the building indicated. There is a lift you may use. Once you get to the top floor, please ring the bell at the Sleep Laboratory door when the night sister will come to welcome you. Please note that there will be no staff in the Sleep Laboratory between 5 pm and 8.30 pm (or on Sundays before 8.30 pm).

You should bring with you to your sleep study your normal night attire and wash things. You will be given a single room to sleep in. In the morning, you will be provided with breakfast. After taking the single blood sample and the x-ray, you will be free to leave at about 10 am. Travel expenses will be paid later by the University of Edinburgh. For that purpose, we would need your National Insurance Number. I will inform you later about the results of your sleep study. If there were something to be concerned about, we would like to inform your GP as well. For that purpose, I would need the name and address of your GP.

With many thanks

Yours sincerely

Dr Renata Riha BMedSc MBBS FRACP



H.C.P.
PAY CAR PARK

WOODS ASSAULT

SECURITY

CARS
IN
ONLY

WEST
GATE

LAURISTON PLACE

WARD 4B
SLEEP LAB

RESPIRATORY FUNCTION LAB

SIMPSON'S
MATERNITY

HURDIS
HOME

WARD 4B
PAY CAR PARK

WARD 4B
SLEEP LAB

WEST
GATE

CARS OUT ONLY

PEDESTRIANS IN

X RAY DEPT

RESPIRATORY FUNCTION LAB

P

FLOWER SHOP

LAURISTON PLACE

EAST GATE

HERRIOTS

MEADOW BALK



Edinburgh

Dear

We wrote to you in the summer regarding participation in some sleep research. Unfortunately, due to our miscalculation with your and our summer breaks, we failed to reach you by telephone in a reasonable period of time.

I am therefore writing again in the hope that you may reconsider our study and am enclosing the initial letter and information which were sent to you.

We hope that by doing so, we are not inconveniencing you and thank you for giving us the time to consider participating.

I shall try to contact you within about a week of mailing out this letter.

Thank you again!

Best regards,

Renata L Riha BMedSc MBBS FRACP
Clinical Research Fellow to Professor Neil J Douglas
Scottish National Sleep Laboratory



Dear

I would like to thank you very much for agreeing to participate in our study of sleep and genetics.

I am enclosing a list of dates available for an overnight sleep study in the next little while and would greatly appreciate it if you could circle the date that suits you best and return it to me in the stamped self-addressed envelope.

When I receive your reply, I shall call to confirm that date and send you out an information letter about where to come.

If you have any queries or concerns, please do not hesitate in contacting me on 0131 5362360.

Thank you again for your assistance – I look forward to meeting you.

With kind regards

Renata L Riha
BMedSc MBBS FRACP
Clinical Research Fellow to Prof. Neil J Douglas

Encl. Information sheet; dates for study; envelope

NAME:

CONTACT NUMBER:

PREFERRED TIMES TO BE CONTACTED:

WOULD PREFER NOT TO BE CONTACTED
(please tick above if this is the case)



Sleep Laboratory Tel: 0131 536 2355 (Secretary) Fax: 0131 536 2362
Director: Professor N J Douglas Consultant Physician: Dr T Mackay

Dear

Thank you very much for completing and also returning our sleep questionnaire.

I have asked one of your other siblings to come to our laboratory in the next few weeks – it just means that we feel that you do not have an appreciable problem with snoring or undue sleepiness at present!!

Once again, thank you for your time and trouble.

With best wishes

Dr Renata L Riha
BMedSc MBBS FRACP

Appendix 5:

Scottish Sleep Centre Questionnaire

PAST MEDICAL HISTORY

If you have had the following illnesses, please give details:

Illness

| | | |
|---------------------|--------|-------|
| Asthma | yes/no | |
| Bronchitis | yes/no | |
| Emphysema | yes/no | |
| Diabetes | yes/no | |
| Heart attacks | yes/no | |
| High blood pressure | yes/no | |
| Ankle swelling | yes/no | |
| Tonsillitis | yes/no | |
| Hay fever | yes/no | |
| Broken nose | yes/no | |
| Nose operations | yes/no | |
| Throat operations | yes/no | |
| Nerve problems | yes/no | |

1. How often do you wake between going to bed and getting up in the morning?

never 1-3 times 3-6 times more than 6 times per night

2. Do you snore during sleep?

Yes/No

If yes:

a) How long have you snored loudly?

Always since childhood / last 5 years / 3 years / 1 year

Appendix 6:

DNA Extraction Protocols

PROTOCOLS FOR DNA EXTRACTION

The following procedure was based on the Promega™ DNA extraction kit utilised in Oxford.

DNA was extracted from frozen 10ml sample volumes of blood:

1. 30 ml of Cell Lysis Solution was added to a sterile 50ml centrifuge tube.
2. The tube of blood was then gently rocked until thoroughly mixed and then 10 ml of the blood were transferred to the tube containing the Cell Lysis Solution. The tube was then inverted 5-6 times to mix.
3. The mixture was incubated for 10 minutes at room temperature (inverted again 2-3 times during the incubation) to lyse the red blood cells. The solution was then centrifuged at 2,000xg for 10 minutes at room temperature.
4. Supernatant was then removed and discarded with approximately 1.4 ml of residual liquid remaining.
5. The tube was then vortexed until the white blood cells were resuspended (10-15 seconds).
6. 10mls of Nucleic Lysis Solution was then added to the tube containing the resuspended cells. The solution was pipetted 5-6 times to lyse the white blood cells. The solution became viscous.
7. 3.3mls of Protein Precipitation Solution were added to the nuclear lysate and vortexed vigorously for 10 – 20 seconds.
8. The solution was then centrifuged at 2,000xg for 10 minutes at room temperature.

9. The supernatant (dark brown protein pellet and supernatant) was transferred to a 50 ml centrifuge tube containing 10mls of room temperature isopropanol.
10. The solution was then gently missed by inversion until strands of DNA formed a visible mass.
11. The mixture was then centrifuged at 2,000xg for one minute at room temperature and the DNA became visible as a small white pellet.
12. The supernatant was decanted and 10mls of room temperature 70% ethanol was added to the DNA. The tube was gently inverted several times to wash the DNA pellet and the sides of the centrifuge tube and the solution was centrifuged again as in step 11 above.
13. The ethanol was then carefully aspirated and the DNA pellet allowed to air dry for 10 – 15 minutes.
14. 800ul of DNA Rehydration Solution was then added to the tube and the DNA rehydrated by incubating at 65C for one hour. The solution was periodically mixed by gently tapping the tube.

Blood samples collected in 2001 – 2002 were processed directly at the Edinburgh Wellcome Trust Clinical Research Facility Genetics Core laboratory using the Nucleon Extraction and Purification Protocol (Product Code: Nucleon B ACC3 RPN 8512 for extraction of DNA from 10 ml of whole blood; © Amersham International plc). The procedure involved cell lysis, deproteinisation, DNA extraction; DNA precipitation; DNA washing and DNA resuspension.

1. Blood was taken out of storage and allowed to defrost at room temperature. Before the extraction procedure was commenced, 100ul of blood of each

sample was spotted onto a Whatman FTA card, numbered and dated. The cards were stored at room temperature in sealed bags containing silica desiccant.

2. Using aseptic technique, 40 ml of dilute Reagent A was added to the 10 ml blood sample in a labelled 50 ml Greiner tube and rotary mixed for 4 minutes at room temperature.
3. The solution was then centrifuged for 5 minutes at 2,600 rpm (1300g) and the supernatant discarded.
4. To the residual pellet, 2 ml of Reagent B was added and the solution vortexed to resuspend the pellet. The suspension was then transferred to a 15 ml screw capped propylene centrifuge tube.
5. 500 μ l of sodium perchlorate solution was added to the suspension, which was then mixed by hand – the capped tube was inverted at least 12 times.
6. 2 ml of chloroform was then added and the solution mixed by hand as above.
7. Without remixing the phases, 300 μ l of Nucleon resin was then added to the solution and it was centrifuged at 1300g (2,600 rpm) for 3 minutes.
8. Without disturbing the Nucleon resin layer, the upper phase (approximately 2.5 ml) was transferred to a clean tube of 10 ml volume.
9. 2 volumes of cold absolute ethanol were added to the solution and the tube mixed by inversion until DNA began to precipitate. The precipitated DNA was hooked out using a Pasteur pipette and placed in 1 ml of 70% ethanol and washed.
10. DNA was removed from the wash and allowed to air dry for a few minutes, and then placed in 1 ml of TE buffer.

11. The above solution was then placed on a rotary wheel and allowed to re-dissolve (one week) prior to storage at -2° - -8°C in the refrigerator.

Appendix 7:

Publications and Presentations arising from this Thesis

PRESENTATIONS AND PUBLICATIONS ARISING FROM THE THESIS

ABSTRACTS

Apolipoprotein E4 – A role in the Obstructive Sleep Apnoea/Hypopnoea Syndrome?

Riha RL, Brander P, Vennelle M, Douglas NJ

Presented at:

- British Thoracic Society Winter Meeting 2002 (poster)
- American Thoracic Society Conference 2003 (poster)

Tumour Necrosis Factor Alpha Gene Polymorphisms in OSAHS.

Riha RL, Brander P, Vennelle M, Mc Ardle N, Douglas NJ

Presented at:

- Obstructive Sleep Apnoea Conference in Helsinki 2003 (poster)
- European Respiratory Society Conference 2003 (oral presentation)
- American Thoracic Society Conference 2004 (oral presentation)

Obstructive Sleep Apnoea/Hypopnoea Syndrome and Snoring: A Cephalometric Comparison

Riha RL, Brander P, Vennelle M, Douglas NJ

Presented at:

- Australasian Sleep Association Annual Meeting 2003 (poster)
- American Thoracic Society Conference 2004 (poster)

The Beta2-Adrenoceptor Gene Polymorphism (C+79G) is associated with Sleep Apnoea

Riha RL, Brander P, Vennelle M, Douglas NJ

Presented at:

- American Thoracic Society Conference 2004 (poster)

No Association of a Serotonin 2A Receptor Gene Polymorphism with Sleep Apnoea

Riha RL, Brander P, Vennelle M, Douglas NJ

Presented at:

- American Thoracic Society Conference 2004 (poster)
- European Sleep Research Society Conference 2004 (poster)

Association of the Growth Hormone Receptor with Sleep Apnoea and Obesity Riha
RL, Brander P, Vennelle M, Douglas NJ

Presented at:

- American Thoracic Society Conference 2004 (poster)
- European Respiratory Society Meeting 2004 (poster)

MANUSCRIPTS UNDER SUBMISSION

The -308 (A/G) Tumour Necrosis Factor Alpha Gene Polymorphism is associated with OSAHS.

Obstructive Sleep Apnoea/Hypopnoea Syndrome and Snoring: A Cephalometric Comparison in Siblings (accepted for publication in *Sleep*)