Gestational and Newborn Screening Markers of Cystic Fibrosis

SALEH ALI ALGHAMDI

Thesis submitted for the degree of Doctor of Philosophy

University of GLASGOW

DIVISION OF DEVELOPMENTAL MEDICINE

OCTOBER 2011

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

BACKGROUND

Newborn Screening for Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive disorder that results in a shortened lifespan if appropriate treatment is not initiated sufficiently early. Approximately one in every 2,000 to one in every 3,500 Caucasians born in Europe is affected by CF, which manifests itself in severe disorders of the lungs and the digestive system. Newborn screening for CF based on the analysis of bloodspot immunoreactive trypsinogen (IRT) has recently been introduced in a number of countries, including the UK. Also, it has been reported that pancreatitis associated protein (PAP) is elevated in bloodspots from neonates with CF and a strategy involving a combination of IRT and PAP may offer enhanced specificity. The study aims to develop an algorithm based on universal IRT measurements and subsequent PAP measurements in newborns with elevated IRT levels which will allow high detection rates to be maintained while at the same time decreasing the number of cases referred for DNA analysis and the accompanying detection of CF carriers.

Prenatal Screening

The aim of antenatal screening programmes is to offer couples reproductive choice, for example, termination of the pregnancy if the foetus is found to have a serious disorder such as CF. In this study, the physiological effects on women pregnant with a baby affected by CF were investigated. Several maternal serum markers were tested in this project in blood samples from women carrying a CF foetus. These markers are known to be associated with a number of adverse outcomes in pregnancy such as low birth weight, pre-term birth, pre-eclampsia, and stillbirth. The markers tested were alpha-fetoprotein (AFP), pregnancy-associated plasma protein (PAPP-A), human chorionic gonadotrophin (hCG), free beta subunit of hCG (FβhCG), unconjugated estriol (UE3) and Inhibin-A.

MATERIALS AND METHODS

Newborn

In this project, blood spot samples with elevated immunoreactive trypsinogen (IRT) samples were from 314 infants together with 2886 controls. In addition there were 189 blood spots from premature babies, 182 from low birth weight babies, 29 from confirmed CF cases and 32 from CF carriers. The samples therefore comprised a total of 3636 blood spots.

Currently, in Scotland, the routine newborn screening test for CF uses the Delfia fluoroimmunoassay method (Perkin Elmer, Finland) to measure IRT. Also available from the routine newborn bloodspot screening programme were the results from tandem mass spectrometry (MS/MS) testing for phenylketonuria (PKU) measuring phenylalanine (PHE) and tyrosine (TYR). In this project, pancreatitis-associated protein (PAP) in all the 3636 blood spots was also measured using a commercially-available enzyme linked immunoassay (Dynabio SA, Marseille, France).

Pregnancy

In the present study, a group of 55 women with CF pregnancies was identified from antenatal screening records and serum samples taken for routine prenatal screening were recovered from frozen storage. Serum samples from an additional 330 pregnancies, both first trimester (10-13 weeks) and second trimester (15 to 20 weeks) cases were randomly chosen as controls. The Dissociation Enhanced Lanthanide Fluro Immuno Assay method (DELFIA) was used to measure AFP, HCG, PAPP-A, and free βHCG. The assays for unconjugated estriol (UE3) and Inhibin-A were conducted using the Access 2 immunoassay system (Beckman Coulter).

RESULTS

Newborn Markers and Detection of CF cases

The main findings in the study are the following. Median PAP concentrations in the CF cases and carriers were significantly elevated over the control median (x 9.9 and x 2.3 respectively). If referral for DNA testing was based on an IRT level ≥99.5th centile and a PAP level ≥1.2 multiples of the control median, 25% fewer DNA analyses would be required and the number of carriers detected reduced from 32 to 24 with no loss of detection of CF cases. Therefore, carrying out PAP assays only on infants with elevated IRT results improves specificity by reducing the number of cases requiring DNA analysis.

The predictive value of IRT and PAP measurements were examined using the Likelihood Ratio (LR) method. IRT and PAP results showed no significant correlation in CF cases (Pearson correlation $p = 0.669$). The product of the likelihood ratios can therefore be calculated thus combining the information from two markers. Setting a threshold LR of 30 as the action point for DNA testing would result in a 43% reduction in DNA testing and a 50% reduction in the number of CF carriers identified. One CF case would be missed. If a likelihood ratio cut off of 500 was chosen this would reduce detection of carriers by 88 % and referrals for DNA analysis by 75%. However detection of 3 CF cases would be missed. Therefore, selecting cases for DNA analysis based on subsequent measurement of PAP in infants with elevated IRT offers a potentially cost-effective method for reducing the number of DNA tests and incidental detection of carriers without the need to measure PAP in all infants.

Marker levels and mutation type in CF Cases

Data presented by Sarles et al (2005) suggest that PAP levels may appear lower in cases where the mutation types are associated with a milder CF phenyotype. Thirteen out of the 29 CF cases examined in this research were homozygous for the common mutation delta F508 with the remaining 16 cases having other combinations of mutation. IRT, PAP, PHE, and TYR concentrations were compared between the two groups of mutation types (Delta F508 homozygous vs. other mutations). It was found that both IRT and PAP medians were considerably lower in the "other" mutation group than in the delta F508 homozygous group (IRT: 147.8 ng/ml vs. 187.7 ng/ml), (PAP: 1.03 ng/ml vs. 2.54 ng/ml) and that only two of the 10 CF cases with the lowest PAP results were homozygous for the delta F508 mutation. However, no statistically significant differences in median level between the two mutation groups for any markers were found using the Mann-Whitney test. This may reflect the relatively small number of cases compared and it is recommended that studies on a more extensive series of CF cases and mutation types should be conducted to investigate this possibility further.

Marker Levels and Mutation type in CF carriers

The delta F508 mutation was found in 22 of the CF carriers from a total of 32 carriers. The remaining 10 carriers had other mutations. Little difference was found in the median concentration for each marker between the two types of mutation. A comparison of IRT, PAP, PHE and TYR concentrations between the two groups of mutation types (ΔF508 vs. other mutation) using Mann-Whitney test showed no significant differences in median level for any markers between these two groups.

Age at bloodspot sampling

The variations in the levels of blood spot markers with day of sampling were examined in this study. In the control group of 2876 samples, the regression analysis at age of

sampling showed significantly reduced PAP levels ($p < 0.001$) with earlier age at sampling. No significant associations were found for IRT ($p = 0.959$), PHE ($p=0.276$) or TYR ($p = 0.249$). In Scotland, most bloodspot samples for newborn screening are collected when the baby is between 5 and 8 days old, with around 5% of samples being collected later. However, in many other countries, bloodspot sampling is carried out earlier, when the baby is between 1 and 3 days old. As published data from centres which use earlier sampling will be unreliable, it is therefore important to establish normal ranges and distribution parameters for each marker locally.

Premature Babies

Marker studies were conducted in 182 infants born prematurely (that is, before 37 weeks of gestation). A linear regression analysis was carried out to discover any relationship between premature birth and levels of IRT, PAP, PHE and TYR concentrations. All markers showed a diminishing trend with advancing gestation, which was statistically significant for IRT ($p < 0.001$), PAP ($p < 0.001$) and TYR ($p = 0.015$) but not statistically significant for PHE ($p = 0.226$). Hence, there will be a risk of false positive results in premature infants when using IRT and PAP to screen for CF unless higher IRT and PAP cut-offs are used.

Low Birth Weight Babies

In this study of 3632 blood spot samples, 189 were from babies who had been born with low birth weight (i.e. less than 2500 grams). Linear regression was used to examine the relationship between low birth weight and levels of IRT, PAP, PHE and TYR concentration. This showed that there was a significant association with PAP ($p=0.002$), where the level decreased with increasing birth weight, whereas no significant changes were found for IRT ($p = 0.212$), PHE ($p = 0.180$) and TYR ($p = 0.436$). Hence, there will

be a tendency to overestimate the risk in very low birth weight infants in any screening programme for CF which uses PAP.

Ethnicity

The data was divided into two groups to discover whether there was any difference in the IRT, PAP, PHE and TYR markers between different ethnic groups. Because numbers were small, two ethnic categories were defined. Group 1 included all babies from British/Irish (A) and any other Caucasian background (C). Group 2 included all babies from other ethnic backgrounds. The median was calculated for IRT, PAP, PHE and TYR in both these groups. The results revealed that the only statistically significant difference between these groups was for TYR in the controls. In fact, there was an insufficient number of babies in Group 2 in the CF and CF carriers samples groups for the comparison to be meaningful.

Gestational Markers and Detection of Cystic Fibrosis

The majority of pregnant women in the UK take up the offer of a prenatal screening test for Down syndrome and other foetal abnormalities based on the analysis of a series of serum markers of placental and foetal origin. These markers are known to vary in concentration across a range of other foetal and maternal abnormalities thus providing a useful alert for improving the management and delivery of such pregnancies. Using the Mann-Whitney test, an analysis of markers AFP ($p = 0.335$), hCG, ($p = 0.539$), PAPP-A, ($p = 0.632$), Free β-hCG ($p = 0.824$), UE3, ($p = 0.840$) and Inhibin-A ($p = 0.650$) showed that these concentration levels remained unchanged in the serum of mothers of children affected by CF. Thus, none of these routinely used screening markers offers any potential for the identification of CF pregnancies.

Ethical approval

Ethical approval for this study was granted by the West of Scotland Research Ethics Service (WoS Rec Committee 2).

REC Reference: 10/S0709/25

Study title: Newborn and Gestational Screening Markers of Cystic Fibrosis

DECLARATION

I declare that this thesis does not contain material previously published or written by another person except who due reference is made in the text and the results presented in this thesis have not been submitted for any other degree or diploma.

All the work contained within this thesis was carried out by myself, Saleh Ali Alghamid.

Saleh Ali Alghamdi

July 2011

TABLE OF CONTENTS

ABSTRACT i
DECLARATION viii
TABLE OF CONTENTS ix
LIST OF TABLES xv
LIST OF FIGURES xvii
LIST OF FIGURES xvii
ACKNOWLEDGEMENTS xxiv
PUBLICATIONS xxv
LIST OF ABBREVIATIONS xxvi
CHAPTER 1 : INTRODUCTION 1
1.1 BACKGROUND 2
1.2 SCREENING 5
1.2.1 Types of Screening 7
1.2.2 Prenatal screening 7
1.2.3 Adult screening 8
1.2.4 Carrier screening 8
1.2.5 Newborn screening 8
1.2.6 The Development of Newborn Screening 9
1.2.6.1 The Bacterial Inhibition Assay 9
1.2.6.2 The Dried Bloodspot Sample 10
1.3 SCREENING FOR IEMs 11
1.3.1 Analytical Techniques Used in Bloodspot Screening 16
1.3.2 Disorders Detected By Newborn Bloodspot Screening 17
1.3.2.1 Phenylketonuria (PKU) 17
1.3.2.2 Galactosaemia 19
1.3.2.3 Congenital hypothyroidism (CHT) 20
1.3.2.4 Sickle Cell Disorder (SCD) 21
1.3.2.5 Congenital adrenal hyperplasia (CAH) 23
1.3.2.6 Medium Chain Acyl-CoA Dehydrogenase Deficiency (MCADD) 24
1.3.3 Screening for Cystic Fibrosis 25
1.3.3.1 Genetics of CF 25
1.3.3.2 Clinical Characteristics of CF 28
1.3.3.3 Treatment of CF 28

1.3.3.4 Gestational Characteristics of CF 30
1.4 NEWBORN SCREENING MARKERS FOR CF 30
1.4.1 Immuno reactive trypsinogen (IRT) 30
1.4.2 Pancreatitis Associated Protein (PAP) 31
1.4.3 Sweat Tests 32
1.4.4 Faecal Elastase 32
1.4.5 Prenatal Screening for CF (Couple Screening) 33
1.5 GESTATIONAL MARKERS OF CF 35
1.5.1 Alpha-fetoprotein (AFP) 36
1.5.2 Human Chorionic Gonadotrophin (HCG) 37
1.5.3 Pregnancy-Associated Plasma Protein A (PAPP-A) 38
1.5.4 Free Beta Human Chorionic Gonadotrophin (FβHCG). 38
1.5.5 Unconjugated Estriol (UE3) 39
1.5.6 Inhibin-A 39
1.6 NEWBORN SCREENING PROTOCOLS FOR CF 39
1.6.1 IRT/IRT Protocol 40
1.6.2 IRT/DNA/IRT Protocol 40
1.6.3 IRT / PAP Protocol 41
1.7 AIMS 42
CHAPTER 2 : MATERIALS 44
2.1 Subjects 45
2.2 Dried Blood Sample 45
2.3 Retrospective IRT Study for CF Affected Babies 50
2.4 Retrospective IRT Study for CF Carriers 50
2.5 Maternal Serum 51
CHAPTER 3 : METHODS 54
3.1 NEWBORN MARKER MEASUREMENTS 55
3.1.1 Measurement of Immunoreactive Trypsinogen (IRT) 55
3.1.2 Principle of IRT assay 58
3.1.3 Measurement of Pancreatitis-Associated Protein (PAP) 59
3.1.3.1 Blood Spot Samples 59
3.1.3.2 Plate Map 59
3.1.3.3 PAP Assay 60
3.1.4 Measurement of Phenylalanine (PHE) and Tyrosine (TYR) 63
3.1.4.1 Method of Operation 64
3.2 Gestational Characteristics of CF Pregnancies 67

3.2.1 Serum Marker 67
3.2.2 AutoDELFIA Immunoassay System Theory of Operation 68
3.2.3 The Access 2 Immunoassay System Theory of Operation 69
3.2.4 Measurement of AFP 69
3.2.5 Measurement of HCG 71
3.2.6 Measurement of PAPP-A 72
3.2.7 Measurement of FβHCG 73
3.2.8 Measurement of UE3 74
3.2.9 Measurement of Inhibin-A 75
3.3 Statistical Methods 76
3.3.1 Descriptive Statistics 76
3.3.1.1 Mean 76
3.3.1.2 Percentiles 76
3.3.1.3 Standard Deviation 76
3.3.1.4 Coefficient of Variation 77
3.3.1.5 Correlation Coefficient (r) 77
3.3.1.6 Median 77
3.3.2 Inferential Statistics 78
3.3.2.1 Mann-Whitney Test 78
3.3.2.2 Regression Analysis 78
3.3.3 Multiple of Median of the Appropriate Gestation 78
3.3.4 Likelihood Ratios (LR) for CF Derived from IRT and PAP 79
3.3.5 EasyPlot Software 79
CHAPTER 4 : RESULTS 81
4.1 NEWBORN BLOOD SPOT MARKERS OF CYSTIC FIBROSIS 82
4.2 Control Blood Spot Samples 83
4.2.1 Male and Female Babies in the Control Sample 90
4.2.1.1 Male Babies 90
4.2.1.2 Female Babies 91
4.2.1.3 Variation in Blood Spot Marker Levels with Day of Sampling. 92
4.3 Elevated IRT Blood Spot Sample Analysis 97
4.3.1 IRT 97
4.3.2 PAP 99
4.3.3 PHE 100
4.3.4 TYR 101
4.3.5 Birth Weight 102

4.3.6 Gestation at Delivery 102
4.3.7 Age at Sampling 102
4.4 PREMATURE BABIES 109
4.5 LOW BIRTH WEIGHT BABIES 119
4.6 MARKER LEVELS AND ETHNICITY WITHIN EACH SAMPLE GROUP 129
4.6.1 Distribution of Ethnic Groups 129
4.6.2 Median Marker levels in Different Ethnic Groups. 131
4.7 BABIES WITH CYSTIC FIBROSIS. 133
4.7.1 Marker Levels in Cystic Fibrosis Cases 133
4.7.2 Mutations Identified in Confirmed CF Cases 136
4.8 MARKER LEVELS IN CYSTIC FIBROSIS CARRIERS 139
4.8.1 Mutations Identified in CF Carriers 142
4.9 BIRTH WEIGHT AND GESTATION IN CF CASES AND CARRIERS. 144
4.9.1 BIRTH WEIGHT STUDY 144
4.9.2 GESTATION STUDY 145
4.10 RETROSPECTIVE STUDY OF IRT IN CF CASES DETECTED RY ROUTINE NEWBORN SCREENING 146
4.10.1 Retrospective Study of IRT Levels in CF Carriers According to Mutation. 146
4.11 DISCRIMINATORY POWER OF PAP MEASUREMENTS 149
4.11.1 PAP and IRT Levels in Relation to Type of Mutation 151
4.12 USE OF LIKELIHOOD RATIOS METHOD IN THE NEWBORN SCREENING OF CF 153
4.13 GESTATIONAL MARKERS OF CYSTIC FIBROSIS 162
4.13.1 AFP Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF 162
4.13.2 HCG Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF 165
4.13.3 PAPP-A Concentration Level in Maternal Serum Samples from Mothers of Children Affected With CF 168
4.13.4 Free β-hCG Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF 170
4.13.5 UE3 Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF 173
4.13.6 Inhibin-A Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF 176

4.13.7 Maternal Serum Marker Levels in CF Pregnancies 179
CHAPTER 5 : DISCUSSION 180
5.1 NEWBORN BLOODSPOT MARKERS OF CF 181
5.1.1 Current CF Screening 181
5.1.1.1 Workload and Cost 181
5.1.1.2 Alternative Newborn Bloodspot Markers 183
5.1.1.3 Pancreatitis Associated Protein (PAP) 184
5.1.2 Marker Levels in Confirmed CF Cases 186
5.2 PREDICTIVE VALUE OF IRT AND PAP 187
5.2.1 Use of Cut-offs for IRT and PAP 187
5.2.2 Use of Likelihood Ratio Method for IRT and PAP 187
5.3 Other PAP Studies 188
5.3.1 Study 1 188
5.3.2 Study 2 189
5.3.3 Study 3 190
5.3.4 Study 4 190
5.4 PAP / IRT Screening Performance 191
5.5 Alternative Screening Strategies 192
5.5.1 Improving Specificity 192
5.5.2 Improving Sensitivity 192
5.6 Disadvantage of the Sequential IRT / PAP Approach 193
5.7 Factors Affecting Bloodspot Marker Measurements 194
5.7.1 Marker levels and Mutations in CF Cases 194
5.7.2 Marker Levels and Mutations in CF carriers 195
5.7.3 Age at bloodspot sampling 195
5.7.4 Premature Babies 196
5.7.5 Low Birth Weight Babies 196
5.7.6 Ethnicity 196
5.8 Gestational Markers of CF 197
5.8.1 AFP 199
5.8.2 HCG 199
5.8.3 PAPP-A 199
5.8.4 FREE β-HCG 200
5.8.5 UE3 200
5.8.6 Inhibin-A 200
5.9 Prenatal Screening Markers in CF Pregnancies 201

CHAPTER 6 : CONCLUSION 202
6.1 CONCLUSION 203
APPENDIX 204
REFERENCES 220

LIST OF TABLES

Table 1-1: Classification, incidence and inheritance of some inborn errors of metabolism 4
Table 1-2: Wilson and Jungner screening criteria. 6
Table 1-3: Advantages of using the DBS method instead of venepuncture 10
Table 1-4: Different types of screened disorders in the Asia Pacific region 13
Table 1-5: Newborn screening development in the Middle East region. 15
Table 1-6: Incidence of CFTR mutations other than ΔF508 with high incidence in specific populations 27
Table 2-1: Demographic information requested on the dried blood spot card 46
Table 2-2: Baby ancestry code description 47
Table 2-3: Summary of the bloodspot specimen used in the study. 50
Table 2-4: Number of control and CF sample available at each gestation for each marker....53
Table 4-1: The lowest, highest and median levels of IRT, PAP, PHE and TYR in 2886 blood spot control samples 84
Table 4-2: Mean and standard deviation Log_{10} for IRT, PAP, PHE and TYR in the blood spot control samples 85
Table 4-3: Lowest, highest and median levels of IRT, PAP, PHE and TYR in baby boys in the control group. 90
Table 4-4: The lowest, highest and median of levels of IRT, PAP, PHE and TYR in female babies in the control group 91
Table 4-5: Mean and median at age of sampling PAP in increased IRT group in 103
Table 4-6: Mean and median of IRT in increased IRT group in 313 samples in female neonates and male neonates at the age of specimen collection.....104
Table 4-7: Median IRT, PAP, PHE, and TYR in premature female babies. 110
Table 4-8: Median IRT, PAP, PHE and TYR in premature male babies. 110
Table 4-9: Distribution of ethnic groups within each sample group.....131
Table 4-10: Median of marker levels in ethnic groups in each sample group. 132
Table 4-11: Demographic data, marker data and mutations in 29 CF cases.....134
Table 4-12: IRT, PAP, PHE and TYR medians with p value in 29 cases and 2886 control samples. 136
Table 4-13: Comparison of levels of 4 markers between common delta F508 mutation and other mutation types in CF cases 137
Table 4-14: Demographic data, marker data and mutations in 32 CF carriers. 140

Table 4-15: IRT, PAP, PHE and TYR medians with p value in 32 CF carriers and 2886 control sample 142
Table 4-16: Comparison of levels of 4 markers between delta F508 mutation and other mutation types in CF carriers 143
Table 4-17: Median birth weight and gestation medians with p value in CF cases, CF carrier and control sample. 145
Table 4-18: Gestation medians with p value in CF cases, CF carrier and control sample. ... 145
Table 4-19: Comparison of IRT concentration between Homozygosity for the common delta F508 mutation and other mutation types in CF cases 147
Table 4-20: Comparison of IRT concentration between common delta F508 mutation and other mutation in CF carriers. 148
Table 4-21: PAP, IRT and mutations in 29 CF cases arranged in ascending order of PAP concentration. 150
Table 4-22: IRT and PAP concentrations in 8 CF carrier samples located below the suggested PAP cut-off of 0.24 ng/ml. 151
Table 4-23: Overall risk of CF from IRT and PAP levels in 29 CF cases. 157
Table 4-24: Overall risk of CF from IRT and PAP levels in 32 CF carriers. 159
Table 4-25: Distributions of likelihood ratios in CF cases, CF carriers and babies with elevated IRT, derived from IRT and PAP results 161
Table 4-26: Control median at each week of gestation for AFP. 163
Table 4-27: Median values at each week of gestation for hCG. 166
Table 4-28: Median values at each week of gestation for PAPP-A 168
Table 4-29: Median values at each week of gestation for Free β-hCG 171
Table 4-30: Median values at each week of gestation for UE3 175
Table 4-31: Median values at each week of gestation for Inhibin-A. 178
Table 4-32: AFP, hCG, PAPP-A, Free β-hCG, UE3 and Inhibin-A medians with p value in CF serum sample and control serum sample 179

LIST OF FIGURES

Figure 2-1: The front of the blood spot card used in the Scottish screening programme 49
Figure 2-2: The reverse of the blood spot card used in the Scottish screening programme....49
Figure 2-3: Semi-automated bloodspot multi-puncher used in the Scottish screening programme 49
Figure 3-1: Flowchart, explaining the CF protocol in Scottish newborn babies. 57
Figure 3-2: AutoDELFIA IRT principle of the assay. 58
Figure 3-3: Plate protocol for blood spot samples. Wells 1-2 are blanks; 3-14 are standards, 15, 16, 17, 93, 94, 95, 96 are controls. Wells R1-R8 contain blood spots from babies with elevated IRT plus matched controls, P contain premature blood spot sample and L is low birth weight sample. 61
Figure 3-4: Example of PAP experiment standard curve. 62
Figure 3-5: An enzyme-linked immunoassay for PAP: principle of the assay. 63
Figure 3-6: MS/MS (Waters Micromass QuattroMicro API tandem mass spectrometer). Screen shot from Waters software. 66
Figure 3-7: AutoDELFIA system for maternal serum screening sample 67
Figure 3-8: Access 2 immunoassay system. 68
Figure 3-9: AutoDELFIA AFP: principle of the assay. 70
Figure 3-10: AutoDELFIA hCG: principle of the assay. 71
Figure 3-11: AutoDELFIA PAPP-A: principle of the assay. 72
Figure 3-12: AutoDELFIA FβhCG: principle of the assay. 73
Figure 3-13: Access 2 Unconjugated estriol principles. 74
Figure 3-14: Inhibin-A principle of the assay. 75
Figure 4-1: Untransformed distributions of IRT concentration in 2886 unaffected newborn babies 85
Figure 4-2: Untransformed distributions of PAP concentration in 2836 unaffected newborn babies 86
Figure 4-3: Untransformed distributions of PHE concentration in 2886 unaffected newborn babies 86
Figure 4-4: Untransformed distributions of TYR concentration in 2886 unaffected newborn babies 87
Figure 4-5: Log-transformed distributions of IRT concentration in 2886 unaffected newborn babies 87

Figure 4-6: Log-transformed distributions of PAP concentration in 2886 unaffected newborn babies....88
Figure 4-7: Log-transformed distributions of PHE concentration in 2886 unaffected newborn babies....88
Figure 4-8: Log-transformed distributions of TYR concentration in 2886 unaffected newborn babies....89
Figure 4-9: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 2893 control samples at the age of sample collection....93
Figure 4-10: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 2893 control samples at the age of sample collection....94
Figure 4-11: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 2893 control samples at the age of sample collection....95
Figure 4-12: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration levels in 2893 control samples at the age of sample collection....96
Figure 4-13: Box plot of IRT (log_{10}) marker for female (169 samples) and male babies (147 samples). Asterisk marks indicate extreme IRT values while circle mark indicates outliers IRT values....98
Figure 4-14: Box plot of PAP (log_{10}) marker for female (169 samples) and male babies (144 samples). Circle mark indicates outlier PAP values....99
Figure 4-15: Box plot of PHE ($log1_0$) marker for female (169 samples) and male babies (144 samples). Asterisk marks indicate extreme PHE values while circle mark indicates outlier PHE values....100
Figure 4-16: Box plot of TYR (log_{10}) marker for female (169 samples) and male babies (144 samples). Asterisk marks indicate extreme TYR values while circle mark indicates outliers TYR values....101
Figure 4-17: Box plot of IRT in increased IRT (log_{10}) group in female neonates and male neonates at the age of specimen collection. Asterisk marks indicate extreme values while circle mark indicates outlier values....105
Figure 4-18: Box plot of PAP in increased PAP (log_{10}) group in female neonates and male neonates at the age of specimen collection. Circle mark indicates outlier values....105

Figure 4-19: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and IRT (log_{10}) concentration level in 314 babies with elevated IRT.....106
Figure 4-20: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and PHE (log_{10}) concentration level in 314 babies with elevated IRT.....107
Figure 4-21: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and TYR (log_{10}) concentration level in 314 babies with elevated IRT.....108
Figure 4-22: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 182 premature samples and gestation at birth.....111
Figure 4-23: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 182 premature samples and gestation at birth.....112
Figure 4-24: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 182 premature samples and gestation at birth.....113
Figure 4-25: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration levels in 182 premature samples and gestation at birth.....114
Figure 4-26: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 182 premature samples at the age of sample collection.....115
Figure 4-27: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 182 premature samples at the age of sample collection.....116
Figure 4-28: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 182 premature samples at the age of sample collection.....117
Figure 4-29: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration levels in 182 premature samples at the age of sample collection.....118
Figure 4-30: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 189 low birth weight infants (less than 2500 grams) and age at sample collection.....120

Figure 4-31: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 189 low birth weight infants (less than 2500 grams) and age at sample collection. 121
Figure 4-32: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 189 low birth weight infant (less than 2500 grams) and age at sample collection. 122
Figure 4-33: Scatter plot with estimated regression line showing the relationship between TYR(log_{10}) concentration levels in 189 low birth weight infants (less than 2500 grams) and age at sample collection. 123
Figure 4-34: Box plot shows the median of IRT (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circle mark indicates outlier values.... 125
Figure 4-35: Box plot shows the median of PAP (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circle mark indicates outlier values.... 125
Figure 4-36: Box plot shows the median of PHE (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circle mark indicates outlier values.... 126
Figure 4-37: Box plot shows the median of TYR (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circle mark indicates outlier values.... 126
Figure 4-38: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration level and low birth weight (less than 2500 grams).... 127
Figure 4-39: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and low birth weight (less than 2500 grams)... 127
Figure 4-40: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration level and low birth weight (less than 2500 grams)... 128
Figure 4-41: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration level and low birth weight (less than 2500 grams). . 128
Figure 4-42: Distribution of IRT concentration according to the type of mutation present. 137
Figure 4-43: Distribution of PAP concentration according to the type of mutation present. 138
Figure 4-44: Distribution of IRT concentration in CF carriers according to the type of mutation present. 143
Figure 4-45: Distribution of PAP concentration in CF carriers according to the type of mutation present. 144

Figure 4-46: IRT median level in CF cases born with homozygous common delta ΔF508 mutation and other mutation. 147
Figure 4-47: IRT median levels in carrier babies born with ΔF508 mutation and other mutation. 148
Figure 4-48: Log_{10} Gaussian distributions for IRT in 29 CF cases. 154
Figure 4-49: Overlapping Log_{10} Gaussian distributions for IRT in affected and unaffected babies..... 155
Figure 4-50: Log_{10} Gaussian distributions for PAP in 29 CF cases..... 155
Figure 4-51: Overlapping Log_{10} Gaussian distributions for PAP in affected and unaffected babies..... 156
Figure 4-52: Median AFP concentration level at each week of gestation in maternal serum control samples..... 163
Figure 4-53: AFP MoM in samples from CF pregnancies. 164
Figure 4-54: Box plot comparison of AFP in 55 CF samples and 330 control serum sample. Circle mark indicates outlier AFP values. 164
Figure 4-55: Median hCG concentration level in maternal serum control samples. 166
Figure 4-56: HCG MoM in maternal serum samples from CF pregnancies..... 167
Figure 4-57: Box plot comparison of hCG in 55 CF samples and 330 control serum samples. Asterisk marks indicate extreme hCG values while circle mark indicates outlier hCG values. 167
Figure 4-58: Median PAPP-A concentration level in maternal serum control samples. 169
Figure 4-59: PAPP-A MoM in maternal serum samples from CF pregnancies. 169
Figure 4-60: Box plot comparison of PAPP-A in 55 CF samples and 330 control serum samples. Asterisk marks indicate extreme PAPP-A values while circle marks indicate outlier PAPP-A values..... 170
Figure 4-61: Median Free β-hCG concentration level in maternal control serum samples... 172
Figure 4-62: Free β-hCG (MoM) in maternal serum samples from CF pregnancies. 172
Figure 4-63: Box plot comparison of Free β-hCG in 55 serum CF samples and 330 control serum samples. Circle mark indicates outlier Free β-hCG values. 173
Figure 4-64: Median UE3 concentration level in maternal control serum samples..... 174
Figure 4-65: UE3 MoM in maternal serum samples from CF pregnancies..... 175
Figure 4-66: Box plot comparison of UE3 in 53 serum CF samples and 325 control serum sample. Asterisk marks indicate extreme UE3 values while circle marks indicate outlier UE3 values. 176
Figure 4-67: Median Inhibin-A concentration level in maternal control serum samples. 177
Figure 4-68: Inhibin-A in maternal serum samples and CF samples from pregnancies..... 178

Figure 4-69: Box plot comparison of Inhibin-A in 53 serum CF samples and 325 control serum samples. Circle mark indicates outlier Inhibin-A values. 179

بسم الله الرحمن الرحيم

" وَعَسَى أَنْ تَكْرَهُوا شَيْئًا وَهُوَ خَيْرٌ

لَكُمْ وَعَسَى أَنْ تُحِبُّوا شَيْئًا وَهُوَ

شَرٌّ لَكُمْ وَاللَّهُ يَعْلَمُ وَأَنْتُمْ لَا تَعْلَمُونَ"

صدق الله العظيم

سورة البقرة الأيه 216

“Yet, you may detest a thing, though it is good for you. And you might love a thing, though it is evil for you, for God knows and you do not know.’’

The Holy Koran

Chapter 2, Part 2, Verse 216

ACKNOWLEDGEMENTS

I am grateful to the Ministry of Health in Saudi Arabia for awarding me a scholarship to study for a PhD.

I would also like to extend my gratitude to Professor J.M Connor for providing me with access to the Department. I would also like to thank my supervisors Dr David Aitken and Dr Jenny Crossley for their close guidance; they have been a great source of encouragement and support throughout all of my PhD study. Thank you so much for your extreme patience in guiding me to complete this thesis.

My thanks also go to Moira Fitch, Gary McCaw, Gordon Graham, Denise Bannon, Thenmalar, Ekram, Suzie and Sandy, for their friendship, kindness and assistance and encouragement as well as to the rest of staff in the Biochemical Genetic department.

I would also like to thank Ann Theriault for her friendliness and for ensuring all orders came on time.

But most of all, I would like to thank my wonderful wife for putting up with me and supporting me throughout all of my studies that have lead to this PhD. Special thanks go to my children, Anas, Aseel and Abdulilah for their endless love and care. Thank you so much for everything and for just being you: this degree is dedicated to you. I would also like to thank my rest of family and friends for their prayers, concern and support during my studies. I wish so much that my parents and my older brother were still alive to see this day.

PUBLICATIONS

Ghamdi S, Fitch M, Crossley J, Aitken D. 2011. Screening for Cystic Fibrosis using IRT and PAP. (In preparation).

ORAL PRESENTATIONS

Ghamdi S, Fitch M, Crossley J, Aitken D. 2011. Newborn screening for Cystic Fibrosis using likelihood ratios derived from bloodspot Immunoreactive Trypsinogen (IRT) and Pancreatitis Associated Protein (PAP) measurements. 7th ISNS European Regional Meeting in Geneva, Switzerland.

POSTER PRESENTATIONS

Ghamdi S, Fitch M, Crossley J, Aitken D. 2010. Reducing incidental carrier detection in newborn screening for Cystic Fibrosis. Association for Clinical Biochemistry National Focus meeting, Glasgow, UK, May 2010

Ghamdi S, Fitch M, Crossley J, Aitken D. 2010. Newborn screening for Cystic Fibrosis using a combination of Immunoreactive trypsinogen and Pancreatitis Associated Protein. 7th Asia Pacific Regional Meeting of the International Society of Neonatal Screening. Bali, Indonesia.

Scientific Awards

2ND Best Free Paper Poster Presenter, 2010. 7th Asia Pacific Regional Meeting of the International Society of Neonatal Screening, Bali, Indonesia.

Scientific Networking Award 2010. Faculty of Medicine, University Glasgow.

Distinguished Achievement 2011, 5th Saudi International Conference. University of Warwick, Coventry, UK, 23rd-26th of June 2011.

LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
BIA	Bacterial inhibition assay
CA	Cellulose acetate electrophoresis
CAH	Congenital adrenal hyperplasia
CF	Cystic fibrosis
CHT	Congenital hypothyroidism
CV	Coefficient of variance
CVS	Chorionic villus sampling
DBS	Dried blood spot samples
DELFIA	Dissociated enhanced lanthanide fluoroimmunoassay
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immmunosorbent assay
FBC	Full blood count
FIA	Fluoro-immunoassay
FβhCG	Free beta human chorionic gonadtrophin
GALT	Galactose-1-phosphate uridyltransferase
Hb S	Haemoglobin S
HCG	Human chorionic gonadotropin
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IEM	Inborn errors of metabolism

Inbihin A	Inhibin A
IRT	Immunoreactive trypsinogen
kDa	Kilodalton
LBC	Liquid-based cytology
LC-MS/MS	Liquid chromatography tandem mass spectrometry
log	Logarithm
LR	Likelihood ratios
m/z	Mass/charge ratio
mAb	Monoclonal antibody
MCADD	Medium-chain acyl-coenzyme A dehydrogenase deficiency
mL	Millilitre
MoM	Multiple of the median
MSMS	Tandem mass spectrometry
MSUD	Maple syrup urine disease
mU/L	Milliunits per litre
ng/ml	Nanograms per millilitre
PAH	Phenylalanine hydroxylase
PAP	Pancreatitis associated protein
PAPP-A	Pregnancy associated plasma protein-A
PBS	Phosphate buffered saline
PHE	Phenylalanine
PKU	Phenylketonuria
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
SCD	Sickle-cell disorders
SD	Standard deviation

TSH	Thyroid Stimulating Hormone
TYR	Tyrosine
U/ml	Unit per millilitre
uE3	Unconjugated estriol
µL	Microlitre

CHAPTER 1 :
INTRODUCTION

1.1 BACKGROUND

Sir Archibald Garrod (1857-1936) was a physician who coined the term 'Inborn Errors of Metabolism' (IEM). He first used this term to describe alkaptonurea (Scriver, 1995). This is a rare inherited disorder of organic acid metabolism that causes urine to turn from yellow to brown and then to black when exposed to air. This is due to the build up of a storage product homogentisic acid which accumulates as a result of a metabolic block caused by deficiency of an enzyme, homogenetisic acid oxidase. Patients affected with this disorder are likely to suffer from arthritis in the spine and joints. Moreover, they may also suffer from heart, kidney and prostate problems. Although Garrod did not know the identity of the deficient enzyme, he established the principle underlying the cause of the majority of inborn errors. Garrod also suggested that the disease followed an autosomal pattern of inheritance as it was found in both males and females (Scriver, 1995).

Later, as the pattern of inheritance became better understood, it was recognised that genes are a significant determinant of human health, with a mutated gene following a predictable pattern of inheritance within families. Single gene inheritance is also referred to as Mendelian inheritance. There are four types of single-gene (Mendelian) inheritance: autosomal recessive, autosomal dominant, X-linked dominant and X-linked recessive. Other types of inheritance include mitochondrial inheritance and multifactorial inheritance.

There is a large number of IEMs whose clinical consequences vary from benign to lethal (Table 1.1). Multiple mutations in a gene e.g. coding for a specific enzyme may lead to heterogeneity within individual IEMs. Some are treatable but many are not: e.g. phenylketonuria (PKU) is a treatable disease when it is diagnosed early in the newborn period whereas Tay-Sachs disease is a non treatable disorder.

If a child is born with a serious metabolic disorder, the child may appear healthy at birth but will at some point become ill. Medical investigation into the illness can establish that the child is affected with a specific metabolic disorder, e.g. an amino acid disorder. Therefore, if all amino acids are tested and elevated phenylalanine levels are found, PKU may be diagnosed. However, this diagnosis will come too late for effective treatment to be given to the child, as it may be days, months or even years before the clinical symptoms indicating a metabolic disorder become apparent. Hence, there is a need for a system to identify at-risk families before it is too late and the damage is done to an affected baby.

With our knowledge of enzymes and understanding of the pathways in many diseases, it is possible to diagnose many inborn errors of metabolism prenatally through biochemical or DNA tests. Therefore, if it is known that a particular disease runs in the family and a couple wish to have a baby, prenatal diagnosis can be offered. The parents can then be given the choice of continuing or terminating the pregnancy if the baby is affected. They can also be told if the baby is a carrier of the disorder, which may be of benefit when the child reaches adulthood allowing appropriate testing to be carried out on their partner before they start a family. Prenatal diagnosis is only possible in families where there is a known history of a particular condition. For the majority of babies born with an IEM, this is an unexpected occurrence and it would be impossible to test for all possible conditions. Thus it is impossible to significantly reduce the burden of inherited metabolic disorders through prenatal diagnosis. A more practical approach is to test newborn babies for a limited number of conditions which can be treated using techniques which can be applied to the whole population. This is newborn screening.

Table 1-1: Classification, incidence and inheritance of some inborn errors of metabolism (Scriver, 1995)

Classification	IEM	Incidence	Mode of Inheritance
Carbohydrate:	(GSD I) Type Ia (Von Gierke)	~1:100,000	AR*
	(GSD I) Type Ib	~1:200,000	AR
	(GSD I) Type III	~1:125,000	AR
	(GSD I) Type IV (Andersen disease)	~1:1,000,000	AR
Amino acid	PKU	~1:10,000	AR
	Maple syrup urine disease	~1:180,000	AR
Fatty Acid Oxidation	Medium-chain acyl-co A dehydrogenase deficiency	~1:20,000	AR
organic Acid	Alcaptonuria	1:100,000-million	AR
Purine	Lesch-Nyhan syndrome	Rare	XLR*
Steroid metabolism	Congenital adrenal hyperplasia due to 21-hydroxylase deficiency (21-OH CAH),	1:10,000 to 1:18,000	AR
Lysosomal storage	Gaucher's disease	Very rare	AR
Membrane Transport Disorders	Cystic Fibrosis	1:2000-3000	AR

*AR: autosomal recessive. *XLR: X-linked recessive

1.2 SCREENING

Screening takes place in two stages. First, the primary screening test is done to detect early disease or risk factors in large numbers of apparently healthy people. Second, a diagnostic test is carried out on individuals who are screen positive to confirm the presence or absence of specific disease as a basis for treatment decisions.

In 1968 Wilson and Jungner proposed several criteria for screening diseases (Table 1.2) (Wilson and Jungner, 1968). These proposed principles were to be followed when establishing any screening programme in order for it to succeed.

The condition, severity, prevalence, mortality or morbidity of the disease should be important and well-defined within the community, and there must also be a need to understand its natural history. Before the implementation of a screening programme, diagnosis and treatment facilities should be available for affected individuals. When introducing a screening programme, the advantages and disadvantages for those being screened should be carefully considered, and only when it has been decided that the advantages outweigh the disadvantages should the screening programme go ahead. Considerations of outcome and treatment are vital factors to be taken into account. The test must be acceptable to the population, and it must also be safe, sensitive, accurate, and cost-effective. The technology employed should ideally be capable of screening the population for more than one disease at a time, and there should be as little delay as possible in obtaining the results of the test so that medical intervention is possible when required. Furthermore, precise monitoring of the effectiveness of a test and a quality assessment procedure should be in place.

Table 1-2: Wilson and Jungner screening criteria (Wilson and Jungner, 1968).

Understanding the nature of the disease
• The condition should be a significant health problem
• There must be a distinctive latent or early symptomatic phase
• There should be a clear understanding of the history of the condition, including development from latent to declared disease.
Understanding the test
• Availability of appropriate test or examination
• The test or examination have to be acceptable to the population
• The case finding needs to be ongoing process rather than a 'one-off' project
• Outcome affected by early diagnosis
Treating the disease
• There should be a recognised treatment for patients diagnosed with the disease
• Facilities for diagnosis and treatment should be accessible
• A policy should be an established regarding whom to treat as patients
Cost considerations
• Cost of screening test
• Cost of follow-up diagnostic test
• The costs of the case finding, including diagnosis and treatment of diagnosed patients, balanced in economic terms relative to potential expenditure on overall medical care.

A Quality Assessment process has to be established, and the effectiveness of the test must be carefully monitored. Finally, a screening programme should be evaluated. This should include the rate of uptake of the test, its acceptability to the population and its total cost (Murray and Clarke, 2002).

There are four important parameters for measuring the effectiveness of a screening test. The first of these is sensitivity, which is the proportion of true positive cases among all affected cases; in other words, the proportion of cases which are detected by a test. The second parameter is specificity, which is the proportion of true negative results among all

non-affected cases, i.e. the extent to which a test detects only affected cases. The third is positive predictive value, being the proportion of true positive results within the high risk group, that is, the proportion of cases actually affected among cases considered screen positive. The final parameter is the negative predictive value which is the proportion of true negative results within the low risk group, which is to say the proportion of individuals, which are actually unaffected among cases considered negative in a test. Thus both the positive and negative aspects of the programme must be evaluated (Grimes and Schulz, 2002).

1.2.1 Types of Screening

1.2.2 Prenatal screening

Various types of screening are carried out at different stages in an individual's life. For example, there is screening during the pregnancy to detect neural tube defects and chromosomal abnormalities. Various infectious diseases are also screened for during pregnancy. In the UK these include rubella, syphilis, HIV and hepatitis B. The aim of screening for some of these disorders, for instance, syphilis, is to detect maternal infection which can be treated with antibiotics and followed by treatment for baby after birth (Wald and Leck, 2000).

Screening for inherited haematological disorders is also carried out during pregnancy. These include the sickling disorders and thalassaemia. Screening, especially in early pregnancy, is important in at-risk ethnic groups so that prenatal diagnosis and termination can be offered if that is the parents' wish. The second type comprises red cell enzyme deficiencies such as glucose-6-phosphate dehydrogenase deficiency. The third type of screening is for the bleeding disorders, such as haemophilia (Wald and Leck, 2000).

1.2.3 Adult screening

Adult pre-symptomatic screening programmes for various types of cancer, such as breast cancer, are offered. Breast cancer screening aims to diagnose women in the early stage of breast cancer, which results in an increase in the chance to cure the disease. The screening test involves the use of a mammography X-ray (Kuhl et al., 2005). Cervical cancer can be screened for using liquid based cytology (LBC). Cells contained in a sample collected from the cervix of the women are spread out as a thin layer of cells on a slide and examined using a microscope to see if there are any malignant cells present (Beerman et al., 2009).

1.2.4 Carrier screening

Screening for carriers of Tay-Sachs disease is offered for specific ethnic groups (e.g Ashkenazi Jews) who are at high risk of being carriers of this disorder. Tay-Sachs disease is an autosomal recessive trait with 1 in 30 Ashkenazi Jews being a carrier. In other ethnic groups however, the carrier rate is much less, around 1 in 300. The screening test is performed by measuring serum-beta-N-acetylhexosaminidase A or by DNA analysis in couples. If both of the partners are carriers, prenatal diagnosis can be offered (Connor & Ferguson-Smith, 2004).

1.2.5 Newborn screening

Inherited metabolic disorders are generally rare conditions which may lead to serious physical and mental handicap in the affected child. The majority are recessively inherited, and there is a 1 in 4 chance of a recurrence in any future pregnancy. Many of these conditions may not be suspected clinically at birth and the characteristic signs and symptoms appear later. Genetic screening for such conditions aims for the early

identification of individuals and the provision of treatment that will allow the child to grow and develop normally.

Over the last 40 years, different screening tests have been designed to detect many inborn errors of metabolism and for certain other conditions which strictly do not fall within the classical definition of inborn errors of metabolism.

1.2.6 The Development of Newborn Screening

1.2.6.1 The Bacterial Inhibition Assay

In 1963 a method for screening newborn babies for metabolic disorders was developed by an American microbiologist, Dr. Robert Guthrie (Guthrie and Susi, 1963). The test consists of two principal parts. The first is a simple, semi-quantitative method of measuring an increased level of phenylalanine in blood to detect children with the amino acid disorder PKU. The second is the development of the specimen in the form of a dried blood spot collected on a special absorbent card (henceforth known as the "Guthrie Card").

The principle underlying the screening test is a bacterial inhibition assay which stems from the finding that bloodspots with phenylalanine in increased concentration can overcome the inhibition of growth of the bacterium bacillus subtilis ATCC 6051 by B-2-thienylalanine on an agar plate.

Guthrie developed the BIA principle to screen for other metabolic disorders, e.g. galactosaemia and maple syrup urine disease. Millions of newborn babies across the world have been screened using this method.

1.2.6.2 The Dried Bloodspot Sample

The second component of Guthrie's strategy for newborn screening is the use of the dried blood spot samples (DBS). Blood is collected from the heel of a baby in their first week of life and applied within circles on a dry filter paper and allowed to dry. There are several advantages to using DBS compared to using other methods like venepuncture and serum collection. Table 1.3 briefly illustrates some of these advantages.

Table 1-3: Advantages of using the DBS method instead of venepuncture (Kirby, et al., 1981; Mei et al., 2001).

Risk aspect	Venepuncture & serum	Dried blood spots (DBS)
	Venepuncture can be quite troublesome to perform (e.g. poor veins)	Simple to perform
	Requires training	Requires minimal training
	Risks of needle stick injury	Minimal risks
	Increased risk if an infectious hazard e.g. HIV & HCV is present.	Represents a low infectious hazard
	Possibility of leakages and breakages in transit, requirement for transportation at controlled (low) temperature.	Cannot leak or be broken in transit and no requirement for transportation in non-ambient condition
Economic aspect	Collection & processing of samples is expensive	Collection & processing of samples is cheap
	Needles and syringes are costly	DBS cards are inexpensive
	Whole blood requires to be packed in unbreakable containers sent by special delivery.	Easily shipped in sealed envelopes by post
	Transportation of frozen sera may need the use of dry ice or liquid nitrogen	Easy to transport, with no need for dry ice or liquid nitrogen

Since Guthrie developed the use of the dried filter paper card to screen newborn babies, there have been many suggestions regarding improving the quality of the filter paper card and standardising the collection of the specimen. The National Committee for Clinical Laboratory Standards in the USA (NCCLS) isued a written standard on the collection of dried blood spot specimens on filter paper (Hannon, 1997).

The bloodspot card is also a medical record as it provides information on the baby; for example, baby's ID number, baby's name, mother's name, address, birth weight, gender, date of first milk feed, type of feed (breast/formula/other), whether the baby has had a blood transfusion or medication, and the baby's family history. Other information is required, such as the date of the specimen collection and the name of the person who took the specimen. The babies' ancestry should also be recorded. A barcode may also be printed on the card which can be used for sample tracking and analysis.

1.3 SCREENING FOR IEMs

Newborn screening for PKU and other IEMs is established in many developed countries. The range of tests varies between different countries. In the UK, 5 disorders are currently screened for: PKU, Congenital Hypothyroidism (CHT), Sickle-cell Disorders (SCD), Medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD), and Cystic Fibrosis (CF). Programmes in other countries may also include Galactosaemia (GALT), Maple Syrup Urine Disease (MSUD), Congenital Adrenal Hyperplasia (CAH) and up to twenty additional amino acid and fatty acid disorders, through the use of tandem mass spectrometry (MSMS) technology, notably in the USA. Electrospray tandem mass spectrometry is used to screen for 10 conditions in Germany (Jeannette, 2011). In the UK, MS/MS is used only to screen for PKU and MCADD. In the USA, the American College of Medical Genetics recommended a core panel of 29 screening conditions with 25 additional secondary targets in every state (Klein, 2011). Screening is also being introduced and developed in other countries, e.g. countries in the Asia Pacific Region (table 1.4) and in the Middle East (table 1.5), with some countries offering selective screening programmes appropriate to their ethnic populations. However, in many countries across the world, establishing newborn screening programmes poses various

challenges. For instance poor economies, differences in language, religion and culture, geographic extremes, unstable governments and various public health priorities lead some governments to focus more on problems of malnutrition or the control of infectious disease than on newborn screening.

Table 1-4: Different types of screened disorders in the Asia Pacific region (Padilla and Therrell, 2007).

Jurisdiction	Program demographics							
	Population, 2004 (000)	Thousand births 2005	Infant mortality rate (under 1) 2005	Life expectancy at birth 2005	Date NBS began	Reported program coverage in 2006	Cost or screening paid by	Cost in US$
Australia	20 155	250	5	81	1967	100%	Govt	6.00
Bangladesh	141 822	3 747	54	64	1999	<1%	Govt	?
Cambodia	14 071	429	98	57	–	?	?	?
China	1 315 844	17 310	21	72	1981	25%	Family	5.50
Hong Kong (China)					1984	99%	Govt	20.00
India	1 103 371	25 926	43	64	1980	<1%	Family	?
Indonesia	222 781	4 495	18	68	1999	<1%	Family	2.50
Japan	128 085	1 162	2	82	1967	>99%	Govt	18.33
Korea (South)	47 817	457	3	78	1991	94%	Govt	17.50
Korea (North)	22 488	342	22	64	–	?	?	?
Laos	5 924	205	35	55	–	?	?	?
Malaysia	25 347	547	5	74	1980	95%	Govt/private	?
Mongolia	2 646	58	26	65	2000	<1%	Grant	?
Myanmar	50 519	976	40	61	2000	<1%	Govt	?
Nepal	27 133	787	40	62	–	?	?	?

Table 1-4: Continued

Jurisdiction	Program demographics							
	Population, 2004 (000)	Thousand births 2005	Infant mortality rate (under1) 2005	Life expectancy at birth 2005	Date NBS began	Reported program coverage in 2006	Cost or screening paid by	Cost in US$
New Zealand	4 028	54	4	79	1966	100%	Govt	15.00
Palau	20	0	14	–	2007	0	Govt	No fee
Pakistan	157 935	4 773	57	64	2000	<1%	?	?
Philippines	83 054	2 018	15	71	1996	10%	Family/ins.	10.00
Singapore	4 326	39	1	79	1965	>99%	Family 40%	32.00
Sri Lanka	20 743	329	11	74	2005	<1%	Govt	?
Taiwan					1985	>99%	Family	26.00
Thailand	62 233	1 009	13	71	1992	97%	Govt	?
Vietnam	84 238	1 648	15	71	2000	<1%	Govt	?
Totals	3 544 580	66 561	25	69				
World statistics	6 449 371	133 449	30	68				

Table 1-5: Newborn screening development in the Middle East region (Saadallah and Rashed, 2007).

Country	Population (million)	Birth rate/1000	Fertility rate/woman	Estimated number of infants/year
Algeria	32.93	17.10	1.89	563 103
Bahrain	0.70	17.80	2.60	12 460
Egypt	78.89	22.94	2.83	1 809 737
Iran	68.69	17.00	1.80	1 167 730
Iraq	26.78	31.98	4.18	856 424
Jordan	5.91	21.25	2.63	125 588
Kuwait	2.42	21.94	2.91	53 095
Lebanon	3.87	18.52	1.90	71 672
Libya	5.90	26.49	3.28	156 291
Morocco	33.2	21.98	2.68	729 736
Oman	3.10	36.40	5.77	112 344
Palestinians[a]	1.43	39.50	5.78	56 485
Palestinians[b]	2.46	31.67	4.28	77 908
Qatar	0.89	15.56	2.81	13 848
Saudi Arabia	27.02	29.34	4.00	792 767
Somalia	8.86	45.13	6.76	399 851
Sudan	41.24	34.53	4.72	1 424 017
Syria	18.88	27.76	3.4	524 108
Tunisia	10.18	15.52	1.74	157 994
UAE	2.60	18.96	2.88	49 296
Yemen	21.46	42.89	6.58	920 419
Total (million)	397.41		**Total**	10 074 873

a Gaza Strip.
b West Bank.

1.3.1 Analytical Techniques Used in Bloodspot Screening

There are several analytical techniques used in bloodspot screening. For example thalassaemia and SCD screening is usually carried out by biochemical techniques such as cellulose acetate electrophoresis (CA), isoelectric focusing (IEF), high performance liquid chromatography (HPLC) and by DNA methods. These techniques are the most commonly used methods to detect variant haemoglobin worldwide (Davies et al., 2000).

The measurement of immunoreactive trypsinogen (IRT) levels in dried blood spots to screen for CF was originally carried out by radioimmunoassay (RIA) with polyclonal antibodies (Crossley et al., 1979). This was replaced by an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies as an alternative approach, and using dried blood spots in a 96-well microtitre plate. This method was more efficient for large sample numbers than RIA, which involves the use of tubes and required radioactive materials (Bowling and Brown, 1988). Further developments in laboratory techniques have led to automated methods of measuring IRT as a primary test to detect CF with dried blood spots using the automated dissociated enhanced lanthanide fluoroimmunoassay (DELFIA) technique (Soini and Kojola, 1983). This technique has been automated in the AutoDELFIA immunoassay system, which is started by loading the microtitration plates containing punched dried blood spot samples and assay reagents. It includes an external PC which controls the running of the system. The principal advantage of this technique is that it has a high sample throughput with good accuracy and precision of bloodspot IRT measurement.

Other ELISAs have been developed for blood spot screening, e.g. for CHT measuring thyroxin (T4) or thyroid stimulating hormone (TSH) and for CAH measuring 17-OH progesterone and these have also been adapted to the DELFIA method.

A recent method used to screen newborn babies is (MS/MS) (Chace et al., 2003). This technique has a high rate of sensitivity and specificity and can be used to screen for a large range of different conditions, all from a single dried blood spot specimen in a microtitre plate.

The system consists of two mass spectrometers coupled in tandem by a collision chamber. Samples are extracted, ionised and introduced into the first mass spectrometer where ions with a specific mass/charge ratio (m/z) are selected and pass into the collision chamber where they are fragmented by collision with molecules of the inert gas, argon. The product ions then enter the second mass spectrometer which is tuned to allow through only the principal daughter ions of interest for the analyte under investigation. These have specific mass/charge ratios and are characteristic of the metabolites of interest, e.g. phenylalanine and its internal standards which are included in the extraction solution in each well of the microtitre plate. As the internal standards are of known concentration, the signal responses from the standards can be compared with the native species and used to calculate their relative concentrations.

1.3.2 Disorders Detected By Newborn Bloodspot Screening

1.3.2.1 Phenylketonuria (PKU)

PKU an autosomal recessive disorder caused by deficiency in the enzyme phenylalanine hydroxylase (PAH). PAH converts the amino acid phenylalanine to tyrosine, and if PAH is missing phenylalanine is converted instead to phenylpyruvic acid, and brain damage and mental retardation will occur. The PAH gene is located on chromosome 12 (q22-q24.1); it is considered to be a homopolymer and it has about 90,000 nucleotide base pairs with 13 exons. Seventy haplotypes were identified by the restriction fragment length

polymorphism (RFLP) method. Of these 70 haplotypes, six haplotypes account for more than 90 per cent of classical PKU in European families (Wald and Leck, 2000) .

PKU occurs in about 1:10,000 live births, with great ethnic variations. In Turkey, due to high consanguinity within the population, PKU incidence is one in every 4000 births, while Finland has the lowest prevalence in Europe with 1 case per 100,000 births. Asians, Africans and Americans can also be affected with PKU disorder (Blau et al., 2010).

There are two types of PKU: classical PKU, and non-PKU type which is due to deficiencies of other enzymes such tetrahydrobiopterin. Affected individuals with classical PKU type will suffer from mental retardation if the disease is untreated. Other clinical findings include microcephaly, decreased pigmentation of hair and skin, eczematous rash and vomiting (Wald and Leck, 2000).

The first test used to identify individuals affected with PKU was a ferric chloride test on urine often carried out due to the presence of a''mousey'' smell in the nappy. Its disadvantage, however, is that it is unreliable in newborn screening due to the variable appearance of characteristic metabolites. The bacteriological inhibition assay was the mainstay in newborn screening for the many years. The Guthrie test is a semi-quantitative technique. The test's advantage is its low cost. Fluorometric assay is also used to detect elevated levels of phenylalanine in the blood (Wald and Leck, 2000). This quality of this technique is easier to control. However the BIA method for phenylalanine has been largely superseded in recent years by the introduction of MSMS (Millington et al., 1990). This powerful technology can be used to screen for multiple disorders from a single blood spot punch.

1.3.2.2 Galactosaemia

Galactosaemia is an inherited autosomal recessive disorder caused by a deficiency of the enzyme galactose-1-phosphate uridyltransferase (GALT). This enzyme acts as a catalyst for the production of glucose-1-phosphate and uridyldiphosphate from galactose-1-phosphate and UPD-glucose. In the United Kingdom the incidence of galactosaemia is around 1:44,000 while, in Japan the incidence of newborn babies affected by galactosaemia is 1:667,000 (reviewed by Seymour et al., 1997).

Various clinical symptoms appear in individuals affected with this disorder, for instance, jaundice, hepatosplenomegaly, and cataracts, which manifest themselves in the few days following the birth of a baby. Individuals affected with galactosaemia are also more susceptible to infections, amino acidaemia and albuminuria. Cirrhosis of the liver which can lead to liver failure and death results from the severe form of galactosaemia (reviewed by Seymour et al., 1997). The early symptoms of galactosaemia can be resolved by excluding glucose from the diet, and this is the basis of the initial management of the disorder.

To detect galactosaemia, neonatal dried blood spot screening can be employed alone, or combined with galactose-1-phosphate levels. Schickling and his colleagues (reviewed by Seymour et al., 1997) conducted a study which involved the screening of 350,000 newborn babies. Their results demonstrated that the use of colorimetric enzymatic micro assay for galactose and galactose-1-phosphate had a false-positive rate of 0.028%, of which 40 % were compound hetrozygotes and were associated mainly with the Duarte variant. However, this study did not show any false negatives, and also demonstrated that the test is inexpensive to carry out.

Thibodeau and his co-workers (reviewed by Seymour et al., 1997) conducted another study in which they screened 299,420 newborn babies using the Beutler assay to determine GALT activity alone. This study also reported no false negatives, and revealed a false-positive rate which averaged 0.16% over 3 years. However, certain disadvantages to employing the Beutler assay were demonstrated in this study; for instance, humidity and temperature affected the assay during transportation of the blood spots, and, in addition, the method was affected by the degree of drying of the blood spots. Galactosaemia screening has been abandoned in the UK as a clinical diagnosis was often made before the newborn screening result was available. However, a raised phenylalanine level secondary to liver damage may be detected by MSMS.

1.3.2.3 Congenital hypothyroidism (CHT)

The thyroid gland is one of the large endocrine glands located in the middle of the lower neck, below the larynx. The function of the thyroid gland is to regulate the rate of body metabolism and developmental function in other body systems, through secreting thyroxin (T4) and triiodothyronine (T3) hormones. In addition, there is another important function of thyroid hormone, which is the development of the central nervous system. Congenital hypothyroidism can be found in many countries all over the world (Park and Chatterjee, 2005).

CHT is one of most important and prevalent disorders which affect newborn babies. It occurs sporadically and the incidence rate of this disorder is around 1 in 3000 to 4000 live births. CHT can cause mental retardation due to the failure of the thyroid gland to grow if the patient is not treated with an adequate thyroxin supplement. In addition, iodine deficiency can cause CHT in areas where iodine is not present in adequate quantities. However, in areas where iodine is present in adequate amounts, CHT is caused by a defect

or dysfunction of the thyroid gland. The thyroid gland may well be present, be it large or small, but production of the thyroid hormone is reduced (hypoplastic) or absent (athyreosis) in CHT patients. In certain cases these abnormal glands are the result of genetic defects such as iodine trapping defect, thyrotropin resistance, thyroglobulin, and iodotyrosine deiodinase deficiency. In some cases, however, there is no identifiable cause for the dysfunction (Park and Chatterjee, 2005). In certain cases, hypothyroidism detected by screening may be transient. This is usually due to the presence of maternal antibodies which temporarily impair thyroid function for a few weeks.

CHT symptoms vary considerably from one patient to another. Some have no clinical sign at 5 days which may be due to the extent of thyroid hormone deficiency and the length of time the body has been deprived of the thyroid hormone. Infants born with CHT which is left untreated will suffer from various illnesses, which can include constipation, hypothermia, umbilical hernia, growth retardation and jaundice. However, most children born with congenital hypothyroidism and treated with thyroxin grow and develop normally in all respects (Rastogi and LaFranchi, 2010).

A number of clinical laboratory tests are available to screen for CHT, for example, fluoro-immunoassay (FIA), (ELISA) and (RIA) (Rose and Brown, 2006).

1.3.2.4 Sickle Cell Disorder (SCD)

Sickle-cell anaemia belongs to a group of genetic diseases known as haemoglobinopathies, which can be passed on to children by their parents via the recessive pattern of inheritance. SCD is more prevalent in certain ethnic groups, such as in tropics and sub-tropical, areas. Due to global migration, the disease has now spread to other parts of the world. The seriousness of its symptoms means that SCD requires

specialized treatment and critical care. The economic and psychosocial aspects of SCD therefore put great pressure not only on affected individuals, but also on their families and the wider community.

SCD is a type of anaemia characterized by the presence of haemoglobin S (Hb S). Under a microscope, red blood cells appear crescent-shaped instead of round biconcave cells. SCD is prevalent in Africa, and also in Central America and the Middle East. Affected individuals look pale with swollen hands and feet, and they are more susceptible to respiratory infections. In severe cases, it can be fatal. Early diagnosis and treatment with prophylactic penicillin reduces morbidity and mortality (Hoffbrand and Pettit, 1993).

This disease is cased by a point mutation in the amino-acid at position 6 of the beta globin chain which leads to the substitution of the amino-acid valine for glutamic acid. SCD is a recessive disease; that is, it is passed on by parents, both of whom have one gene for sickle haemoglobin and one normal allele. Numerous other variants have been identified which are associated with a range of clinical severity e.g. HbC, Hb, HbDPunjab, HbE. The life span of a normal blood cell is approximately one hundred and twenty days, whereas a sickle cell stays viable for only about twenty days, and because of this short life span the bone marrow cannot reproduce sufficient blood cells, eventually resulting in anaemia (Hoffbrand and Pettit, 1993).

SCD can be diagnosed by haemoglobin electrophoresis test, which identifies the particular types of haemoglobin, that is, haemoglobin A and F (adult and foetal) and any variants. A provisional diagnosis can be achieved by carrying out full blood count (FBC). This measures the status of several features of the blood, for example, the number of red blood cells in the blood and the amount of haemoglobin; the percentage of blood cells as a proportion of the total blood volume; the average amount of haemoglobin in the red blood

corpuscles; the volume of red blood cells; the number of white blood cells, and the percentages of the various types of these, as well as the number of platelets. Hence, the presence of anaemia can be revealed by an FBC. Reticulocyte count can also be employed in testing for sickle cell anaemia (Hoffbrand and Pettit, 1993).

SCD screening is usually carried out by biochemical techniques such as electrophoresis on cellulose acetate (CA), isoelectric focusing (IEF), high performance liquid chromatography (HPLC) and DNA methods. These techniques are the most commonly used methods to detect variant haemoglobin worldwide. The limitation of the electrophoretic methods is that they are manual, labour-intensive procedures. The advantage of using the HPLC technique is that it provides an automated and quantitative analysis, and requires only a small sample, while its disadvantage is that it is unable to detect 'fast-moving' variants e.g. HbBarts Both methods have high sensitivity and specificity rate (Clark and Thein, 2004).

1.3.2.5 Congenital adrenal hyperplasia (CAH)

CAH is an inherited recessive disorder which is caused by a deficiency in one of the important enzymes required for the synthesis of cortisol in the adrenal cortex. Deficiency of the 21-Hydroxylase enzyme is the most frequent cause, and accounts for about 95 % of all cases affected with CAH (van der Kamp and Wit, 2004).

The screening test for CAH disorder is performed using filter paper blood spots obtained by heel puncture during the fifth day after the birth of the baby. The metabolite 17-hydroxy progesterone (17-OHP) is then assayed using one of the following techniques. First, there is the ELISA assay used in Japan, second, the RIA which is used in the United States, and third, the FIA used in Europe. It has been found that there is a significant variation in the cut-off levels from one programme to another. These differences are due

to the characteristics of the reference population (birth weight and gestational age), while other variations are due to the varying thicknesses of the filter paper employed and the different antibodies and reagents which are used (van der Kamp and Wit, 2004).

Immunoassay tests for 17-OHP are generally known to produce high false positive rates. Another method has been developed, the steroid profiling method, which uses liquid chromatography tandem mass spectrometry (LC-MS/MS) on blood spots to determine 17-OHP, androstenedion and cortisol simultaneously, This method can identify the false positive results often found in newborn screening for CAH (Lacey et al., 2004).

1.3.2.6 Medium Chain Acyl-CoA Dehydrogenase Deficiency (MCADD)

Medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD) is a common disorder that causes a defect in mitochondrial β-oxidation. In genetics, the MCADD gene is located on chromosome 1 at position p31; the most common MCADD mutation is A985G. It is inherited as an autosomal recessive pattern (Wang et al., 1999).

Deficiency of MCADD, occurring in 1:15 000 (Hsu et al., 2008), is a key enzyme involved in the metabolism of mitochondrial fatty acids. The bodies of those affected with MCADD cannot metabolise certain fats of medium chain length (C6-C14) into energy. This may occur during a period of fasting where glucose stores are depleted, and so individuals with this disease should avoid fasting (Hsu et al., 2008).

The symptoms of MCADD vary from patient to patient, with signs generally appearing after a period of fasting. In severe cases, the disease may lead to hypoglycaemia or acute encephalopathy. Symptoms include clamminess of skin, fever, fatigue; diarrhoea,

behavioural changes and vomiting, and these symptoms may appear in infants or young children (Kompare and Rizzo, 2008).

Individuals affected with MCADD can be detected through tandem mass spectrometry (MS/MS). Blood spot samples are collected and then analysed to detect abnormal accumulation of C8 (Kompare and Rizzo, 2008). A positive result is confirmed by a DNA mutation test. The MS/MS technique is suitable for a large-scale analysis. Today MS/MS is used in many countries such as the USA, UK and Australia for newborn screening programmes.

1.3.3 Screening for Cystic Fibrosis

Newborn screening for CF is a recent addition to screening programmes in many countries including UK based on the analysis of bloodspot immunoreactive trypsinogen (IRT). Elevated levels of IRT are associated with CF. The use of sensitive immunoassays methods (e.g. DELFIA) to measure IRT followed by DNA mutation analysis and/or a sweat chloride test are required to confirm or exclude the presence of CF. However specificity is poor with a relatively large number of false positives. Other screening markers which could help reduce the false positive rate would be useful.

1.3.3.1 Genetics of CF

The cystic fibrosis transmembrane conductance regulator (CFTR) gene, which consists of 27 coding exons spanning over 250 kb on chromosome 7q31.2, with a transcript of 6.5 kb, was cloned in 1989 (Kerem et al., 1989; Rommens et al., 1989; Riordan et al., 1989). A 3- base pair (bp) deletion is the most widespread CF mutation and leads to phenylalanine loss at position 508 of the protein (F508del). The CFTR gene encodes a protein forming the chloride (Cl-) channel in the apical membrane of exocrine epithelial cells and contains 1480 amino acids, with a molecular weight of ~170kDa.

More than 1000 mutations have been identified (Ahmed et al., 2003). F508 del is found in about 70% of the CF chromosomes worldwide, although its frequency varies considerably among different ethnic groups, ranging between 100% in the Danish Faroe Islands to 18% in Tunisia (Hodson, et al., 2007). The gradient in the frequency of F508del diminishes notably from northeast to southwest Europe. The majority of the other mutations are uncommon, with only 11 being found in over 100 patients. However, there is a high incidence of a number of the other uncommon mutations in isolated populations (Hodson et al., 2007) (Table 1.6).

Table 1-6: Incidence of CFTR mutations other than ΔF508 with high incidence in specific populations (Hodson et al., 2007).

Mutation	Frequency (%) in specific population	Frequency (%) in the general population
Q359K/T360K	Georgian Jews (88)	
M1101K	Hutterite Brethren (69)	
S549K	United Arab Emirates (61.5)	
W1282X	Ashkenazi Jews (48) Tunisian Jews (17) Israeli Arabs (10.6)	1.2
405+1G→A	Tunisian Jews (48) Libyan Jews (18)	
3120 +1G→A	Bantu, Africa (46.4) South African (17.4) African American, USA (13.9) African American, Africa (12.2) Saudi Arabia (10)	
N1303K	Egyptian Jews (33) Israeli Arabs (21) Algeria (20) Lebanon (10)	1.3
G85E	Turkish Jews (30)	
1898+5G→T	Taiwan (30)	
394delTT	Finland (28.8) Estonia(13.3)	
621+1G→T	Saguenay Lac-Saint-Jean, Canada (24.3) Northern Greece (12.1)	0.7
Y122X	Reunion Island (24)	
3905insT	Amish, Mennonite (16.7) Switzerland (9.8)	
Y569D	Pakistani, UK (15.4)	
T338I	Italy, Sardinia (15.1)	
1548delG	Saudi Arabia (15)	
R553X 3120+1kb del8.6kb	Switzerland (14) Israeli Arabs (13)	0.7
I1234V	Saudi Arabia (13)	
R347P	Turkish population, Bulgaria (11.7)	0.2
Q98X	Pakistani, UK (11.5)	
G542X	South Spain (11.4)	2.4
711+1G→T	Algeria(10)	
4010del4	Lebanon (10)	
R1162X	Northeast Italy (9.8)	0.3
1525—1G→A	Pakistani, UK (9.6)	

*The mutations are listed in a decreasing order of their frequency (in the case of more than one population, the frequency was listed according to the highest).

1.3.3.2 Clinical Characteristics of CF

CF is an autosomal recessive disorder that leads to a shortened lifespan unless it is treated with appropriate medication sufficiently early. It is a disorder that involves severe pulmonary and gastrointestinal manifestations and it is found in approximately one in every 2,000 to one in every 3,500 Caucasians born in Europe. In North America and Hawaii it occurs in one in every 8,400 births to Hispanics, one in every 89,000 births to the Asian population of Hawaiian-Americans and the rate among African-Americans is one in every 15,000 births (Rodrigues et al., 2008). CF shows considerable heterogeneity and often there are no clinical signs apparent at birth.

All the body's exocrine glands are affected by CF, resulting in the production of abnormal mucus. Mucus acts in the body to lubricate and protect the lining of several organs; for example, the lungs and stomach. However, due to the thickness and viscosity of the mucus of CF patients, they are subject to bacterial infections. Bronchiolar obstruction and pulmonary infection eventually lead to damage to the lungs (Proesmans et al., 2008).

In addition, the pancreatic ducts in the digestive system are blocked by this viscous mucus, which can lead to hepatic cirrhosis, intestinal obstruction, steatorrhoea, malnutrition, diarrhoea, and meconium ileus. This symptom last is often the first indication of CF. Furthermore, CF can be the cause of growth problems and male infertility, which manifest themselves in late childhood or early adulthood (Proesmans et al., 2008).

1.3.3.3 Treatment of CF

There is currently no cure for cystic fibrosis. The treatment strategy is based primarily on the treatment of lung infections, loosening and removing the thick and sticky mucus from the lungs, by physical therapy, antibiotic medication, and exercise. Chest physiotherapy

(CPT) is an essential part of treatment patient affected with CF. CPT helps to improve ventilation and mucociliary clearance secretions (Fauroux et al., 1999). Exercise is important for CF patients, as it prevents deterioration of the lungs as well as being good for overall physical condition (Wilkes et al., 2009). Many patients with cystic fibrosis are malnourished when diagnosed. Nutritional therapy provides strength and improves growth. The nutritional therapy includes supplements of vitamins A, E, D, and K, oral pancreatic enzymes, a high-salt diet or salt supplements (Sinaasappel et al., 2002). Other medication includes antibiotics, and anti-inflammatories. These medicines may be inhaled or oral. Treatment of end-stage organ disease, such as heart-lung transplant may be an option (Proesmans et al., 2008).

A large study in Wisconsin, USA examined the impact of newborn screening on clinical outcomes in children with CF (Farrell et al., 1997). A total of 650,341 newborn infants were screened for cystic fibrosis by measuring immunoreactive trypsinogen on dried blood spots between April 1985 and June 1991 or, between July 1991 and June 1994, by the trypsinogen test combined with DNA analysis. Cystic fibrosis was diagnosed in 74 of the 325,171 infants in the early-diagnosis group. Nutritional status was evaluated using anthropometric and biochemical methods for up to ten years in 56 of the infants in the early-diagnosis group and in 40 of those in the control group, who had been diagnosed by standard methods. Study result showed that the early-diagnosis group had considerably higher height and weight percentiles and a higher head-circumference percentile at the time of diagnosis. In the follow-up period, the infants in the early-diagnosis group also had appreciably higher anthropometric indexes. It was concluded that newborn screening and early treatment offer the opportunity to prevent malnutrition in infants with cystic fibrosis (Farrell et al., 1997).

1.3.3.4 Gestational Characteristics of CF

CF is a disorder which affects most organs of the body. The main affected parts are the lungs and pancreas but it can also affect the liver, reproductive organs and sweat glands. About 59 % of affected newborn babes and 90 % of those with one year of age have pancreatic insufficiencies which result in poor growth and malnutrition in the first few weeks of the baby's life and into child hood (Bronstein et al., 1992). The function of the placenta is to provide the infant with the nutrients required during pregnancy (Rama and Jagannadha Rao, 2003). These nutrients come from the mother through the umbilical cord. CFTR gene function is an ion channel across the cell membrane, and any alteration on this gene may cause alteration in placental function. Two studies (Faller et al., 1995; Mylona et al., 1996) demonstrate that CFTR is present in the human placental tissue, and it has been suggested that any defect in the function of the CFTR protein in the placenta will affect the baby's health, for instance by premature birth or low birth weight.

It has been shown that there is reduced birth weight in newborns with CF compared with normal healthy babies. This was demonstrated a cohort study carried out in the Florence region of Italy in 2005 by (Festini et al., 2005) over a period of 11 years, through studies of gestational and neonatal characteristics of children with CF.

1.4 NEWBORN SCREENING MARKERS FOR CF

1.4.1 Immuno reactive trypsinogen (IRT)

IRT is a pancreatic product found to be elevated in the majority of babies born with CF (Crossley et al., 1979). IRT is one of the secretory products of the human pancreas. Pancreas acinar cells produce two major isoenzymes of enzymatically inactive trypsinogen which are activated to trypsin upon cleavage of a hexapeptide from the N-

terminus (Paju and Stenman, 2006). Trypsinogen is the major form in the blood and the AutoDELFIA Neonatal IRT Kit only measures the major forms. In CF newborns, the IRT level increase is probably caused by a blockage of the ductile secretions in the pancreas (Crossley et al., 1979).

1.4.2 Pancreatitis Associated Protein (PAP)

It was in 1984 that Keim and his group first discovered the biochemical marker pancreatic associated protein (PAP) in the secretory protein of rat pancreatic juice (Keim et al., 1984). PAP is a 16 kDa protein and in 1992 its human orthologue was identified from the pancreatic juice of diabetic patients who had kidney and pancreas transplantation (Keim et al., 1984). PAP was found to be greatly over-expressed in the pancreas during acute pancreatic disease and to remain expressed for about 3 to 4 days following pancreatitis (Closa et al., 2007).

PAP expression takes place in the pancreatic acinar cells in response to many injuries for example, hypoxia, toxins, diabetes, transplanted tissues, lipopolysaccharides, acute and chronic pancreatitis. The physiological role of PAP in the pancreas and other organs is still a matter of debate. Several studies have shown that PAP has certain physiological activities; for example, anti-inflammatory, anti-apoptotic, anti-bacterial and proliferative effects (Closa et al., 2007). PAP expression can be found in other organs, such as in the primary hepatocarcinomas in the liver; in the uterus, particularly in the luminal epithelial cells; chronic inflammation in the intestine as a result of Crohn's disease; and in the brain tissue of patients with Alzheimer's disease (Closa et al., 2007).

Recent pilot studies in France (Sarles et al., 2005) measuring PAP in addition to IRT, suggest that a combination of IRT and PAP measurements can identify infants for follow-

up testing with improved sensitivity and specificity. Where bloodspot analysis gives both IRT >50 ng/ml and PAP >1.0 ng/ml it is proposed that mutation testing can be avoided and diagnoses confirmed by sweat test alone. These data have been obtained in bloodspot specimens collected at 3 days of age. If this can be confirmed in other studies on specimens taken at 5 days of age (as in UK) and different protocols for combining PAP and IRT are explored, this may offer the potential to reduce the false positive rate associated with CF/DNA screening.

1.4.3 Sweat Tests

A sweat test is a confirmatory test used in newborns suspected of having CF. Babies affected with CF produce larger quantities of sweat chloride than unaffected babies. The method used to measure the sweat in the babies is to put pilocarpine, which is a chemical causing the skin to produce sweat, on a small area of the arm or leg and then place an electrode on the skin at two points. A weak electric current is then applied to help to draw the pilocarpine into the skin where it stimulates the sweat glands to produce sweat. The test area is cleaned and dried and absorbent filter paper which soaks up the sweat is placed on the skin. The filter paper is removed after 30 minutes and is then sent to the laboratory to determine the sodium and chloride concentrations (Hodson et al., 2007).

1.4.4 Faecal Elastase

Human elastase was first described in 1950 (Balo and Banga, 1950) and characterised in 1975 by Mallory and Travis as protease E. (Mallory and Travis, 1975). In the pancreas, human elastase is synthesised by the acinar cells. Human elastase is composed of 240 amino acids with a molecular weight of 26 kd. Human elastase has a special affinity for the carboxyl groups of alanine, valine and lecine (Dominguez-Munoz et al., 1995; Sziegoleit et al., 1989).

Faecal elastase 1 (FE1) concentration level in the pancreatic juices ranges between 170 and 360 μg/ml, representing about 6% of all secreted pancreatic enzymes. FE1 is bound to bile salts without degradation during the intestinal passage. FE1 is concentrated about five-fold to six-fold, as water is reabsorbed, making FE1 easer to measure in the stool (Sziegoleit, 1984). FE1 is stable for a wide range of pH and can be stored for 5 days at room temperature without influencing immunological quantification in the stool (Nandhakumar and Green, 2010).

1.4.5 Prenatal Screening for CF (Couple Screening)

Prior to the Wisconsin study which provided evidence to justify neonatal screening for CF, a number of prenatal screening programmes were started based on the identification of specific DNA mutations in one or both members of a couple. If both were found to be carriers, prenatal diagnosis could be offered. This give couples the choice of avoiding the birth of a child with CF through selective termination of affected foetuses.

There has been considerable debate about the suitability and design of antenatal screening programmes for CF. Some geneticists support the view that CF screening programmes in pregnancy should include the woman's partner if she gives a positive carrier result in the DNA test. In contrast, other geneticists suggest that screening programmes for CF during pregnancy will place an unacceptable burden of decision-making on the couples concerned.

There are two types of antenatal screening programme; the first is a two-step screening programme which tests women first, and if a woman is found to be a positive carrier for CF her partner will be also tested for CF alleles. The second model of testing is the

"couple" model. In this model both partners should agree to participate before laboratory analysis starts (Wald and Leck, 2000).

A prenatal screening programme has been offered in the Edinburgh Maternity Hospital and is a good example of a two-step screening model (Mennie et al., 1992). In this model information about the CF disorder and prenatal screening is sent to the pregnant women in a specially designed leaflet, along with the antenatal clinic appointment. The test is based on extraction of DNA from a buccal swab followed by mutation analysis for a panel of the most common mutations.

If the pregnant woman is found to be a positive carrier for a CF allele, her partner is also tested for CF alleles. If he is also a carrier, they will have 1 chance in 4 of having a child affected with CF disease. Where both are carriers prenatal diagnosis is offered to the pregnant woman to discover the status of the foetus. If the foetus is affected a couple may decide to terminate the pregnancy. If she is a carrier and her partner is negative, no further action will be taken.

The two-step model does have certain disadvantages, such as the anxiety evoked for a carrier partner, while they wait for the result of the status of the other, to determine whether they are both carriers or not. The second problem is that the appointment at the antenatal clinic may well be too late to allow the required action to be taken in the first trimester of pregnancy. To solve this dilemma, it has been suggested that a heterozygote test should be offered to women at the time when they have a positive pregnancy test (Mennie et al., 1992).Significant cost would be incurred using this approach in the whole pregnant population.

Time is very important in the antenatal screening programme and the test must be conducted early enough to give the couples sufficient time to think about taking the decision to terminate the foetus or not. Screening during pregnancy can be argued to be the most effective process for CF screening programmes as it avoids the birth of an affected child. On the other hand, early detection of CF through newborn screening and the introduction of well designed treatment regimes can reduce the burden of CF disease in affected children.

1.5 GESTATIONAL MARKERS OF CF

Antenatal screening programmes and newborn screening programmes both have the same goal, which is to identify individuals at risk of diseases. Antenatal screening aims to provide couples with reproductive choice, e.g. termination of the foetus if the mother is found to be pregnant with an untreatable disorder. Hence, prenatal diagnosis for CF can be offered to discover whether the foetus is affected or not. While this can be carried out by offering carrier screening by mutation analysis to parents and the analysis of CVS from at risk pregnancies, it would be of interest to study the effect of CF on the physiology of pregnancy. This latter approach may provide a non-invasive method of assigning the risk of CF in the foetus.

Several maternal serum markers have been shown to be linked to a variety of adverse outcomes in pregnancy. Among these are alpha-fetoprotein (AFP), pregnancy-associated plasma protein (PAPP-A), Human chronic gonadtrophin (hCG), Free βhCG, Unconjugated estriol (UE3) and Inhibin-A. Serum concentrations of these markers show various changes in the case of low birth weight, early delivery, pre-eclampsia, spontaneous preterm birth, and still birth (Muttukrishna et al., 1997; Smith et al., 2002; Smith et al., 2004; Smith, et al. 2006). It is not known if any of these markers which are

synthesised or transported by the placenta may show changes in pregnancies in which the foetus is affected by CF.

1.5.1 Alpha-fetoprotein (AFP)

AFP is synthesised by the embryonic yolk sac cells and subsequently by the foetal liver (Tomasi, 1977). Several studies have suggested that AFP is synthesised by villous tissue in the first trimester of pregnancy (Lafuste et al., 2002)

AFP levels in the foetus increase up to 13 weeks of gestation to reach a maximum level and then decrease to term. During the second trimester of pregnancy, the AFP level present in amniotic fluid is about 1% of the foetal serum levels, and in maternal serum, AFP levels increase to a peak at 32 weeks of gestation (reviewed by Newby et al., 2005).

The function of the placenta is to provide the infant with the nutrients required during pregnancy. AFP reaches the maternal circulation from the foetal circulation across the placenta and from the amniotic fluid across the foetal membranes and decidua (review by Newby, et al., 2005). CFTR gene function is an ion channel across the cell membrane (Welsh and Smith, 1993), and any alteration on this gene may lead to changes in the tissues that produce mucus, sweat, saliva and tears. Moreover, it has been proposed that any defect in the function of the CFTR protein in the placenta may have an effect on the health of the infant; for example, by premature birth or low birth weight. However, the physiological mechanism of AFP interchange between foetus and maternal circulation remains unclear and it is not known if maternal serum AFP levels are affected when the foetus has CF.

Any variation in AFP from the normal concentration level in the maternal circulation during pregnancy indicates a complication in the pregnancy; for example, AFP

concentration levels increase in amniotic fluid and maternal serum with foetal malformation, in particular neural tube defects (Ryynänen, et al., 1983). In the second trimester, the maternal serum level of AFP concentration is decreased in pregnancies affected by foetal Down syndrome (Cuckle et al., 1984; Merkatz et al., 1984; Tabor et al., 1984).

1.5.2 Human Chorionic Gonadotrophin (HCG)

HCG is member of the glycoprotein hormone (GPH) family that also includes leutinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). GPHs are heterodimers consisting of an α-subunit and a β-subunit which are non-covalently bound to each other. The α-subunit is nearly identical to other glycoprotein hormones such as TSH, FSH and LH.

HCG has a molecular weight of 36,000 – 46,000 Da. hCG is synthesised in the syncytiotrophoblast cells (Albertini et al., 1982). HCG is found in various tissues of human body; for instance, the uterus, lungs, pancreas, pituitary glands, gonads, placenta and foetal membranes (Braunsteins et al., 1979).

The corpus luteum plays an important role in the production of oestrogen and progesterone. The function of hCG is to maintain the progesterone production of the corpus luteum during early pregnancy, a function it shares with LH. In addition, its other functions include stimulating steroidogenesis in the placenta (Keay, et al., 2004).

In early pregnancy, to determine whether the women is pregnant or not, hCG is used as a chemical marker in pregnancy tests (Hussa, 1982). At 6-8 days after conception, hCG will appear in the serum and reach a peak level 9-10 weeks after the last menstrual period.

In the second trimester, increased hCG levels in maternal serum levels have been found to be linked with foetal Down syndrome (Bogart et al., 1987) but it is not known if maternal serum hCG levels are altered when the foetus has CF.

1.5.3 Pregnancy-Associated Plasma Protein A (PAPP-A)

Pregnancy-Associated Plasma Protein A (PAPP-A) is secreted from the trophoblastic tissue of placenta. It is a glycoprotein with a structural gene located in chromosome 9. During a normal pregnancy, PAPP-A increases rapidly in the first trimester, and then increases at a lower rate in the second trimester. However, in pregnancies affected by Down syndrome, PAPP-A concentration is decreased and this effect in more marked in the first trimester than in the second trimester (Fialova and Malbohan, 2002) .

PAPP-A is used in the Down syndrome screening protocol as an additional marker for the identification of pregnancies at high risk of the condition. In addition, PAPP-A has been found to decrease on average in pregnancies affected with Trisomy 18, acute coronary syndromes and Cornelia de Lange syndrome (Fialova and Malbohan, 2002) and it is not known if maternal serum PAPP-A levels are affected when the foetus has CF.

1.5.4 Free Beta Human Chorionic Gonadotrophin (FβHCG).

If the foetus is affected with Down syndrome, the FβhCG level is found to be elevated in 1st and 2nd trimesters of pregnancy (Cuckle, 1996; Wald, et al., 1996). FβhCG with other biochemical markers such as AFP and uE3 with maternal age are effective methods in prenatal screening for Down syndrome in the second trimester (Cuckle, 1996). This marker can also be found to be elevated during the first trimester of pregnancies where a foetus is affected with Down syndrome (Macri, et al., 1993). It is not known if maternal serum FβhCG level are affected when the foetus has CF.

1.5.5 Unconjugated Estriol (UE3)

Unconjugated estriol (UE3) is a steroid hormone synthesised by the placenta from the foetal precursor molecule, 16 alpha-hydroxy-dehydroepiandrosteronesulphate (16 α-OH DHEAS). UE3 concentration has been shown to be lower in pregnant women with a Down syndrome foetus (Jorgensen and Trolle, 1972). It is not known if the UE3 level of maternal serum is affected when the foetus has CF.

1.5.6 Inhibin-A

Inhibin is glycoprotein produced by gonads and placental tissues. Inhibins are divided into two subunits, α and β. The β subunit is divided into form βA and βB, to form Inhibin-A or Inhibin-B (Aitken, et al., 1996). Inhibin-A concentration level increases in the second trimester and shows elevation in DS pregnancies (Van Lith et al., 1992). Inhibin A levels have been reported as elevated in pregnancies with pre-eclampsia (Cuckle et al., 1998). It is not known if maternal serum Inhibin-A level are affected when the foetus has CF.

Data on the above markers are available for a proportion of pregnant women in the west of Scotland who opt for prenatal screening for Down syndrome and neural tube defects through the regional prenatal screening programme. In this study, the outcome of these pregnancies will be audited to identify any case of cystic fibrosis and to make a comparison of the level of each marker in the CF and unaffected pregnancies.

1.6 NEWBORN SCREENING PROTOCOLS FOR CF

Currently, various protocols are used in CF screening programmes, with each having its advantages and disadvantages.

1.6.1 IRT/IRT Protocol

These programmes use the IRT marker as a primary test. If it is found to be elevated e.g ≥ 99th centile of the normal range in dried blood spots, confirmation of this elevation will be obtained by repetition of the test on a second dried blood sample. If both samples show elevated IRT, a sweat test is used to confirm a diagnosis.

The advantages to using the IRT/IRT protocol without using a DNA test are that its specificity and sensitivity are good in the second sample test and carriers are not detected. However, its disadvantages are poor specificity in the first sample test leading to a relatively high number of repeated blood spot samples, which leads to increasing the anxiety of the baby's family, and a high sweat test rate for definitive diagnosis (Wilcken, 2007).

1.6.2 IRT/DNA/IRT Protocol

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene was identified in 1989 (Riordan et al., 1989), and since then many mutations have been identified contributing to the diagnosis of the CF. Delta F508 is the most common mutation for CF in the Northern European Caucasian population and is included in all mutation panels used in the diagnosis of CF.

This protocol is used in several countries around world. First, an IRT sample test is carried out and if elevated (i.e. above a chosen cut off e.g. 60 ng/ml), a repeat IRT on the same card is carried out in duplicate. If the mean of all 3 tests shows a high level of the marker (≥ 99.5th centile), a further blood spot sample is used for DNA analysis to test for the common mutation ΔF508, as well as for other selected CF mutations, depending on the

ethnic mix of the population. The DNA test is performed with the same dried blood spots. If two mutations are identified, the baby is diagnosed as having CF, while if only one mutation is identified, the baby either has CF due to the presence of a second unidentified mutation (which can be revealed by sequencing) or may be a carrier for the disease. A further bloodspot sample is taken at around 21-30 days of age where there is an initial raised IRT and one mutation. Depending on the result an extended mutation test panel and / or a sweat test are used to provide a definitive diagnosis.

The advantages of using IRT/DNA/IRT compared with IRT/IRT are increased positive predictive value (PPV), a reduction in the number of sweat test analyses and good sensitivity and specificity. The disadvantages of this strategy are the detection of CF carriers, and missing CF in certain ethnic groups. CF carrier detection poses significant reporting problems and may lead to genetic counselling and cascade testing in families (Wilcken, 2007).

1.6.3 IRT / PAP Protocol

Various studies have indicated that PAP concentration level is elevated in the blood of newborn babies affected with CF. Sarles and his group (Sarles et al., 2005) investigated the use of a combination of PAP and IRT as a screening protocol in newborn babies in France. Over 200,000 babies in five regions in France were screened for CF using the PAP test in parallel with the routine IRT test. Using two different combinations of PAP and IRT cut off values, they showed that the addition of PAP measurement in dried blood spots for screening newborn babies for CF gave the same performances as the IRT / DNA protocol but without the need for DNA mutation analysis. The protocol advantage is the reducing workload and cost associated with avoiding of DNA mutation analysis and consequently no detection of CF carriers. The disadvantage of this approach is that PAP

testing is carried out in all newborn babies in addition to IRT. This significantly raises the cost of the primary screening test which is unlikely to be offset by the reduction in DNA mutation testing.

1.7 AIMS

Programmes with the purpose of screening for and detecting particular conditions in unborn children and neonates are not a recent innovation. Nonetheless, the tests for certain conditions are not sufficiently sensitive or specific and their predictive value is low. For instance, it is possible for bloodspot screening for cystic fibrosis may fail to identify affected infants or may identify carriers, who are not the intended target of screening.

1. Newborn Bloodspot Screening

Research Question

Is the sensitivity and specificity of blood spot screening for CF improved when an additional screening marker, PAP, is used in combination with an existing marker (IRT)?

Specifically:

1. Can the rate of detection of affected infants be maintained or improved?
2. Can the rate of carrier detection be reduced by the addition of PAP measurements?
3. Can the requirement for DNA analysis for confirmation of a diagnosis of CF be reduced by the addition of PAP measurements?

2. Prenatal Maternal Serum Screening

Research Question: Does the measurement of a range of maternal serum markers in samples from women carrying a child with CF have any predictive value for the birth of an affected child?

Specifically:

1. Are there any concentration changes in the maternal serum markers AFP, hCG, PAPP-A, Free β-hCG, UE3 or Inhibin-A which may be predictive of a CF pregnancy?

2. Are there any other gestational characteristics (low birth weight and prematurity) which are associated with CF pregnancies?

CHAPTER 2 :

MATERIALS

2.1 Subjects

Two types of samples were used in the study. First, dried blood spot samples were examined from babies with cystic fibrosis (CF) to determine whether pancreatitis-associated protein (PAP) in combination with immuno-reactive trypsinogen (IRT) can improve the sensitivity and specificity of CF screening and reduce the amount of DNA testing required to confirm a diagnosis of CF.

Second, maternal serum samples from mothers of children affected with CF were studied for variations in the concentration of the range of serum markers known to be associated with adverse outcomes of pregnancy in order to determine whether they have any predicative value for CF.

2.2 Dried Blood Sample

The Scottish Neonatal Screening Programme started in 1965. Around sixty thousand dried blood spots (on Guthrie cards) are received annually from all regions of Scotland by mail. The programme aims to test newborn babies for phenylketonuria (PKU), congenital hypothyroidism (CHT) and cystic fibrosis (CF) in the early stage of the baby's life to allow the affected infants to be treated appropriately. In October 2010, screening for SCD and MCADD was added. Many types of filter paper cards are used for newborn screening; for example, Schleicher & Schuell # 903, Whatman BFC180, and Toyo Roshi 545. The filter paper type used in the Scottish programme is the SNS 903.The design of the card is shown in Figures 2.1 and 2.2. The blood spot card includes basic details of the baby on the front and on the reverse of the card (table 2-1).

Table 2-1: Demographic information requested on the dried blood spot card

Baby's information	Baby's surname	
	Baby's first name	
	Mother's or baby's alternative surname (not maiden)	
	Address	
	Post Code	
	Place of Birth	
	Baby's date of birth	
	Gender	
	Gest. Age at Birth	
	Birth weight	
	Ancestry	
G.P.'s information	G.P.'s Name	
	G.P.'s Address	
	G.P.'s Practice Code	
Mother's information	Mother born in UK	
	Mother's date of birth	
	Date of first full milk feed	
	Type of feed at blood spot collection	Breast Milk Formula Other
Specimen information	Date Specimen Taken	
	Specimen taken by	
	Contact Telephone Number	
Other information	Is the baby in	Hospital Premature On Antibiotics
	Has baby had a	Blood transfusion; if yes, date of last transfusion Drug therapy
	Comments, e.g.	Family history

Five circles are presented on the absorbent part of the card to receive the blood spots. The heel is punctured with a lancet or similar device and the blood allowed to flow within the area of each circle. Layering should be avoided. The spots are allowed to dry and can then be safely sent to the laboratory by post. The standard method for collecting the blood spots is summarised on the reverse of the card and include the following instructions:

Baby's heel needs to be warm.

Clean and dry, then squeeze skin taut

Puncture firmly in area shown, wait for drop to form, wipe away first drop and then obtain the second large hanging drop, collect one drop onto centre of circle and for the other remaining circles.

Allow the card to air dry before placing in glassine bag and then immediately post in pre-paid envelope.

On the back of the card the baby's ancestry code is described in (Table 2.2).

Table 2-2: Baby's ancestry code description

Ancestry	Code	Description
White	A	British/Irish
	C	Any other White background
Mixed	D	White and Black Caribbean
	E	White and Black African
	F	White and Asian
	G	Any other mixed background
Asian	H	Indian
	J	Pakistani
	K	Bangladeshi
	L	Any other Asian background
Black	M	Caribbean
	N	African
	P	Any other Black background
Other	R	Chinese
	S	Any other ethnic category
	Z	Not stated

Blood spots are generally collected on day five (ideally within a range of 5-8 days) of the baby's life. The demographic information required on the card should be completed at the time of collection. Following analysis, all negative screening results and any repeat specimens are required to be notified to the Department of Child Health for the area where the child was born, within 48 hours. Positive screening results are reported by telephone to the appropriate paediatrician /child health department within 24 hours.

CF screening in Scotland based on the measurement of IRT is carried out in the Newborn Screening Laboratory in the Biochemical Genetics Department, Yorkhill Hospital, and Glasgow. For this study, blood spots for pancreatitis associated protein (PAP) evaluation were obtained by punching an extra dried blood spot from all samples showing an elevated IRT level (i.e. $\geq$ 70 ng /ml). In addition, 4 dried blood spots received before, and another 4 dried blood samples received after each card with an increased IRT were used as controls. Moreover, extra dried blood samples were taken from 189 babies born with low birth weight and from 182 babies born prematurely (< 37 weeks of gestation).

The sample discs of 3.2 mm in diameter are punched out using a semi-automated punching machine (multi-puncher, PerkinElmer). (Figure 2.3). When the cards are punched, it is important to select cards with both sides of the spots filled with blood to ensure accuracy of the analysis. All the punched dried blood spots were then collected in Eppendorf tubes and stored in a freezer at minus 20°C, pending analysis.

A review of the available blood spots with elevated IRT showed that there were 314 available, along with 2886 controls, plus 189 from premature and 182 from low birth weight babies. There were 29 identified CF cases and 32 CF carriers. Therefore, the total study samples were 3632 blood spots (Table 2.3).

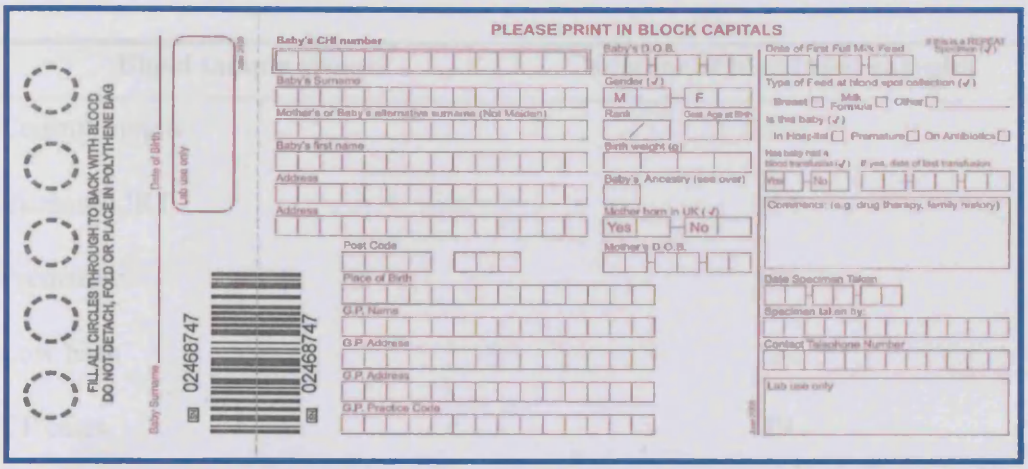

Figure 2-1: The front of the blood spot card used in the Scottish screening programme.

Figure 2-2: The reverse of the blood spot card used in the Scottish screening programme

Figure 2-3: Semi-automated bloodspot multi-puncher used in the Scottish screening programme.

Table 2-3: Summary of the bloodspot specimens used in the study.

Blood sample groups	Number of blood spot samples
Control samples	2886
Increased IRT	314
Premature	189
Low birth	182
CF cases	29
CF carriers	32
Total	**3632**

2.3 Retrospective IRT Study for CF Affected Babies

All 198 babies affected with CF identified by the routine newborn screening programme in Scotland between 2003 and December 2010 were reviewed. Homozygosity for the common delta F508 mutation was found in 88, with another 110 having various other combinations of mutations. All cases were gathered from the records of the Biochemical Genetics Department. The IRT concentration levels were identified for each of the babies with CF. The IRT median for the cases with common delta F508 mutation and that for the cases with one of the various other mutations were analysed using the SPSS statistics programme.

2.4 Retrospective IRT Study for CF Carriers

The IRT concentration levels were identified for all CF carrier babies born during the period 2003 December 2010. From the total number of these babies, 148 babies with the common delta F508 mutation and another 82 with other mutations were identified, making 230 babies in all with either delta F508 or one of several other mutations. The data were

obtained from the Biochemical Genetics Department records. The SPSS statistics programme was used to analyse the IRT medians for the common delta F508 cases and the cases with different mutations.

2.5 Maternal Serum

The Duncan Guthrie Institute of Medical Genetics, Biochemical Genetics Department, at Yorkhill Hospital provides a prenatal screening service for Down syndrome (DS) and neural tube defects (NTD) for pregnant women in the west of Scotland. The screening test is based on the measurements of several maternal serum markers and the aim is to give an estimate of the likelihood that a foetus will be affected with Down syndrome (DS) or neural tube defect (NTD).

Blood samples (5-10mls) were collected at the antenatal clinic during the first (11-13 weeks) or second trimester (15-20 weeks), for prenatal screening. All samples were sent to the Biochemical Genetics department for the measurement of alpha-fetoprotein (AFP), human chronic gonadotrophin (hCG), free β-human chorionic gonadotropin (β-hCG), pregnancy-associated plasma protein A (PAPP-A), unconjugated oestriol UE3, and inhibin-A as appropriate.

Collected blood samples received at the Biochemical Genetics department were labelled with a laboratory number and aliquot of serum was separated by centrifugation and used for analysis. Samples were assigned different coloured forms. Yellow forms for second trimester hCG,AFP screening for DS, green forms for 1st trimester Free β-hCG and PAPP-A screening for DS and white forms for 2nd trimester AFP screening for NTD. The remainder of the serum, about 1-3 mls, was stored in numbered tubes in a freezer at -20 °C

and details of each sample were entered in a computerised data base (Lifecycle Laboratory Information Management System).

To identify the blood samples for the study, a series of pregnant women who had given birth to a baby affected with CF were identified by cross-checks with the prenatal screening database, which contains information on the mother's name, address, and date of birth. Another database was constructed for affected babies, identified by cross-checks with the newborn screening database, which contains information on the baby's name, address and date of birth, as well as the mother's name and date of birth. This was followed by matching all the information of mothers and their CF-affected babies and transferring all the matched information to a single database containing all the basic information on the mothers and their affected babies.

A study group of 55 women with CF pregnancies was found and the corresponding serum samples recovered from frozen storage. An additional 330 pregnancies, both second trimester (15-20 weeks) and first trimester cases (10-13 weeks) were randomly selected as controls. All the samples together were transferred and arranged in a special tray and kept at minus 20 °C until analysis (table 2.4).

A number of samples of both first and second trimester cases were rejected for various reasons, including there being an insufficient volume of blood to give an adequate volume for assay (<0.4ml serum) or no identification on the sample tube, or haemolysed sample.

At the time of analysis, serum samples were recovered from frozen storage, allowed to thaw at room temperature, and vortex mixed. The samples were then labelled with computer-generated lab numbers to identify each sample, together with matching computer-generated bar codes on the aliquot for assay.

Table 2-4: Number of controls and CF samples available at each gestation for each marker

	Markers	WEEK												TOTAL
		9	10	11	12	13	14	15	16	17	18	19	20	
Control samples	AFP	1	0	4	24	8	1	121	117	30	12	8	4	
	HCG	1	0	4	24	8	1	121	117	30	12	8	4	
	B-hCG	1	0	4	24	8	1	121	117	30	12	8	4	
	PAPP-A	1	0	4	24	8	1	121	117	30	12	8	4	**330**
	UE3	1	0	4	24	8	1	121	117	30	12	8	4	
	Inihibn-A	1	0	4	24	8	1	121	117	30	12	8	4	
CF SAMPLES	AFP	0	0	3	2	1	0	21	19	4	3	1	1	
	HCG	0	0	3	2	1	0	21	19	4	3	1	1	
	B-hCG	0	0	3	2	1	0	21	19	4	3	1	1	
	PAPP-A	0	0	3	2	1	0	21	19	4	3	1	1	**55**
	UE3	0	0	3	2	1	0	21	19	4	3	1	1	
	Inihibn-A	0	0	3	2	1	0	21	19	4	3	1	1	

CHAPTER 3 : METHODS

3.1 NEWBORN MARKER MEASUREMENTS

The current routine newborn screening test for CF is based on the measurement of immunoreactive trypsinogen (IRT) using the Delfia fluoroimmunoassay method (Perkin Elmer, Finland). In this study pancreatitis-associated protein (PAP) was also measured using a commercially-available enzyme linked immunoassay (Dynabio SA, Marseilles, France). The results from tandem mass spectrometry (MS/MS) testing for PKU in neonates measuring phenylalanine (PHE) and tyrosine (TYR) were also available.

3.1.1 Measurement of Immunoreactive Trypsinogen (IRT)

IRT is a pancreatic product found to be elevated in the majority of babies affected with CF. Where the initial IRT level is $\geq$ 60 ng /ml, the test will be repeated within the laboratory in duplicate using blood from the same card and if the mean of these 3 results is less than 70ng /ml, CF is not suspected. Faecal contamination may be suspected when there is large variation between the 3 individual IRT levels. When results of 70 ng /ml or above are found, the dried blood sample is sent to the Molecular Genetics lab for CFTR mutation analyses. During the course of this study, a DNA diagnostic kit containing a panel of 30 of the most frequent mutations was used (CV-kit V.3, Tepnel Diagnostics).

In the DNA lab, if two mutations are found, CF is diagnosed and a telephone call is made to the nominated CF physician and a letter sent to Child Health Department, the CF physician, G.P. and hospital. If no mutation is detected and the baby is of Caucasian ethnicity, the report will be that CF is not suspected, but if the child is from a non-Caucasian ethnic group, there will be a request for a repeat of the sample at day 27 of the baby's life.

When only one mutation is detected, this indicates a probable carrier but it is possible that it may be a case of CF as another rare (untested) mutation might be present. The routine

procedure is to request another sample to be taken at day 27 of the baby's life. If the result is 60ng/ml or above the report will be that there is a high risk of CF, and a sweat test and genetic counselling will be required. If the result is less than 60ng/ml, the report will be that there is one mutation carrier and that genetic counselling should be offered (Figure 3.1).

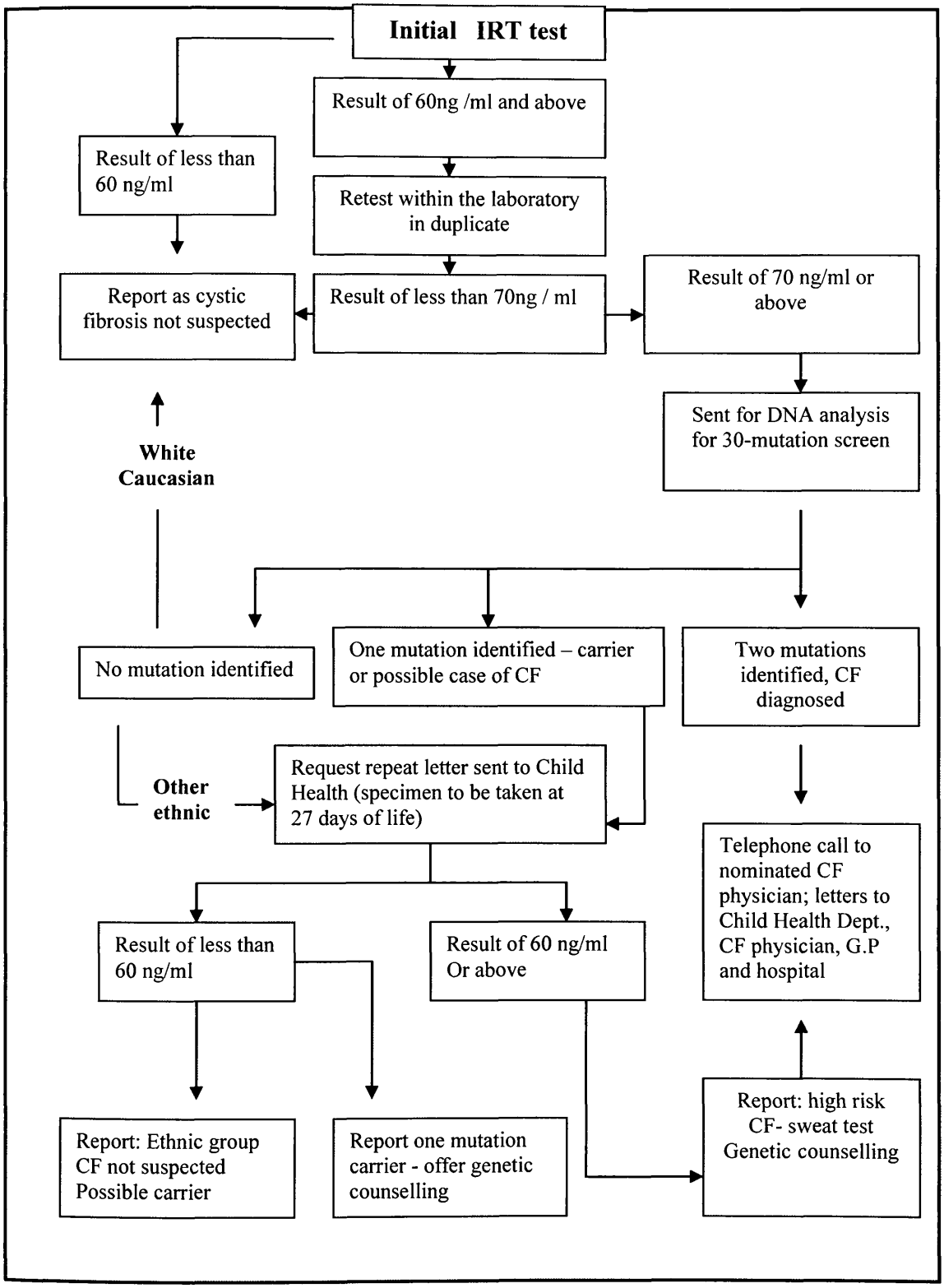

Figure 3-1: Flowchart explaining the CF protocol for Scottish newborn babies.

3.1.2 Principle of IRT assay

The AutoDELFIA fluoroimmunometric assay system was used to measure IRT in newborn samples. This system is based on a direct sandwich technique which utilises two monoclonal antibodies directed against different epitopes on the IRT molecule. The first anti-IRT monoclonal is coated on to the wells of microtitre plates. Blood spots are punched into each well and the IRT in the sample binds to the monoclonal antibodies and is immobilised. Europium (Eu) labelled anti-IRT IgG is then added to the reaction, which binds to a different epitopic site on the IRT molecule. After incubation, this is washed to remove unbound Eu-labeled IRT IgG and excess sample eluate. The addition of an enhancement solution, which is an acidic chelating detergent, dissociates Europium ions from labelled antibodies into a solution where they form fluorescent chelates with components of the enhancement solution. The fluorescence is proportional to the quantity of IRT in the sample (Figure 3.2). The analysis is semi-automated using the AutoDELFIA immunoassay analyser.

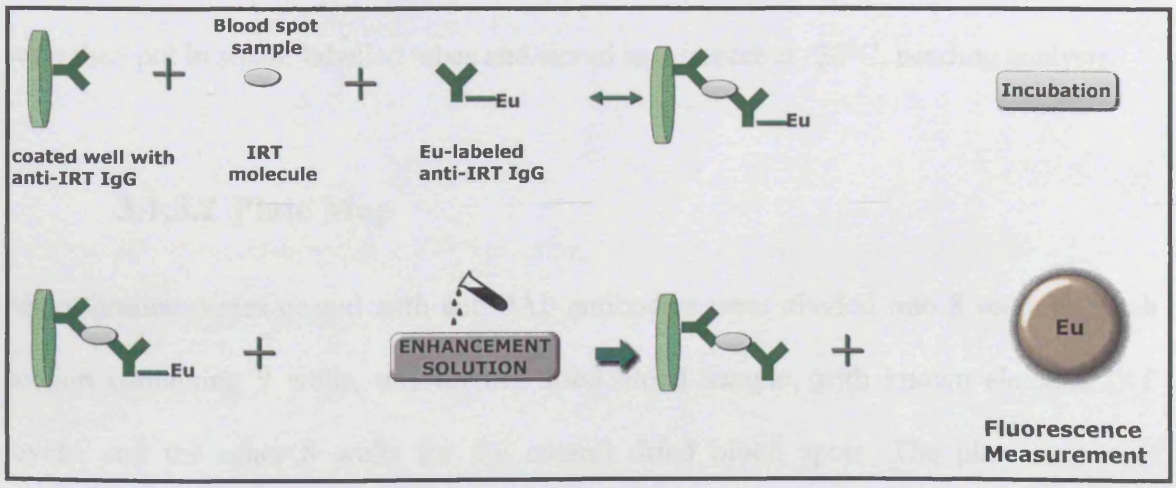

Figure 3-2: AutoDELFIA IRT principle of the assay.

3.1.3 Measurement of Pancreatitis-Associated Protein (PAP)

3.1.3.1 Blood Spot Samples

Over the course of the study, PAP was measured prospectively in dried blood spots from a total of 3632 samples. There were 2886 infants with normal IRT (<70 ng/ml) and 314 infants with elevated IRT (≥70 ng/ml). In addition, samples from low birth weight babies (< 2,500g) (total 189), samples from premature babies (< 37 weeks gestation) (total 182), samples from confirmed CF cases (total 29), and samples from CF carriers (total 32) were tested. In addition there was one bloodspot sample from a child with a confirmed diagnosis of CF which had a low level of IRT (20 ng/ml) and had therefore been missed by screening. Four dried blood spots received before and another four dried blood samples received after each spot with an increase in IRT were used as controls (total 2886).

The sample discs of 3.2 mm in diameter were punched out using a semi-automated punching machine (multi-puncher, PerkinElmer). The punched spots were selected from cards with both sides of the spots filled with blood. All the punched dried blood spots were then put in small, labelled tubes and stored in a freezer at -20°C, pending analysis.

3.1.3.2 Plate Map

Microtitration plates coated with anti-PAP antibodies were divided into 8 sections, each section containing 9 wells, one for the dried blood sample, with known elevated IRT levels, and the other 8 wells for the control dried blood spots. The plate map also contained two wells, one for a sample from a baby already known to have low birth weight and another for a premature baby (Figure 3.3).

Seventy-four samples were run in each assay, 8 of which were samples known to have elevated IRT, with the remaining 66 being matched as control samples. In the plate, 12

wells were used for the standard solutions, 8 wells for quality control sera and 2 wells for the blanks.

3.1.3.3 PAP Assay

An enzyme-linked immunoassay (ELISA sandwich) was used for the analysis of the dried blood samples using the MucoPAP kit, (manufactured by DYNABIO France). Punched dried blood spots from labelled tubes were placed in the wells of 96-well U-bottom plates according to the plate map, to which 150 ul of PBS buffer is added.

Sixteen hours were then allowed for the elution of the blood spots at 4°C, and then the standard prepared from freeze-dried recombinant PAP (rhPAP), was reconstituted with 487µl volume H_2O to obtain a solution of 4 ng/ml. The standard solution concentrations used to construct the standard curve were: 0.0075, 0.015, 0.03, 0.06, 0.125 and 0.25 ng/ml (Figure 3.4).

The control serum was reconstituted with 800 µl H_2O to obtain a solution of 2.4ng/ml. The high value was 1.2ng/ml and the low value control was 0.15ng/ml. Using an electronic multichannel pipette, 100 µl of the samples were deposited in the 96-well microtitration plate coated with anti-PAP antibodies (100µl/well), followed by 3h incubation at room temperature. After washing, the biotinylated antibody (100µl/well) was distributed, and then incubated for 30 min at room temperature . After 4 washes, Avidin-POD (100µl/well) was allocated, and then incubated for 15 min at room temperature, followed by distribution of the substrate-chromogen after washes and incubation of the plate for 10-15 min in the dark. The reaction was then stopped with H_2SO_4 (100µl/well). The absorbance of the wells was read by a spectrophotometric plate reader at 450nm with a 630 nm filter used as a reference (figure 3.5). PAP concentrations in eluates must be multiplied by a factor of 65 to obtain PAP blood concentrations. The factor 65 was

calculated because the 3.2 mm punched card corresponds to 3.1µl of blood with the dilution being 3.1/200=1/65.

Blank	Blank	CON	CON	33 B2 19	34 B3 27	49 B4 35	50 B5 43	**65** **L** 51	66 B6 59	81 B7 67	82 B8 73
STD	STD	19 B1 5	20 B1 12	35 B2 20	36 **R3** 28	51 B4 36	52 B5 44	67 B6 52	68 B6 60	83 B7 68	84 **R8** 74
STD	STD	21 B1 6	22 B1 13	37 B2 21	38 B3 29	53 **R4** 37	54 B5 45	69 B6 53	70 B7 61	85 B7 69	86 B8 75
STD	STD	23 B1 7	24 B2 14	39 B2 22	40 B3 30	55 B4 38	56 **R5** 46	71 B6 54	72 B7 62	87 B8 70	88 B8 76
STD	STD	25 B1 8	26 B2 15	**41** **P** 23	42 B3 31	57 B4 39	58 B5 47	73 B6 55	74 B7 63	89 B8 71	90 B8 77
STD	STD	27 **R1** 9	28 B2 16	43 B3 24	44 B3 32	59 B4 40	60 B5 48	75 **R6** 56	76 B7 64	91 B8 72	92 B8 78
STD	STD	29 B1 10	30 B2 17	45 B3 25	46 B4 33	61 B4 41	62 B5 49	77 B6 57	78 **R7** 65	CON	CON
CON	CON	31 B1 11	32 **R2** 18	47 B3 26	48 B4 34	63 B5 42	64 B5 50	79 B6 58	80 B7 66	CON	CON

Figure 3-3: Plate protocol for blood spot samples. Wells 1-2 are blanks; 3-14 are standards, 15, 16, 17,18, 93, 94, 95, 96 are controls. Wells R1-R8 contain blood spots from babies with elevated IRT plus matched controls, P contain premature blood spot sample and L is low birth weight sample.

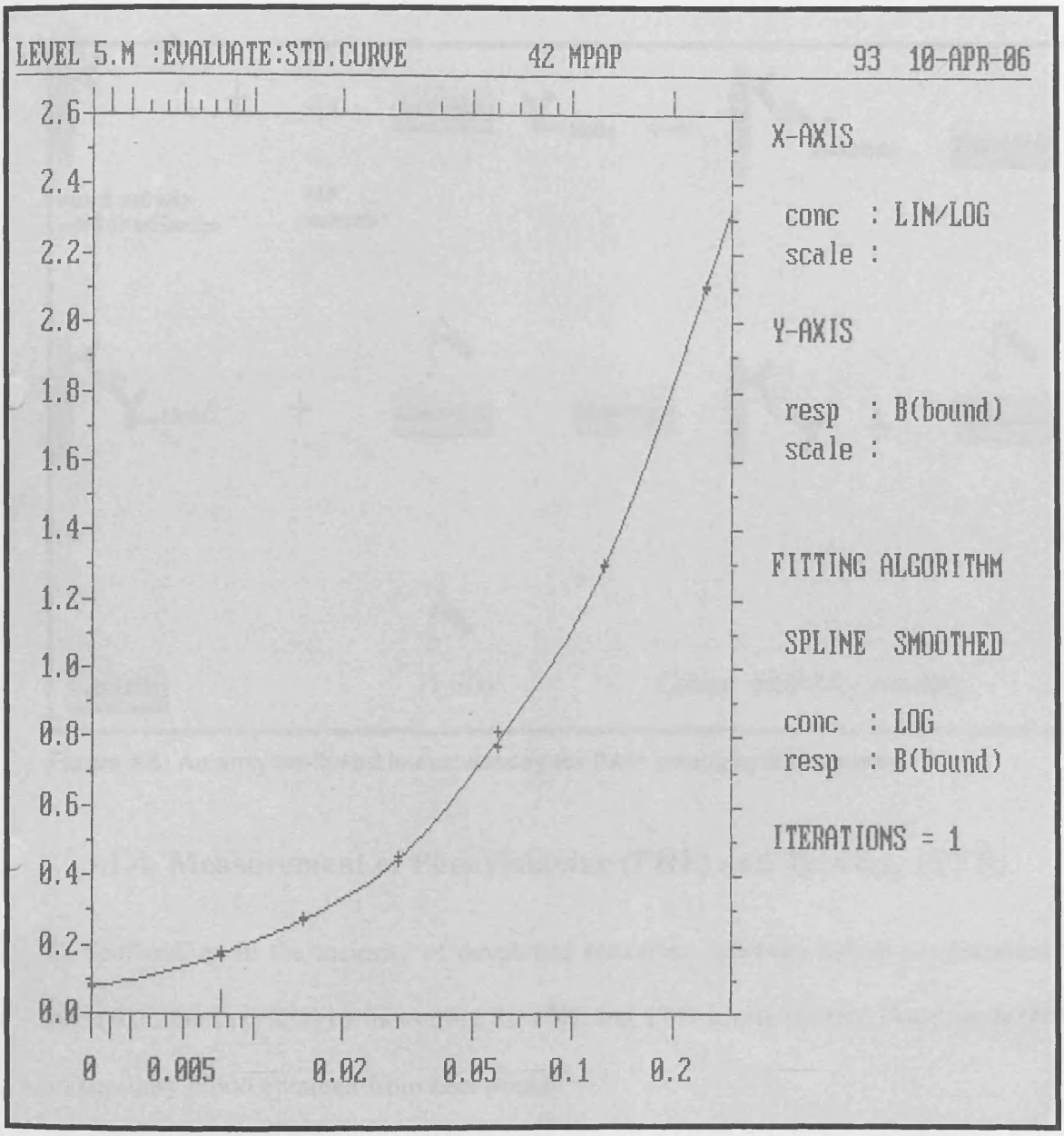

Figure 3-4: Example of PAP experiment standard curve.

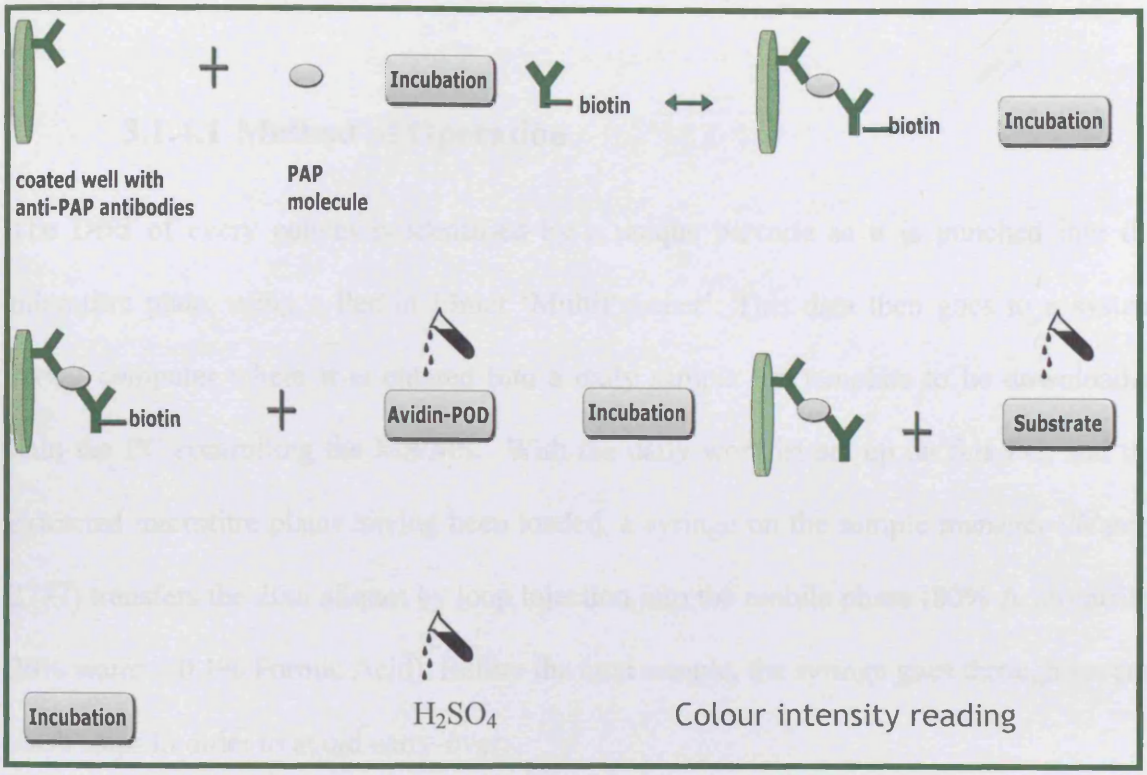

Figure 3-5: An enzyme-linked immunoassay for PAP: principle of the assay.

3.1.4 Measurement of Phenylalanine (PHE) and Tyrosine (TYR)

In Scotland, as in the majority of developed countries, newborn babies are screened for phenylketonuria (PKU) by measuring the PHE and TYR levels in dried blood spots (DBS) of capillary blood obtained from heel pricks.

Testing for PHE and TYR levels is conducted by a simplified form of liquid chromatography tandem mass spectrometry (LC MS/MS). DBS from patients are punched into 96-well microtitre plates with a semi-automated 'multi-puncher'. The DBS are then eluted on a plate shaker for 30 minutes, using methanol which also contains a known concentration of internal standards for both PHE and TYR. These internal standards are isotopomers, which vary in mass from the native species by several Daltons (Da).

A regulated flow of mobile phase is provided by a liquid chromatography (LC) pump. An automated sample manager injects a 20ul aliquot of eluent from one of the microtitre plate wells into the mobile phase, which then 'carries' this aliquot into the MS/MS for analysis.

3.1.4.1 Method of Operation

The DBS of every patient is identified by a unique barcode as it is punched into the microtitre plate, using a Perkin Elmer 'MultiPuncher'. This data then goes to a system server computer where it is entered into a daily sample list template to be downloaded onto the PC controlling the MS/MS. With the daily worklist set up on this PC, and the extracted microtitre plates having been loaded, a syringe on the sample manager (Waters 2777) transfers the 20ul aliquot by loop injection into the mobile phase (80% Acetonitrile: 20% water + 0.1% Formic Acid). Before the next sample, the syringe goes through several wash steps in order to avoid carry-over.

The MS/MS (Waters Micromass QuattroMicro API tandem mass spectrometer) has five separate components (see also Figure 3.6):

1. The ESI and ion source
2. MS1: the first quadrupole analyser
3. Collision cell
4. MS2: the second quadrupole analyser
5. The ion detector

The mobile phase, with the patient sample, enters an ElectroSpray where the dissolved analytes undergo Electro Spray Ionisation (ESI) into a highly charged electric field (3kV). The ions are pulled into the instrument through a small-aperture sample cone in the ion source (1) by a combination of opposite charge on this cone and the high vacuum inside the MS/MS. This ion stream is electromagnetically focussed into an ion 'beam' and passes

through MS1, the first quadrupole analyser (2). Quadrupoles can be tuned so that only ions with a specific mass-to-charge ratio (m/z) will pass through with a stable trajectory, while all others will be dispersed out of the sides.

After passing through MS1, a given ionic species then enters the collision cell (3) containing a low density of an inert gas, generally argon (Ar), where individual ions collide with the gas atoms and fragment in a process known as collision induced dissociation (CID). This process of fragmentation is predictable and consistent for each molecular species and results in product, or 'daughter', ions with a known and specific m/z.

The product ions then enter the MS2, second quadrupole (4), which is tuned to allow through only the principle daughter ion of interest. These 'final' product ions then enter the Ion Detector (5) for measurement.

The mass filtering settings of MS1 and MS2 are combined in such as way that MS2 will scan, at any given point in time, only for the specific daughter ions derived from the ionic species that MS1 is tuned to allow through (the precursor or 'parent' ions). This process, known as multiple reaction monitoring (MRM) (4 channel), is repeated for each ionic species of interest, in this case PHE, TYR and their respective internal standards. As the internal standards are of a known concentration, the response from these species can be compared to that of the native species and employed to calculate their relative concentrations.

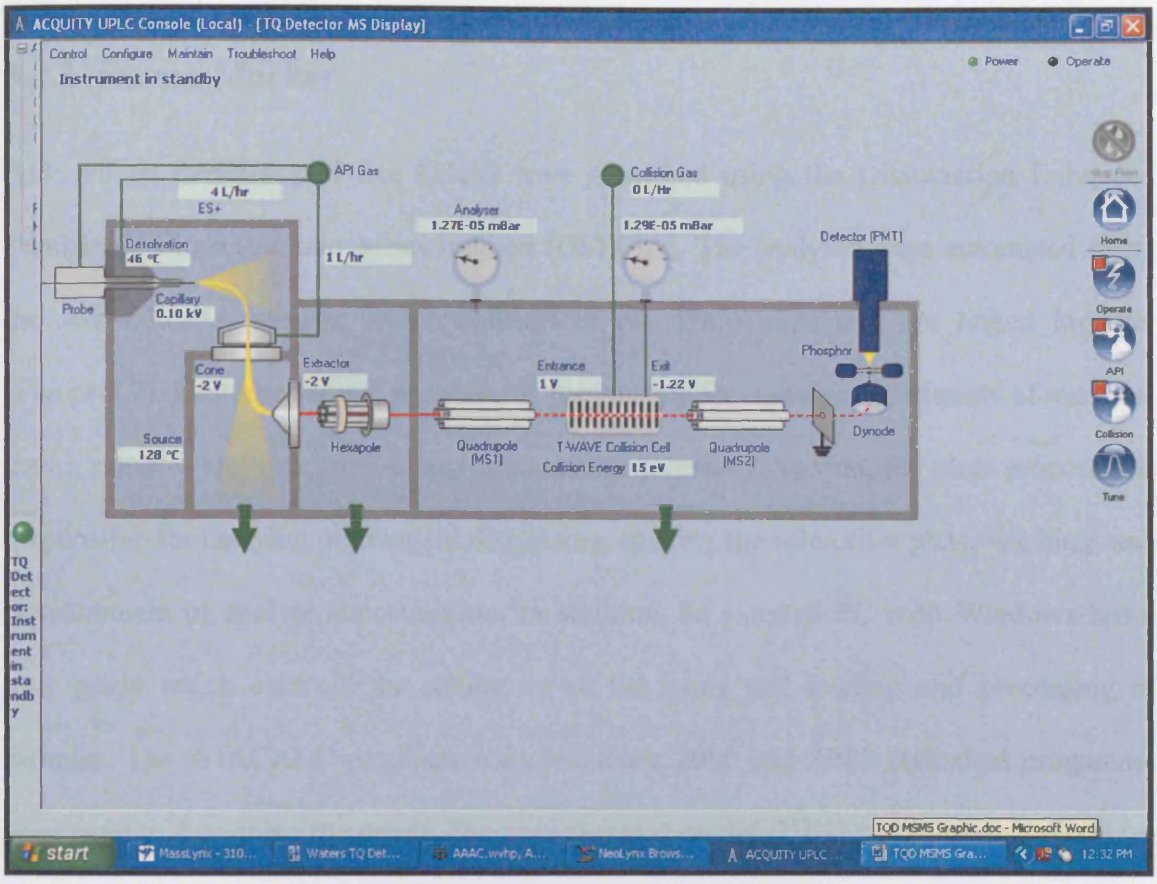

Figure 3-6: MS/MS (Waters Micromass QuattroMicro API tandem mass spectrometer).Screen shot from Waters software.

3.2 Gestational Characteristics of CF Pregnancies

3.2.1 Serum Marker

AFP, HCG, PAPP-A and free βHCG were measured using the Dissociation Enhanced Lanthanide Fluro Immuno Assay method (DELFIA). The analyses were automated using the AutoDELFIA system, which consists of two main units that are linked together (Figure 3.7). First, the sample processor is responsible for transferring aliquots of maternal serum samples and carrying out any dilutions as necessary. Second, the plate processor is responsible for carrying out reagent dispensing, shaking the microtitre plate, washing, and measurement of analyte concentration. In addition, an external PC with Windows has a user guide which controls the setting up of the assay and loading and processing of samples. The WIACALC programme on Multicalc 2000 and SPSS statistical progamme were used to determine the result. The unconjugated estriol (UE3) and inhibin-A (Inhibin-A) assay were carried out on the Access 2 immunoassay system manufactured by Beckman Coulter, as shown in Figure 3.8.

Figure 3-7: AutoDELFIA system for maternal serum screening sample

3.2.2 AutoDELFIA Immunoassay System Theory of Operation

The AutoDELFIA immunoassay system theory of operation is started with

Step 1: Pipetting

- 4 sampling probes equipped with liquid level and clot detection aspirate sample aliquots and dispense them into microtitre plates.
- Buffer and tracer are added using two dispensers

Step 2: Incubation

- Plates are then incubated at 25°C. The system can access any plate if required during incubation time.

Step 3: Add enhancement solution

- After incubation, plates are withdrawn from the shaker for the washing step followed by adding enhancement solution to each well

Step4: Measurements

- Plate then moved to instruments fluorometer for measurement

Step 5: Calculate results

- Result then calculated using MultiCalc software program.

Figure 3-8: Access 2 immunoassay system.

3.2.3 The Access 2 Immunoassay System Theory of Operation

The Access 2 immunoassay system theory of operation is started with

Step 1: Pipetting

- Reaction vessel (RV) containing samples in which estriol and inhibin-A are to be measured is moved from RV to pipetting position

Step 2: Incubation

- RV containing estriol and inhibin-A moves from pipetting position to incubator belt

Step 3: Washing

- After incubation RV moves to wash position on wash /read carousel

Step 4: Substrate Addition

- RV containing estriol and inhibin-A moves from wash position to substrate addition position

Step 5: Washing

- RV moves from substrate to luminometer position

Step 6: Ejection

- RV containing estriol and inhibin-A moves from luminometer position to incubator .belt and then ejected into RV waste bag

3.2.4 Measurement of AFP

The AutoDELFIA fluoroimmunometric assay system was used to measure human alpha-fetoprotein (AFP) in maternal serum samples. The method is based on a direct sandwich technique which utilises two monoclonal antibodies directed against different epitopes on the AFP molecule. The first anti-AFP monoclonal is coated onto the wells of microtitre plates. Serum samples are added to the plates and the AFP in the sample binds to the monoclonal antibodies and is immobilised. Eu-labeled anti-AFP IgG is then added to the

reaction, which binds to a different epitopic site on the AFP molecule. After incubation, the plate this is washed to remove unbound Eu-labeled AFP IgG and excess maternal serum. The addition of an enhancement solution, which is an acidic chelating detergent, dissociates Europium ions from labelled antibodies into a solution where they form fluorescent chelates with components of the enhancement solution. The fluorescence is proportional to the quantity of AFP in the serum sample (Figure 3.9).

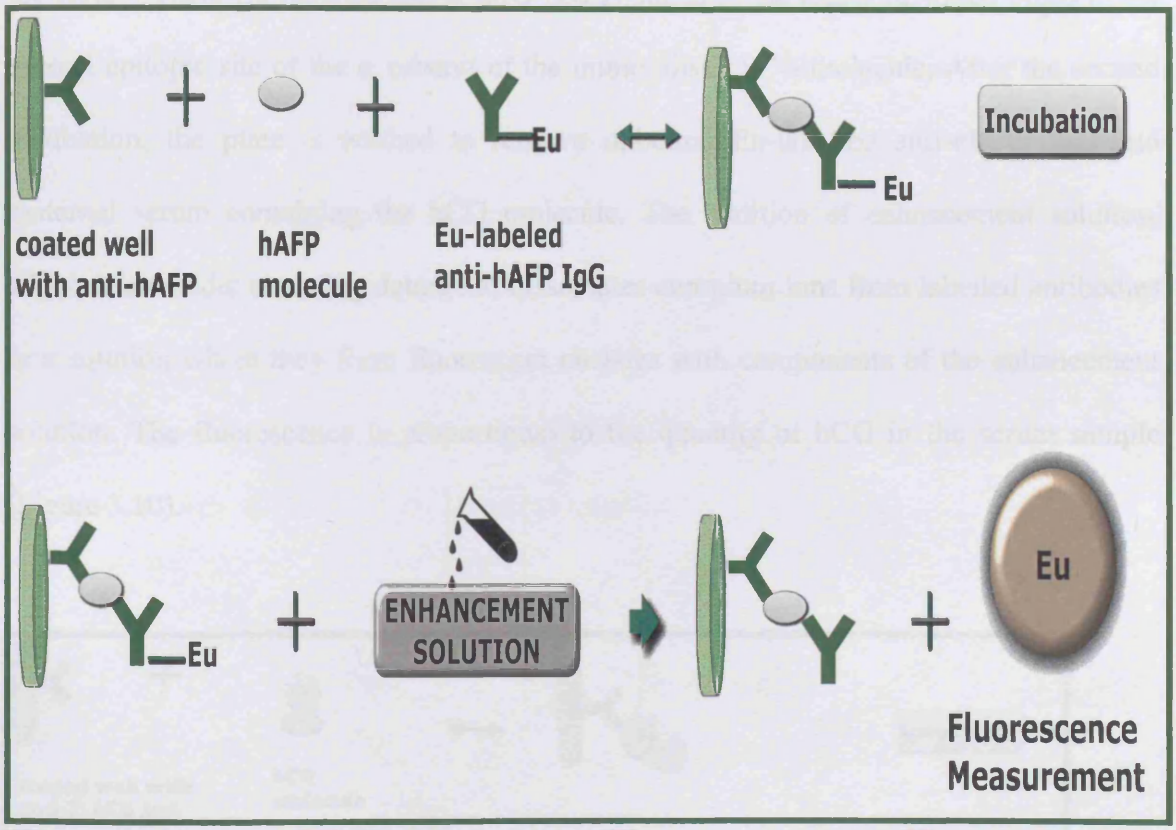

Figure 3-9: AutoDELFIA AFP: principle of the assay.

3.2.5 Measurement of HCG

The AutoDELFIA fluoroimmunometric assay system is used to measure human hCG in maternal serum samples. The system is based on the direct sandwich principle where two monoclonal antibodies directed against different sites on the β- and α hCG subunits are used. Serum samples containing hCG are added to the wells of a microtitre plate coated with an anti-βhCG monoclonal antibody. This binds the dimeric hCG molecules present in the serum. Then, Eu-labelled anti-α hCG IgG is added to the reaction, which binds to the second epitopic site of the α subunit of the immobilised hCG molecule. After the second incubation, the plate is washed to remove unbound Eu-labelled anti-αhCG IgG and maternal serum containing the hCG molecule. The addition of enhancement solution, which is an acidic chelating detergent, dissociates europium ions from labelled antibodies in a solution where they form fluorescent chelates with components of the enhancement solution. The fluorescence is proportional to the quantity of hCG in the serum sample (Figure 3.10).

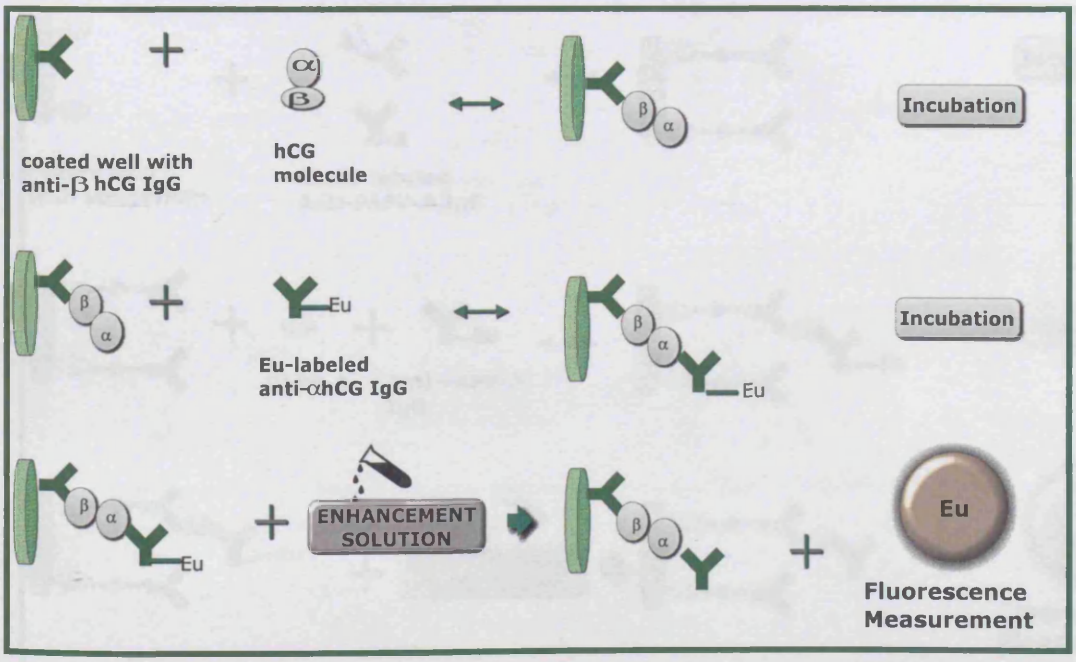

Figure 3-10: AutoDELFIA hCG: principle of the assay.

3.2.6 Measurement of PAPP-A

PAPP-A is found in serum complexed 2 :2 with proMBP. As proMBP is present at 5-10 times the concentration of PAPP-A an assay specific for PAPP-A which does not cross react with proMBP is required.

The AutoDELFIA fluoroimmunometric assay system is used to measure PAPP-A in maternal serum. The system is based on the indirect sandwich technique. In the first incubation, a microtitre plate well coated with streptavidin reacts with biotin-labelled anti-PAPP-A IgG. The microtitration strips are then washed, and in the second incubation, standards, controls and maternal serum samples containing PAPP-A molecules are reacted with tracer antibodies labelled with chelates of europium. Then, the addition of enhancement solution dissociates europium ions from labelled antibodies producing a solution where they form fluorescent chelates. The fluorescence is proportional to the quantity of PAPP-A in the serum sample (Figure 3.11).

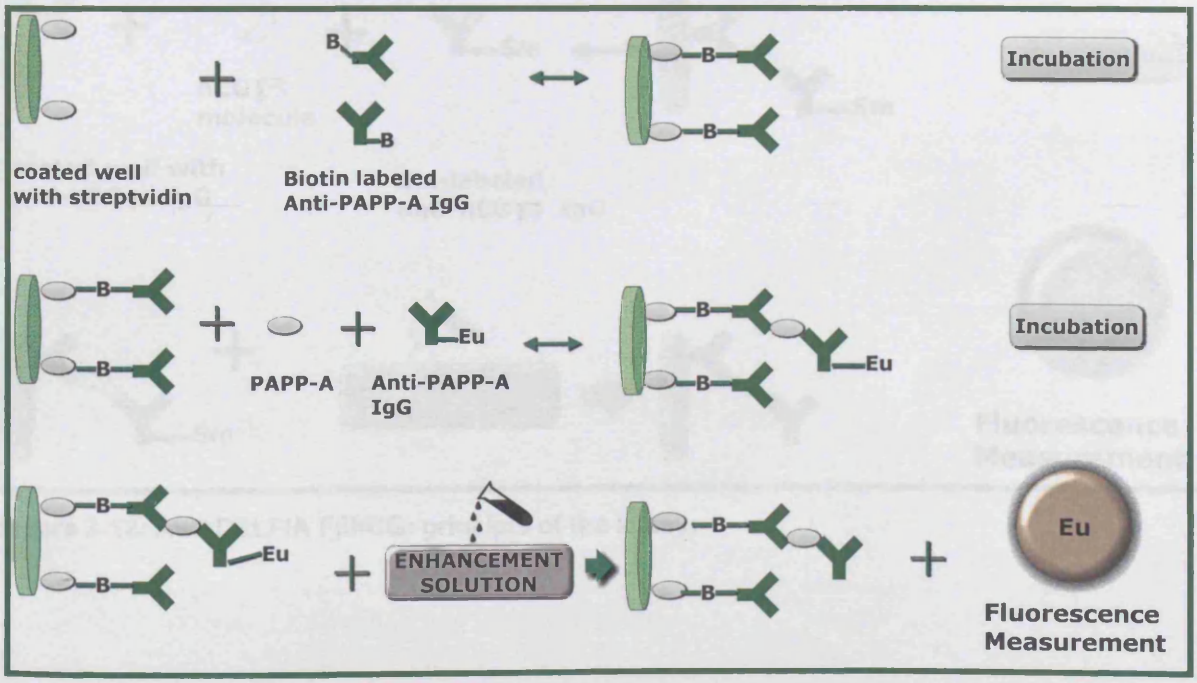

Figure 3-11: AutoDELFIA PAPP-A: principle of the assay.

3.2.7 Measurement of FβHCG

The AutoDELFIA fluoroimmunometric assay system was used to measure free beta subunit of hCG in maternal serum samples. The system is based on the direct sandwich principle. Two monoclonal antibodies detected against different epitopes on the β subunit are used. The first antibody is immobilised on the surface of the wells of a microtitre plate. Serum is added to the wells and the free β subunit present in the samples binds to the monoclonal antibodies. After washing, a second samarium-(Sm) labelled anti-beta subunit antibody is added to the wells and binds to the second epitopic site on the immobilised β-subunit molecule. After incubation, the plate is washed to remove unbound Sm-labelled-anti-hCG IgG and maternal serum containing the βhCG molecule. The addition of enhancement solution, which is an acidic chelating detergent, dissociates samarium ions from labelled antibodies into a solution where they form fluorescent chelates with components of the enhancement solution. The fluorescence is proportional to the quantity of FβhCG in the serum sample (Figure 3.12).

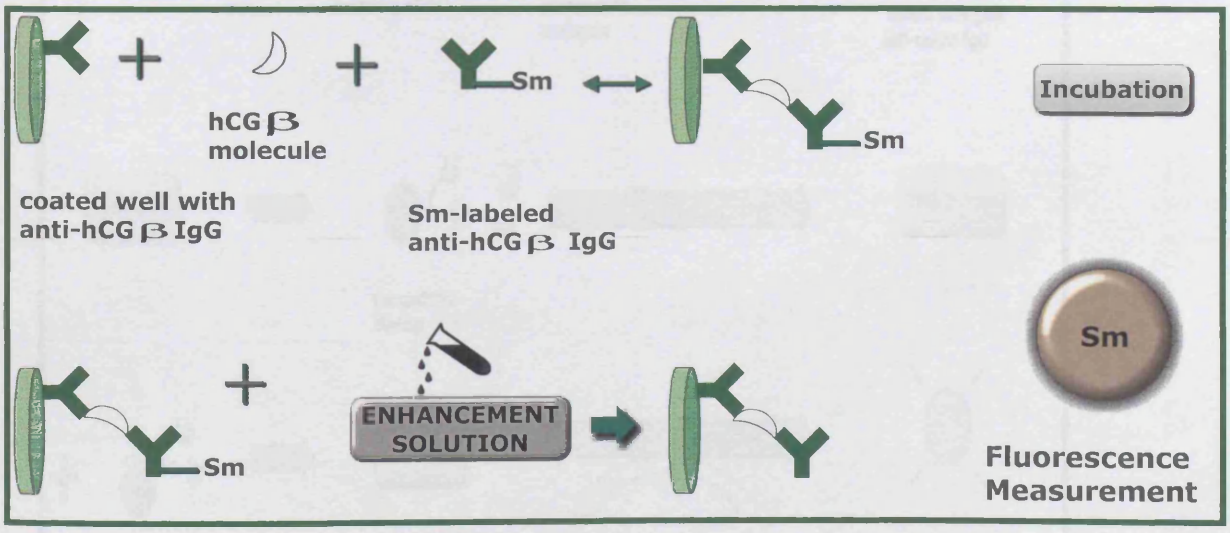

Figure 3-12: AutoDELFIA FβhCG: principle of the assay.

3.2.8 Measurement of UE3

The Access 2 immunoassay system manufactured by Beckman Coulter was used to measure UE3. The Access UE3 assay is 1-step competitive binding technique assay. Rabbit anti-estriol polyclonal antibodies are reacted with serum samples containing estriol, an estriol-alkaline phosphatase conjugate and paramagnetic particles coated with goat anti-rabbit IgG. After incubation at 36.5 °C for 20 minutes, maternal serum samples containing estriol compete with estriol-alkaline phosphatase conjugate for a limited number of binding sites on the specific anti-estriol antibody. Bound materials are held in a magnetic field while unbound materials are washed away. Light generated by the reaction after adding the substrate is measured with a luminometer. Estriol concentration in the maternal serum sample is inversely proportional to the light production (Figure 3.13).

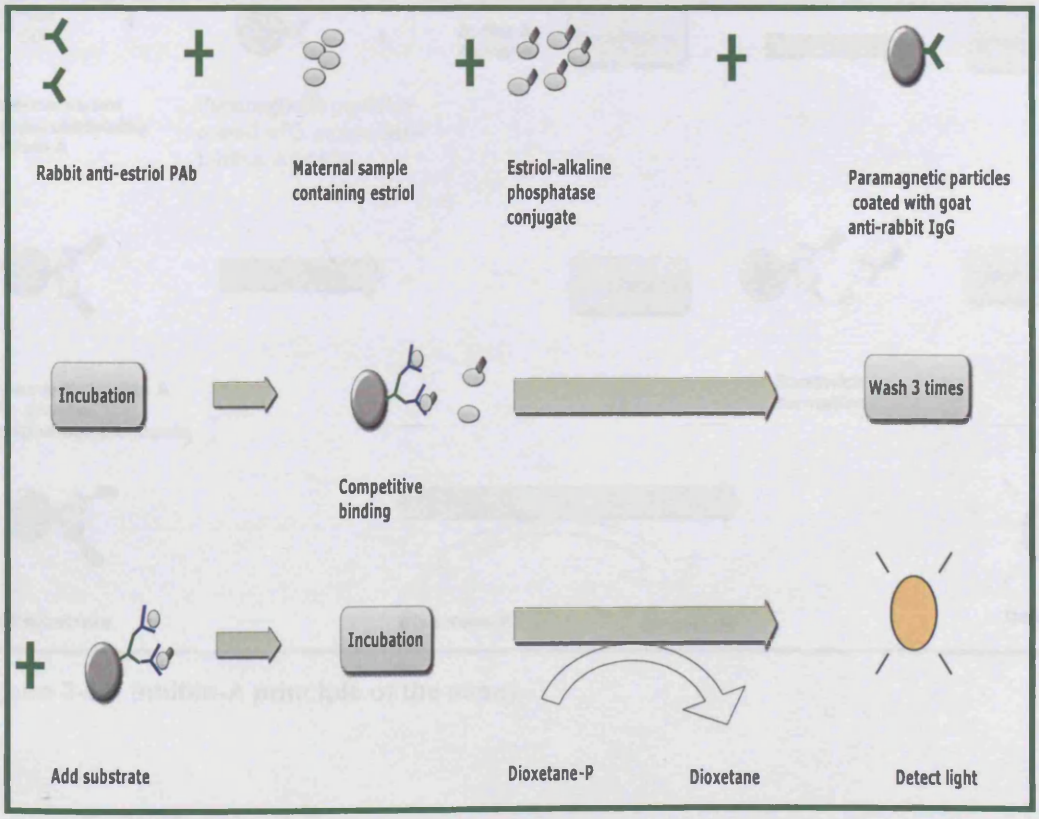

Figure 3-13: Access 2 uncongealed estriol principles.

3.2.9 Measurement of Inhibin-A

The Access 2 immunoassay system, manufactured by Beckman Coulter was used to measure Inhibin-A. The Access Inhibin-A assay is a 2-step sandwich technique assay. Serum samples containing Inhibin-A are reacted with paramagnetic particles coupled an with anti-Inhibin-A monoclonal antibody. After incubation at ~36 °C for 19 minutes followed by a wash step to remove excess sample and reagents, an anti-inhibin, monoclonal antibody-alkaline phosphatase conjugate is added to a reaction mixture and incubated at ~36 °C for 15 minutes. Bound materials are held in a magnetic field while unbound materials are washed away. Light generated by the reaction after adding the substrate is measured with a luminometer. The inhibin-A concentration in the maternal serum sample is directly proportional to the light production (Figure 3.14).

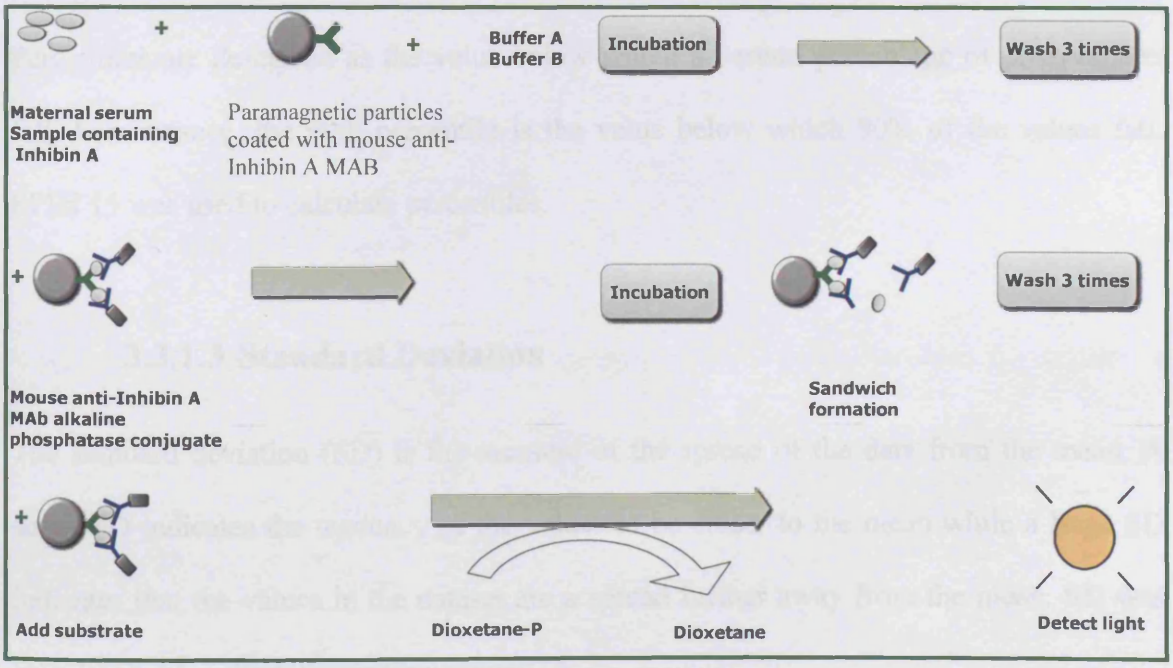

Figure 3-14: Inhibin-A principle of the assay.

3.3 Statistical Methods

Descriptive statistics are used to organise and summarise data. Inferential statistics are used to test one set of data against another or generalise from a sample to a larger group.

3.3.1 Descriptive Statistics

3.3.1.1 Mean

The mean is used to measure the central location but is influenced by outliers. The mean is calculated by adding up all the values and dividing this sum by the number of data points. Means were calculated using the SPSS v15 program.

3.3.1.2 Percentiles

Percentiles are described as the value below which a certain percentage of observations fall. For instance, the 90th percentile is the value below which 90% of the values fall. SPSS 15 was used to calculate percentiles.

3.3.1.3 Standard Deviation

The standard deviation (SD) is the measure of the spread of the data from the mean. A small SD indicates the tendency of the values to be closer to the mean while a large SD indicates that the values in the dataset are a spread further away from the mean. SD was calculated using the following equations:

$$(1)\ SD = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}} \quad \text{or} \quad (2)\ \frac{\log_{10}(90^{th}\ \text{centile}) - \log_{10}(10^{th}\ \text{centile})}{2.56}$$

Where $\bar{x}$ the mean and n is the number of cases. SDs were calculated using SPSS 15

3.3.1.4 Coefficient of Variation

Assay reproducibility for all the markers in the study was measured using the coefficient of variance (CV). CV represents the ratio of the standard deviation (SD) to the mean ($\bar{x}$) expressed as a percentage. The following equation was used to calculate the CV.

$$CV = 100 \times \left(\frac{SD}{\bar{X}} \right)$$

3.3.1.5 Correlation Coefficient (r)

The level of association between two variables, for example X and Y, is called the correlation coefficient. The r value has a range between -1 and +1. A positive value points to a positive correlation and a negative value points to a negative correlation. If there is no association between the X and Y, the r value would be close to 0. The formula to calculate r value for two variables is:

$$r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}}$$

where xi and y_i are the values of X and Y for the individual value. To check for outliers, a simple box-plot was used. SPSS 15 programme was used to calculate correlation coefficients.

3.3.1.6 Median

The median is calculated by arranging the data set in order from the lowest to highest values. The median is the middle value of this ordered set. SPSS 15 programme was used to calculate the medians.

3.3.2 Inferential Statistics

3.3.2.1 Mann-Whitney Test

The Mann Whitney test is based on ranking or ordering the data from two groups. The data from the two groups are combined and ordered from the lowest to the highest. The ranks from each group are summed and the test is based on examining the sum of the ranks in each group divided by the number in that group. SPSS 15 programme was used to calculate Mann Whitney test.

3.3.2.2 Regression Analysis

Regression analysis is used to estimate the relationships between two variables. Regression analysis was used to determine the relationship between IRT and PAP and other variables and between AFP, HCG, FBHCG, PAPP-A, UE3 and inhibin-A levels and gestation in weeks. To estimate the relationship between markers, various models were used for instance, exponential, quadratic and inverse. When choosing the best-fitted model, the overall fit of the data and the adjusted r2 values were considered. This was done using the curve estimation routine in SPSS 15.

3.3.3 Multiple of Median of the Appropriate Gestation

All gestation-related markers levels were converted to a multiple of the control median (MoM) at the appropriate gestational week. This allows changes of marker levels with the gestational age to be compared PAP and IRT levels were also converted to MoM where appropriate. The following equation was used to calculate MoM values:

$$\text{MoM} = \frac{\textbf{Marker concentration}}{\textbf{Regressed median concentration}}$$

The MoM was calculated using the regression equation from the best fitted model for each marker.

3.3.4 Likelihood Ratios (LR) for CF Derived from IRT and PAP

The predictive value of the data for the affected and unaffected samples was examined using the (LR) method. Risks are derived from the overlapping $\log_{10}$ Gaussian distributions for affected babies with CF (Total 29 cases) and with unaffected babies with CF (Total 2886). LR were calculated for CF cases, CF carriers and increased IRT unaffected samples using the following equations.

LR for PAP:

$a = (\log_{10} \text{PAP} - \text{Mean x}) / \text{SD x})^2$

$b = (\log_{10} \text{PAP} - \text{Mean y}) / \text{SD y})^2$

LR = (SDy) / (Dx)* EXP (-0.5 * (PAP-a - PAP-b))

LR for IRT:

$a = (\log_{10} \text{IRT} - \text{Mean x}) / \text{SD x})^2$

$b = (\log_{10} \text{IRT} - \text{Mean y}) / \text{SD y})^2$

LR = (SDy) / (SDx)* EXP (-0.5 * (IRT-a - IRT-b))

Where x = affected and y = unaffected

The overall LR from IRT and PAP was calculated using the following equation:

IRT-LR×PAP-LR

3.3.5 EasyPlot Software

EasyPlot software (version 3.00-11) is used for scientific graphing. EasyPlot was used in the gestational study for drawing the curves using regression equation from the best fitted model for each marker. In the newborn study, the EasyPlot programme was used to

differentiate between the median for CF cases and CF carrier mutations types for both IRT and PAP. The programme was also used in the newborn screening study for plotting the overlapping $\log_{10}$ Gaussian distribution.

CHAPTER 4 :

RESULTS

4.1 NEWBORN BLOOD SPOT MARKERS OF CYSTIC FIBROSIS

The study of newborn screening for cystic fibrosis using dried blood spots was designed to search for ways to make testing more specific and to reduce the number of carriers detected through DNA testing of screen-positive babies. The current routine newborn screening test is based on measurement of immunoreactive trypsinogen (IRT) using the Delfia fluoroimmunoassay method (Perkin Elmer, Finland), followed by DNA analysis to search for mutations in the CF gene. In addition, a new marker of CF, Pancreatitis-Associated Protein (PAP) was measured using a commercially-available enzyme linked immunoassay (Dynabio SA, Marseille, France).

Other data available from routine bloodspot screening were measurements of phenylalanine (PHE) and tyrosine (TYR) by tandem mass spectrometry and these markers are also investigated in this sample series.

A total of 3632 sample were used in this study, of which 1827 (50 %) were from baby boys and 1791 (49%) were from baby girls. There was incomplete gender information on 18 of the babies.

The data is divided into 6 groups which include: control samples (total 2886), samples with an increased IRT level ($\geq$ 70 ng ml)(total 314), samples from low birth weight babies (< 2,500g) (total 189), samples from premature babies (< 37 weeks gestation) (total 182), samples from confirmed CF cases (total 29), and samples from CF carriers (total 32).

Various types of information were collected to allow examination of the effect of several variables in each samples group. These included date of birth, date blood spot collected,

gender, age at blood spot collection, the ethnic background, birth weight, length of gestation, immunoreactive trypsinogen (IRT), phenylalanine (PHE), tyrosine (TYR) result and type of CF mutation. In addition, pancreatitis-associated protein (PAP) was measured in all blood spots and these data analysed and compared with the other marker data in each sample group.

4.2 Control Blood Spot Samples

Out of 3632 blood spot samples, 2886 (79%) samples were control samples, of which 1465 (50 %) were boys and 1407 (48 %) were baby girls, with the gender of the remaining 14 samples not recorded on the dried blood spot card.

The ethnic backgrounds of all 2886 babies in the control group were identified. Most of these babies, i.e. 2528 (87 % of the total sample) were of white ethnic background and 244 (8%) samples belonged to various other ethnic backgrounds. Gender information was missing for 6 samples (i.e., it was not recorded on the dried blood spot card) and in 108 samples the ethnic background was not stated.

In addition, date the specimen was collected was recorded for 2886. For 2659 (91%) of the samples, the dried blood spot specimens were obtained before the tenth day of the baby's life, while 240 samples (8%), were obtained on or after day 10 of the baby's life. The lowest, highest and median values of each marker IRT, PAP, PHE and TYR, were identified and are presented in table 4.1.

The frequency distribution of the IRT, PAP, PHE and TYR levels in 2886 controls blood spot sample are shown in figures 4.1 – 4.4. Each of the above marker distributions was positively skewed and log transformation ($\log_{10}$) was required to normalise the

distributions. These are shown in figures 4.5 - 4.8. The mean and standard deviation values for each marker derived from the $\log_{10}$ distributions are shown in table 4.2.

Table 4-1: The lowest, highest and median levels of IRT, PAP, PHE and TYR in 2886 blood spot control samples

BIOCHEMICAL MARKERS	NO. OF BABIES	CONTROL SAMPLES		
		Lowest	**Highest**	**Median**
IRT (ng / ml)	2886	3.4	71.1	19.3
PAP (ng / ml)	2886	0.001	17.517	0.192
PHE (μmol/L)	2886	25.7	179.5	56.2
TYR (μmol/L)	2886	12.9	968.4	93.8

Table 4-2: Mean and standard deviation Log_{10} for IRT, PAP, PHE and TYR in the blood spot control samples

BIOCHEMICAL MARKERS	NO. OF BABIES	MEDIAN	MEAN OF LOG_{10}	STD. DEVIATION OF LOG_{10}
IRT (ng / ml)	2886	19.3	1.2887	0.203
PAP (ng / ml)	2886	0.192	-0.7389	0.477
PHE (µmol/L)	2886	56.2	1.7549	0.101
TYR (µmol/L)	2886	93.8	1.9794	0.168

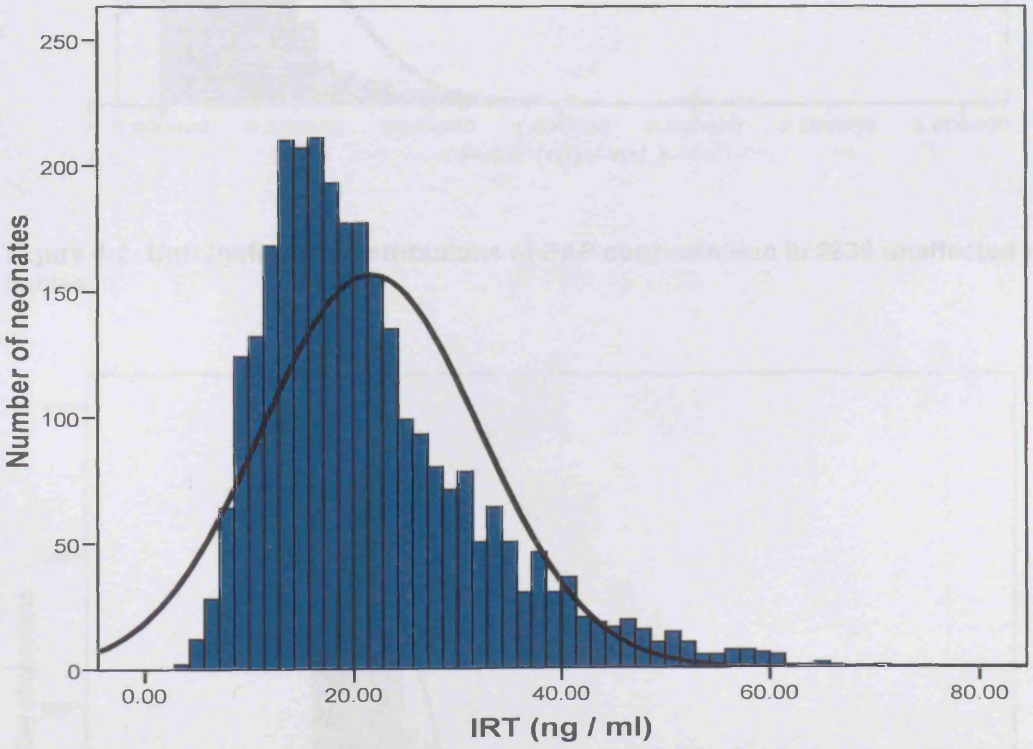

Figure 4-1: Untransformed distributions of IRT concentration in 2886 unaffected newborn babies

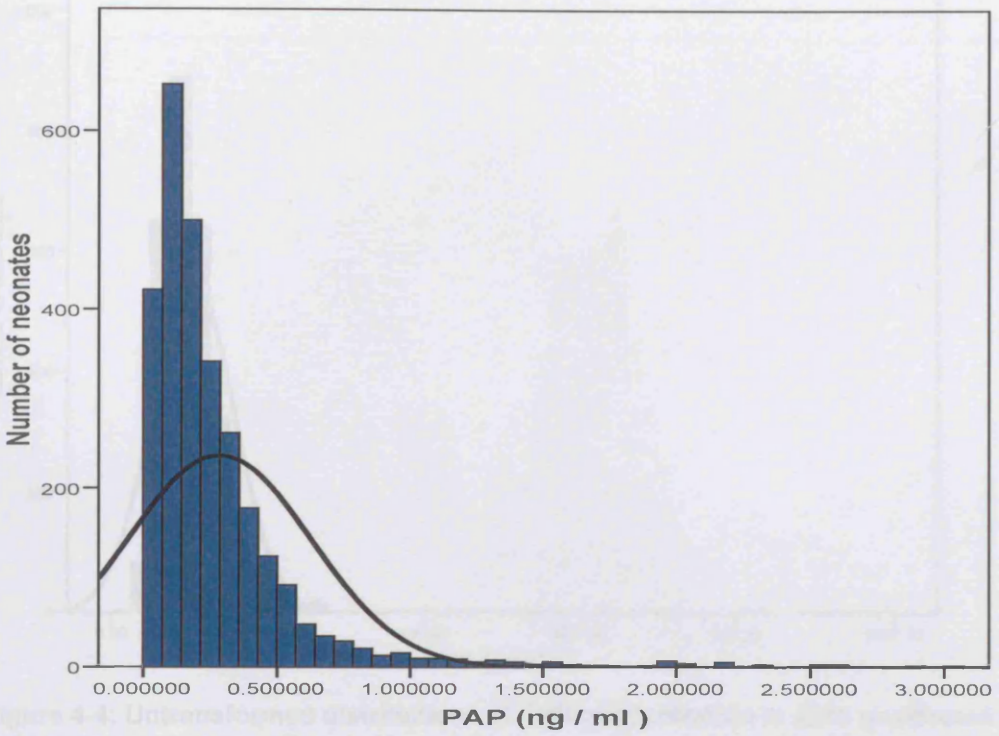

Figure 4-2: Untransformed distributions of PAP concentration in 2836 unaffected newborn babies.

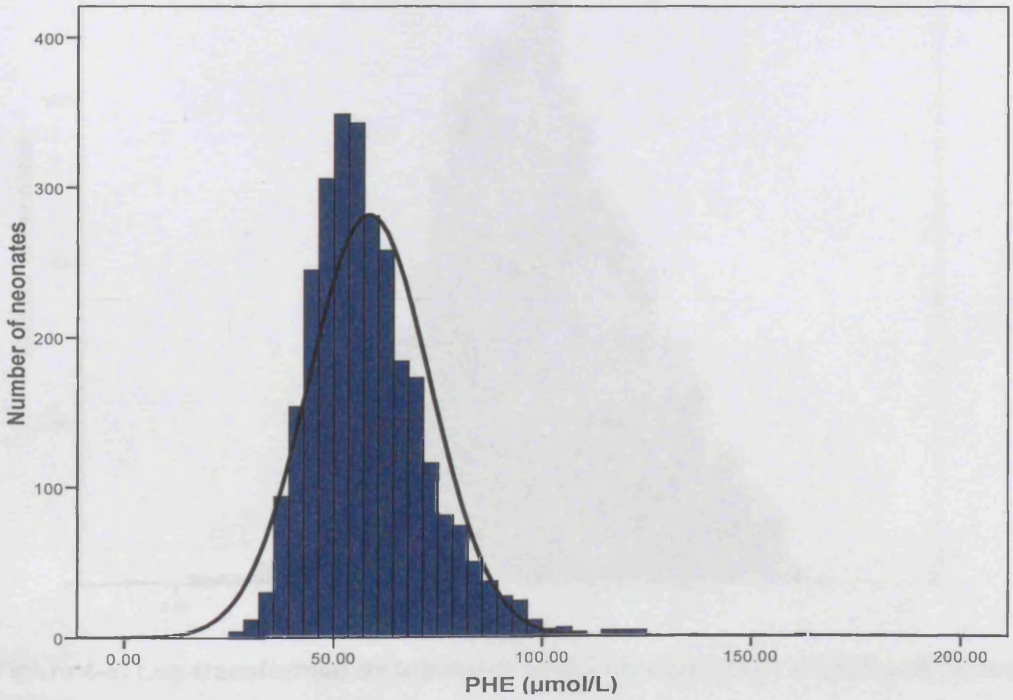

Figure 4-3: Untransformed distributions of PHE concentration in 2886 unaffected newborn babies.

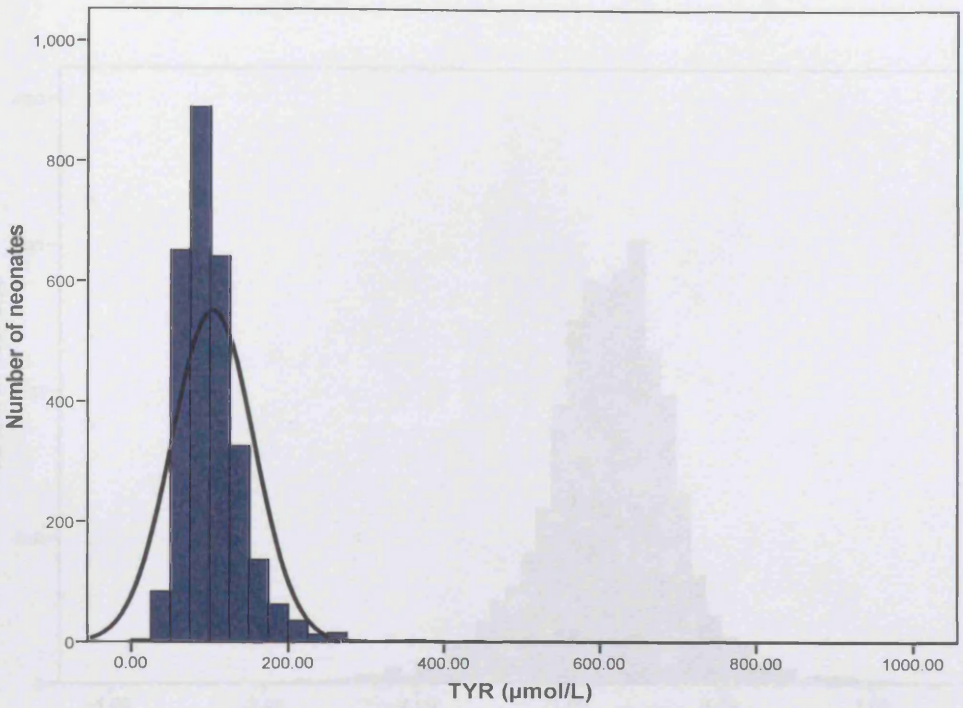

Figure 4-4: Untransformed distributions of TYR concentration in 2886 unaffected newborn babies.

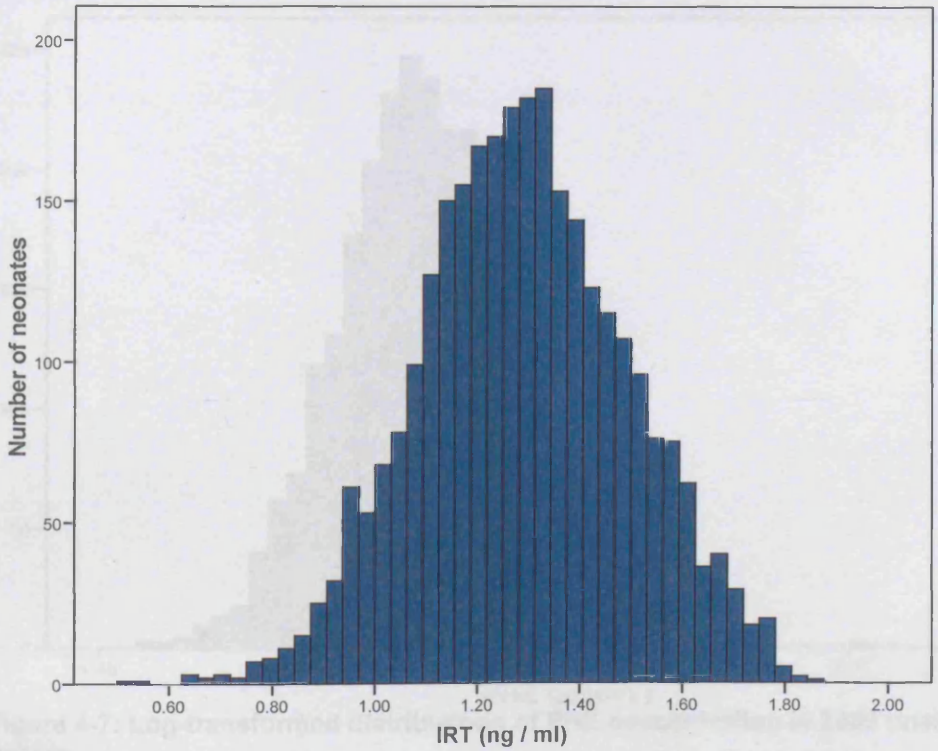

Figure 4-5: Log-transformed distributions of IRT concentration in 2886 unaffected newborn babies.

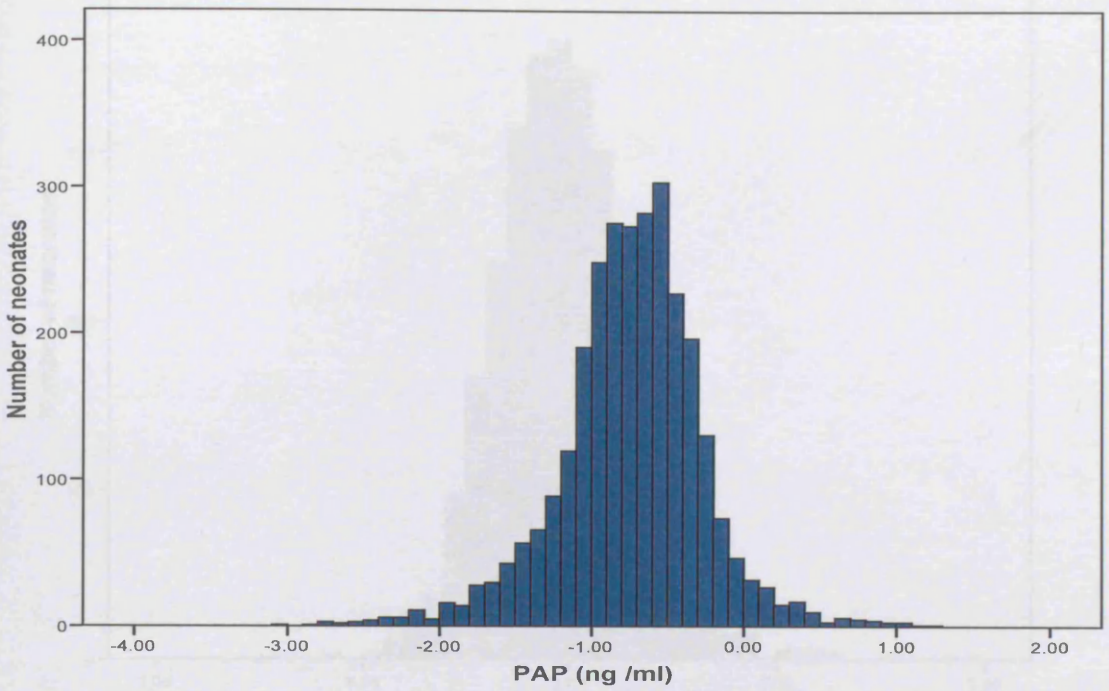

Figure 4-6: Log-transformed distributions of PAP concentration in 2886 unaffected newborn babies.

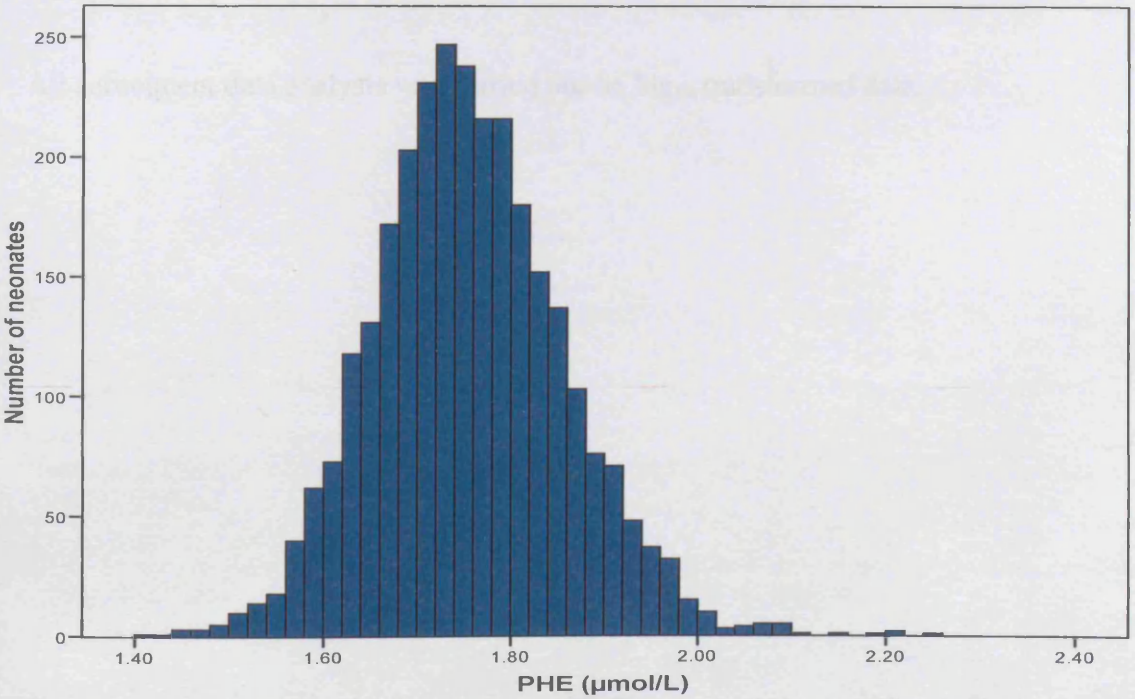

Figure 4-7: Log-transformed distributions of PHE concentration in 2886 unaffected newborn babies.

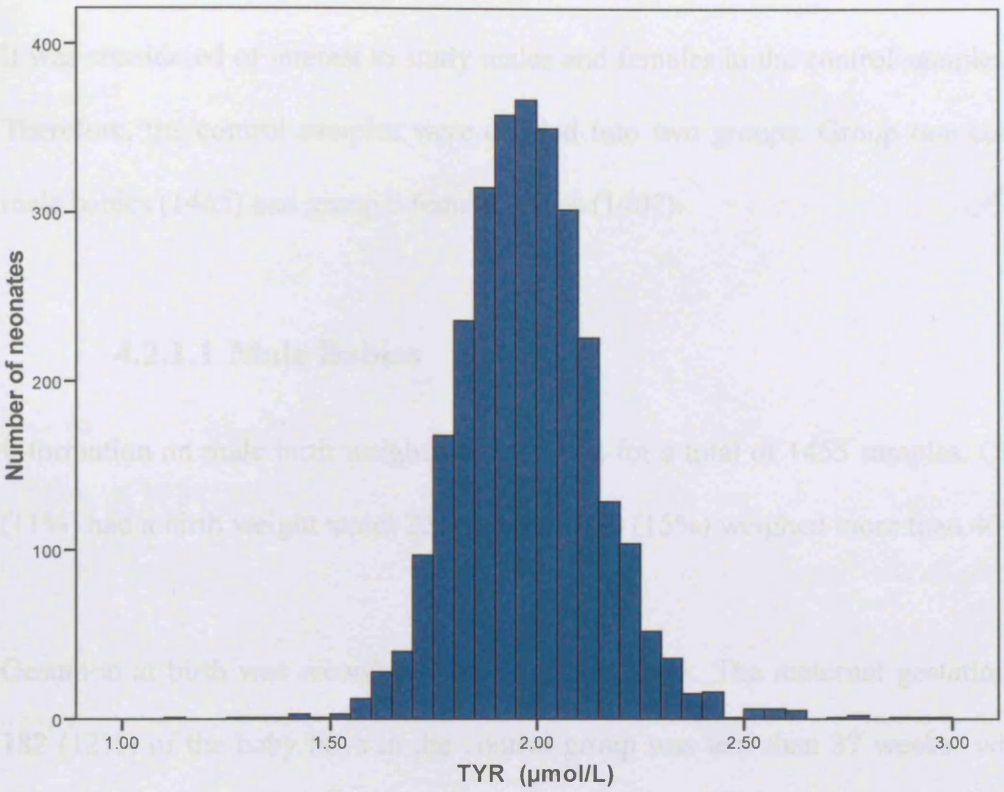

Figure 4-8: Log-transformed distributions of TYR concentration in 2886 unaffected newborn babies.

All subsequent data analysis was carried out on $\log_{10}$ transformed data.

4.2.1 Male and Female Babies in the Control Sample

It was considered of interest to study males and females in the control samples separately. Therefore, the control samples were divided into two groups. Group one comprised the male babies (1465) and group 2 female babies (1407).

4.2.1.1 Male Babies

Information on male birth weight was recorded for a total of 1455 samples. Of these, 169 (11%) had a birth weight under 2500g, while 230 (15%) weighed more than 4000g.

Gestation at birth was recorded for 1365 male babies. The maternal gestation period for 182 (12%) of the baby boys in the control group was less than 37 weeks while for two babies it was more than 42 weeks. The shortest period of maternal gestation was 25 weeks and the maximum gestation period was 43 weeks. 1334 dried blood spot specimens were obtained in the first 10 days of life, while 124 samples were obtained on or after day 10.

For the 1455 baby boys in the control group, the lowest and highest and the median values of IRT, PAP, PHE and TYR markers were identified, and these are shown in table 4.3.

Table 4-3: Lowest, highest and median levels of IRT, PAP, PHE and TYR in baby boys in the control group.

Biochemical markers	No. of babies	Male neonates		
		Lowest	Highest	Median
IRT (ng / ml)	1465	3.4	66.3	18.9
PAP (ng / ml)	1465	0.002	15.06	0.17
PHE (μmol/L)	1465	28.1	179.5	56.4
TYR (μmol/L)	1465	21.7	569.1	92.3

4.2.1.2 Female Babies

The birth weight of 1399 samples from female babies was recorded, while information was missing for 8 samples (i.e., it was not recorded on the dried blood spot card). Out of these 1399 samples, 163 (11%) had a birth weight less than 2500g, while 132 (9%) weighed more than 4000g.

Information on the duration of the gestation at birth was recorded for 1336. The gestation at birth of 157 (11%) of the baby girls in the control group was less than 37 weeks while only one baby girl in this group was born later than 42 weeks. The shortest recorded period of maternal gestation was 25 weeks and the maximum gestation period was 43 weeks.

In 1303 (92 %) of the baby girls in the control group, the dried blood spot specimens were obtained before 10 days of life, while in 98 (7%) of cases, the dried blood spot specimens were obtained on or after the 10th day of the baby's life.

Fore the 1415 baby girls in the control group, the lowest and highest and the median values of IRT, PAP, PHE and TYR markers were identified, and these are shown in table 4.4.

Table 4-4: The lowest, highest and median of levels of IRT, PAP, PHE and TYR in female babies in the control group

Biochemical markers	No. of babies	Female neonates		
		Lowest	Highest	Median
IRT (ng / ml)	1407	3.6	71.0	19.68
PAP (ng / ml)	1407	0.001	17.516	0.20
PHE (μmol/L)	1407	25.7	152.7	55.9
TYR (μmol/L)	1407	12.9	968.4	96.07

Median, IRT, PAP, PHE and TYR levels in both males and females were compared using Mann-Whitney test to see if there are any difference between them. Female babies had statistically significantly higher IRT ($p = 0.013$), PAP ($p< 0.001$) and TYR values ($p = 0.047$) than males. There was no statistically significant different in PHE medians in males and females ($p = 0.280$).

4.2.1.3 Variation in Blood Spot Marker Levels with Day of Sampling.

In the control group (2876 samples) the regression analysis at age of sampling showed PAP level significant decreased ($p < 0.001$) with earlier age at sampling (Figure 4.10). No significant associations were found for IRT ($p = 0.959$), PHE ($p=0.276$) or TYR ($p = 0.249$). (Figure 4.9, 4.11, 4.12)

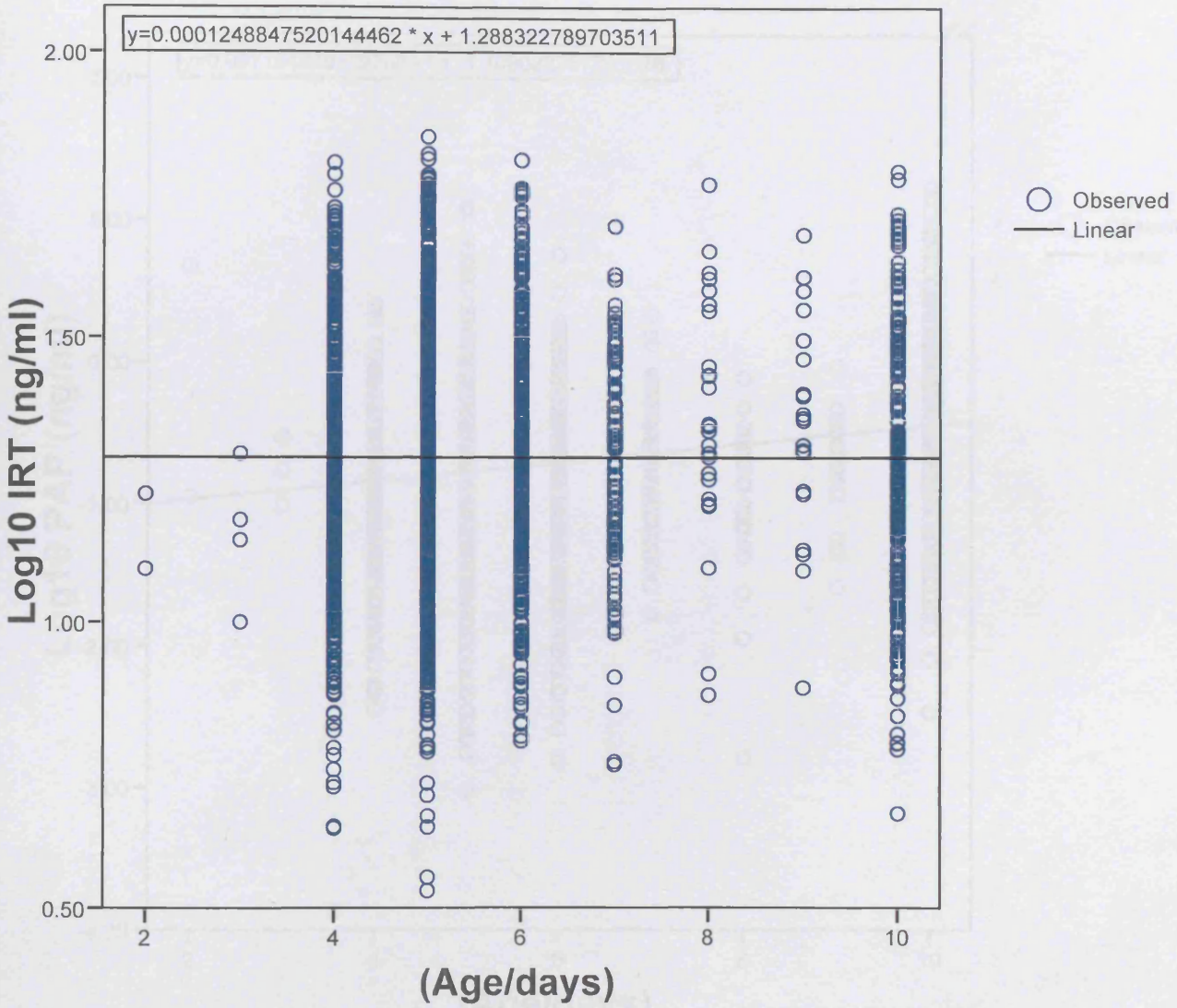

Figure 4-9: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 2876 control samples at the age of sample collection.

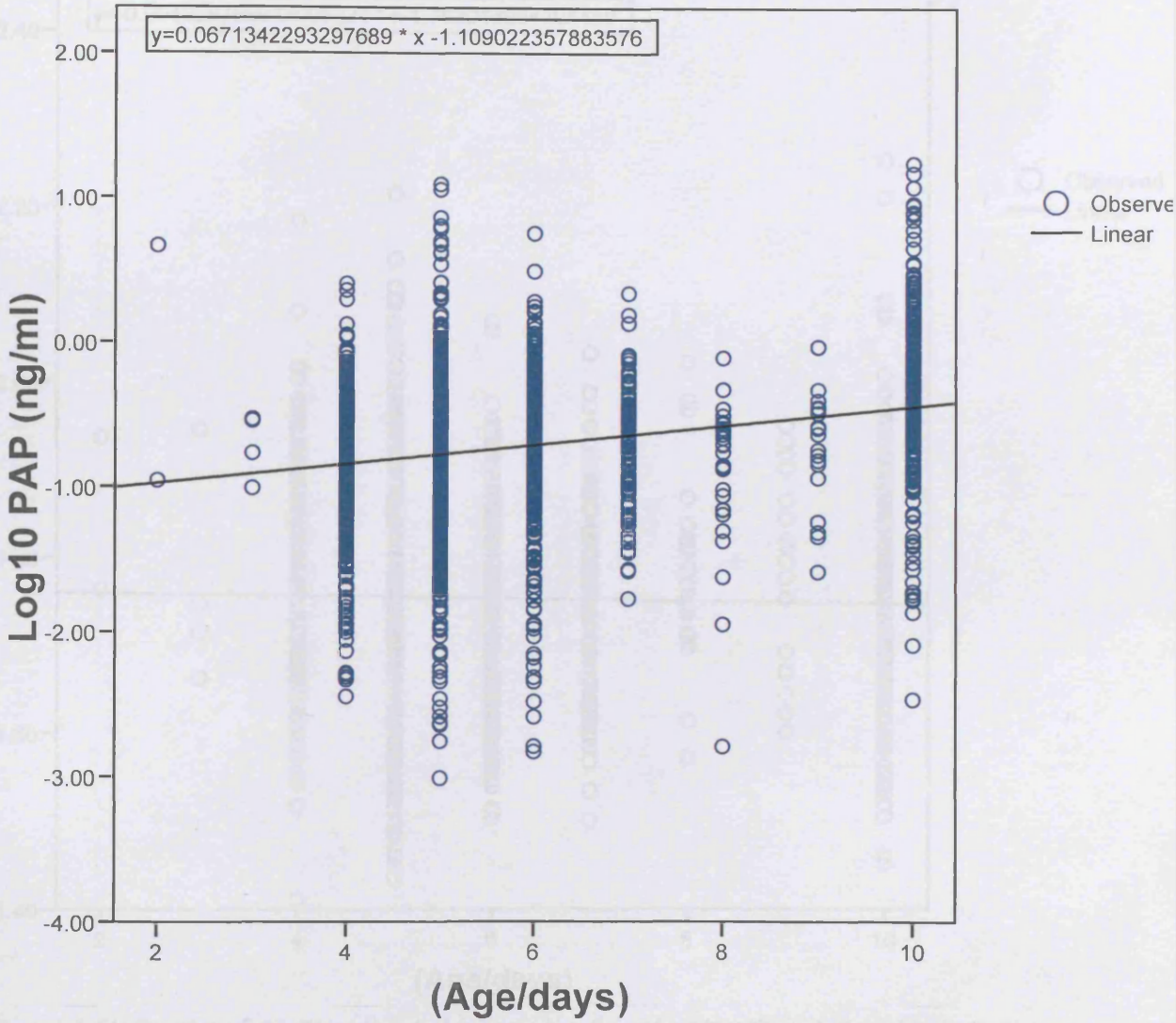

Figure 4-10: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 2876 control samples at the age of sample collection.

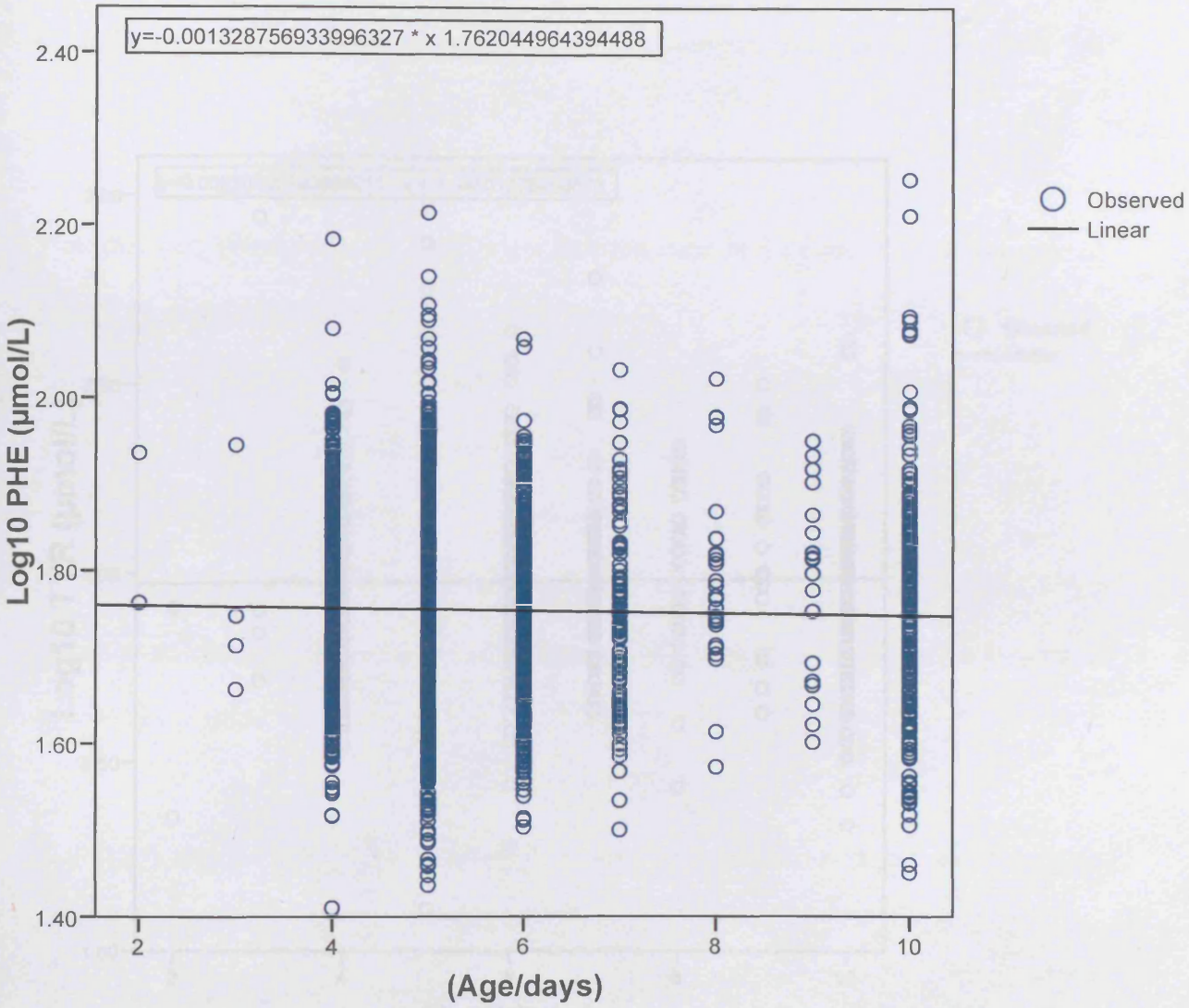

Figure 4-11: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 2876 control samples at the age of sample collection.

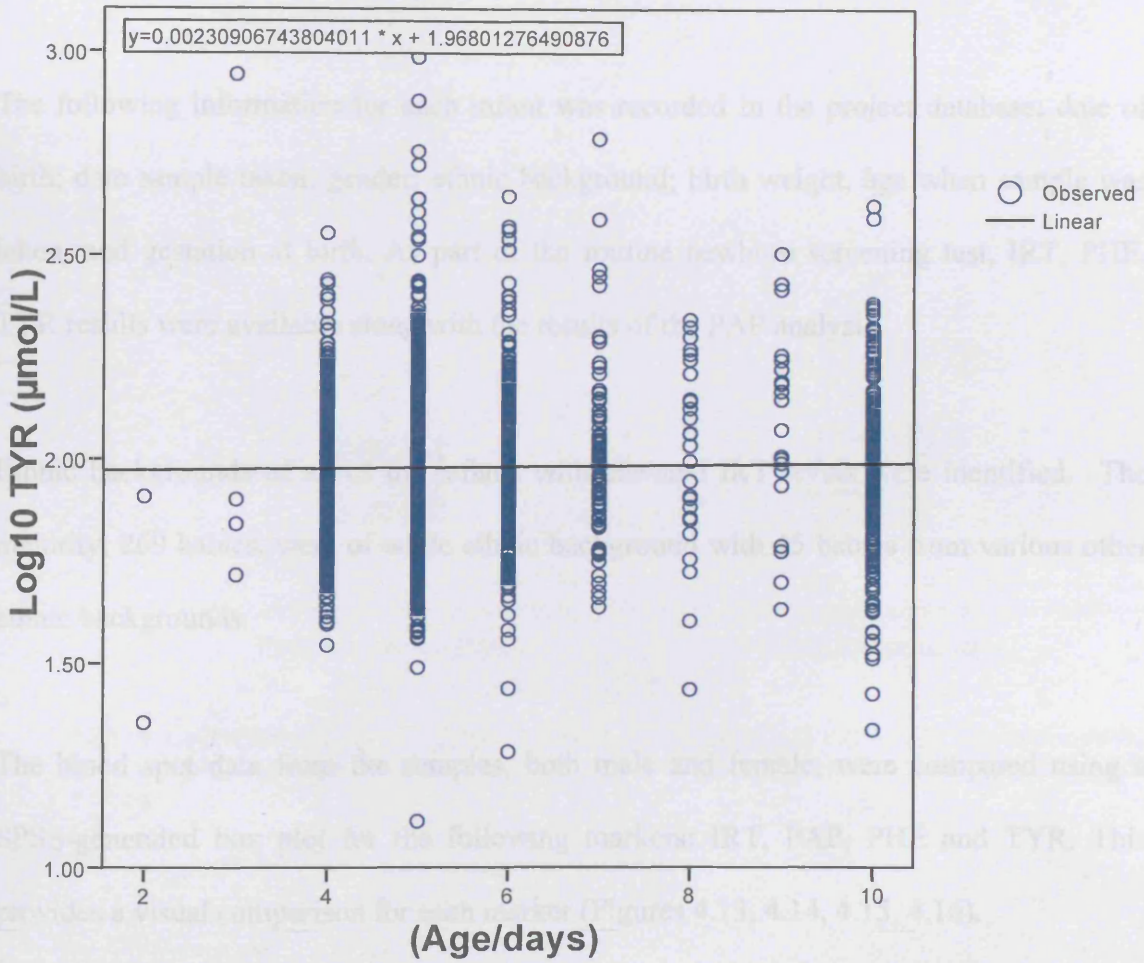

Figure 4-12: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration levels in 2876 control samples at the age of sample collection.

4.3 Elevated IRT Blood Spot Sample Analysis

Of 3632 blood spot samples, 314 (8%) were from infants selected on the basis of an elevated IRT level and absence of any mutation in the CF gene. Of these, 169 were from female infants and 144 from males. The gender of the remaining one sample was not recorded on the dried blood spot card.

The following information for each infant was recorded in the project database: date of birth; date sample taken; gender; ethnic background; birth weight, age when sample was taken, and gestation at birth. As part of the routine newborn screening test, IRT, PHE, TYR results were available along with the results of the PAP analysis.

Ethnic backgrounds of all of the infants with elevated IRT levels were identified. The majority, 269 babies, were of white ethnic background with 45 babies from various other ethnic backgrounds.

The blood spot data from the samples, both male and female, were compared using a SPSS-generated box plot for the following markers: IRT, PAP, PHE and TYR. This provides a visual comparison for each marker (Figures 4.13, 4.14, 4.15, 4.16).

4.3.1 IRT

The result of the SPSS-generated box plot for IRT of female babies showed that 50% of IRT levels were between 78 and 113 ng/ml, with a median of 88.1 ng/ml. The highest value was 653.2 ng/ml and the lowest value was 70.1 ng/ml.

Regarding male babies, the IRT analysis revealed that 50% of IRT levels were between 77 and 120 ng/ml and the median 86.9 ng/ml. The highest value was 503.3 ng/ml and the

lowest set value was 70 ng/ml (Figure 4.13). There was no statistically significant difference in the IRT distributions between males and females with increased IRT ($p = 0.560$).

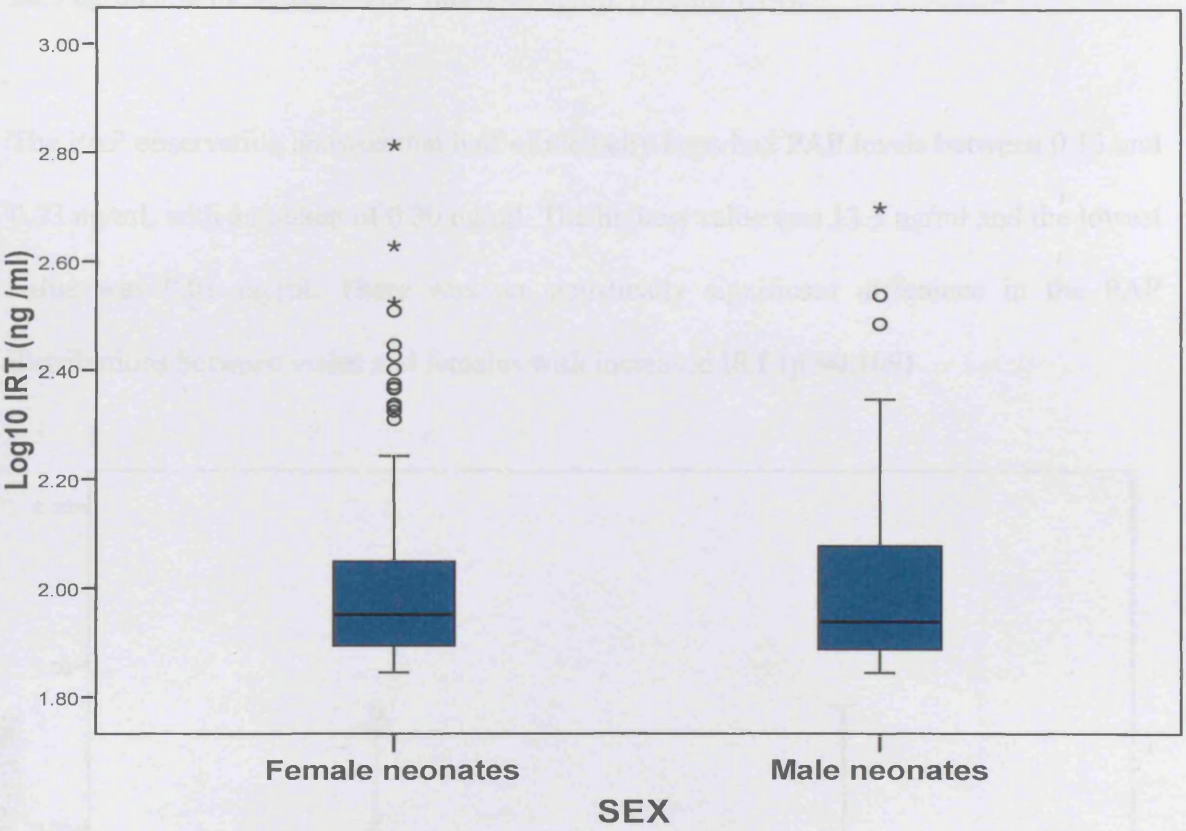

Figure 4-13: Box plot of IRT (log_{10}) marker for female (169 sample) and male babies (144 sample). Asterisks indicate extreme IRT values, while circles indicate outlier IRT values.

4.3.2 PAP

The SPSS-generated box plot for PAP levels of baby girls showed that 50% of PAP levels were between 0.19 and 0.69 ng/ml, with a median of 0.34 ng/ml. The highest value was 28.5 ng/ml and the lowest value was 0.02 ng/ml. (Figure 4.14).

The PAP observation showed that half of the baby boys had PAP levels between 0.13 and 0.73 ng/ml, with a median of 0.30 ng/ml. The highest value was 13.3 ng/ml and the lowest value was 0.01 ng/ml. There was no statistically significant difference in the PAP distributions between males and females with increased IRT (p =0.109)

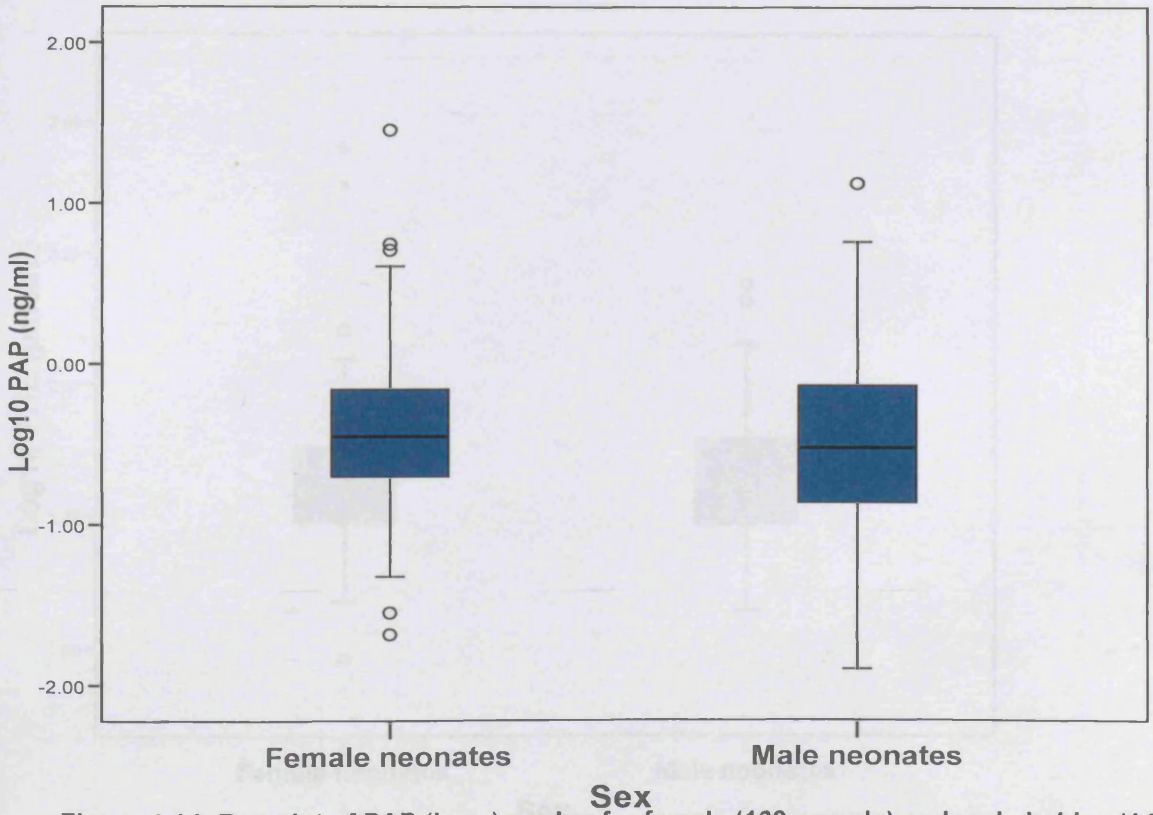

Figure 4-14: Box plot of PAP (log_{10}) marker for female (169 sample) and male babies (144 sample). Circles indicate outlier PAP values.

4.3.3 PHE

Regarding the PHE levels of baby girls, the PHE observation from the SPSS-generated box plot revealed that 50% of these were between 54 and 75 µmol/L and the median was 64.5 µmol/L. The highest value was 280 µmol/L and the lowest value was 30.1 µmol/L (Figure 4.15)

For baby boys, the PHE observation showed that 50% had PHE levels of between 54 and 78 ng/ml and the median was 65.2 µmol/L .The highest value was 155 µmol/L and the lowest set value was 37 µmol/L.

There was no statistically significant differences in the PHE distributions between males and females with increased IRT (p = 0.406).

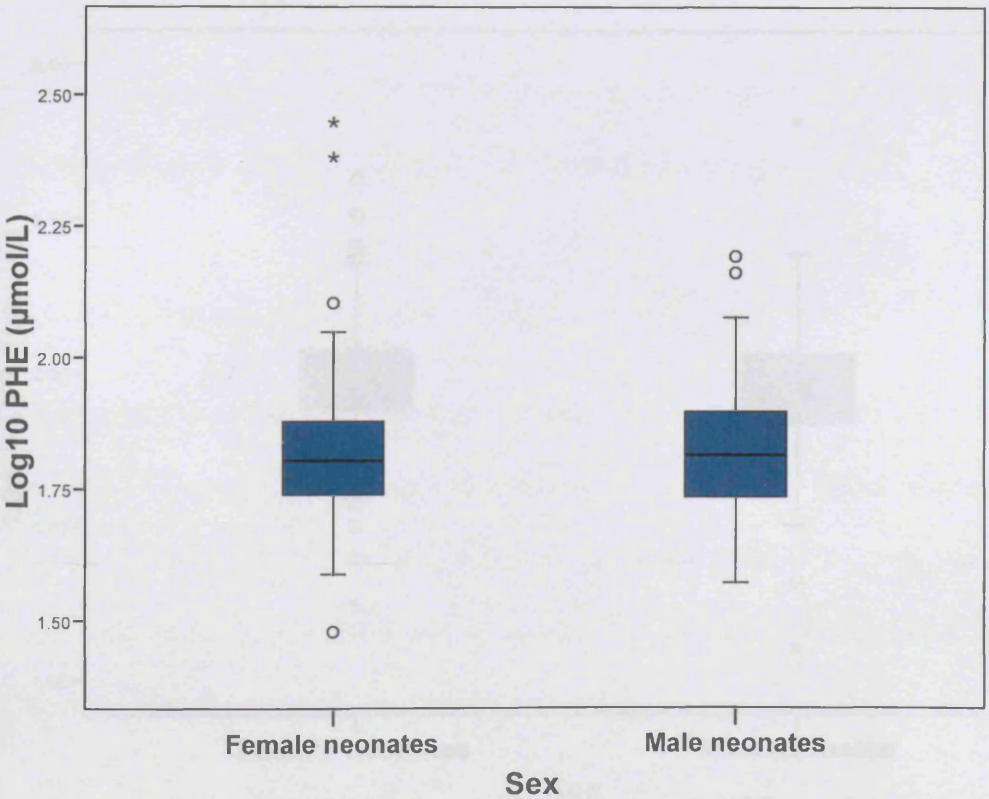

Figure 4-15: Box plot of PHE (log_{10}) marker for female (169 sample) and male babies (144 sample). Asterisks indicate extreme PHE values, while circles mark indicate outlier PHE values.

4.3.4 TYR

The results of the SPSS-generated box plot of TYR levels in baby girls showed that 50% had TYR levels between 75 and 118 μmol/L and a median of 95.5 μmol/L. The highest value was 422 μmol/L and the lowest recorded value was 17 μmol/L (Figure 4.16).

Half of the baby boys had TYR levels of between 68 and 115 μmol/L, and the median was 91.7 μmol/L, The highest value was 654. 5 μ mol/L and the lowest recorded value was 12.5 μ mol/L.

There was no statistically significant differences in the TYR distributions between males and females with increased IRT (p = 0.279).

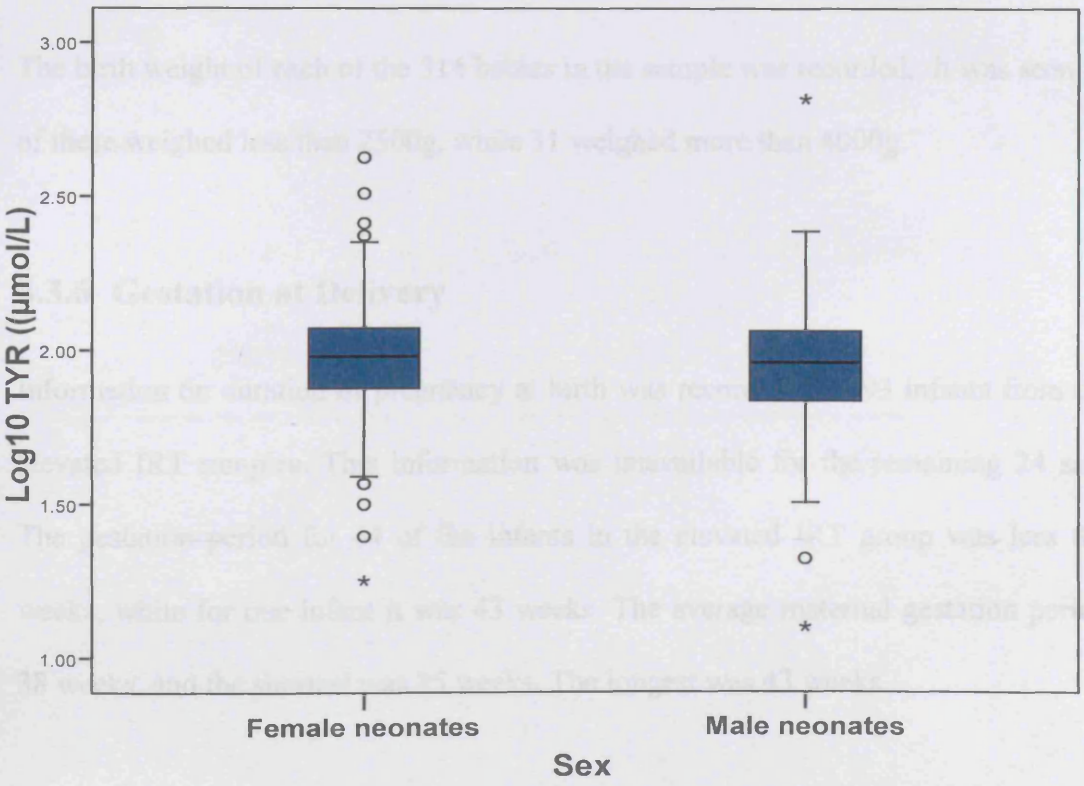

Figure 4-16: Box plot of TYR (log_{10}) marker for female (169 sample) and male babies (144 sample). Asterisks indicate extreme TYR values, while circles mark indicates outliers TYR values.

Using the Mann-Whitney test, the differences in IRT, PAP, PHE and TYR levels were compared in males and females within the high IRT group and with the levels of the same marker in control groups.

This showed that there is a significant difference between level of IRT, PAP and PHE in the increased IRT group and level of IRT, PAP and PHE in the control group. The median level of IRT, PAP and PHE is higher in the high IRT group than the level of IRT, PAP and PHE in control samples.

The p value for each marker was ($p < 0.001$). There was no significant difference between level of TYR in the increased IRT group and level of TYR in the control group ($p = 0.427$).

4.3.5 Birth Weight

The birth weight of each of the 314 babies in the sample was recorded. It was seen that 55 of these weighed less than 2500g, while 31 weighed more than 4000g.

4.3.6 Gestation at Delivery

Information on duration of pregnancy at birth was recorded for 293 infants from the 314 elevated IRT samples. This information was unavailable for the remaining 24 samples. The gestation period for 44 of the infants in the elevated IRT group was less than 37 weeks, while for one infant it was 43 weeks. The average maternal gestation period was 38 weeks, and the shortest was 25 weeks. The longest was 43 weeks.

4.3.7 Age at Sampling

The age of the infant at which the specimen was obtained was recorded for 313 of the 314 infants with elevated IRT. It was recorded that in 285 samples the dried blood spot specimens were obtained before 10 days of life, while 28 samples were obtained on or after day 10 of the baby's life (tables 4.5 and 4.6).

The youngest baby at the time of taking a blood sample was 3-day-old female, born at 32 weeks gestation, with an IRT level of 76.6 ng /ml and PAP level with 0.05 ng/ml. The oldest baby to have a blood sample taken was 91 days of age; this was a female born at 40 weeks gestation, with an IRT level of 88 ng /ml and PAP level of 4.0ng/ ml. Figure 4.17 and 4.18 show the levels of IRT and PAP relative to the age of sampling.

Table 4-5: Mean and median at age of sampling PAP in increased IRT group.

Age /days	3		4		5		6		7		8		9		≥ 10	
Sex	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Number	1	0	24	25	90	70	29	23	7	4	2	6	2	1	13	15
Total	1		49		160		52		11		8		3		28	
Mean PAP (ng/ml)	0.0473		0.3847		0.6236		0.5374		0.6672		0.9343		2.0536		2.6453	
Median PAP (ng/ml)	0.04738		0.2817		0.3073		0.3405		0.6939		0.5598		1.1766		0.9725	

Table 4-6: Mean and median of IRT in increased IRT group in 313 samples in female neonates and male neonates at the age of specimen collection.

Age /days	3		4		5		6		7		8		9		≥ 10	
Sex	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Number	1	0	24	25	90	70	29	23	7	4	2	6	2	1	13	15
Total	1		49		160		52		11		8		3		28	
Mean IRT (ng/ml)	76.58		118.88		106.51		109.54		128.072		90.37		122.95		94.50	
Median IRT (ng/ml)	76.58		89.95		87.56		90.160		92.430		84.86		121.61		82.38	

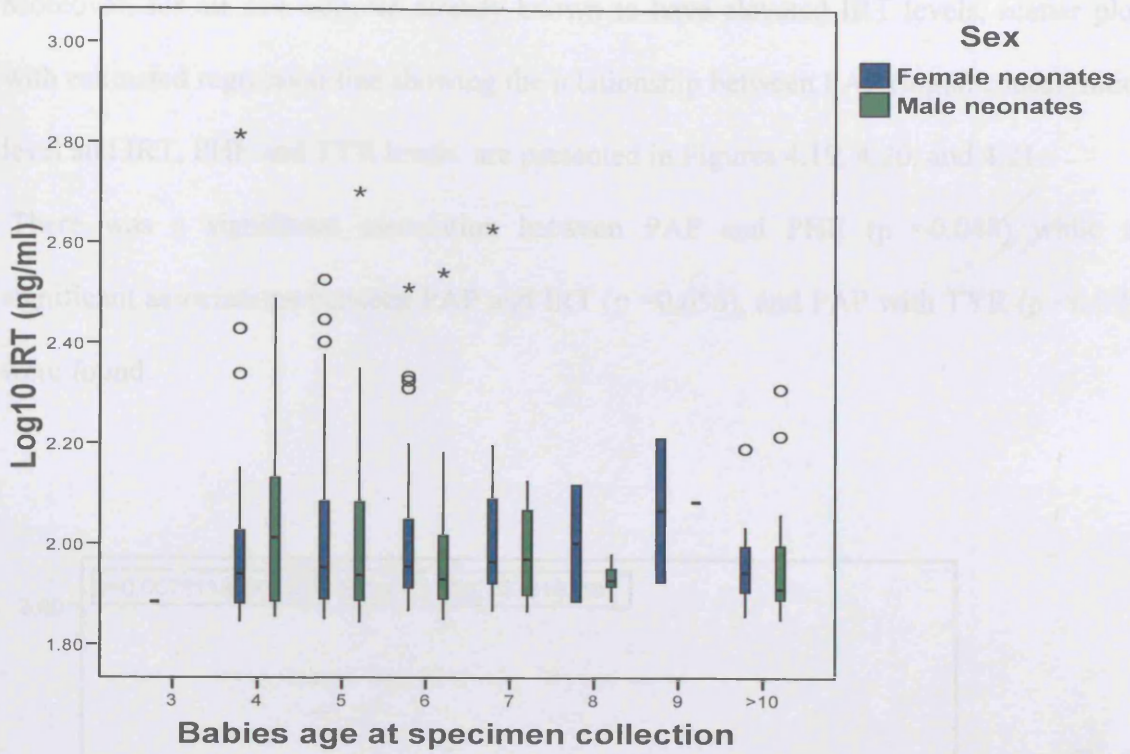

Figure 4-17: Box plot of IRT in increased IRT (log_{10}) group in female neonates and male neonates at the age of specimen collection. Asterisks indicate extreme values, while circles indicate outlier values.

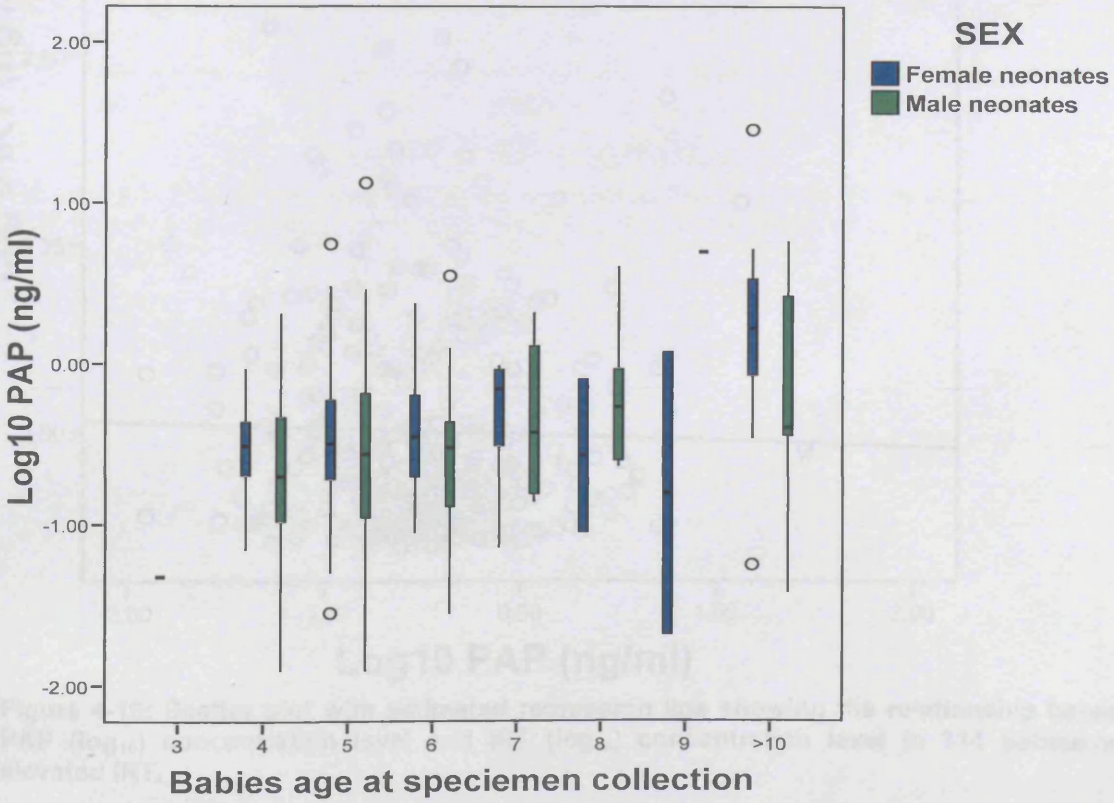

Figure 4-18: Box plot of PAP in increased PAP (log_{10}) group in female neonates and male neonates at the age of specimen collection. Circles indicate outlier values.

Moreover, for all 314 samples already known to have elevated IRT levels, scatter plots with estimated regression line showing the relationship between PAP (log_{10}) concentration level and IRT, PHE and TYR levels are presented in Figures 4.19, 4.20, and 4.21.

There was a significant association between PAP and PHE (p =0.048) while no significant associations between PAP and IRT (p =0.656), and PAP with TYR (p =0.075) were found.

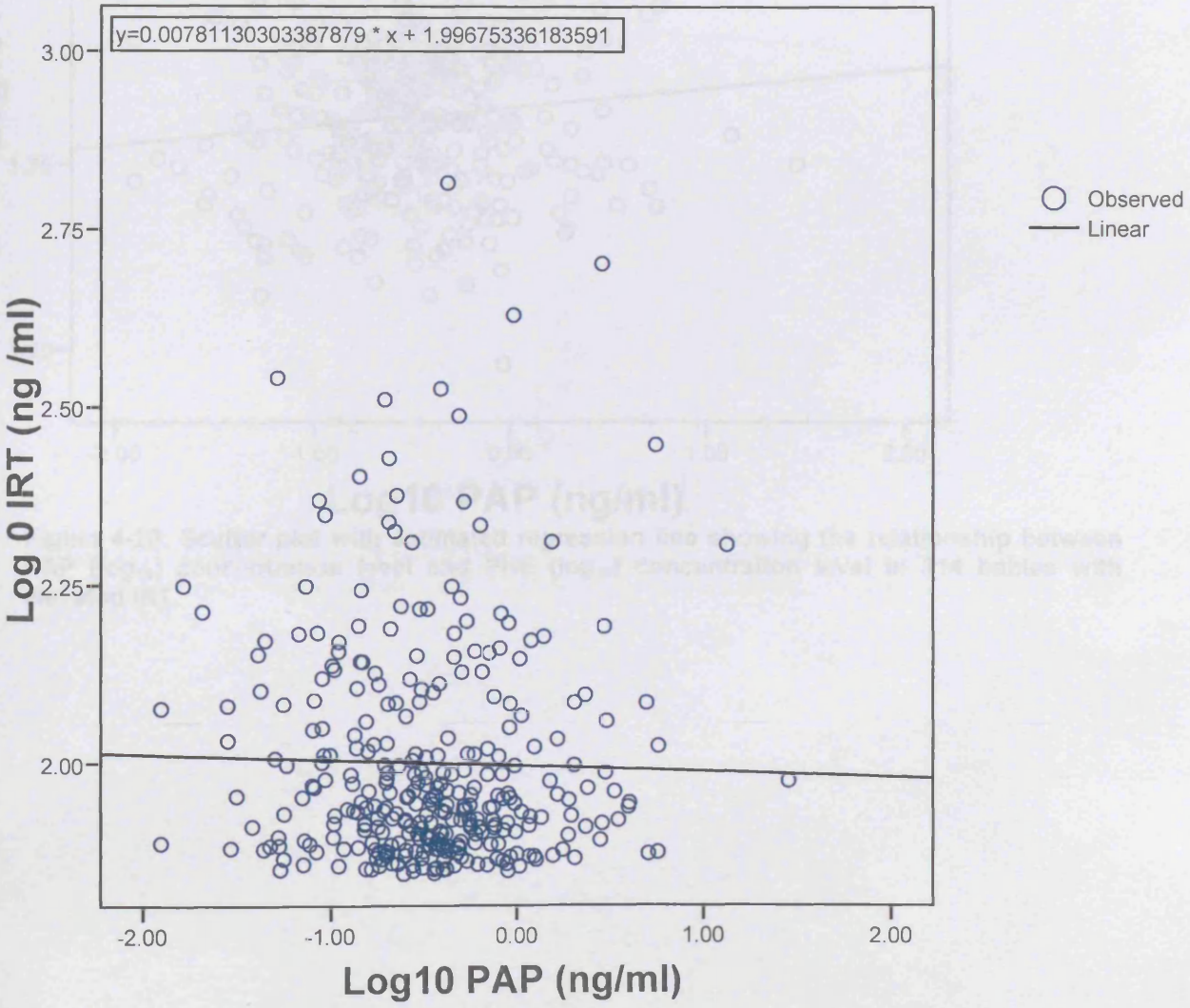

Figure 4-19: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and IRT (log_{10}) concentration level in 314 babies with elevated IRT.

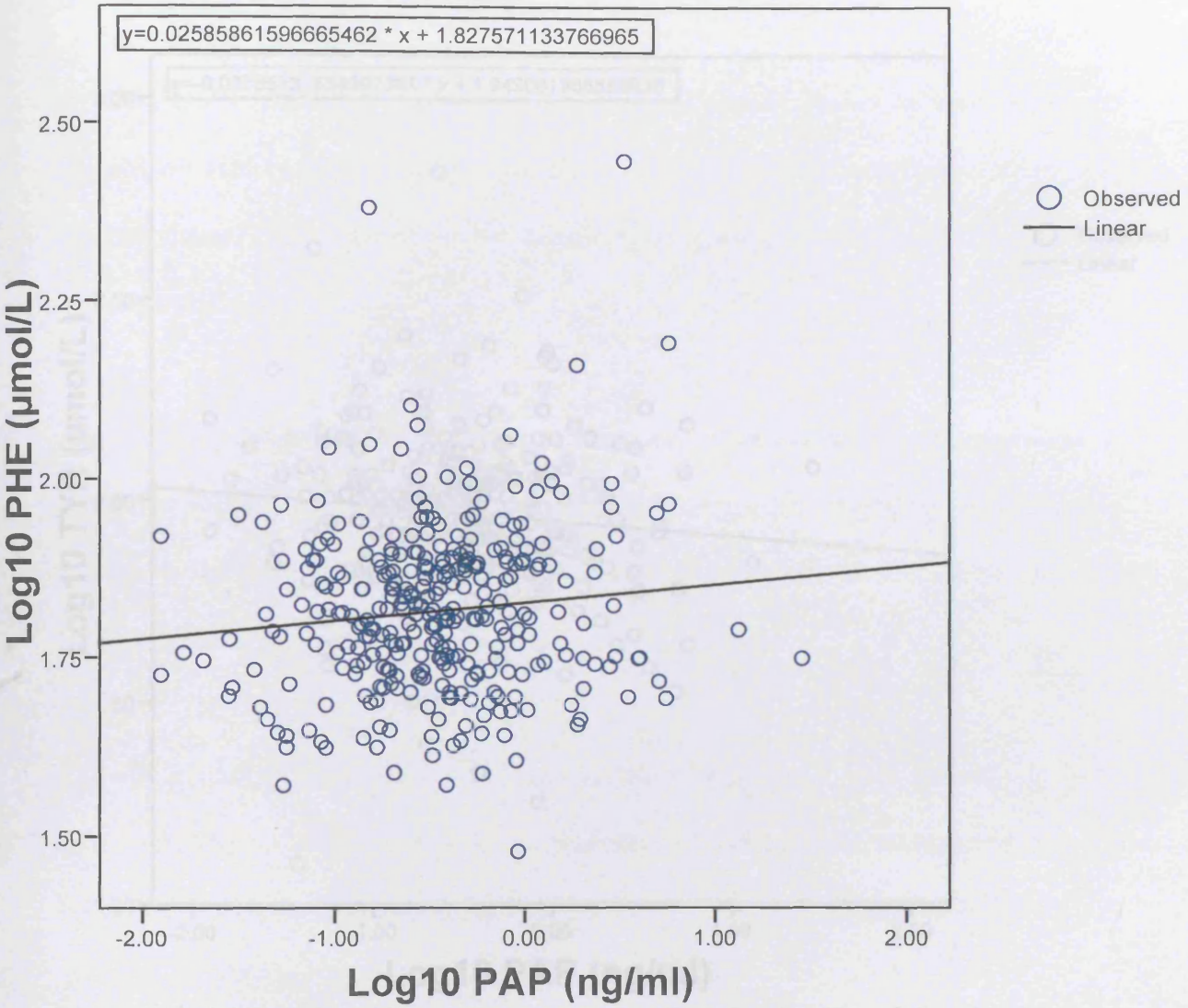

Figure 4-20: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and PHE (log_{10}) concentration level in 314 babies with elevated IRT.

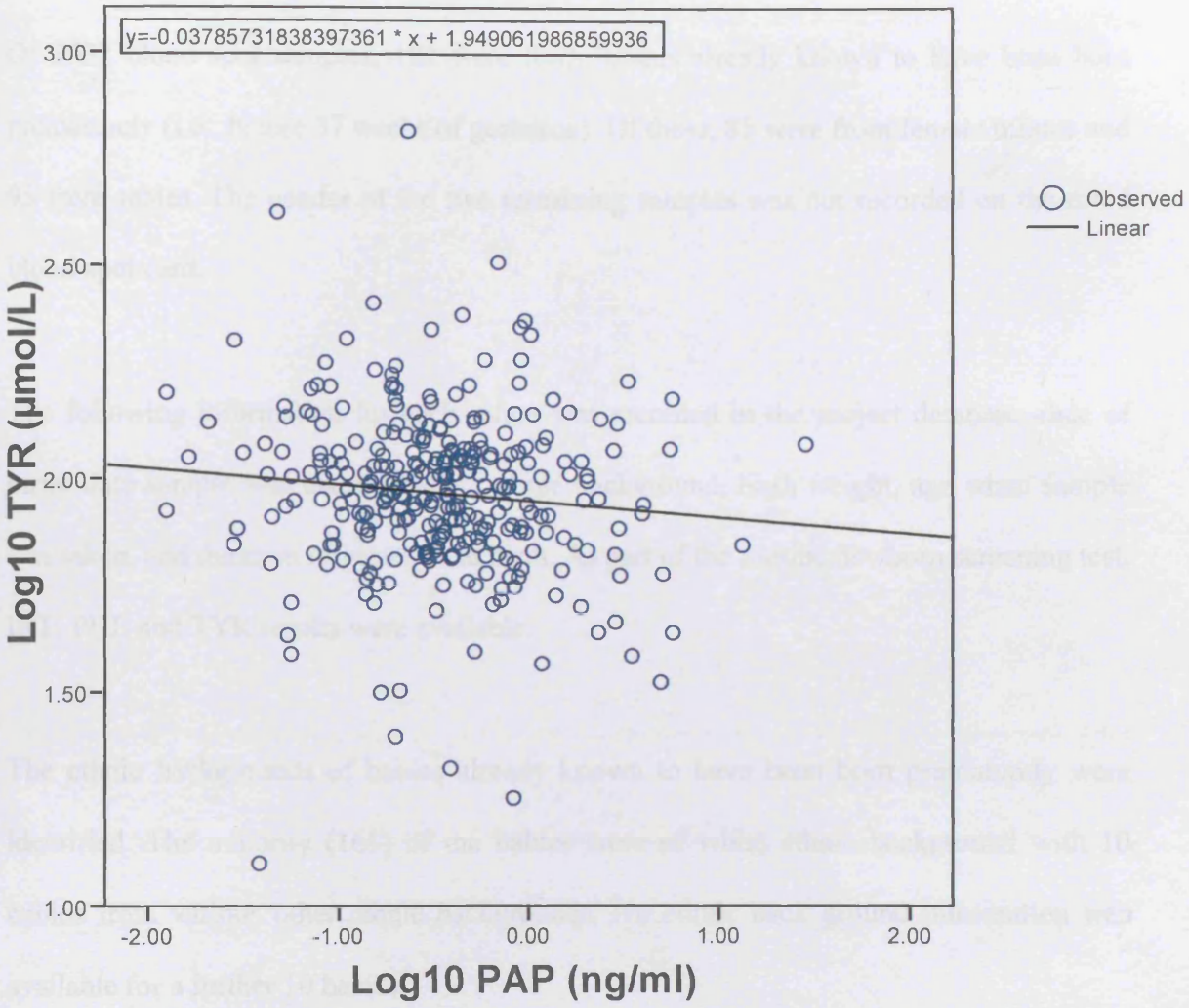

Figure 4-21: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and TYR (log_{10}) concentration level in 314 babies with elevated IRT

4.4 PREMATURE BABIES

Of 3632 blood spot samples, 182 were from infants already known to have been born prematurely (i.e., before 37 weeks of gestation). Of these, 85 were from female infants and 95 from males. The gender of the two remaining samples was not recorded on the dried blood spot card.

The following information for each infant was recorded in the project database: date of birth; date sample was taken; gender; ethnic background; birth weight, age when sample was taken, and duration of gestation at birth. As part of the routine newborn screening test, IRT, PHE and TYR results were available.

The ethnic backgrounds of babies already known to have been born prematurely were identified. The majority (161) of the babies were of white ethnic background with 10 babies from various other ethnic backgrounds. No ethnic back ground information was available for a further 10 babies.

Out of a total of 182 babies, the shortest recorded period of gestation at birth was 25 weeks and the maximum gestation period was 36 weeks. For the 182 babies, median values of IRT, PAP, PHE and TYR markers were calculated for each week of gestation. Table 4.7 and 4.8 show the median levels of the above markers and for each sex at each week of gestation.

Table 4-7: Median IRT, PAP, PHE, and TYR in premature femal babies.

Gestation (weeks)	Number	Median IRT (ng /ml)	Median PAP (ng/ml)	Median PHE (μ mol /L)	Median TYR (μ mol /L)
25	1	21.6	1.1	79.6	72.3
27	3	32.2	2.2	36.8	106.8
28	2	28	3.5	49	119.8
29	2	22.8	1.7	68.5	219.4
30	3	22.2	0.17	65.6	237.1
31	6	19.4	0.2	71.5	281.2
32	8	20.7	0.14	52.9	115.8
33	5	39.9	0.15	57	122.2
34	12	19.6	0.15	72	122
35	17	17.9	0.12	56.1	98.5
36	26	19.9	0.19	54.4	103.1

Table 4-8: Median IRT, PAP, PHE and TYR in premature male babies.

Gestation (weeks)	Number	Median IRT (ng /ml)	Median PAP (ng/ml)	Median PHE (μ mol /L)	Median TYR (μ mol /L)
26	1	47.2	2.1	69.5	50.5
27	1	19.2	0.17	54.8	70
28	1	33.8	0.08	53.3	136.7
29	2	32.9	2.4	41.7	175
30	6	21	1.3	60.3	162.2
31	6	21.6	0.11	58.4	123.7
32	22	22	0.13	64.1	152.8
33	9	22.9	0.10	51.9	133.4
34	9	20.1	0.27	54.5	128.3
35	17	21.5	0.18	56.7	100.7
36	21	16.6	0.20	62.5	91.2

Using the Mann-Whitney test, the differences in IRT, PAP, PHE and TYR levels were compared in males and females within the premature group. The data showed no statistical significant difference between males and females for IRT ($p = 0.534$), PAP ($p = 0.658$), PHE ($p = 0.968$), and TYR ($p = 0.695$).

In addition to discover any relationship between premature (less than 37 weeks) babies and levels of IRT, PAP, PHE and TYR concentrations measured in ng/ml, a linear regression was performed (figure 4.22 , 4.23, 4.24, and 4.25).

All markers showed a downward trend with advancing gestation. This was statistically significant for IRT ($p < 0.001$), PAP ($p < 0.001$) and TYR ($p = 0.015$) but not statistically significant for PHE ($p = 0.226$).

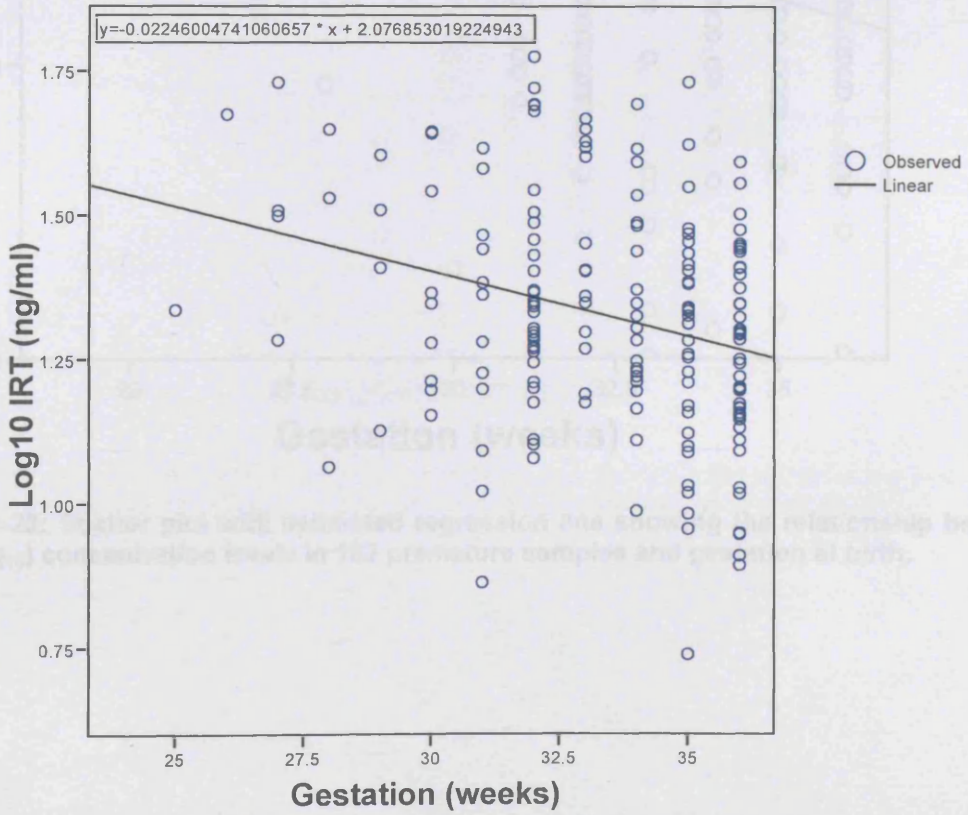

Figure 4-22: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 182 premature samples and gestation at birth.

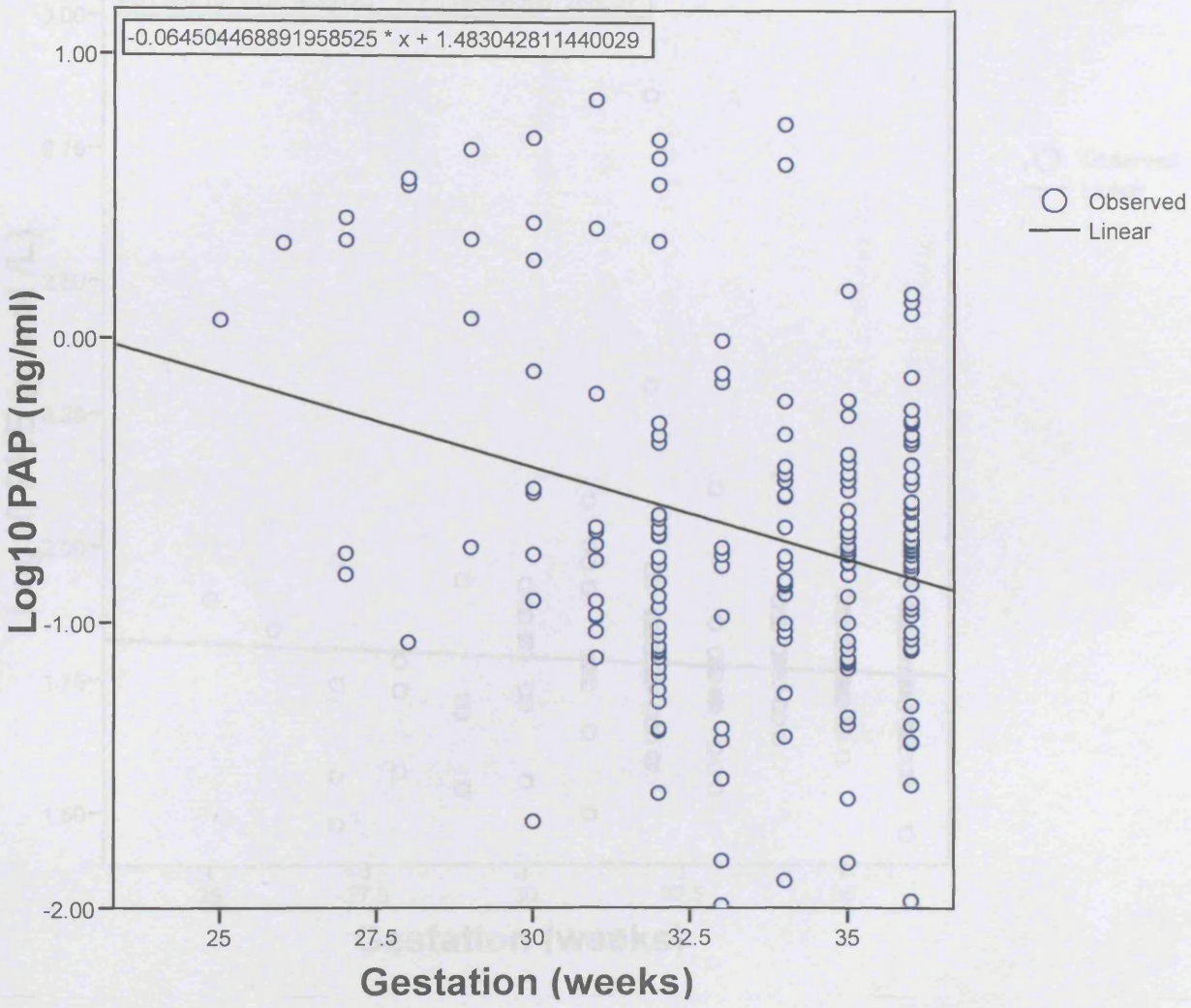

Figure 4-23: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 182 premature samples and gestation at birth.

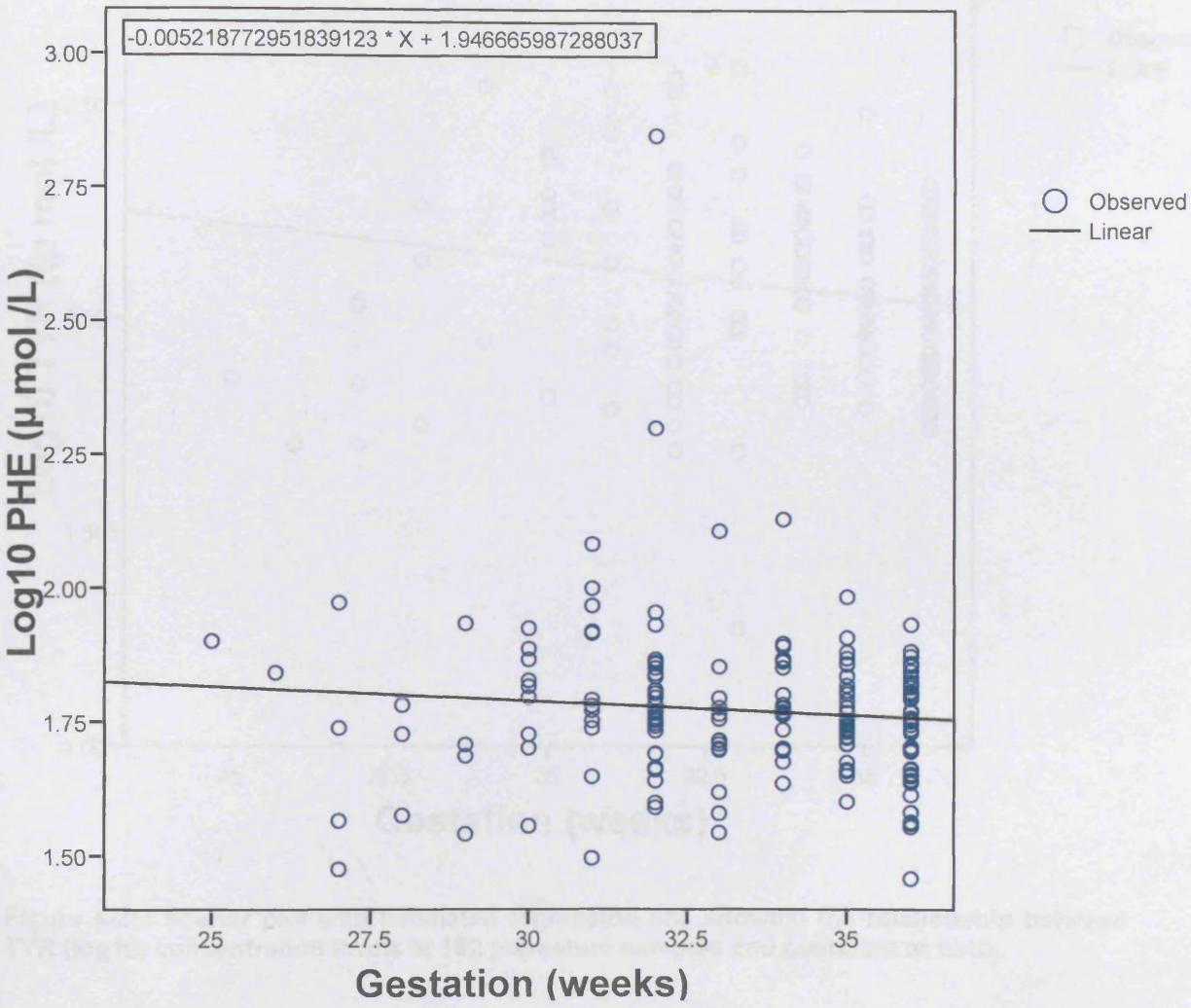

Figure 4-24: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 182 premature samples and gestation at birth.

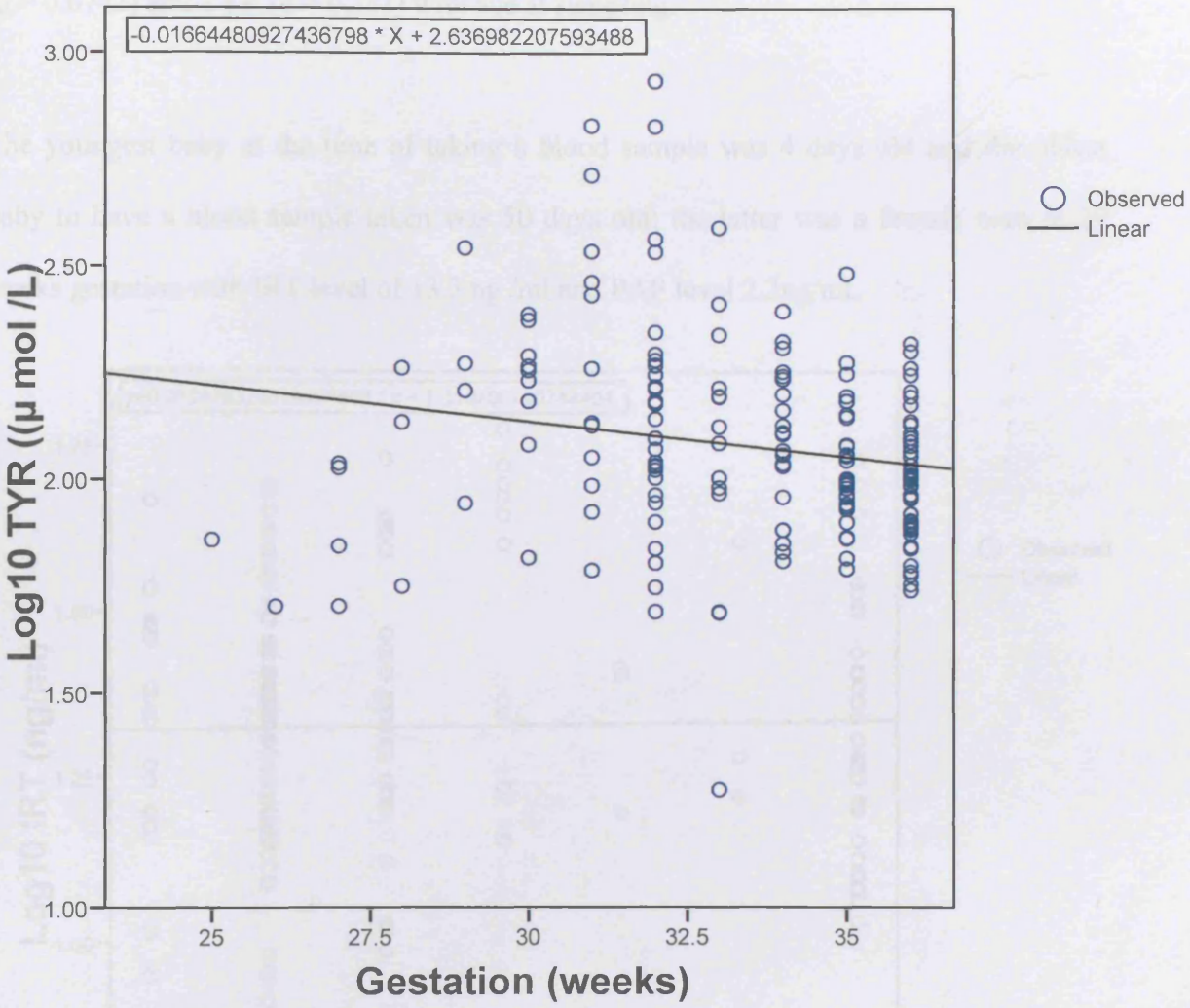

Figure 4-25: Scatter plot with estimated regression line showing the relationship between TYR (log10) concentration levels in 182 premature samples and gestation at birth.

For 152 of the babies, the dried blood spot specimens were taken before the first 10 days of life, while in another 30 dried blood spot samples were collected on or after day 10 day.

In addition, for all 182 babies known to be born prematurely, scatter plots with an estimated regression line showing the relationship between the ages of specimen and IRT, PAP, PHE and TYR concentration are presented (figure 4.26, 4.27, 4.28 and 4.29). Regression analysis showed that PAP levels decreased significantly ($p < 0.001$) with earlier age at sampling whereas there was a significant increase in the PHE level (p

=0.006) associated with earlier sampling. No significant associations were found for IRT ($p = 0.0742$) and TYR ($p = 0.551$) with age at sampling.

The youngest baby at the time of taking a blood sample was 4 days old and the oldest baby to have a blood sample taken was 50 days old; the latter was a female born at 29 weeks gestation with IRT level of 13.3 ng /ml and PAP level 2.2ng/ml.

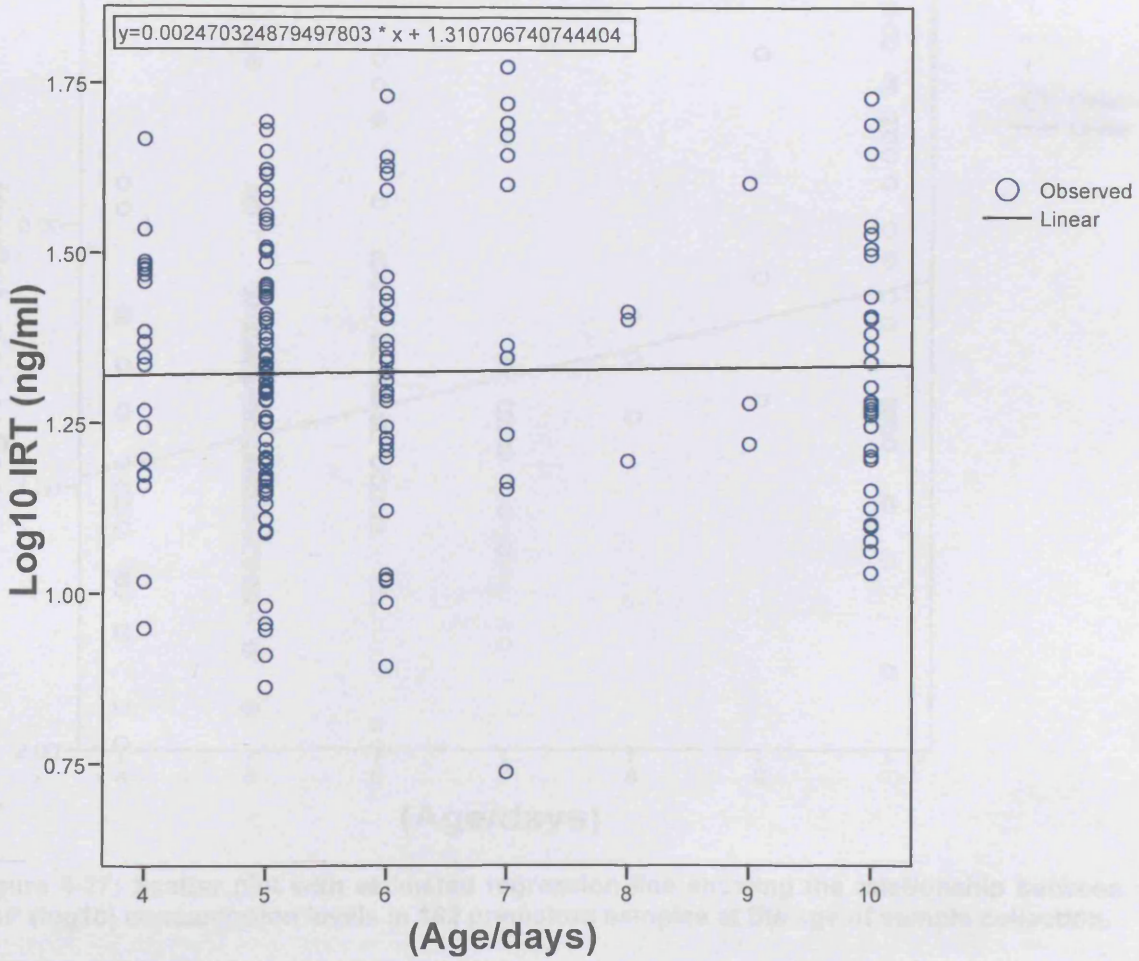

Figure 4-26: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 182 premature samples at the age of sample collection.

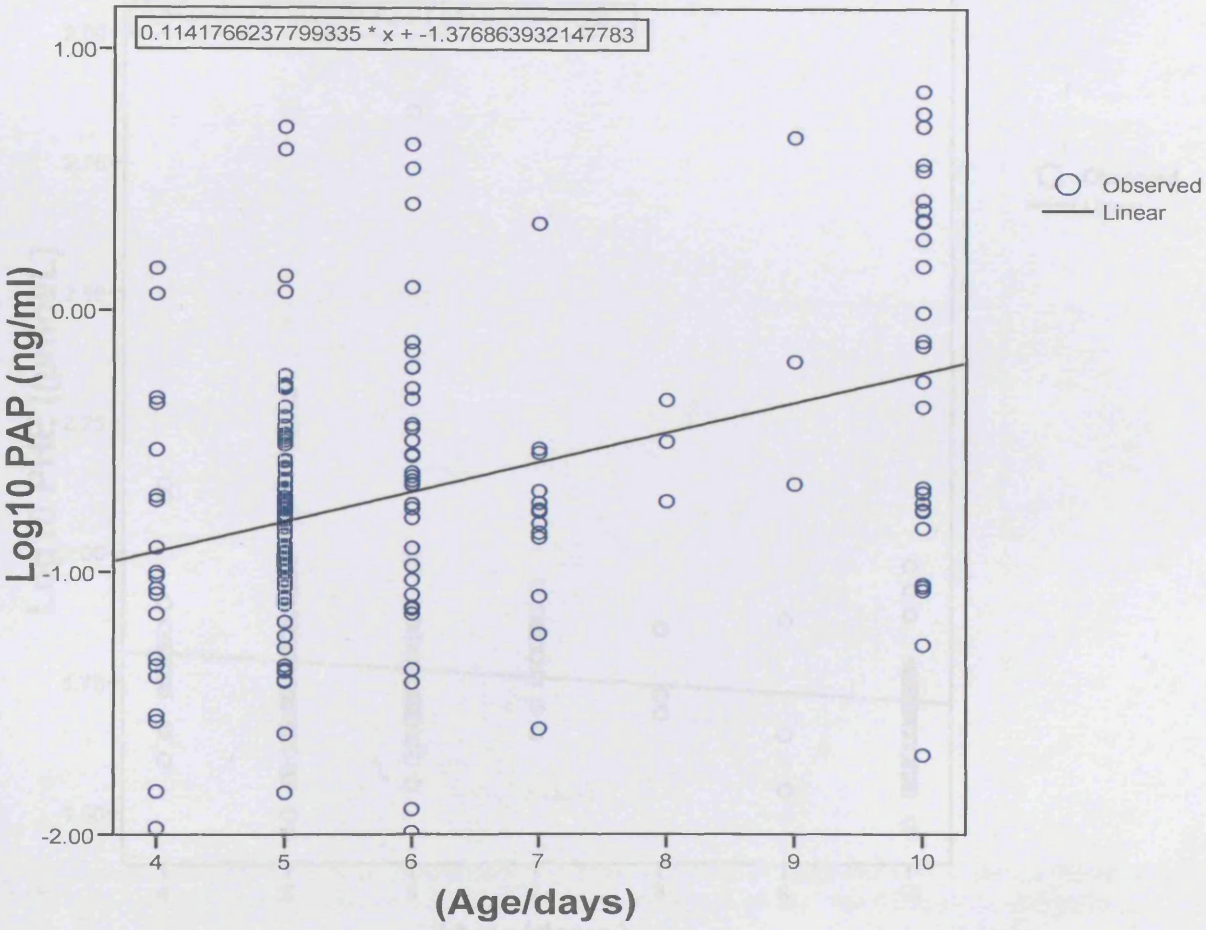

Figure 4-27: Scatter plot with estimated regression line showing the relationship between PAP (log10) concentration levels in 182 premature samples at the age of sample collection.

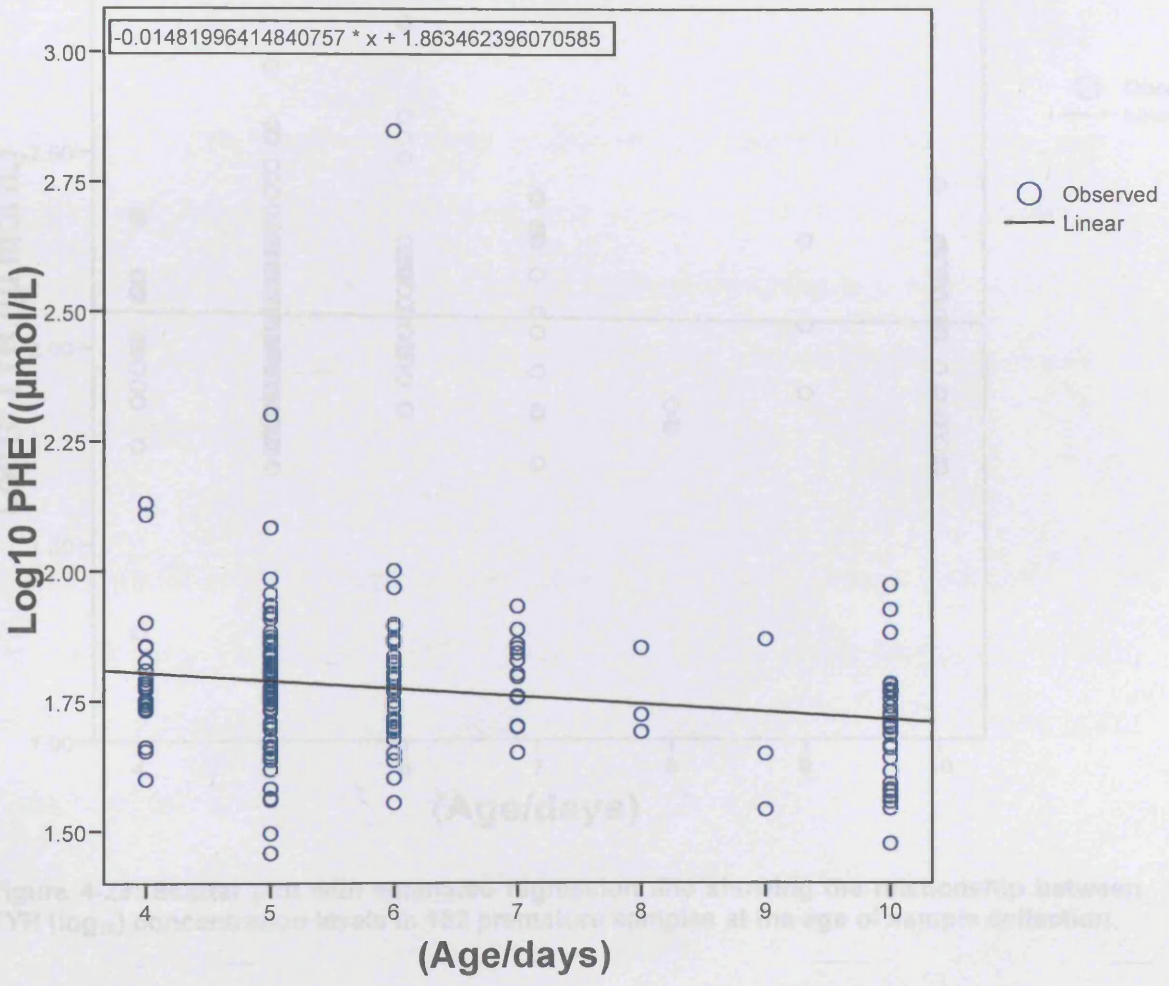

Figure 4-28: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 182 premature samples at the age of sample collection.

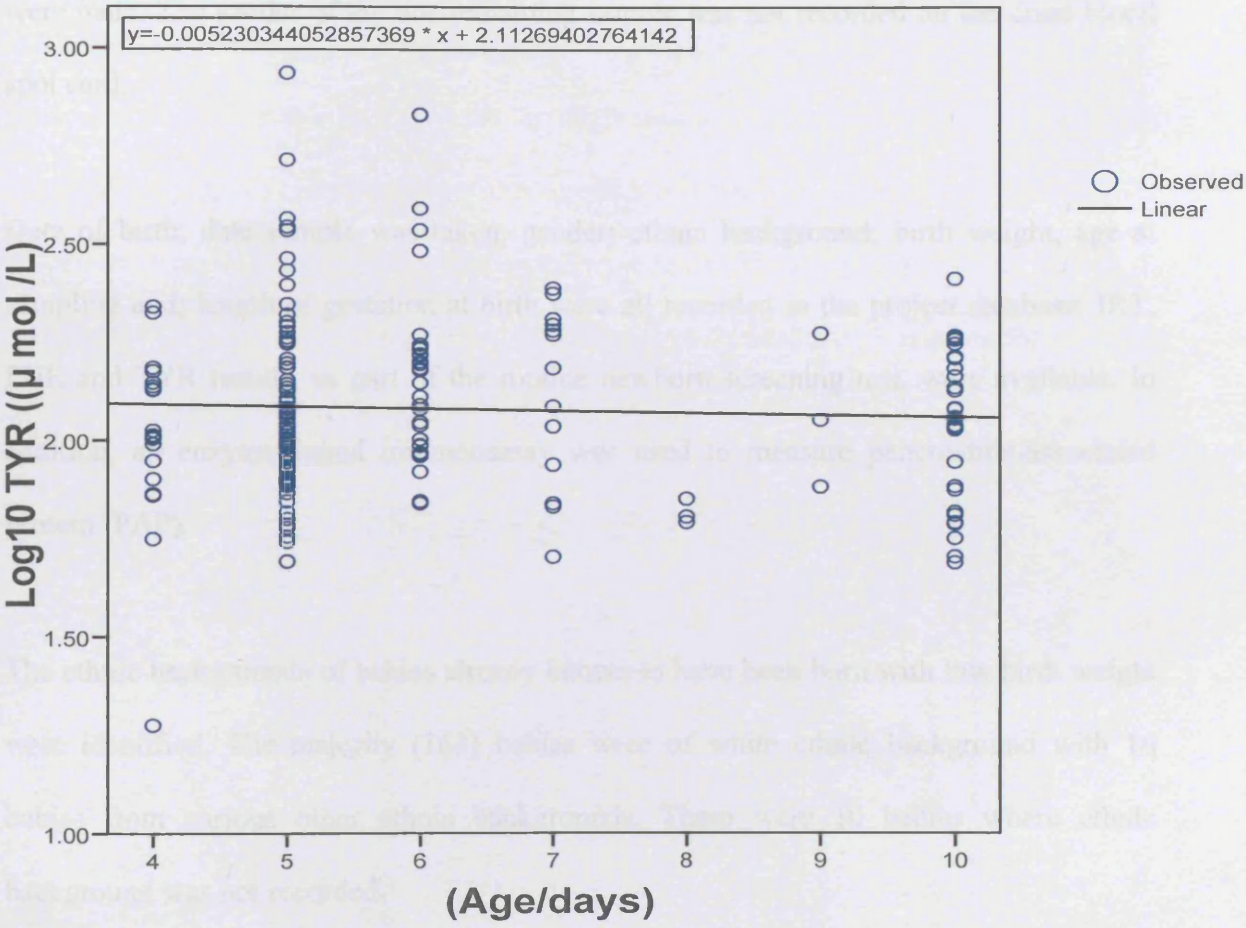

Figure 4-29: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration levels in 182 premature samples at the age of sample collection.

4.5 LOW BIRTH WEIGHT BABIES

Of 3632 blood spot samples, 189 were from infants known to have been born with low birth weight (less than 2500 grams). One hundred of these were female infants, while 88 were male. The gender of the one remaining sample was not recorded on the dried blood spot card.

Date of birth; date sample was taken; gender; ethnic background; birth weight, age at sampling and; length of gestation at birth were all recorded in the project database. IRT, PHE and TYR results, as part of the routine newborn screening test, were available. In addition, an enzyme-linked immunoassay was used to measure pancreatitis-associated protein (PAP).

The ethnic backgrounds of babies already known to have been born with low birth weight were identified. The majority (163) babies were of white ethnic background with 16 babies from various other ethnic backgrounds. There were 10 babies where ethnic background was not recorded.

From a total of 189 low birth weight babies, the shortest recorded period of gestation was 25 weeks, while the maximum gestation period was 42 weeks. 136 of the dried blood spot samples were from babies born at less than 37 weeks and 44 babies were born at 37 weeks or later.

In 140 of these babies, the dried blood spot specimens were obtained before the first 10 days of age while in the other 48, the dried blood spot specimens were collected on or after day 10. The youngest baby was 3 days old. The oldest baby when the sample was collected was 59 days old.

IRT, PAP, PHE and TYR levels at the age of specimen collection were analysed for all 188 babies with low birth weight. Scatter plots with estimated regression lines showing the relationship between the ages of specimen and IRT, PAP, PHE, and TYR .concentration level are presented in figures 4.30, 4.31, 4.32 and 4.33. Regression analysis showed that PAP levels in LBW babies decreased significantly with earlier sampling ($p<0.001$) (figure 4.31) while there was a significant increase in the PHE level ($p = 0.001$) associated with earlier sampling. No significant associations were found for IRT ($p = 0.187$) and TYR ($p =0.888$) with age at sampling. These results are similar to those found for the same analysis in premature babies.

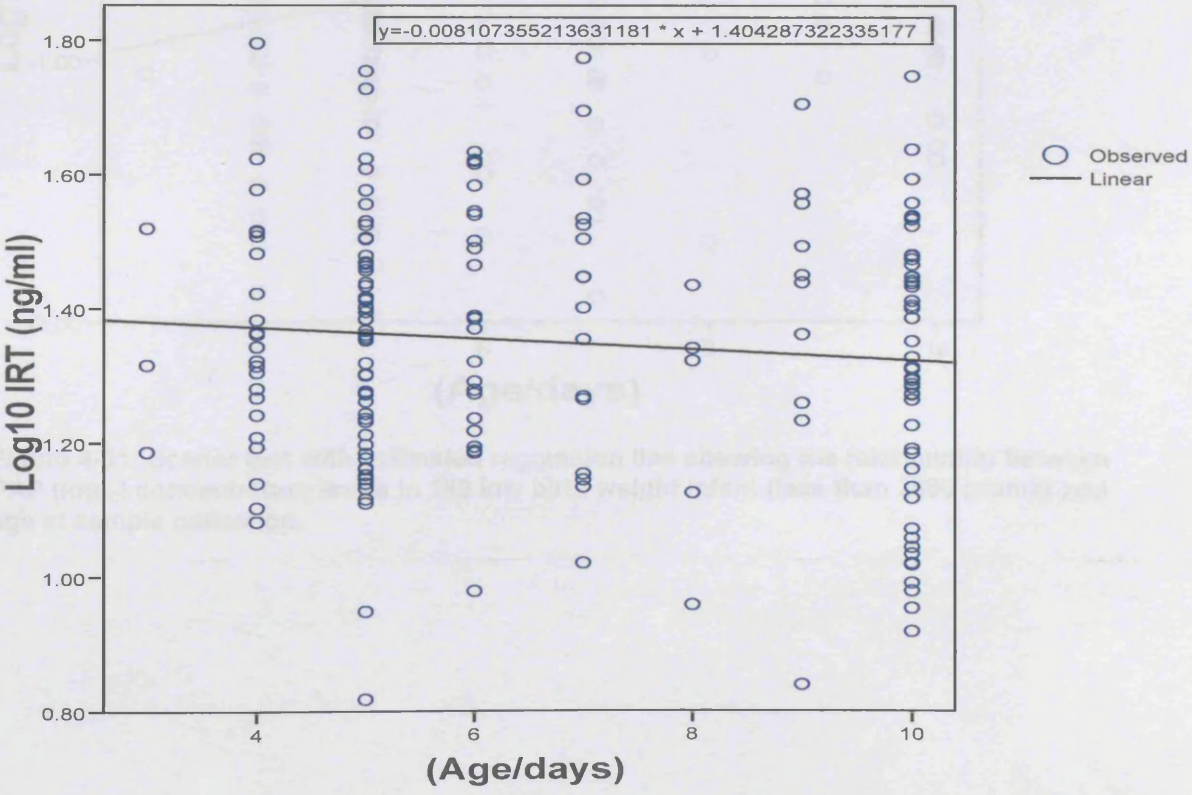

Figure 4-30: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration levels in 189 low birth weight infant (less than 2500 grams) and age at sample collection.

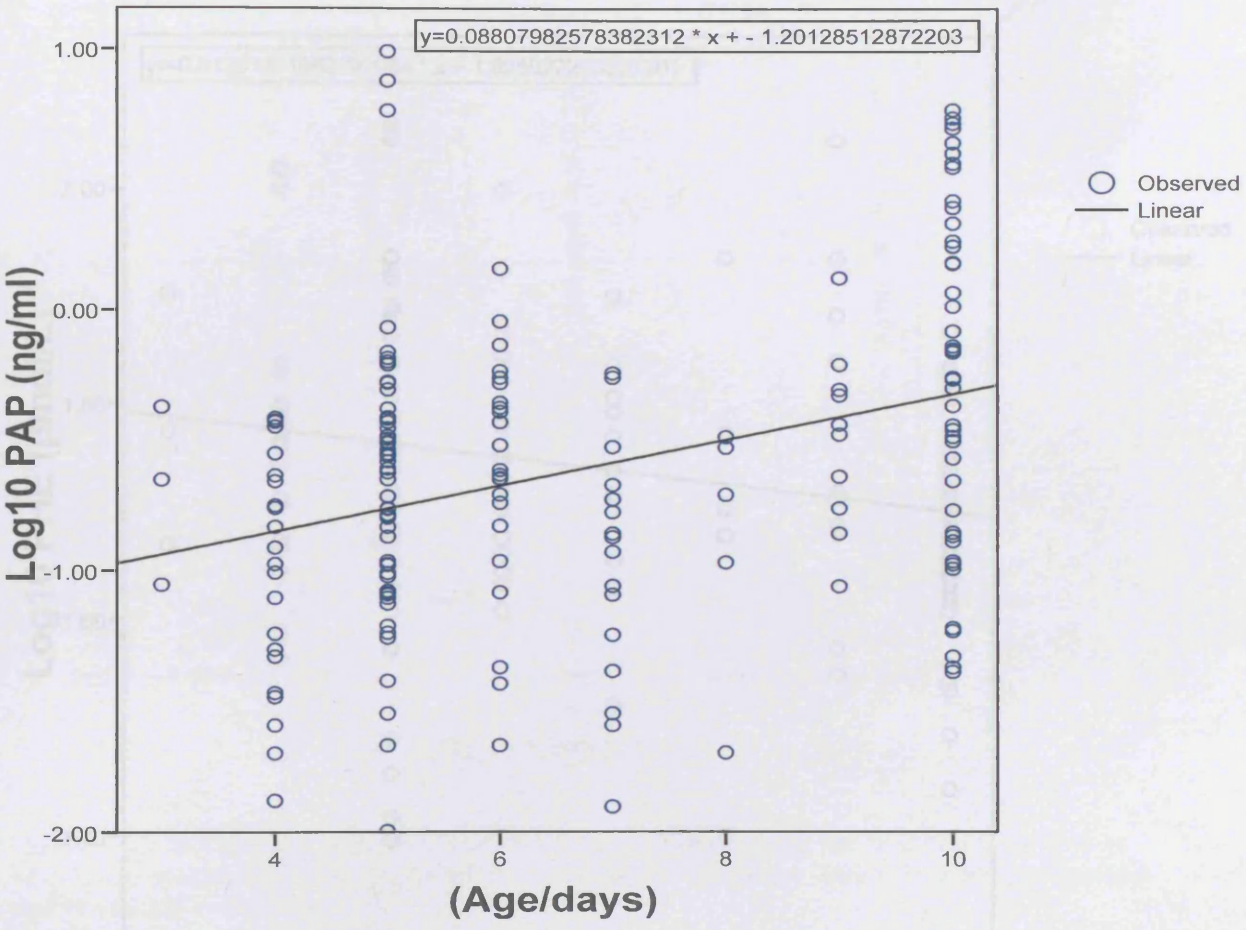

Figure 4-31: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration levels in 189 low birth weight infant (less than 2500 grams) and age at sample collection.

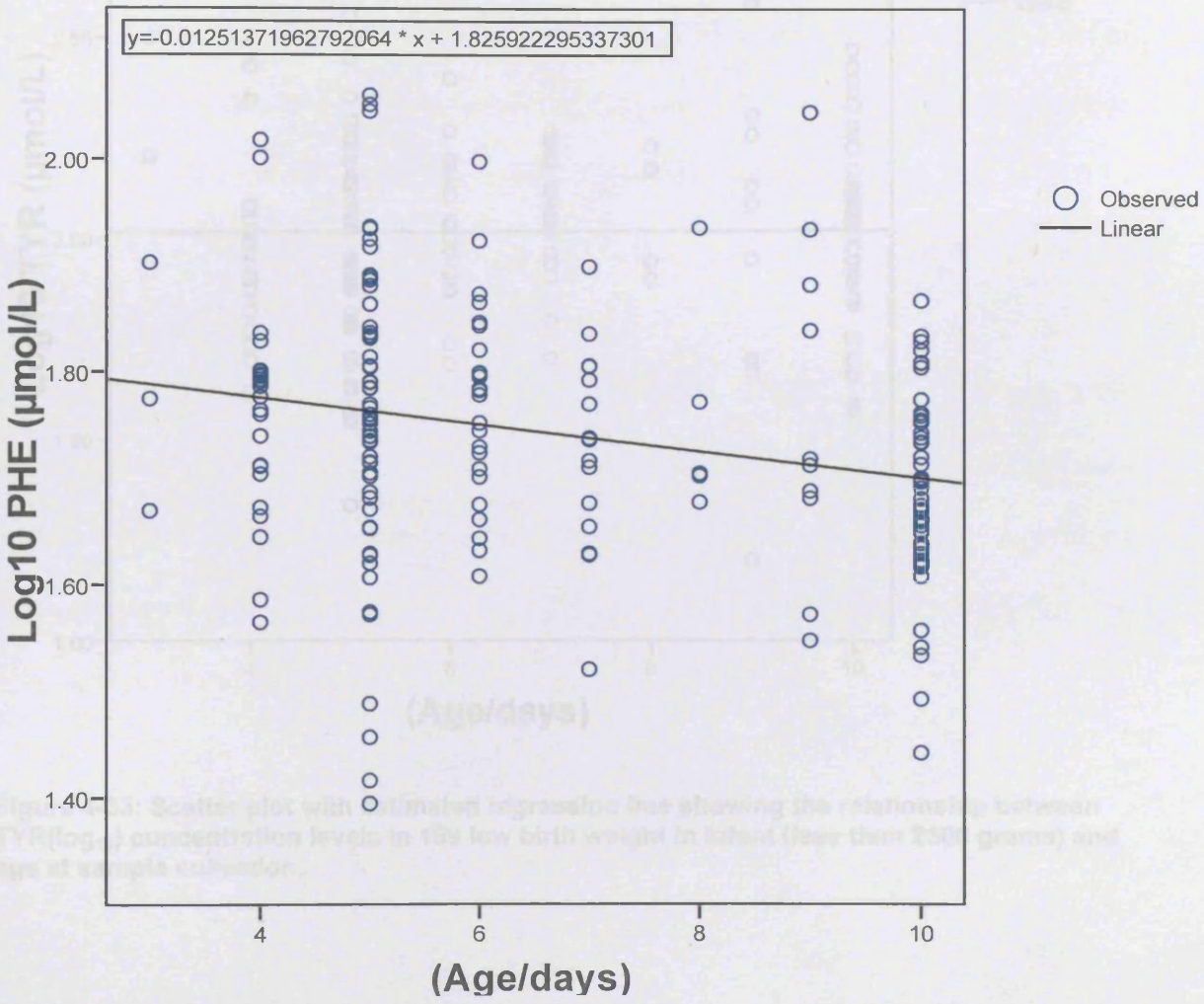

Figure 4-32: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration levels in 189 low birth weight infant (less than 2500 grams) and age at sample collection.

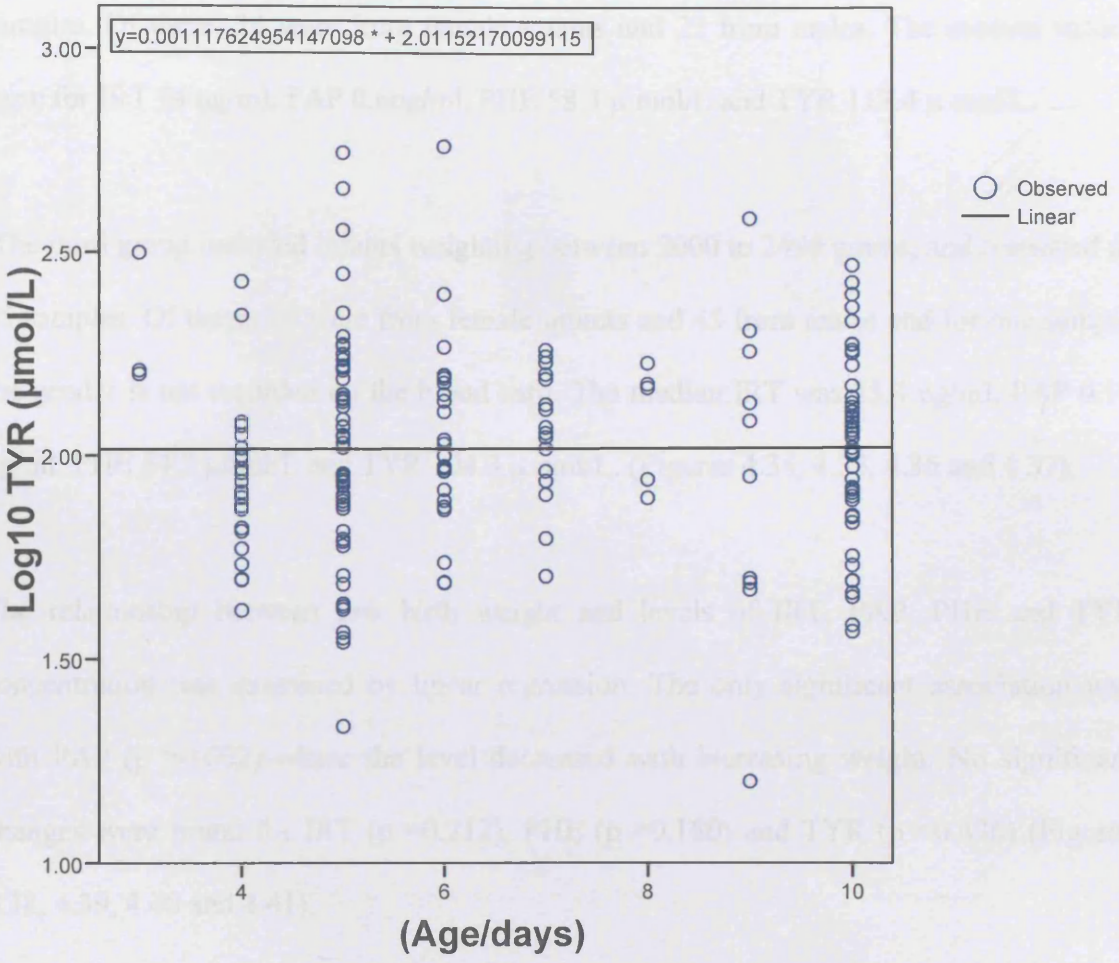

Figure 4-33: Scatter plot with estimated regression line showing the relationship between TYR(log_{10}) concentration levels in 189 low birth weight in infant (less than 2500 grams) and age at sample collection.

The birth weight of all 189 babies in the sample was recorded and all missing samples were excluded, the lowest being recorded as 760g and the highest 2490g. To calculate the median for all above markers, the blood samples were divided into three groups.

The first group included all babies born with a birth weight less than 1500 grams comprising 48 samples. Of these, 27 were female infants and 21 males. The median IRT in this group was 19.7 ng/ml, median PAP was 0.58 ng/ml, median PHE 53.3 µ mol/L and median TYR 86.8µmol/L.

The Second group comprised infant between 1500 to 1999 grams, and included 46 samples. Of these, 24 were from female infants and 22 from males. The median values were for IRT 24 ng/ml, PAP 0.6ng/ml, PHE 58.4 μ mol/L and TYR 117.4 μ mol/L.

The third group included infants weighting between 2000 to 2499 grams, and consisted of 95 samples. Of these, 49 were from female infants and 45 from males and for one sample the gender is not recorded on the blood card. The median IRT was 23.4 ng/ml, PAP 0.19 ng/ml, PHE 54.2 μmol/L and TYR 104.3 μ mol/L. (Figures 4.34, 4.35, 4.36 and 4.37).

The relationship between low birth weight and levels of IRT, PAP, PHE and TYR concentration was examined by linear regression. The only significant association was with PAP ($p = 0.002$) where the level decreased with increasing weight. No significant changes were found for IRT ($p = 0.212$), PHE ($p = 0.180$) and TYR ($p = 0.436$) (Figures 4.38, 4.39, 4.40 and 4.41).

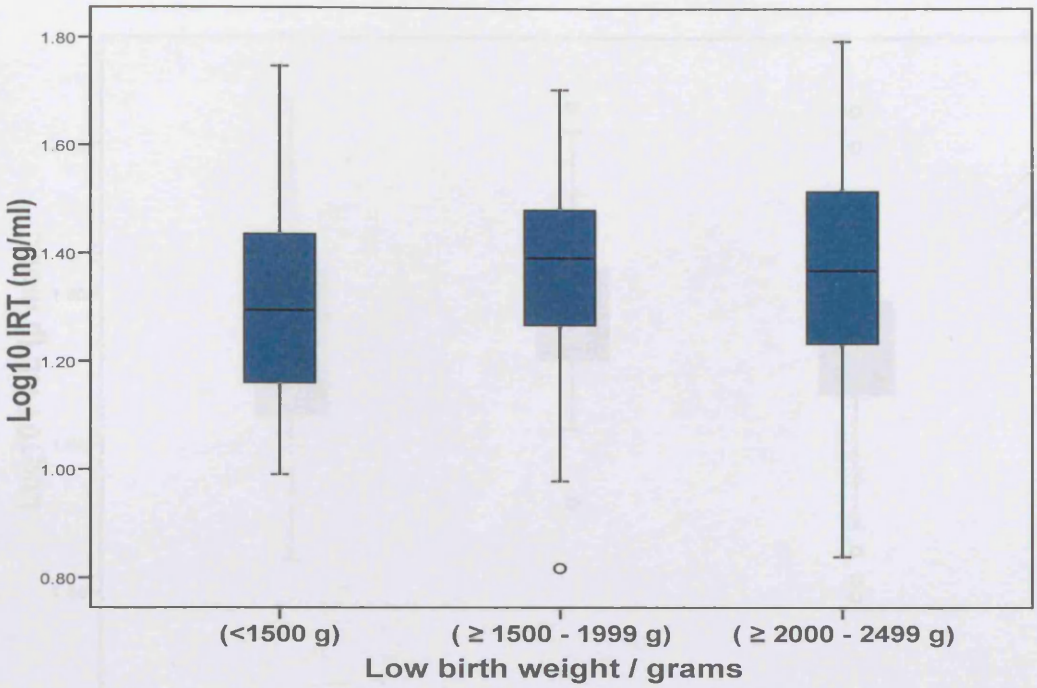

Figure 4-34: Box plot shows the median of IRT (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circles indicate outlier values.

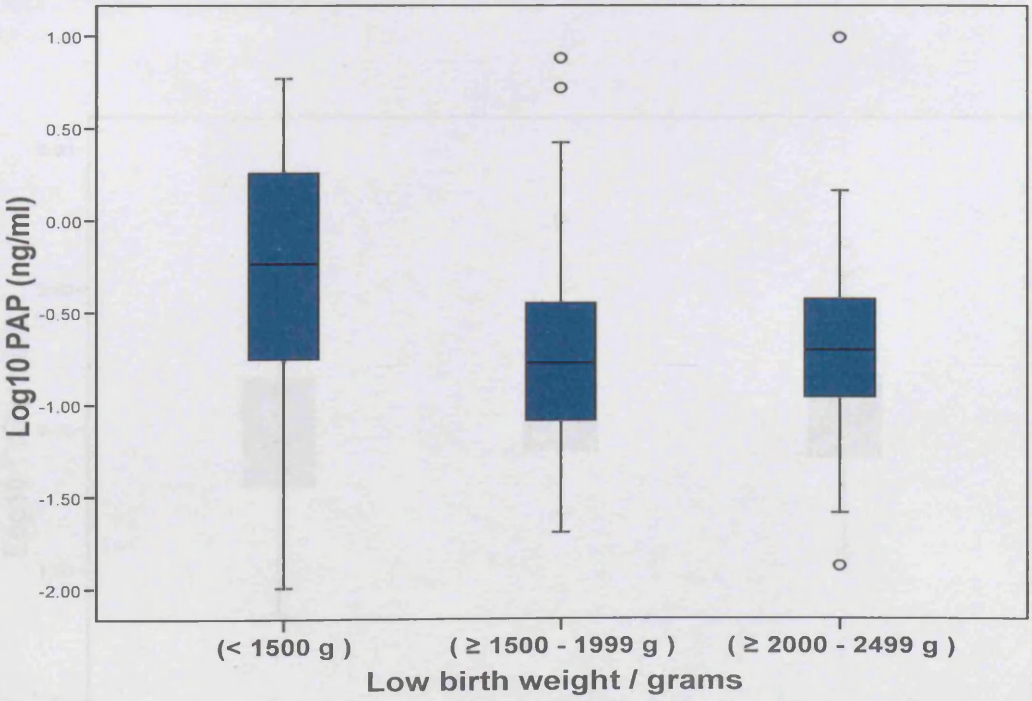

Figure 4-35: Box plot shows the median of PAP (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circles indicate outlier values.

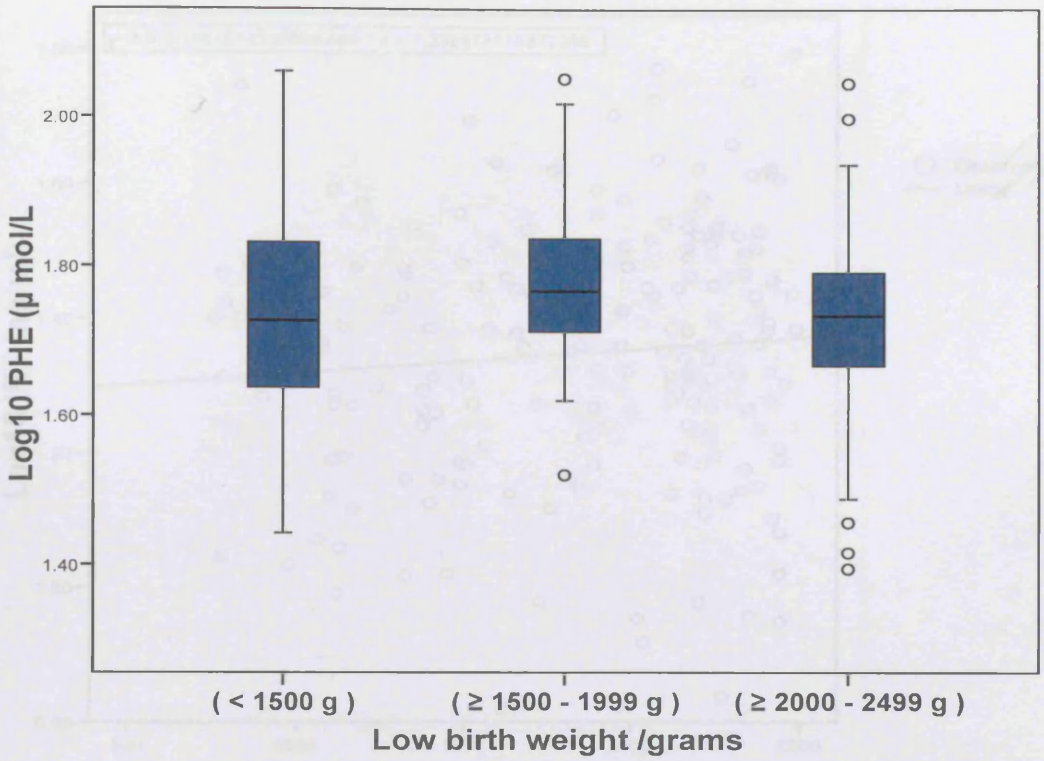

Figure 4-36: Box plot shows the median of PHE (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circles indicate outlier values.

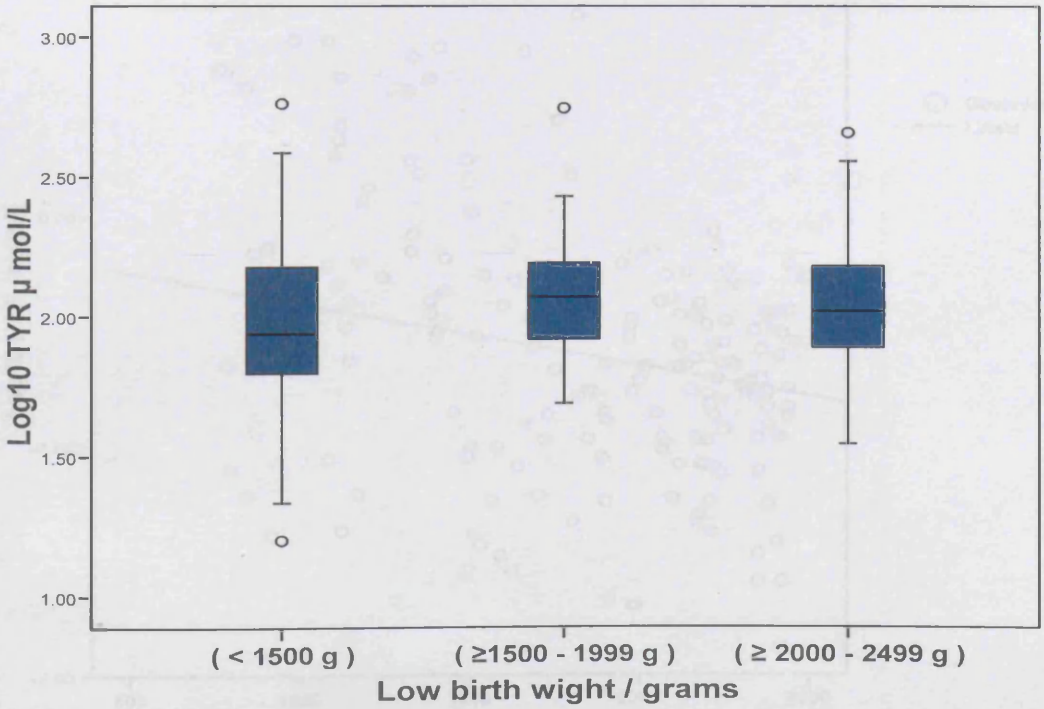

Figure 4-37: Box plot shows the median of TYR (log_{10}) for babies born weighing less than 1500 grams, babies born weighing between 1500 to 1999 grams and those weighing between 2000 to 2499 grams. Circles indicate outlier values.

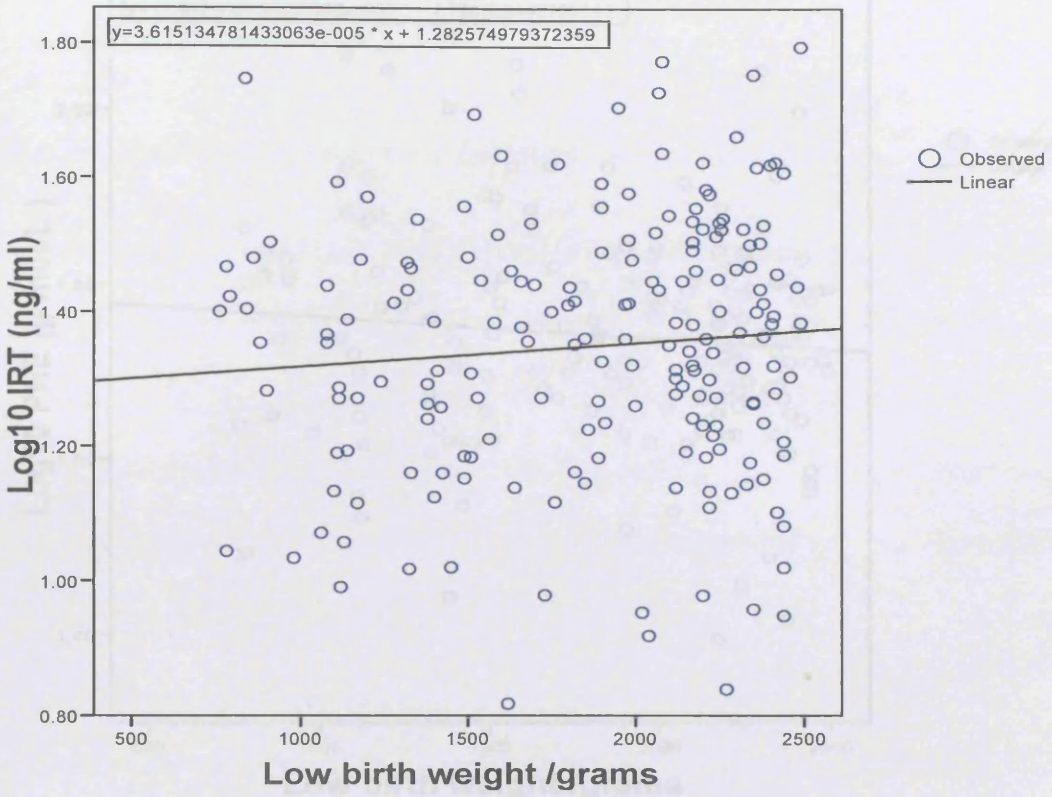

Figure 4-38: Scatter plot with estimated regression line showing the relationship between IRT (log_{10}) concentration level and low birth weight (less than 2500 grams).

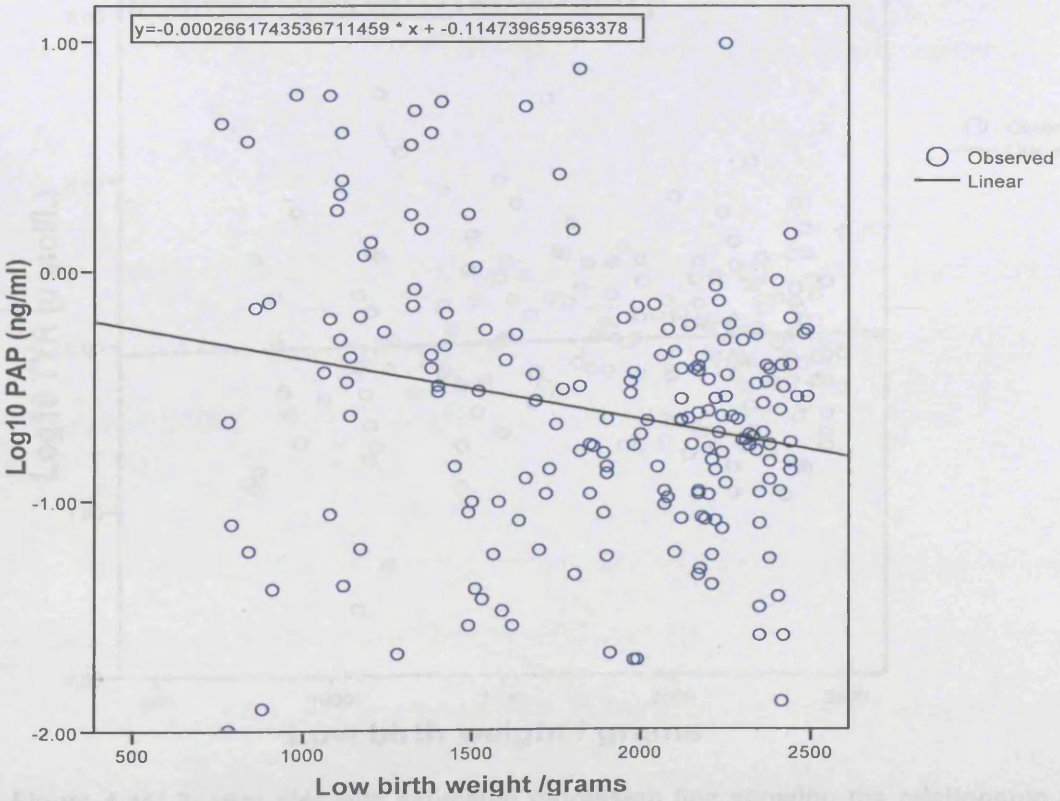

Figure 4-39: Scatter plot with estimated regression line showing the relationship between PAP (log_{10}) concentration level and low birth weight (less than 2500 grams).

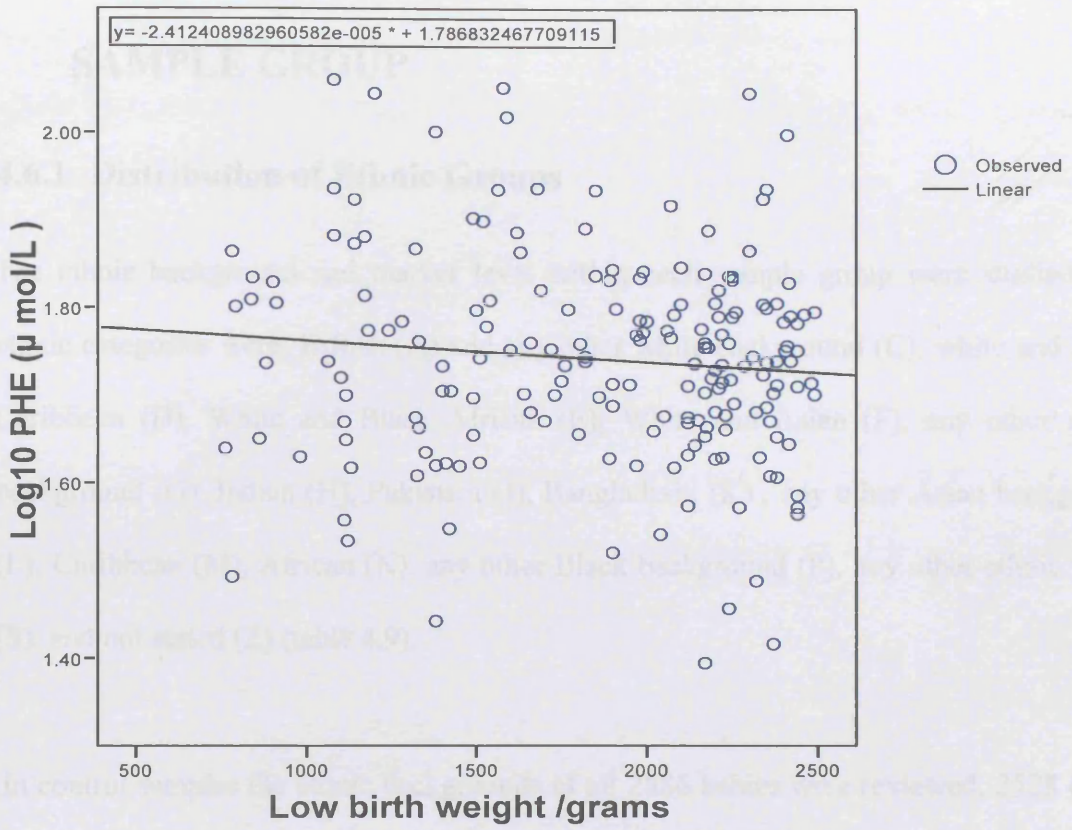

Figure 4-40: Scatter plot with estimated regression line showing the relationship between PHE (log_{10}) concentration level and low birth weight (less than 2500 grams).

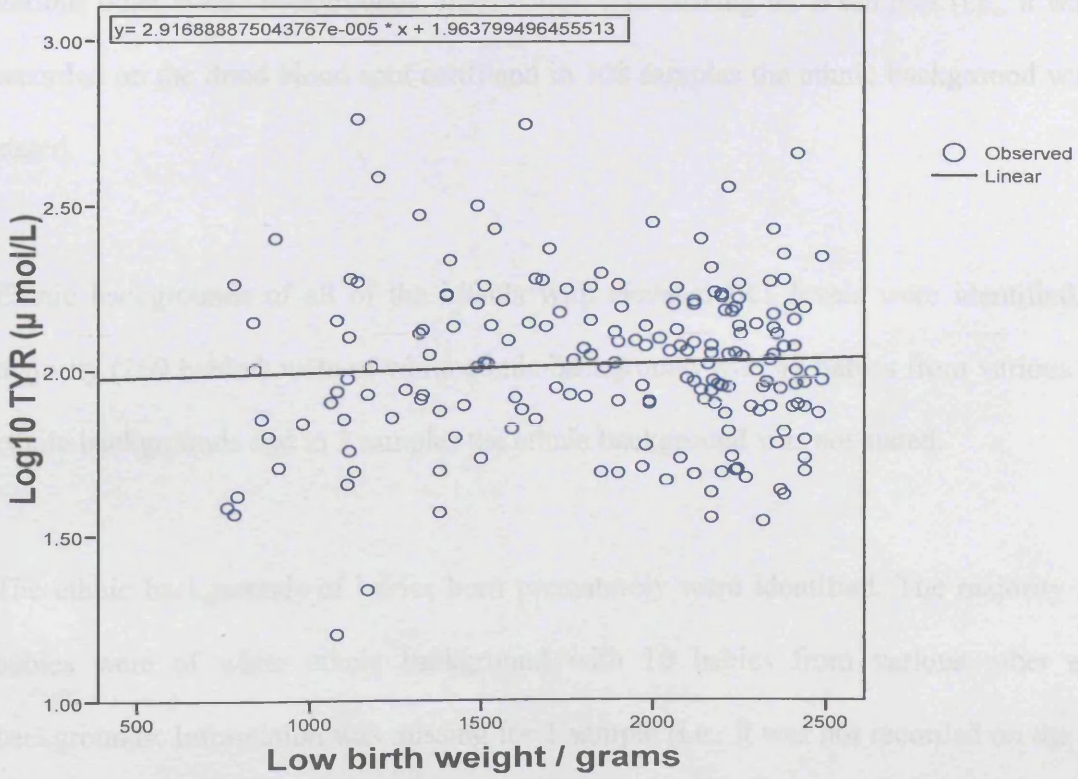

Figure 4-41: Scatter plot with estimated regression line showing the relationship between TYR (log_{10}) concentration level and low birth weight (less than 2500 grams).

4.6 MARKER LEVELS AND ETHNICITY WITHIN EACH SAMPLE GROUP

4.6.1 Distribution of Ethnic Groups

The ethnic background and marker level within each sample group were studied. The ethnic categories were: British (A) and any other white background (C), white and Black Caribbean (D), White and Black African (E), White and Asian (F), any other mixed background (G), Indian (H), Pakistani (J), Bangladeshi (K) , any other Asian background (L), Caribbean (M), African (N), any other Black background (P), any other ethnic group (S), and not stated (Z) (table 4.9).

In control samples the ethnic backgrounds of all 2886 babies were reviewed. 2528 (87 % of the total sample) were of white ethnic background and 244 (8%) samples belonged to various other ethnic backgrounds. Information was missing for 6 samples (i.e., it was not recorded on the dried blood spot card) and in 108 samples the ethnic background was not stated.

Ethnic backgrounds of all of the infants with elevated IRT levels were identified. The majority (269 babies) were of white ethnic background with 42 babies from various other ethnic backgrounds and in 3 samples the ethnic background was not stated.

The ethnic backgrounds of babies born prematurely were identified. The majority (161) babies were of white ethnic background with 10 babies from various other ethnic backgrounds. Information was missing for 1 sample (i.e., it was not recorded on the dried blood spot card). The ethnic background was not stated in 10 babies and information on one baby from total 182 was absent.

The ethnic backgrounds of babies born with low birth weight were identified. The majority (163) babies were of white ethnic background with 16 babies from various other ethnic backgrounds. There another 8 samples where the ethnic back ground was not stated and information was missing for 2 samples (i.e. it was not recorded on the dried blood spot card).

The ethnic backgrounds of the CF cases were as follows: 26 cases were white British (A); 2 cases were Pakistanis in the Asian or Asian British group (J), and there was one case classified as Asian or Asian British or any other Asian Background (L).

Thirty of the 32 CF carriers were of white British (A) ethnic background, while one was white of any other white background (C), and one was in the mixed race group of white and black African (E).

Table 4-9: Distribution of ethnic groups within each sample group.

Ethnic group	Sample group					
	Controls	Increased IRT	Prem	LBW	CF Cases	CF carrier
A & C*	2528	269	161	163	26	30
Other**	244	42	10	16	3	2
No info /not stated	114	3	11	10	0	0
Total	2886	314	182	189	29	32

* Include: British (A) and any other white background (C).
** Include: White and Black Caribbean (D), White and Black African (E), White and Asian (F), any other Mixed background (G), Indian (H) Pakistani (J), Bangladeshi (K) Any other Asian background (L), Caribbean (M), African, (N), any other Black background (P), any other ethnic group (S).

4.6.2 Median Marker levels in Different Ethnic Groups.

To see if there was any difference in the IRT, PAP, PHE and TYR markers among all ethnic categories, the data was divided in two groups. Group 1 included all British (A) and any other white back ground (C). Group 2 included all other ethnic backgrounds (Table 4.10).

Medians were calculated for IRT, PAP, PHE and TYR in the British /Irish and 'other' ethnic groups (Table 4.10). The only statistically significant difference between these groups was for TYR in the controls. There were too few individuals from 'other' ethnic groups in the CF and CF carrier sample groups for meaningful comparison.

Table 4-10: Median of Marker levels in Ethnic Groups in each sample group.

Sample group	Ethnic group	N	Marker medians IRT	PAP	PHE	TYR	Mann-whitney p VALUE IRT	PAP	PHE	TYR
Controls	A&C	2528	19.2	0.19	56.1	93.0	(p= 0.312)	(p=0.297)	(p=0.103)	(p <0.001)
	Other	244	19.9	0.20	58.1	102.8				
↑IRT	A&C	269	89.6	0.33	64.7	91.3	(p= 0.085)	(p=0.400)	(p=0.788)	(p= 0.115)
	Other	42	83.6	0.44	64.5	99.5				
Premature	A&C	161	20.5	0.18	58.7	114.7	(p= 0.674)	(p=0.995)	(p=0.175)	(p= 0.187)
	Other	10	21.2	0.18	50.7	76.6				
LBW	A&C	163	23.2	0.22	56.4	105.3	(p= 0.229)	(p=0.372)	(p=0.078)	(p= 0.161)
	Other	16	19.8	0.18	50.2	90.9				
CF Cases	A&C	26	166.1	1.54	53.4	62.6	*	*	*	*
	Other	3	126.6	12.1	78.7	34.4				
CF Carrier	A&C	30	77.4	0.43	60.4	76.9	*	*	*	*
	Other	2	105.8	0.40	120.5	48.3				
Total		3495								

* Not tested due to small numbers of other Ethnic groups

4.7 BABIES WITH CYSTIC FIBROSIS.

Out of 3632 babies screened, 29 samples were positively identified as CF cases, of which 14 were girls and 15 boys. The ethnic backgrounds of the CF cases were as follows: 26 cases were white British (A); 2 cases were Pakistanis in the Asian or Asian British group (J), and; 1 case in the Asian or Asian British group of any other Asian Background (L). These CF cases data are summarised in table 4.11.

4.7.1 Marker Levels in Cystic Fibrosis Cases

The median PAP concentration in controls was 0.19 ng/ml and in the 29 CF cases 1.91 ng/ml, equivalent to 10 MoM. The corresponding median for IRT was 19.3 ng/ml in the control and 161.5 ng/ml in the CF cases, equivalent to 8.3 MoM. The lowest PAP level in the CF cases was 0.24 ng/ml (1.2 MoM). The median PHE concentration in control was 56.2 μ mol/L and in the 29 CF cases 54.0 ng/ml, equivalent to 0.96 Mom. The median TYR concentration in controls was 93.8 μ mol/L and in the 29 CF cases 59.0 μ mol/L, equivalent to MoM 0.63 (Table 4.12).

Apart from PHE ($p = 0.890$), all markers shows statistically significant differences using Mann-Whitney test. IRT ($p = < 0.001$) and PAP ($p = < 0.001$) levels were significantly elevated while TYR levels were significantly reduced ($p = < 0.001$), (Table 4.12).

Table 4-11: Demographic Data, Marker Data and Mutations in 29 CF cases.

CF CASES	M/F	Ethn	BW	Gest.	Age	IRT		PAP		PHE		TYR		MUTATIONS
						ng /ml	MoM	ng /ml	MoM	µmol/L	MoM	µmol/L	MoM	
1	F	A	3580	40	5	74.88	3.94	0.24	1.25	44.40	0.79	57.81	0.62	R556X/2789+5G>A
2	F	A	3190	.	5	330.00	17.37	0.26	1.39	43.88	0.78	99.58	1.07	F508/G542X
3	F	A	2600	38	5	166.19	8.75	0.27	1.41	42.75	0.76	62.07	0.67	F508/621+1G>T
4	M	A	3820	41	5	98.77	5.20	0.29	1.55	49.40	0.88	58.37	0.63	F508/R117H
5	M	A	4300	40	5	167.45	8.81	0.42	2.21	51.56	0.92	106.68	1.15	F508/2789+5G>A
6	F	A	2100	36	5	236.04	12.42	0.62	3.27	47.28	0.84	56.18	0.60	F508/F508
7	F	A	3545	39	5	80.75	4.25	0.64	3.36	49.37	0.88	87.49	0.94	R117H/G542X
8	F	A	3225	41	5	150.54	7.92	0.70	3.71	66.30	1.18	63.32	0.68	F508/1461INSA5AT
9	M	A	3210	40	6	136.00	7.11	0.75	3.97	62.31	1.11	89.70	0.96	F508/F508
10	F	A	3100	36	5	289.56	15.24	0.76	4.00	62.73	1.12	97.55	1.05	F508/G551D
11	F	A	3145	40	6	166.10	8.74	1.23	6.47	72.38	1.29	90.29	0.97	F508/F508
12	M	A	4930	41	4	96.92	5.10	1.31	6.87	53.62	0.96	77.09	0.83	F508/R117H
13	M	A	2610	39	4	187.72	9.88	1.43	7.52	60.03	1.07	68.88	0.74	F508/F508
14	M	A	2680	38	5	136.08	7.16	1.65	8.69	53.33	0.95	58.79	0.63	F508/R560T
15	F	A	2950	38	4	128.49	6.76	1.90	10.02	51.29	0.92	49.69	0.53	F508/F508
16	F	A	2760	39	5	205.03	10.79	2.09	10.99	48.35	0.86	64.03	0.69	F508/F508
17	M	L	2280	36	6	149.91	7.89	2.22	11.69	78.67	1.40	52.82	0.57	F508/Sweat Test
18	F	A	2960	38	4	138.09	7.27	2.55	13.39	34.91	0.62	42.51	0.46	F508/F508
19	M	A	3506	41	5	188.31	9.91	2.58	13.59	63.69	1.14	63.84	0.69	F508/G551D
20	F	A	3090	40	5	145.63	7.66	2.58	13.60	61.21	1.09	49.40	0.53	G551D/621+1G>T
21	M	A	2420	34	5	237.57	12.50	3.29	17.30	106.26	1.90	403.18	4.34	F508/F508

Table 4.11 (continued).

CF CASES	M/F	Ethn	BW	Gest.	Age	IRT		PAP		PHE		TYR		MUTATIONS
						ng /ml	MoM	ng /ml	MoM	µmol/L	MoM	µmol/L	MoM	
22	F	A	3080	39	5	336.00	17.68	4.31	22.71	59.33	1.06	72.17	0.78	F508/F508
23	M	A	3080	38	5	161.54	8.50	5.32	28.01	81.00	1.45	50.93	0.55	F508/F508
24	M	A	3600	40	5	225.81	11.88	7.66	40.32	55.00	0.98	55.44	0.60	F508/F508
25	M	A	2730	38	5	255.32	13.44	7.69	40.45	51.39	0.92	59.09	0.64	F508/D493X
26	F	A	3130	40	4	134.58	7.08	8.60	45.25	54.21	0.97	56.39	0.61	F508/F508
27	M	A	2810	39	4	221.29	11.65	10.91	57.42	53.07	0.95	38.36	0.41	F508/F508
28	M	J	3260	40	3	126.67	6.67	12.09	63.61	91.88	1.64	34.39	0.37	Y569D/Y569D
29	M	J	2790	38	26	81.00	4.26	19.70	03.68	54.07	0.97	12.59	0.14	Sweat Test

Table 4-12: IRT, PAP, PHE and TYR medians with p value in 29 cases and 2886 control samples.

MARKERS	CONTROL MEDIAN		CF CASES MEDIAN		*p* Value *
	N		N		
IRT (ng /ml)	2886	19.3	29	161.5	*(p <0.001)*
PAP (ng /ml)	2886	0.19	29	1.91	(p <0.001)
PHE (µmol/L)	2886	56.2	29	54.0	p = 0.890
TYR (µmol/L)	2886	93.8	29	59.0	(p <0.001)

* Mann-Whitney test

4.7.2 Mutations Identified in Confirmed CF Cases

Out of 3632 screened babies, 29 cases were identified as positive for CF. In thirteen cases, the infants were homozygous for common mutation delta F508 (Table 4.11). All thirteen cases were of white British (A) ethnic background. There were a total of 16 cases with other combinations of mutations including two cases identified by sweat test. One of these cases had a single mutation (F508), while the other mutation was not identified. No mutations were identified in the second case. Thirteen cases were of white British (A) ethnic background, two CF cases were of Asian or Asian British Pakistani (J) ethnic background and one case was of other Asian background (L) (Table 4.11).

The median IRT, PAP, PHE and TYR concentration in the 13 cases homozygous for delta F508 and 16 cases with other mutations were compared (table 4.13). The IRT marker data are shown in Figure 4.42 and PAP marker data in Figure 4.43. There were no significant differences in median level between the two mutation groups for any markers (table 4.13).

Table 4-13: Comparison of levels of 4 markers between common delta F508 mutation and other mutation types in CF cases

Markers	ΔF508 / ΔF508			OTHER MUTATION			*p* value*
	N	Median	(Range)	N	Median	(Range)	
IRT ng/ml	13	187.7	(128.5-336.0)	16	147.8	(74.9-330)	NS
PAP ng/ml	13	2.54	(0.62 - 10.9)	16	1.03	(0.24-19.7)	NS
PHE µmol/L	13	55.0	(34.9-106)	16	53.5	(42.8-91.9)	NS
TYR µ/mol/L	13	56.4	(38.3-403.1)	16	60.6	(12.6-106.9)	NS

NS = not significant (Mann-Whitney test)

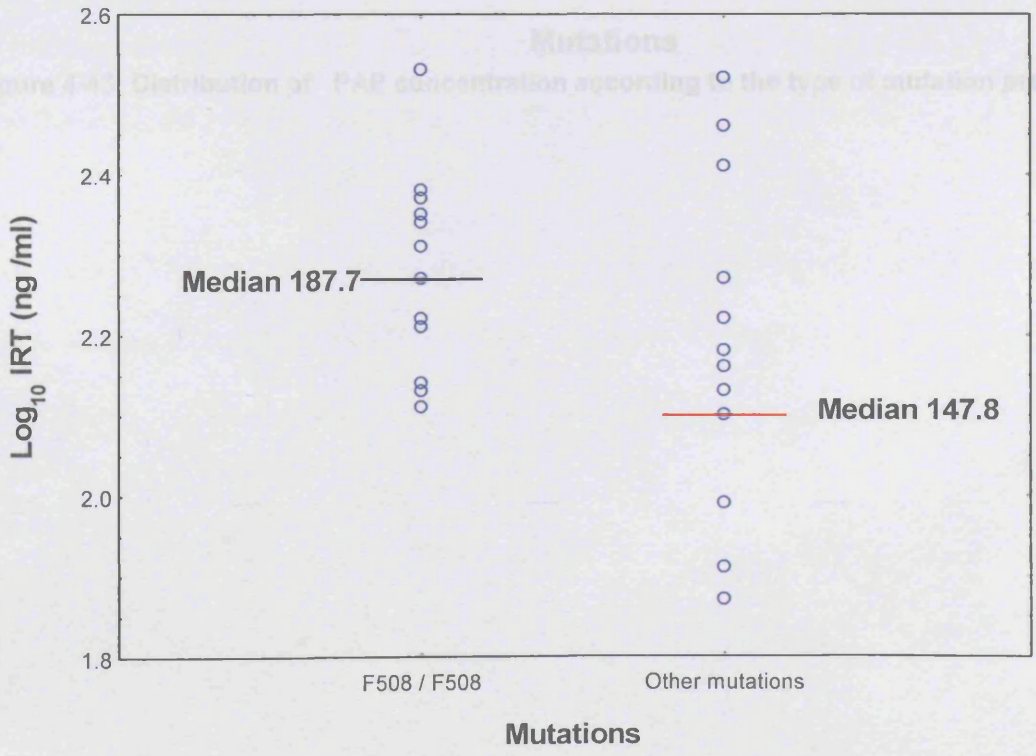

Figure 4-42: Distribution of IRT concentration according to the type of mutation present.

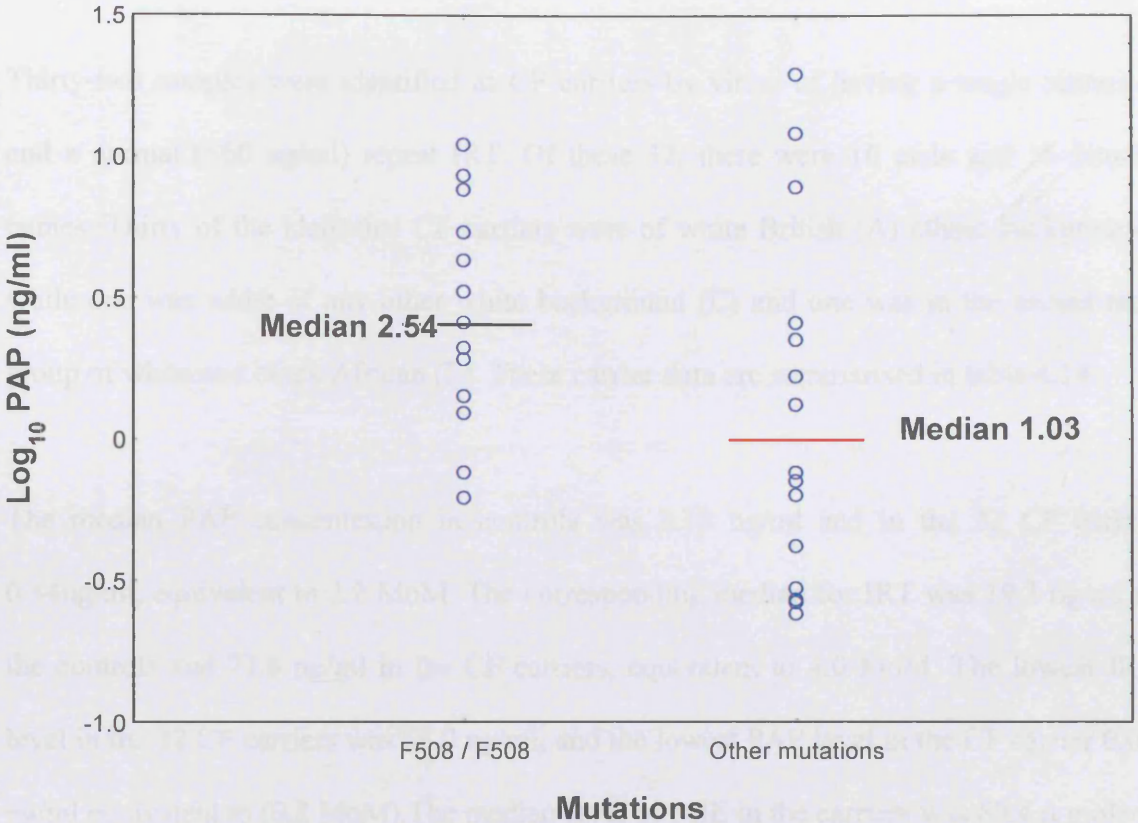

Figure 4-43: Distribution of PAP concentration according to the type of mutation present.

4.8 MARKER LEVELS IN CYSTIC FIBROSIS CARRIERS

Thirty-two samples were identified as CF carriers by virtue of having a single mutation and a normal (<60 ng/ml) repeat IRT. Of these 32, there were 16 male and 16 female babies. Thirty of the identified CF carriers were of white British (A) ethnic background, while one was white of any other white background (C) and one was in the mixed race group of white and black African (E). These carrier data are summarised in table 4.14

The median PAP concentration in controls was 0.19 ng/ml and in the 32 CF carrier 0.44ng/ml, equivalent to 2.2 MoM. The corresponding median for IRT was 19.3 ng/ml in the controls and 77.6 ng/ml in the CF carriers, equivalent to 4.0 MoM. The lowest IRT level in the 32 CF carriers was 68.0 ng/ml, and the lowest PAP level in the CF carrier 0.04 ng/ml equivalent to (0.2 MoM).The median level of PHE in the carriers was 60.4 μ mole/L and median level of TYR was 76.8 μ mole /L (Table 4.15).

Apart from PHE ($p = 0.244$), all markers showed statistically significant differences using Mann-Whitney test. IRT ($p < 0.001$) and PAP ($p = < 0.001$) levels were significantly elevated, while TYR levels were significantly reduced ($p = < 0.001$) (Table 4.15).

Table 4-14: Demographic data, marker data and mutations in 32 CF carriers.

CF CARRIERS	M/F	Ethn	BW	Gest	Age	IRT		PAP		PHE		TYR		MUTATION
						ng /ml	MoM	ng /ml	MoM	µmol/L	MoM	(µmol/L	MoM	
1	F	A	1900	32	5	78.65	4.14	0.04	0.21	41.97	0.75	50.35	0.54	F508
2	M	A	3850	41	5	128.67	6.77	0.05	0.28	125.10	2.23	122.56	1.32	F508
3	M	A	3730	.	5	72.95	3.84	0.07	0.36	32.91	0.59	61.66	0.66	P67L
4	F	A	4180	41	6	72.00	3.79	0.11	0.57	66.99	1.20	68.54	0.74	F508
5	M	A	3700	40	4	89.44	4.71	0.13	0.67	84.70	1.51	85.93	0.92	R560T
6	F	A	3110	39	6	92.77	4.88	0.14	0.74	62.73	1.12	97.11	1.04	F508
7	F	A	3140	.	5	77.54	4.08	0.18	0.93	46.51	0.83	76.99	0.83	F508
8	M	E	550	24	6	108.00	5.68	0.22	1.16	199.87	3.57	32.26	0.35	F508
9	F	A	3720	40	5	74.66	3.93	0.26	1.38	84.11	1.50	133.08	1.43	F508
10	M	A	3520	39	5	84.26	4.43	0.27	1.40	56.28	1.01	82.99	0.89	G551D
11	F	A	3080	38	7	73.95	3.89	0.28	1.47	42.64	0.76	24.29	0.26	F508
12	M	A	4110	42	4	71.03	3.74	0.29	1.55	55.30	0.99	64.86	0.70	F508
13	F	A	2850	39	5	94.23	4.96	0.35	1.82	47.93	0.86	87.71	0.94	F508
14	M	A	2940	39	27	68.00	3.58	0.38	2.01	50.28	0.90	66.60	0.72	F508
15	F	A	3480	39	5	82.25	4.33	0.39	2.04	74.36	1.33	90.90	0.98	R117H
16	F	A	3755	.	6	77.00	4.05	0.42	2.21	69.04	1.23	44.65	0.48	F508
17	M	A	3780	40	5	70.27	3.70	0.45	2.36	61.08	1.09	99.14	1.07	F508
18	M	A	4150	42	5	81.92	4.31	0.49	2.57	84.58	1.51	142.47	1.53	F508
19	F	A	2180	34	5	84.25	4.43	0.50	2.62	62.10	1.11	79.21	0.85	R117H
20	F	A	3750	41	41	85.87	4.52	0.53	2.77	54.19	0.97	76.80	0.83	R117H
21	M	A	2730	35	6	244.78	12.88	0.55	2.92	56.95	1.02	67.19	0.72	G551D

Table 4.14 (continued)

CF CARRIERS	M/F	Ethn	BW	Gest	Age	IRT		PAP		PHE		TYR		MUTATION
						ng /ml	MoM	ng /ml	MoM	µmol/L	MoM	µmol/L	MoM	
22	F	C	3600	41	5	103.69	5.46	0.60	3.15	41.16	0.74	64.34	0.69	R117H
23	F	A	4360	42	5	76.89	4.05	0.69	3.64	60.69	1.08	66.73	0.72	F508
24	M	A	5100	.	4	158.28	8.33	0.70	3.68	88.26	1.58	57.78	0.62	F508
25	F	A	3330	40	6	77.59	4.08	0.78	4.08	65.78	1.17	130.51	1.40	R117H
26	F	A	3690	41	5	74.94	3.94	1.01	5.31	60.51	1.08	69.58	0.75	F508
27	M	A	3500	41	5	70.07	3.69	1.03	5.45	60.33	1.08	78.46	0.84	F508
28	M	A	4070	42	5	75.56	3.98	1.22	6.40	33.17	0.59	64.59	0.69	F508
29	F	A	920	30	22	71.00	3.74	1.25	6.58	43.11	0.77	120.27	1.29	F508
30	M	A	3940	40	27	75.00	3.95	1.25	6.60	58.36	1.04	90.89	0.98	F508
31	M	A	3650	40	13	94.38	4.97	1.42	7.49	54.98	0.98	35.06	0.38	1078delT
32	M	A	1125	28	16	77.36	4.07	3.21	16.90	91.31	1.63	76.89	0.83	F508

Table 4-15: IRT, PAP, PHE and TYR medians with p value in 32 CF carriers and 2886 control sample

MARKERS	CONTROL MEDIAN		CF CARRIER MEDIAN		*p* Value*
	N		N		
IRT (ng /ml)	2886	19.3	32	77.6	*(p <0.001)*
PAP (ng /ml)	2886	0.19	32	0.44	*(p <0.001)*
PHE (µmol/L)	2886	56.2	32	60.4	p = 0.244
TYR (µmol/L)	2886	93.8	32	76.8	*(p <0.001)*

* Mann-Whitney test

4.8.1 Mutations Identified in CF Carriers

The mutations identified in the 32 CF carriers are summarised in table 4.14. The most common mutation, delta F508 was detected in 22 of the CF carriers. Twenty carriers were of white British (A) ethnic background, while one belonged to the white of any other white background group (C) and one was from the white and Black African group (E).

In total, 10 other mutations were found. The other mutations identified in the 32 CF carrier cases were the following: P67L (one sample), R560T (one sample), 1078delT (one sample), G551D (two samples) and R117H (Five samples). All of these were of white British (A) ethnic background.

The median IRT and PAP concentrations were analysed in two groups, those with deltaF508 mutation and those with other mutation. The IRT marker data are shown in figure 4.44 and PAP marker data in figure 4.45.

IRT, PAP, PHE and TYR concentrations were compared between the two groups of mutation types (ΔF508 vs. other mutation) using Mann-Whitney test. There were no

significant differences in median level between the two mutation groups for any markers (table 4.16).

Table 4-16: Comparison of levels of 4 markers between delta F508 mutation and other mutation types in CF carriers

Markers	ΔF508			OTHER MUTATION			*p* value*
	N	Median	(Range)	N	Median	(Range)	
IRT ng/ml	22	77.5	(68.0-244.8)	10	79.9	(70.0 - 94.4)	NS
PAP ng/ml	22	0.47	(0.04-3.21)	10	0.40	(0.17-1.42)	NS
PHE µmol/L	22	60.6	(32.9-199.8)	10	58.3	(42.6 - 84.1)	NS
TYR µmol/L	22	69.0	(32.3-142.5)	10	77.2	(24.3-133.0)	NS

NS = not significant (Mann-Whitney test)

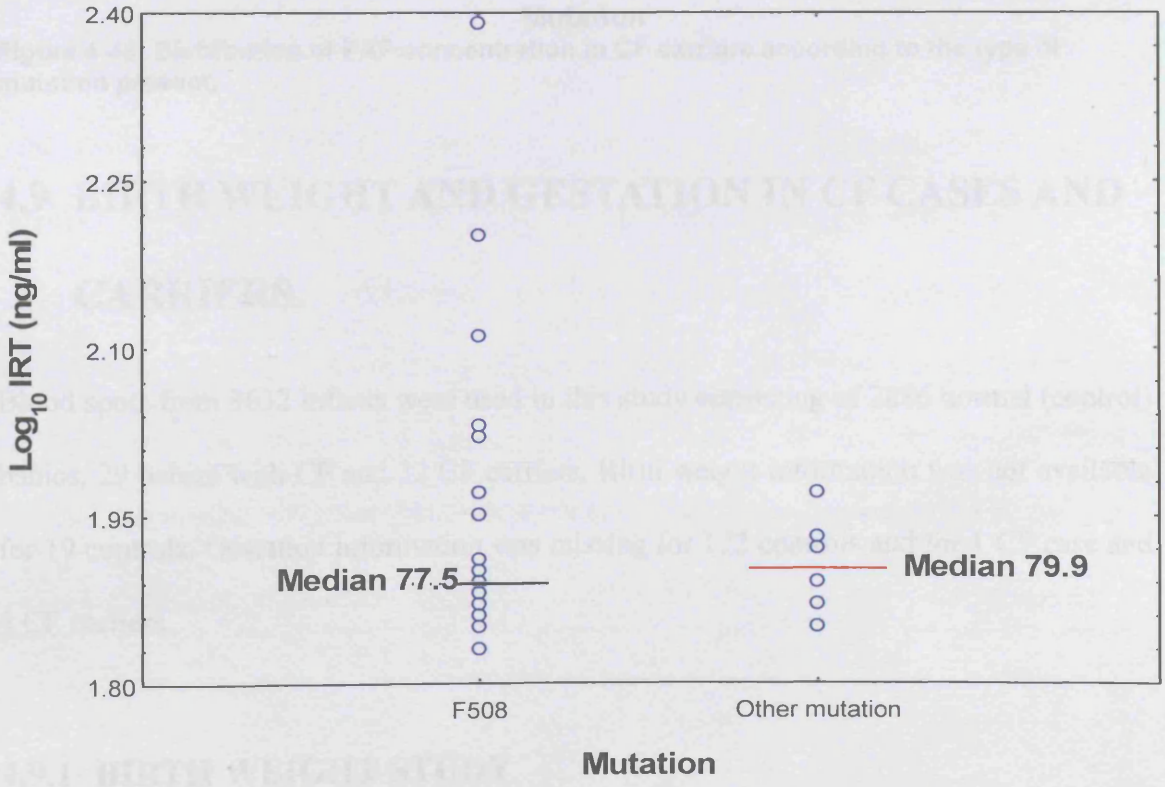

Figure 4-44: Distribution of IRT concentration in CF carriers according to the type of mutation present.

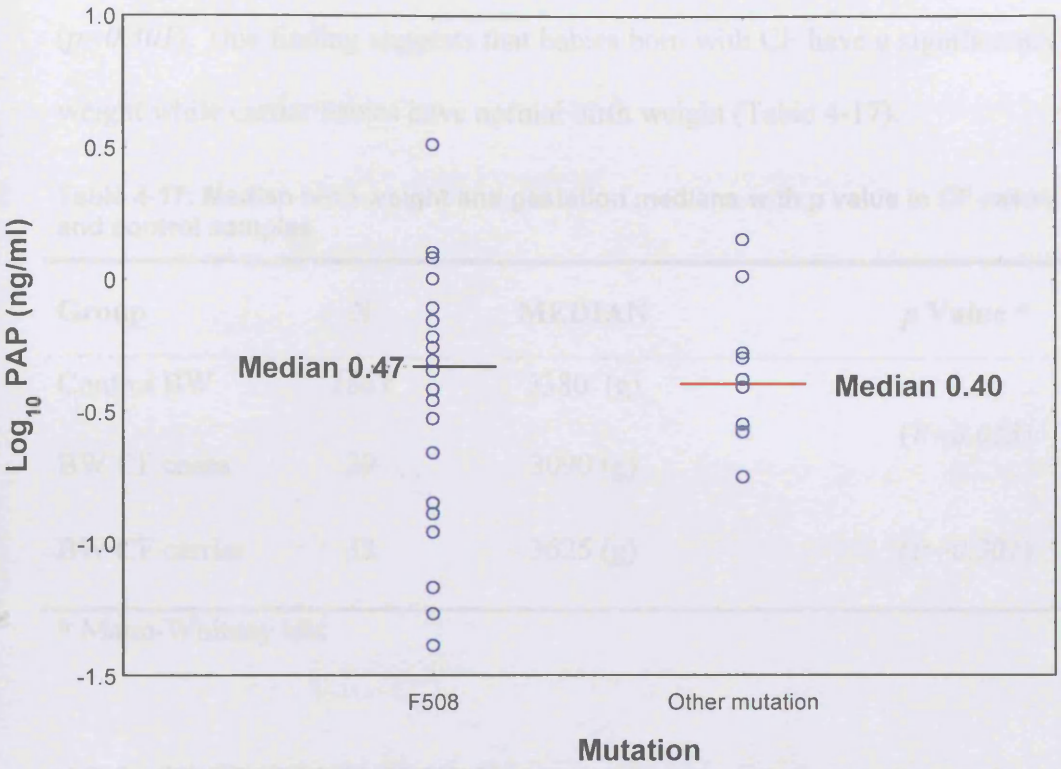

Figure 4-45: Distribution of PAP concentration in CF carriers according to the type of mutation present.

4.9 BIRTH WEIGHT AND GESTATION IN CF CASES AND CARRIERS.

Blood spots from 3632 infants were used in this study consisting of 2886 normal (control) babies, 29 babies with CF and 32 CF carriers. Birth weight information was not available for 19 controls. Gestation information was missing for 172 controls and for 1 CF case and 4 CF carriers.

4.9.1 BIRTH WEIGHT STUDY

It was found that 3 newborns out of 29 CF cases were reported as being of low birth weight, i.e. weighing less 2500 g and out of 32 CF carriers, 4 cases were reported as being of low birth weight. Out of 2867 newborns matched as controls, 338 were reported were

reported as low birth weight. The median value of the birth weight in the control samples is 3380 g, while it is 3090.g in the CF samples (*p=0.013*) and 3625 g in CF carrier (*p=0.301*). This finding suggests that babies born with CF have a significantly lower birth weight while carrier babies have normal birth weight (Table 4-17).

Table 4-17: Median birth weight and gestation medians with p value in CF cases, CF carrier and control samples

Group	N	MEDIAN	*p* Value *
Control BW	2867	3380 (g)	
BW CF cases	29	3090 (g)	*(P=0.013)*
BW CF carrier	32	3625 (g)	*(P=0.301)*

* Mann-Whitney test

4.9.2 GESTATION STUDY

Four newborns out of 28 CF cases were born prematurely (born before 37 weeks of gestation), and 6 newborns out of 28 CF carriers were born prematurely. Out of 2714 newborns matched as controls, 345 were born prematurely. The median value of the gestation in the control samples is 40 weeks, while it is 39 weeks in the CF cases *(p=0.251)* and 40 weeks in CF carriers *(p=0.436)*, which suggests that babies affected with CF, or carriers of CF are born within the normal gestation period (table 4-18).

Table 4-18: Gestation medians with p value in CF cases, CF carrier and control samples

Group	N	MEDIAN	*p* Value*
Control Ges	2714	40 (weeks)	
Ges. CF cases	28	39 (weeks)	*(p=0.251)*
Ges. CF Carrier	28	40 (weeks)	*(p=0.436)*

* Mann-Whitney test

4.10 RETROSPECTIVE STUDY OF IRT IN CF CASES DETECTED RY ROUTINE NEWBORN SCREENING

All 198 babies affected with CF identified by the routine newborn screening programme in Scotland between 2003 and December 2010 were reviewed. Homozygosity for the common delta F508 mutation was found in 88, with another 110 cases having various other combinations of mutations.

IRT concentrations were compared between the two groups of mutation types (homozygous common delta ΔF508 vs. other mutation) using the Mann-Whitney test. There were significant differences in median levels between the two mutation groups (table 4.19). The concentration level of IRT in cases homozygous for the common delta F508 was higher than the IRT level concentration in other mutations (Figure 4.46).

4.10.1 Retrospective Study of IRT Levels in CF Carriers According to Mutation.

The IRT concentration levels were identified for all 230 CF carrier babies born between 2003 and December 2011. From the total number of these babies, 148 carriers had the common delta F508 mutation and a further 82 had other mutations.

IRT concentrations were compared between the two groups of mutation types (ΔF508 vs. other mutation) (figure 4.47). Using the Mann-Whitney test, no significant difference in median level between the two mutation groups in the CF carriers was found (Table 4.20).

Table 4-19: Comparison of IRT concentration between homozygosity for the common delta F508 mutation and other mutation types in CF cases

Mutation	Number	Median (ng/ml)	(Range ng/ml)	*p* value*
Delta F508 Homozygous	88	166.50	(75-337)	(*p <0.001*)
Other mutation	110	133.50	(70-373)	

* Mann-Whitney test.

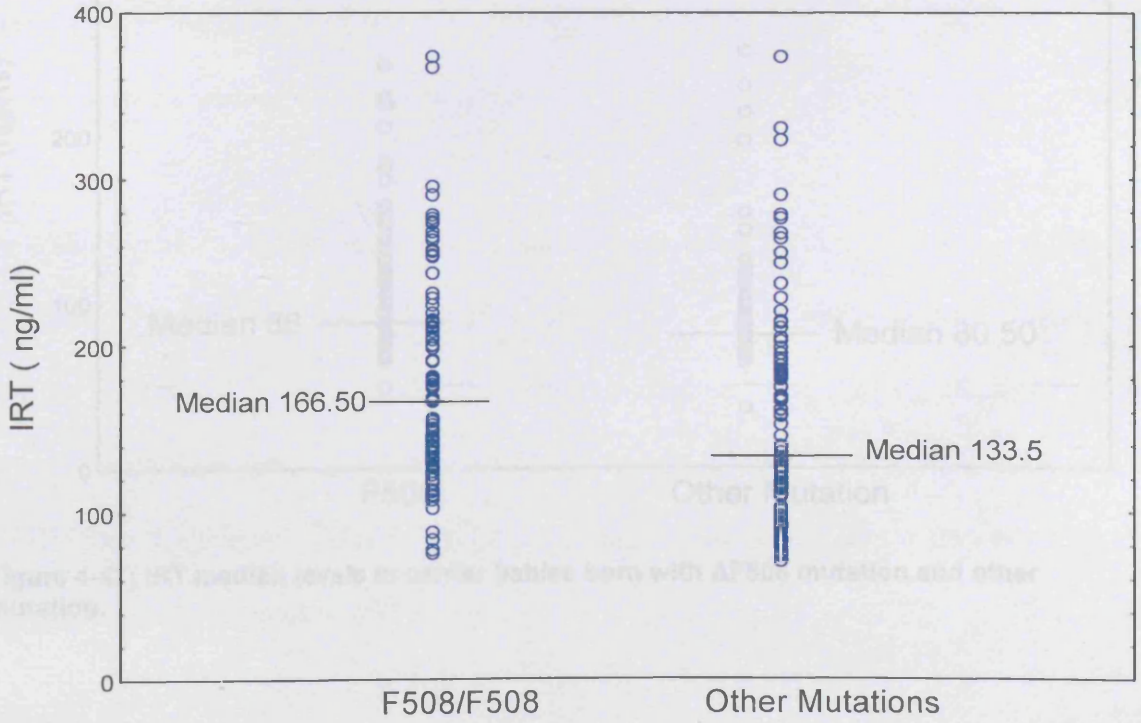

Figure 4-46: IRT median level in CF cases born with homozygous common delta ΔF508 mutation and other mutation.

Table 4-20: Comparison of IRT concentration between common delta F508 mutation and other mutation in CF carriers.

Mutation	Number	Median (ng/ml)	(Range ng/ml)	*p* value
Delta F508	148	88	(69-360)	(*p =0.130*)
Other mutation	82	80.50	(68-251)	

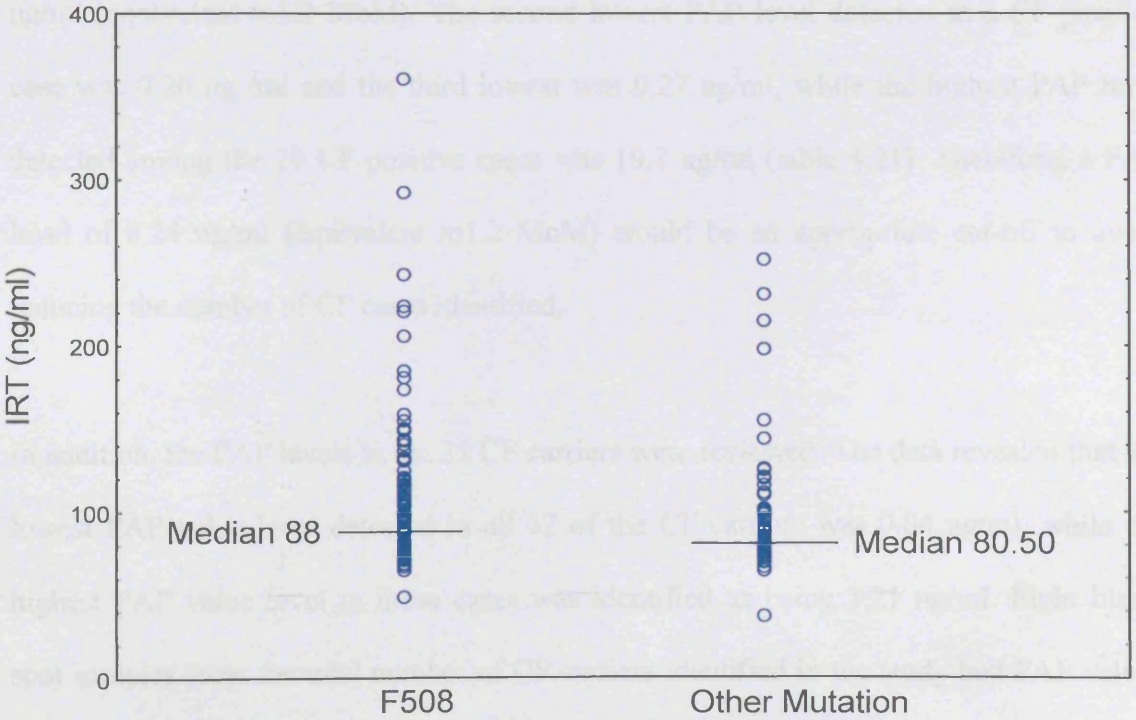

Figure 4-47: IRT median levels in carrier babies born with ΔF508 mutation and other mutation.

4.11 DISCRIMINATORY POWER OF PAP MEASUREMENTS

Data were obtained on PAP analysis on 3632 samples in this project. A key aim of the study was to develop an algorithm using the results of both IRT and PAP markers to minimise the number of samples referred for DNA analysis due to increased IRT levels and therefore reduce the number of carriers detected, whilst maintaining detection of CF cases.

The lowest PAP concentration from the 29 CF positive cases was identified; this was 0.24 ng/ml (equivalent to1.2 MoM). The second lowest PAP level detected in a CF positive case was 0.26 ng /ml and the third lowest was 0.27 ng/ml, while the highest PAP level detected among the 29 CF positive cases was 19.7 ng/ml (table 4.21). Therefore, a PAP level of 0.24 ng/ml (equivalent to1.2 MoM) would be an appropriate cut-off to avoid reducing the number of CF cases identified.

In addition, the PAP levels in the 32 CF carriers were reviewed. The data revealed that the lowest PAP value level detected in all 32 of the CF carriers was 0.04 ng/ml, while the highest PAP value level in these cases was identified as being 3.21 ng/ml. Eight blood spot samples from the total number of CF carriers identified in the study had PAP values of less than 0.24ng/ml (suggested PAP cut-off), and the remaining 24 samples having PAP values above the suggested PAP cut off.

Thus, if selection of infants for DNA mutation analysis was based on both an IRT level $\geq$ 70 ng/ml and a PAP level $\geq$ 0.24 ng / ml , eight fewer CF carriers would be detected and 25% fewer DNA test would be required (See table 4.22) .

Table 4-21: PAP, IRT and mutations in 29 CF cases arranged in ascending order of PAP concentration.

CF cases	IRT ng / ml	PAP ng / ml	Mutation
1	74.88	0.24	R556X/2789+5G>A
2	330.00	0.26	F508/G542X
3	166.19	0.27	F508/621+1G>T
4	98.77	0.29	F508/R117H
5	167.45	0.42	F508/2789+5G>A
6	236.04	0.62	F508/F508
7	80.75	0.64	R117H/G542X
8	150.54	0.70	F508/1461INSA5AT
9	136.00	0.75	F508/F508
10	289.56	0.76	F508/G551D
11	166.10	1.23	F508/F508
12	96.92	1.31	F508/R117H
13	187.72	1.43	F508/F508
14	136.08	1.65	F508/R560T
15	128.49	1.90	F508/F508
16	205.03	2.09	F508/F508
17	149.91	2.22	F508/Sweat Test
18	138.09	2.55	F508/F508
19	188.31	2.58	F508/G551D
20	145.63	2.58	G551D/621+1G>T
21	237.57	3.29	F508/F508
22	336.00	4.31	F508/F508
23	161.54	5.32	F508/F508
24	225.81	7.66	F508/F508
25	255.32	7.69	F508/D493X
26	134.58	8.60	F508/F508
27	221.29	10.91	F508/F508
28	126.67	12.09	Y569D/Y569D
29	81.00	19.70	Sweat test

Table 4-22: IRT and PAP concentrations in 8 CF carrier samples located below the suggested PAP cut-off of 0.24 ng/ml.

Sample No	Gender	Birth weight	Gestation	IRT ng/ml	PAP ng/ml	Mutations
1	Female	1900	32	78.65	0.04	F508
2	Male	3850	41	128.67	0.05	F508
3	Male	3730	?	72.95	0.07	F508
4	Female	4180	41	72.00	0.11	F508
5	Male	3700	40	89.44	0.13	F508
6	Female	3110	39	92.77	0.14	F508
7	Female	3140	?	77.54	0.18	R117H
8	Male	550	24	108.00	0.22	F508

4.11.1 PAP and IRT Levels in Relation to Type of Mutation

The PAP concentration showed a tendency to be higher in the CF cases homozygous for the common delta F508 mutation than the levels in CF cases with other mutations (see table 4.13). The median PAP concentration in CF cases homozygous for the common delta F508 mutation was 2.54 ng/ml, while for other combinations of mutations the median PAP concentration was 1.03 ng/ml.

Furthermore, the IRT concentration levels showed a tendency to be higher in the CF cases homozygous for the common delta F508 mutation than that in CF cases with other mutations. The median IRT concentration in CF cases homozygous for the common delta F508 mutation was 187.7 ng/ml, while in CF cases with other combinations of mutations it was 147.8 ng/ml (table 4.13).

Statistical testing(Mann-Whitney test), however, showed that the differences for PAP and IRT were not significantly different. These data are summarised in table 4.13 along with the PHE and TYR results in the two classes of mutation. Analysis of PAP concentration levels in CF carriers showed a tendency for PAP to be higher in carriers heterozygous for

the common delta F508 mutation than in CF carriers with other mutations. The median PAP concentration in CF carriers heterozygous for the common delta F508 mutation was 0.47 ng/ml, while in cases with other mutations it was 0.40 ng/ml (table 4.16).

Conversely, IRT concentration levels did not tend to be higher in CF carriers heterozygous for the common delta F508 than in CF carriers with other mutations. The median IRT concentration in CF carriers heterozygous for the common delta F508 mutation was 77.5 ng/ml, while in cases with other mutations the median IRT concentration was 79.9 ng/ml (Table 4.16). These data, along with data for PHE and TYR in cases and carriers with the two classes of mutation, are summarised in table 4.13 and 4.16. Mann-Whitney testing showed that there were no statistically significant differences for any markers.

4.12 USE OF LIKELIHOOD RATIOS METHOD IN THE NEWBORN SCREENING OF CF

As noted in section 4.2, IRT and PAP concentrations are log10 normally distributed in controls (Figures 4.1, 4.2 and 4.5, 4.6). Using the parameters of the IRT and PAP in CF cases (table 4.12) shows that they also fit a $\log_{10}$ Gaussian distribution (Figure 4.48, 4.50). However, there is no complete separation of the levels of either marker between normal and affected infants (Figures 4.49 and 4.51). Therefore nether marker is an absolute predictor of CF.

Using Gaussian statistics, the likelihood that a particular IRT or PAP level is associated with a CF infant rather than an unaffected infant can be estimated from the overlapping distributions. For example, in Figure 4.49 an IRT concentration of 70.80 ng/ml ($\log_{10}$ 1.85) is 8.2 times (i.e. a:b) more likely to be associated with a CF infant than with unaffected infant. Similarly in figure 4.51, a PAP level of 1.26 ng/ml ($\log_{10}$ 0.1) gives a likelihood ratio of 4.1 times (i.e., a:b) more likely to be associated with a CF infant than with an unaffected infant. Moreover, the product of the individual likelihood ratios can be calculated thus combining the information from two markers due to their being only a low correlation between IRT and PAP levels in CF cases (Pearson correlation $p = 0.669$).

Table 4.23 shows the likelihood ratio derived from IRT and PAP levels in 29 CF cases. The likelihood ratios for both markers are multiplied together to give the overall risk for each affected CF baby. The lowest overall risk for both markers is 4.84 and the highest value is > 50,000

Table 4.24 shows the likelihood ratios derived from the IRT and PAP levels in 32 carriers. The likelihood ratios for both markers are multiplied together to give the overall risk for

each CF carrier. The lowest CF carrier overall risk for both markers is 1.45 and the highest value is > 50,000.

Individual likelihood ratios were also calculated for the 314 increased IRT samples using the IRT and PAP levels. The likelihood ratios for both markers are multiplied together to give the overall risk for each increased IRT sample. The lowest overall risk for both markers is 1.10 and the highest value is > 50,000.

Table 4.25 shows the distribution of the overall likelihood ratios in CF cases, CF carriers and babies with increased IRT level. The suggested cut-off risk for IRT and PAP of 30 for CF cases, CF carrier, and Increased IRT would be an appropriate risk to select and would result in reducing the number of CF carriers from 32 to 16 (50%) and reduce further DNA analysis from 314 to 180 (43%) with losing one CF case only. Setting a likelihood ratio and referrals for DNA analysis by 500 would reduce the number of DNA test by 75% and carriers by 88%. However, detection of 3 CF cases would be missed.

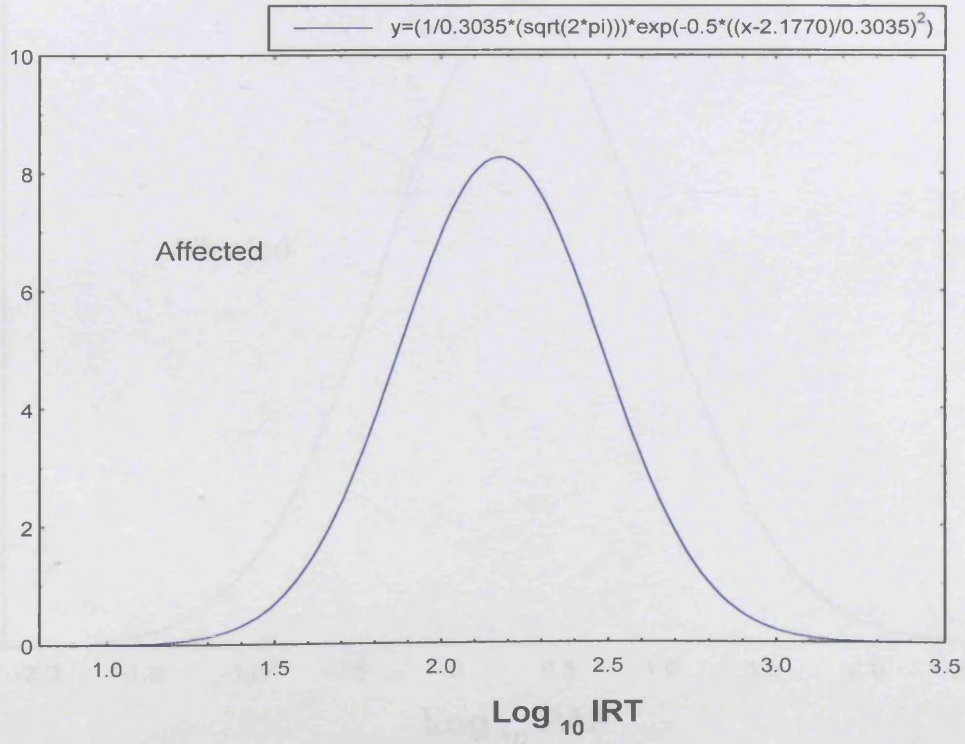

Figure 4-48: Log_{10} Gaussian distributions for IRT in 29 CF cases.

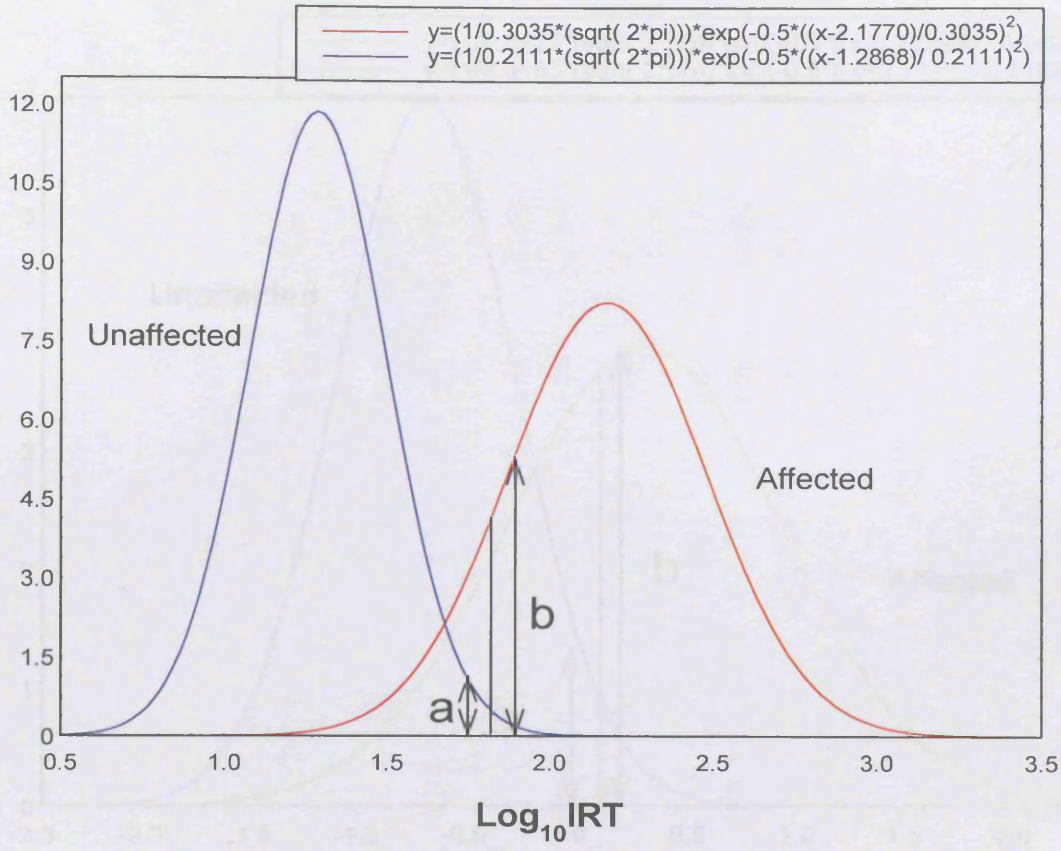

Figure 4-49: Overlapping Log_{10} Gaussian distributions for IRT in affected and unaffected babies.

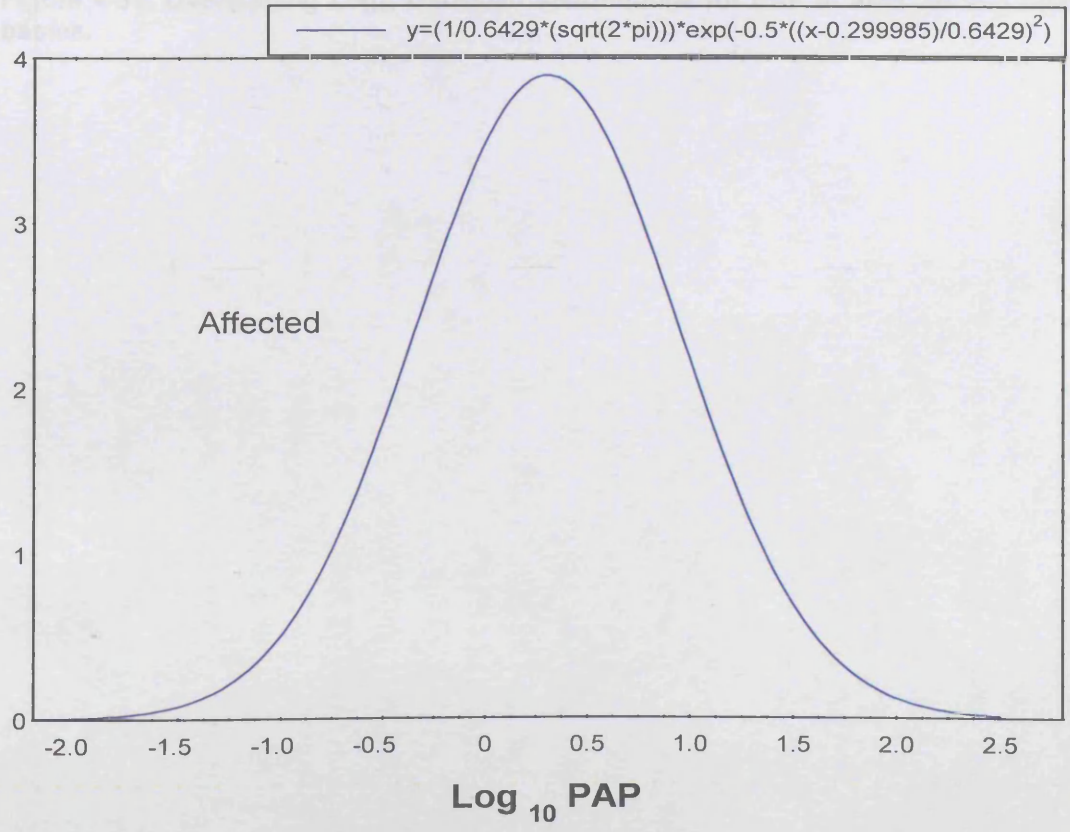

Figure 4-50: Log_{10} Gaussian distributions for PAP in 29 CF cases

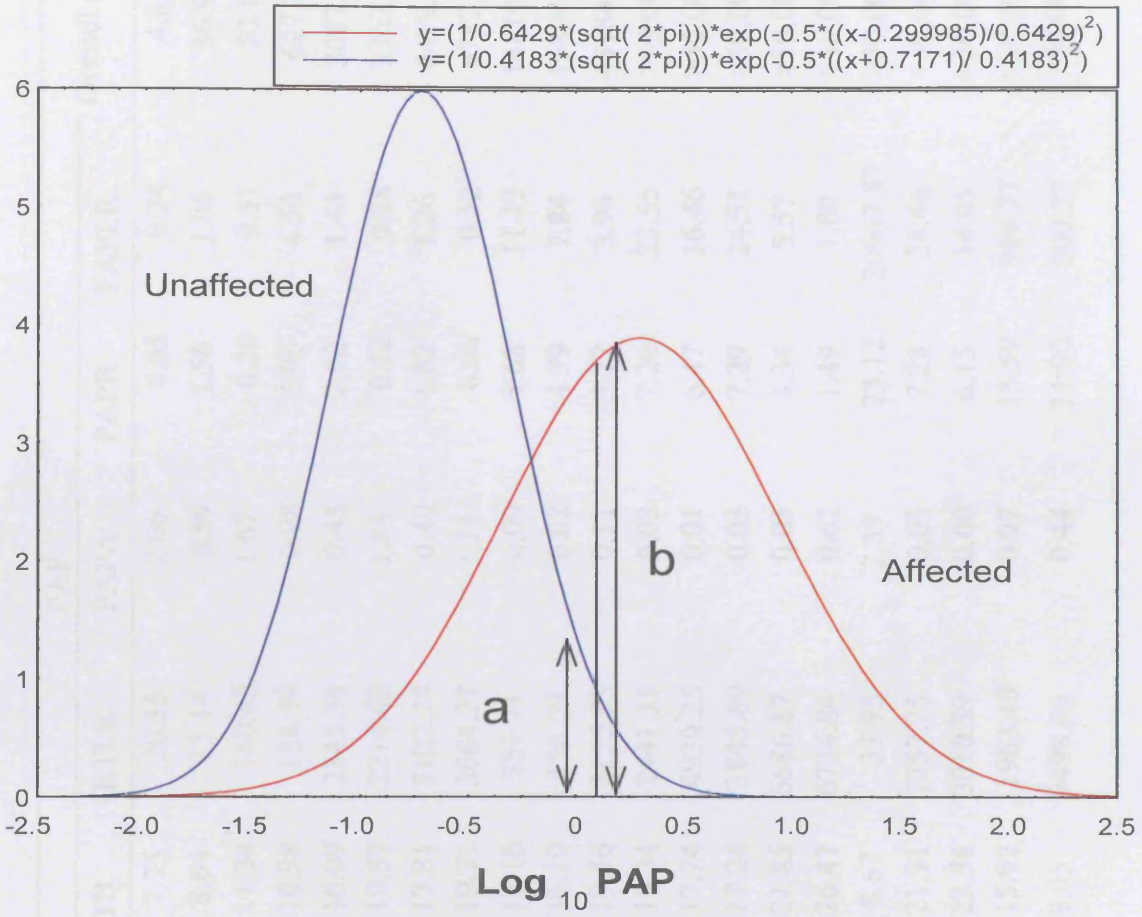

Figure 4-51: Overlapping Log_{10} Gaussian distributions for PAP in affected and unaffected babies.

Table 4-23: Overall risk of CF from IRT and PAP levels in 29 CF cases.

CF CASES	IRT (ng /ml)	PAP (ng /ml)	IRT Log_{10} IRT	Log_{10} PAP	PAP IRTA	IRTB	IRTLR	PAPA	PAPB	PAPLR	Overall risk
1	74.88	0.238030	1.8744	-0.6234	0.99	7.75	20.35	2.06	0.05	0.24	4.84
2	80.75	0.637715	1.9071	-0.1954	0.79	8.64	35.14	0.59	1.56	1.05	36.99
3	98.77	0.294970	1.9946	-0.5302	0.36	11.24	160.42	1.67	0.20	0.31	50.10
4	96.92	1.30501	1.9864	0.1156	0.39	10.98	138.59	0.08	3.96	4.53	627.70
5	136.00	0.754065	2.1335	-0.1226	0.02	16.09	2145.39	0.43	2.02	1.44	3087.89
6	166.19	0.267800	2.2206	-0.5722	0.02	19.57	2214.63	1.84	0.12	0.28	3362.33
7	150.54	0.704470	2.1777	-.1521	0.00	17.81	5122.24	0.49	1.82	1.26	6479.21
8	167.45	0.419445	2.2239	-0.3773	0.02	19.71	3064.27	1.11	0.66	0.52	6787.15
9	128.49	1.90450	2.1089	0.2798	0.05	15.16	331.71	0.00	5.68	11.13	14819.37
10	136.08	1.65055	2.1338	0.2176	0.02	16.10	156.09	0.02	4.99	7.84	16894.49
11	166.10	1.22967	2.2204	0.0898	0.02	19.56	2155.93	0.11	3.72	3.96	48184.76
12	138.09	2.54500	2.1402	0.4057	0.01	16.34	2441.35	0.03	7.20	23.55	>50,000
13	149.91	2.22131	2.1758	0.3466	0.00	17.74	4939.25	0.01	6.47	16.46	>50,000
14	145.63	2.58343	2.1633	0.4122	0.00	17.24	3845.89	0.03	7.29	24.51	>50,000
15	187.72	1.42831	2.2735	0.1548	0.10	21.85	6686.87	0.05	4.34	5.57	>50,000
16	236.04	0.621595	2.3730	-0.2065	0.42	26.47	6734.84	0.62	1.49	1.00	>50,000
17	81.00	19.6986	1.9085	1.2944	0.78	8.67	35.95	2.39	23.12	20667.87	>50,000
18	188.31	2.58121	2.2749	0.4118	0.10	21.91	7757.25	0.03	7.28	24.46	>50,000
19	205.03	2.08897	2.3118	0.3199	0.20	23.58	3010.39	0.00	6.15	14.05	>50,000
20	134.58	8.59781	2.1290	0.9344	0.03	15.92	1963.40	0.97	15.59	969.77	>50,000
21	161.54	5.32200	2.2083	0.7261	0.01	9.05	9498.06	0.44	11.90	200.77	>50,000

Table 4-23 (continued)

CF CASES	IRT (ng /ml)	PAP (ng /ml)	IRT $Log_{10\ IRT}$	PAP $Log_{10\ PAP}$	IRT IRT^A	IRT IRT^B	IRT IRT^{LR}	PAP PAP^A	PAP PAP^B	PAP PAP^{LR}	Overall risk
22	330.00	0.264485	2.5185	-0.5776	1.27	34.04	9119804.36	1.86	0.11	0.27	>50,000
23	289.56	0.759980	2.4617	-0.1192	0.88	30.98	2387777.64	0.43	2.04	1.46	>50,000
24	126.67	12.0861	2.1027	1.0823	0.06	14.94	182.71	1.48	18.50	3235.79	>50,000
25	237.57	3.28725	2.3758	0.5168	0.43	26.61	7151.93	0.11	8.70	47.67	>50,000
26	225.81	7.66084	2.3537	0.8843	0.34	25.55	6879.26	0.83	14.66	655.33	>50,000
27	221.29	10.9100	2.3450	1.0378	0.31	25.13	0576.79	1.32	17.60	2235.47	>50,000
28	255.32	7.68554	2.4071	0.8857	0.57	28.16	0878.08	0.83	14.68	662.46	>50,000
29	336.00	4.31450	2.5263	0.6349	1.32	34.48	11002031.5	0.27	10.45	105.43	>50,000

* $IRT^A = (\log_{10} IRT - Mean\ x) / SD\ x)^2$

$IRT^B = (\log_{10} IRT - Mean\ y) / SD\ y)^2$

IRT^{LR} Likelihood ratio for CF derived from the following equation: $IRT^{LR} = (SDy) / (SDx)$* EXP (-0.5 * (IRT-a - IRT-b))

*$PAP^A = (\log_{10} PAP - Mean\ x) / SD\ x)^2$

$PAP^B = (\log_{10} PAP - Mean\ y) / SD\ y)^2$

PAP^{LR} Likelihood ratio for CF derived from CF cases and controls using the following equation: $PAP^{LR} = (SDy) / (SDx)$* EXP (-0.5 * (PAP-a - PAP-b))

*Overall risk: the overall risk of the CF from both IRT and PAP using the following equation: IRT-LR×PAP-LR

Table 4-24: Overall risk of CF from IRT and PAP levels in 32 CF carriers.

CF CARRIERS	IRT (ng /ml)	PAP (ng /ml)	IRT $Log_{10\ IRT}$	IRT $Log_{10\ PAP}$	IRT IRT^{A}	IRT IRT^{B}	IRT IRT^{LR}	PAP PAP^{A}	PAP PAP^{B}	PAP PAP^{LR}	Overall risk
1	72.95	0.068250	1.86	-1.17	1.07	7.45	16.90	5.20	1.15	0.09	1.45
2	72.00	0.108940	1.86	-0.96	1.11	7.30	15.40	3.86	0.35	0.11	1.73
3	78.65	0.040755	1.90	-1.39	0.86	8.32	29.00	6.91	2.59	0.07	2.17
4	71.03	0.294320	1.85	-0.53	1.15	7.15	14.00	1.67	0.20	0.31	4.36
5	77.54	0.176150	1.89	-0.75	0.90	8.15	26.16	2.69	0.01	0.17	4.46
6	68.00	0.382655	1.83	-0.42	1.29	6.68	10.32	1.24	0.51	0.45	4.66
7	74.66	0.262860	1.87	-0.58	1.00	7.71	19.93	1.87	0.11	0.27	5.36
8	73.95	0.279435	1.87	-0.55	1.03	7.60	18.62	1.76	0.15	0.29	5.41
9	70.27	0.449215	1.85	-0.35	1.18	7.04	12.98	1.01	0.78	0.58	7.51
10	89.44	0.128245	1.95	-0.89	0.55	9.92	75.10	3.44	0.17	0.13	9.56
11	77.00	0.420810	1.89	-0.38	0.92	8.07	24.88	1.11	0.67	0.52	12.99
12	84.26	0.265070	1.93	-0.58	0.69	9.16	48.07	1.86	0.11	0.27	13.06
13	92.77	0.140530	1.97	-0.85	0.48	10.39	99.08	3.21	0.10	0.14	13.63
14	82.25	0.387790	1.92	-0.41	0.74	8.86	40.22	1.22	0.53	0.46	18.53
15	81.92	0.487500	1.91	-0.31	0.75	8.81	39.05	0.91	0.94	0.66	25.81
16	76.89	0.691795	1.89	-0.16	0.92	8.05	24.62	0.51	1.77	1.22	30.10
17	84.25	0.497185	1.93	-0.30	0.69	9.16	48.03	0.88	0.98	0.68	32.80
18	70.07	1.034670	1.85	0.01	1.19	7.01	12.72	0.20	3.06	2.73	34.67
19	77.59	0.775190	1.89	-0.11	0.90	8.16	26.29	0.41	2.10	1.52	39.90
20	85.87	0.526500	1.93	-0.28	0.64	9.39	55.34	0.81	1.10	0.75	41.60
21	94.23	0.346450	1.97	-0.46	0.45	10.60	111.63	1.40	0.38	0.39	43.57

Table 4.24 (continued)

CF CARRIERS	IRT (ng /ml)	PAP (ng /ml)	IRT Log_{10} IRT	Log_{10} PAP	IRT^A	IRT^B	IRT^{LR}	PAP^A	PAP^B	PAP^{LR}	Overall risk
22	74.94	1.009450	1.87	0.00	.99	7.76	20.47	0.21	2.97	2.59	52.96
23	71.00	1.249365	1.85	0.10	1.15	7.15	13.95	0.10	3.78	4.11	57.31
24	108.00	0.220025	2.03	-0.66	0.22	12.51	323.64	2.22	0.02	0.22	70.17
25	75.56	1.216085	1.88	0.08	0.97	7.85	21.72	0.11	3.68	3.87	83.99
26	75.00	1.253655	1.88	0.10	0.99	7.77	20.59	0.10	3.80	4.14	85.21
27	128.67	.053235	2.11	-1.27	0.05	15.19	1347.34	5.99	1.77	0.08	106.22
28	103.69	.599040	2.02	-0.22	0.28	11.92	234.52	0.66	1.40	0.94	220.60
29	94.38	1.423305	1.97	0.15	0.44	10.62	113.00	0.05	4.33	5.52	624.16
30	77.36	3.211520	1.89	0.51	0.90	8.12	25.73	0.10	8.56	44.63	1148.09
31	158.28	0.698555	2.20	-0.16	0.01	18.69	7936.43	0.50	1.80	1.25	9881.50
32	244.78	0.553865	2.39	-0.26	0.49	27.25	50692.97	0.75	1.21	0.82	> 50,000

* $IRT^A = (\log_{10} IRT - Mean\ x) / SD\ x)^2$

$IRT^B = (\log_{10} IRT - Mean\ y) / SD\ y)^2$

IRT^{LR} Likelihood ratio for CF derived from the following equation: $IRT^{LR} = (SDy) / (SDx)$* EXP (-0.5 * (IRT-a - IRT-b))

* $PAP^A = (\log_{10} PAP - Mean\ x) / SD\ x)^2$

$PAP^B = (\log_{10} PAP - Mean\ y) / SD\ y)^2$

PAP^{LR} Likelihood ratio for CF derived from CF carrier and controls using the following equation: $PAP^{LR} = (SDy) / (SDx)$* EXP (-0.5 * (PAP-a - PAP-b))

*Overall risk: the overall risk of the CF from both IRT and PAP using the following equation: IRT-LR×PAP-LR

Table 4-25: Distributions of likelihood ratios in CF cases, CF carriers and babies with elevated IRT, derived from IRT and PAP results

	LR	CF Cases	CF carrier	↑ IRT unaffected
Threshold risk 1	<20	1	14	116
	21-30	0	2	18
	31-40	1	3	22
	41-50	1	2	12
	51-60	0	2	7
	61-70	0	1	7
	71-80	0	0	7
	81-90	0	2	6
	91-100	0	0	1
	101-200	0	1	19
	201-300	0	1	9
	301-400	0	0	10
Threshold risk 2	401-500	0	0	3
	501-600	1	0	3
	601-700	1	1	2
	701-800	0	0	3
	801-900	0	0	2
	901-1000	0	0	3
	1001-2000	0	1	11
	2001-3000	0	0	6
	3001-4000	1	0	4
	4001-5000	0	0	4
	>5000	23	2	39

4.13 GESTATIONAL MARKERS OF CYSTIC FIBROSIS

Maternal serum samples from mothers of children affected with CF were studied for variations in the concentration of a range of serum markers known to be associated with adverse outcomes of pregnancy to determine whether they have any predictive value for CF. A total of 55 serum samples taken during pregnancies for which the outcome was the live birth of a child with CF were identified. An additional total of 330 pregnancies were randomly selected as controls matched to the same gestational range. Both second trimester (15 to 20 weeks) and first trimester cases (10 to 13 weeks) were included. The serum markers were AFP, hCG, PAPP-A, Free β-hCG, UE3 and Inhibin-A.

4.13.1 AFP Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF

In the 330 control pregnancy serum samples, the lowest AFP concentration was 4.80 U/mL (equivalent to 0.35 MoM) while the highest concentration level was 99.30 U/mL (equivalent to 2.22 MoM). The median AFP concentration level in the 330 control serum samples was 27.1 U/mL (equivalent to 0.98 MoM). Figure 4.52 shows the median AFP concentration level in maternal serum control samples and table 4.26 present the median values at each week of gestation. Figure 4.53 shows AFP (MoM) in samples from CF pregnancies. Figure 4.54 shows a box plot comparison of AFP level in controls and CF pregnancies.

The lowest AFP concentration level in the 55 serum samples of pregnancies where the outcome was the live birth of a child with CF was 5.00 U/mL (equivalent to 0.47 MoM), while the highest concentration level was 86.60 U/mL (equivalent to 3.47 MoM). The median AFP concentration level in the 55 maternal serum samples from mothers of children affected with CF was 29.27 U/mL (equivalent to 1.11 MoM).

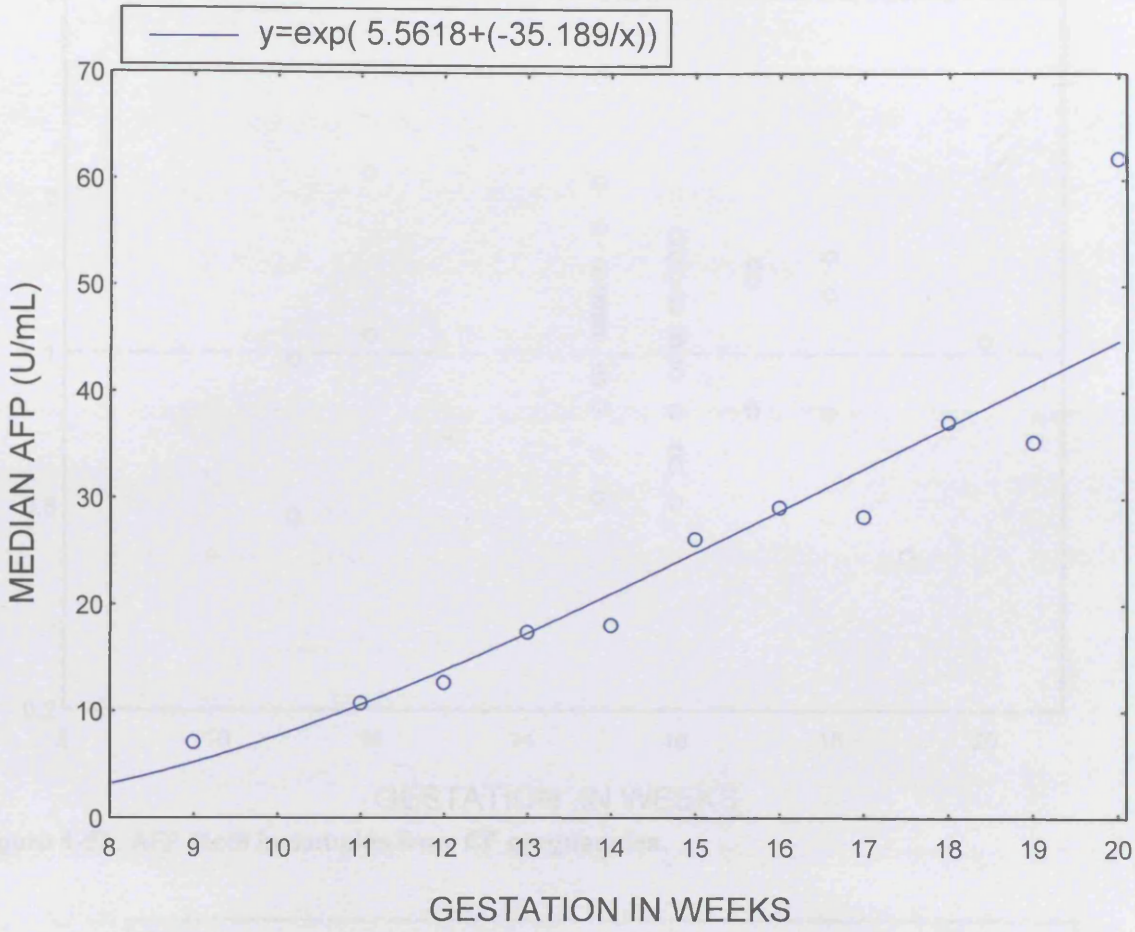

Figure 4-52: Median AFP concentration level at each week of gestation in maternal serum control samples.

Table 4-26: Control median at each weeks of gestation for AFP.

Gestation	AFP MEDIAN (U/mL)
9	7.00
11	10.65
12	12.60
13	17.35
14	18.00
15	26.10
16	29.10
17	28.22
18	37.12
19	35.23
20	61.90

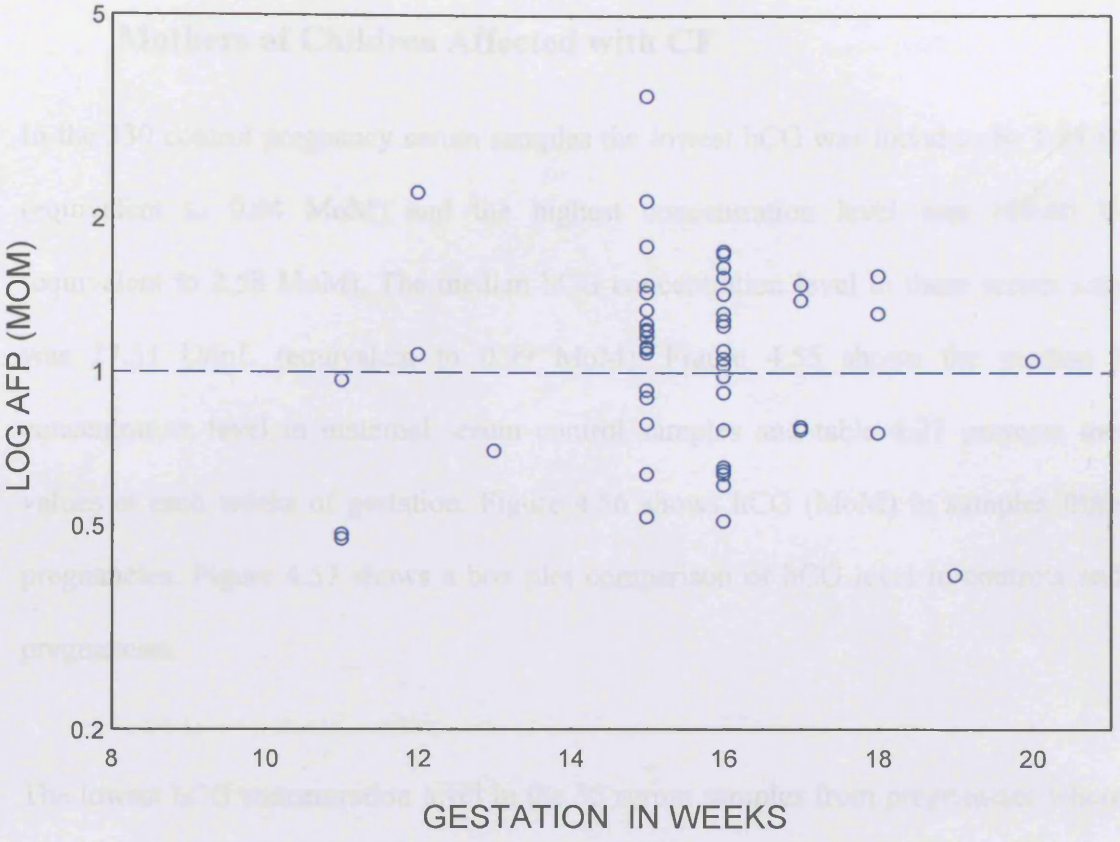

Figure 4-53: AFP MoM in samples from CF pregnancies.

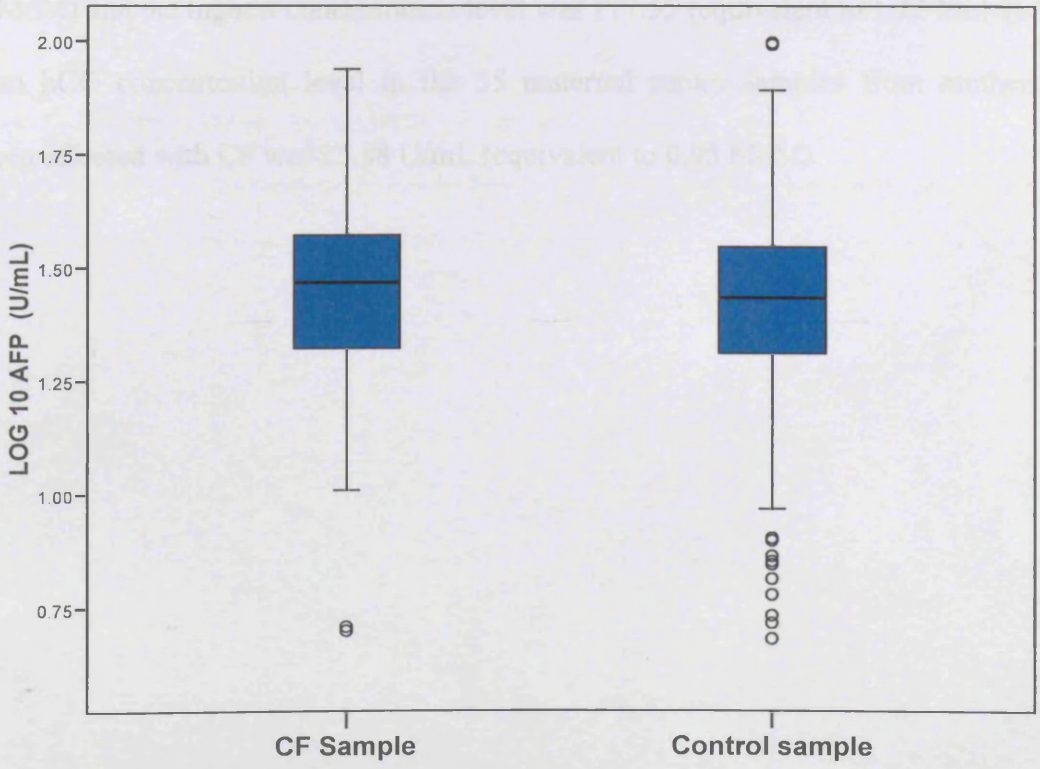

Figure 4-54: Box plot comparison of AFP in 55 CF samples and 330 control serum sample. Circles indicate outlier AFP values.

4.13.2 HCG Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF

In the 330 control pregnancy serum samples the lowest hCG was found to be 1.34 U/mL (equivalent to 0.04 MoM) and the highest concentration level was 180.60 U/mL (equivalent to 2.58 MoM). The median hCG concentration level in these serum samples was 27.51 U/mL (equivalent to 0.99 MoM). Figure 4.55 shows the median hCG concentration level in maternal serum control samples and table 4.27 presents median values at each weeks of gestation. Figure 4.56 shows hCG (MoM) in samples from CF pregnancies. Figure 4.57 shows a box plot comparison of hCG level in controls and CF pregnancies.

The lowest hCG concentration level in the 55 serum samples from pregnancies where the outcome was the live birth of a child with CF was found to be 8.50 U/mL (equivalent to 0.35 MoM) and the highest concentration level was 117.55 (equivalent to 1.22 MoM). The median hCG concentration level in the 55 maternal serum samples from mothers of children affected with CF was 25.88 U/mL (equivalent to 0.95 MoM).

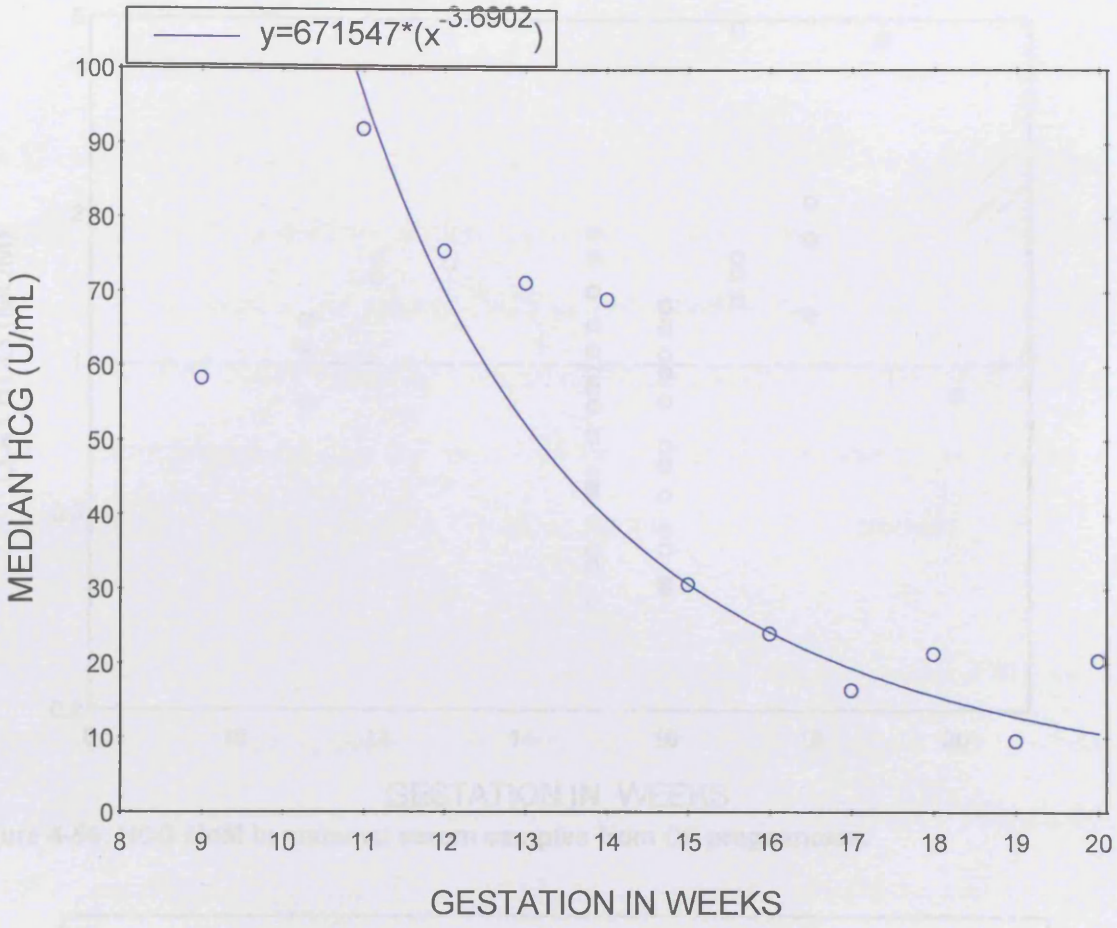

Figure 4-55: Median hCG concentration level in maternal serum control samples.

Table 4-27: Median values at each weeks of gestation for hCG.

Gestation	hCG MEDIAN (U/mL)
9	58.20
11	91.82
12	75.45
13	71.12
14	68.93
15	30.50
16	23.91
17	16.30
18	21.20
19	9.49
20	20.25

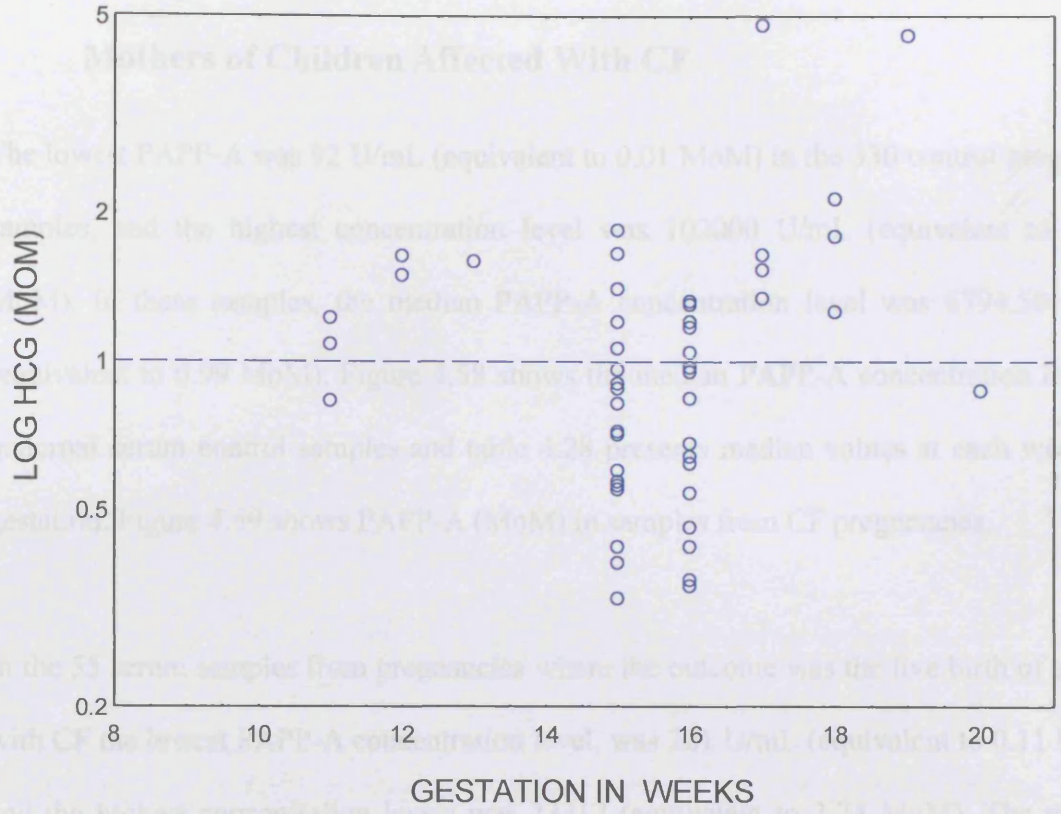

Figure 4-56: HCG MoM in maternal serum samples from CF pregnancies.

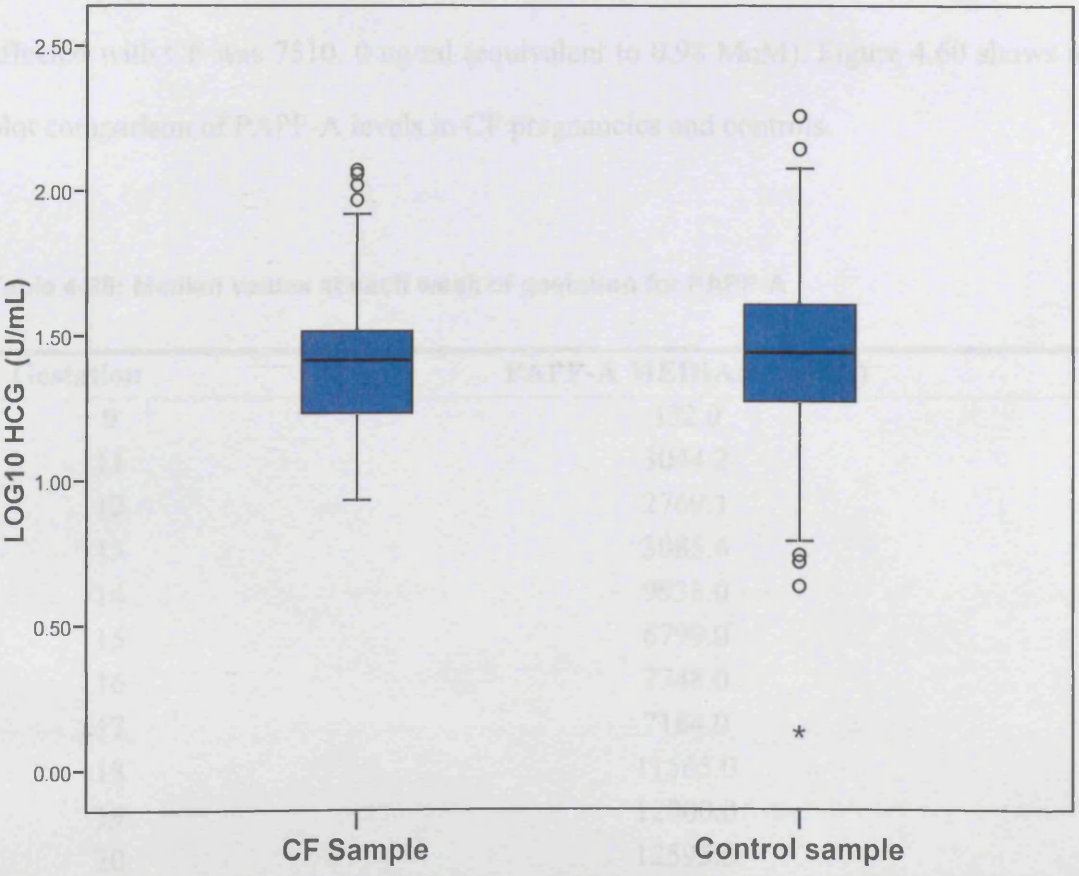

Figure 4-57: Box plot comparison of hCG in 55 CF samples and 330 control serum samples. Asterisks indicate extreme hCG values while circles indicate outlier hCG values.

4.13.3 PAPP-A Concentration Level in Maternal Serum Samples from Mothers of Children Affected With CF

The lowest PAPP-A was 92 U/mL (equivalent to 0.01 MoM) in the 330 control pregnancy samples, and the highest concentration level was 102000 U/mL (equivalent to 16.04 MoM). In these samples, the median PAPP-A concentration level was 6794.50 U/mL (equivalent to 0.99 MoM). Figure 4.58 shows the median PAPP-A concentration level in maternal serum control samples and table 4.28 presents median values at each weeks of gestation. Figure 4.59 shows PAPP-A (MoM) in samples from CF pregnancies.

In the 55 serum samples from pregnancies where the outcome was the live birth of a child with CF the lowest PAPP-A concentration level, was 221 U/mL (equivalent to 0.11 MoM) and the highest concentration levels was 23717 (equivalent to 3.74 MoM). The median PAPP-A concentration level in 55 maternal serum samples from mothers of children affected with CF was 7510. 0 ng/ml (equivalent to 0.98 MoM). Figure 4.60 shows a box plot comparison of PAPP-A levels in CF pregnancies and controls.

Table 4-28: Median values at each week of gestation for PAPP-A

Gestation	PAPP-A MEDIAN (mU/L)
9	332.0
11	3044.2
12	2769.1
13	3085.6
14	9938.0
15	6799.0
16	7748.0
17	7184.0
18	11565.0
19	12000.0
20	12595.0

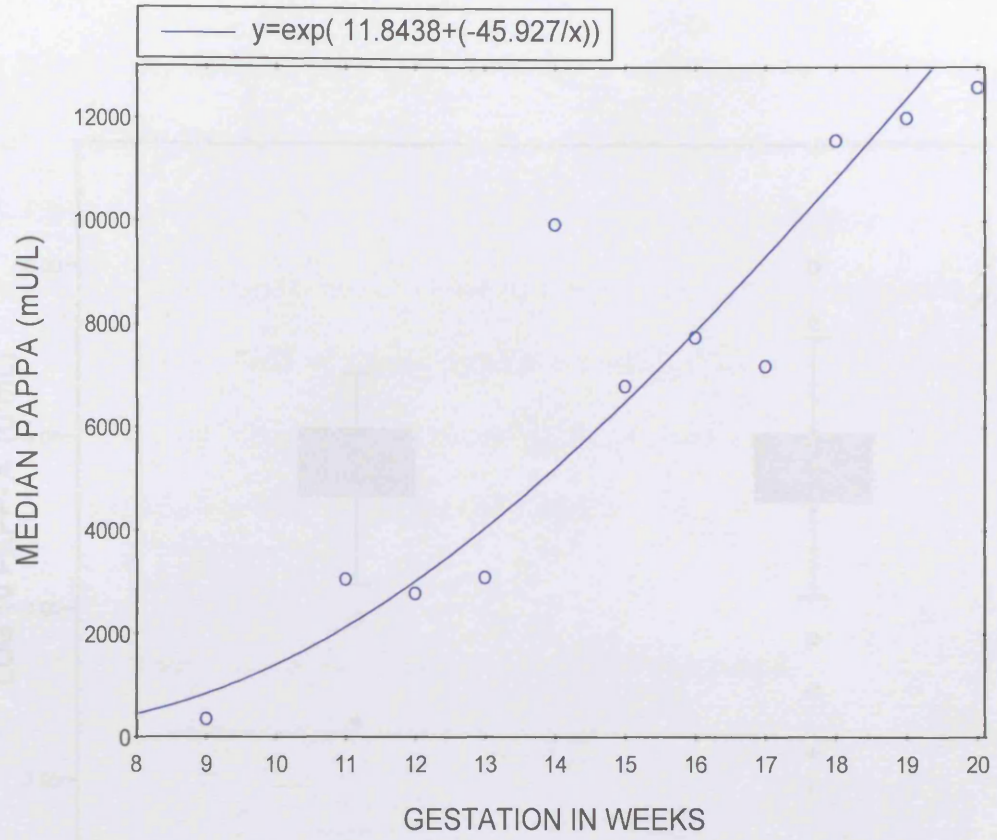

Figure 4-58: Median PAPP-A concentration level in maternal serum control samples.

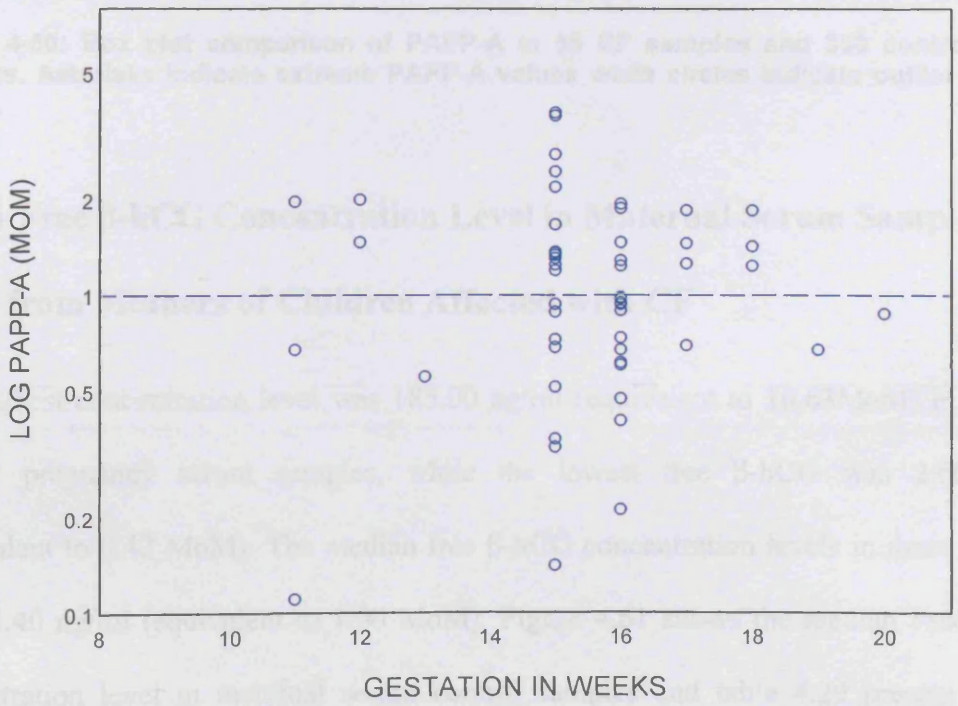

Figure 4-59: PAPP-A MoM in maternal serum samples from CF pregnancies.

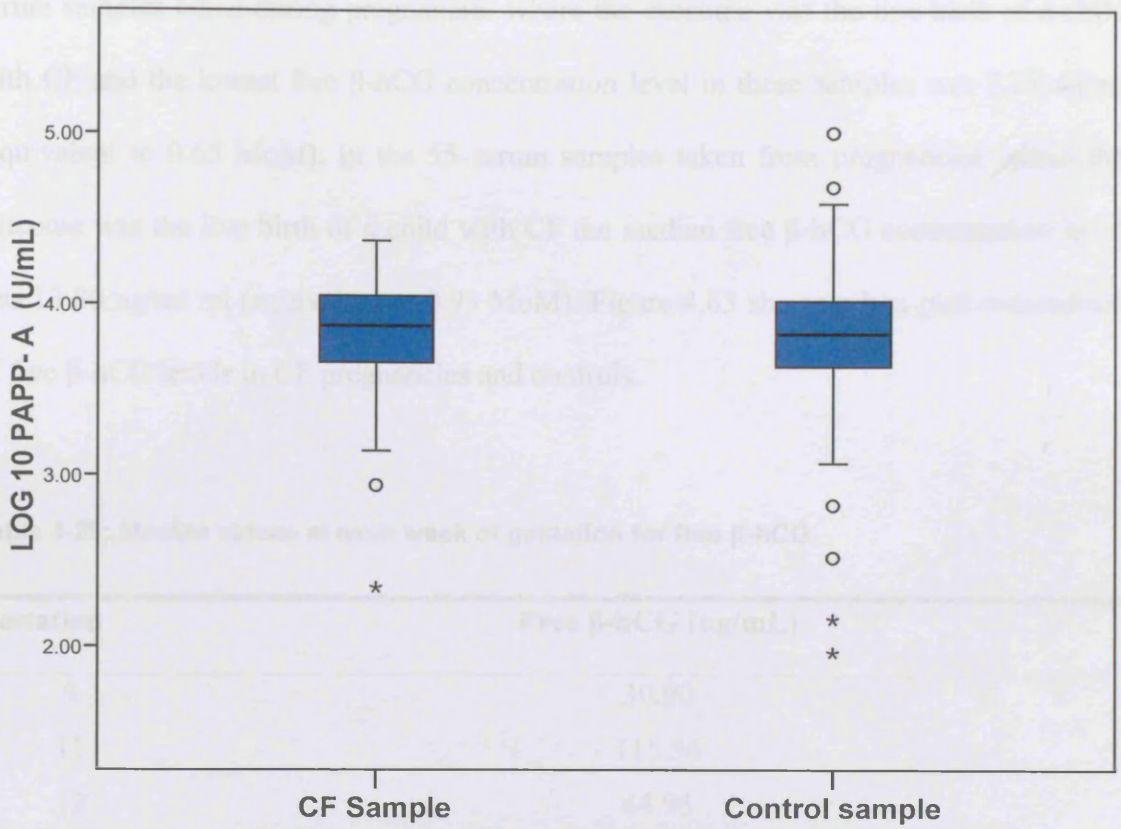

Figure 4-60: Box plot comparison of PAPP-A in 55 CF samples and 330 control serum samples. Asterisks indicate extreme PAPP-A values while circles indicate outlier PAPP-A values.

4.13.4 Free β-hCG Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF

The highest concentration level was 185.00 ng/ml (equivalent to 10.63MoM) in the 330 control pregnancy serum samples, while the lowest free β-hCG was 2.60 ng/ml (equivalent to 0.42 MoM). The median free β-hCG concentration levels in these samples was 14.40 ng/ml (equivalent to 1.00 MoM). Figure 4.61 shows the median Free β-hCG concentration level in maternal serum control samples and table 4.29 present median values at each week of gestation. Figure 4.62 shows free β-hCG (MoM) in samples from CF pregnancies.

The highest concentration level was 122.96 ng/ml (equivalent to 1.85 MoM) in the 55 serum samples taken during pregnancies where the outcome was the live birth of a child with CF and the lowest free β-hCG concentration level in these samples was 3.27 ng/ml (equivalent to 0.65 MoM). In the 55 serum samples taken from pregnancies where the outcome was the live birth of a child with CF the median free β-hCG concentration level was 13.80 ng/ml ml (equivalent to 0.93 MoM). Figure 4.63 shows a box plot comparison of free β-hCG levels in CF pregnancies and controls.

Table 4-29: Median values at each week of gestation for free β-hCG.

Gestation	Free β-hCG (ng/mL)
9	30.90
11	115.56
12	44.93
13	54.85
14	44.28
15	17.00
16	12.90
17	7.86
18	15.25
19	4.71
20	8.53

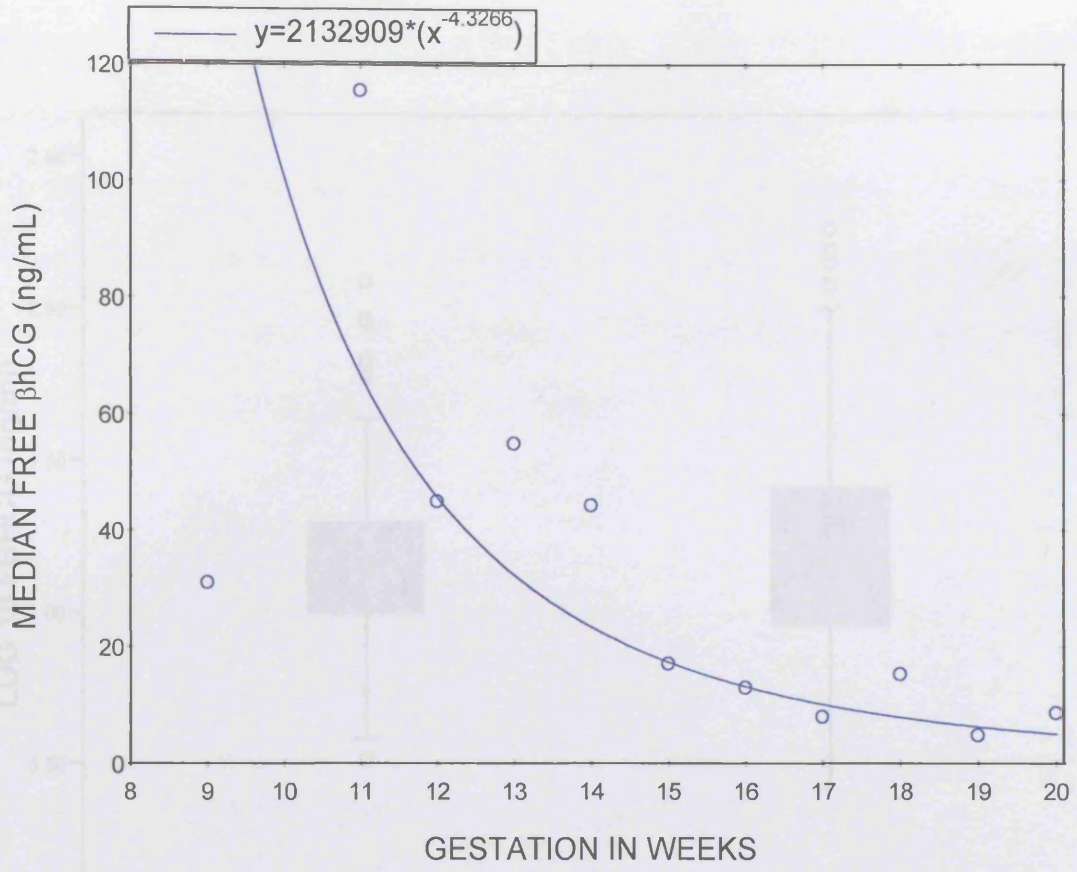

Figure 4-61: Median free β-hCG concentration level in maternal control serum samples.

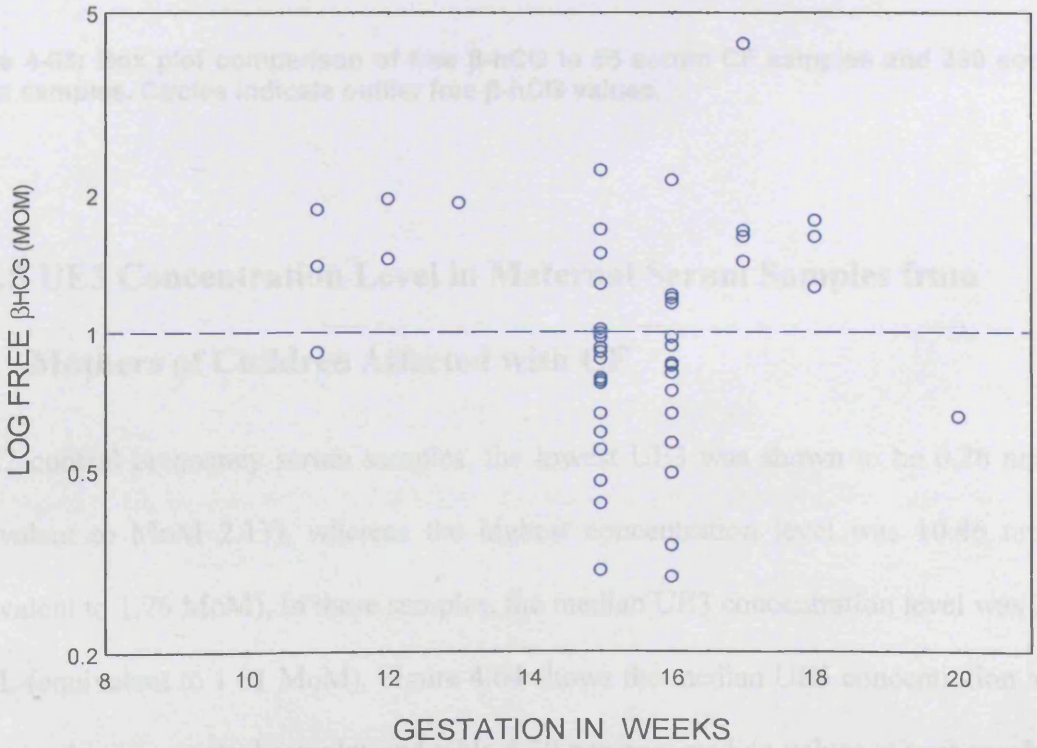

Figure 4-62: Free β-hCG (MoM) in maternal serum samples from CF pregnancies.

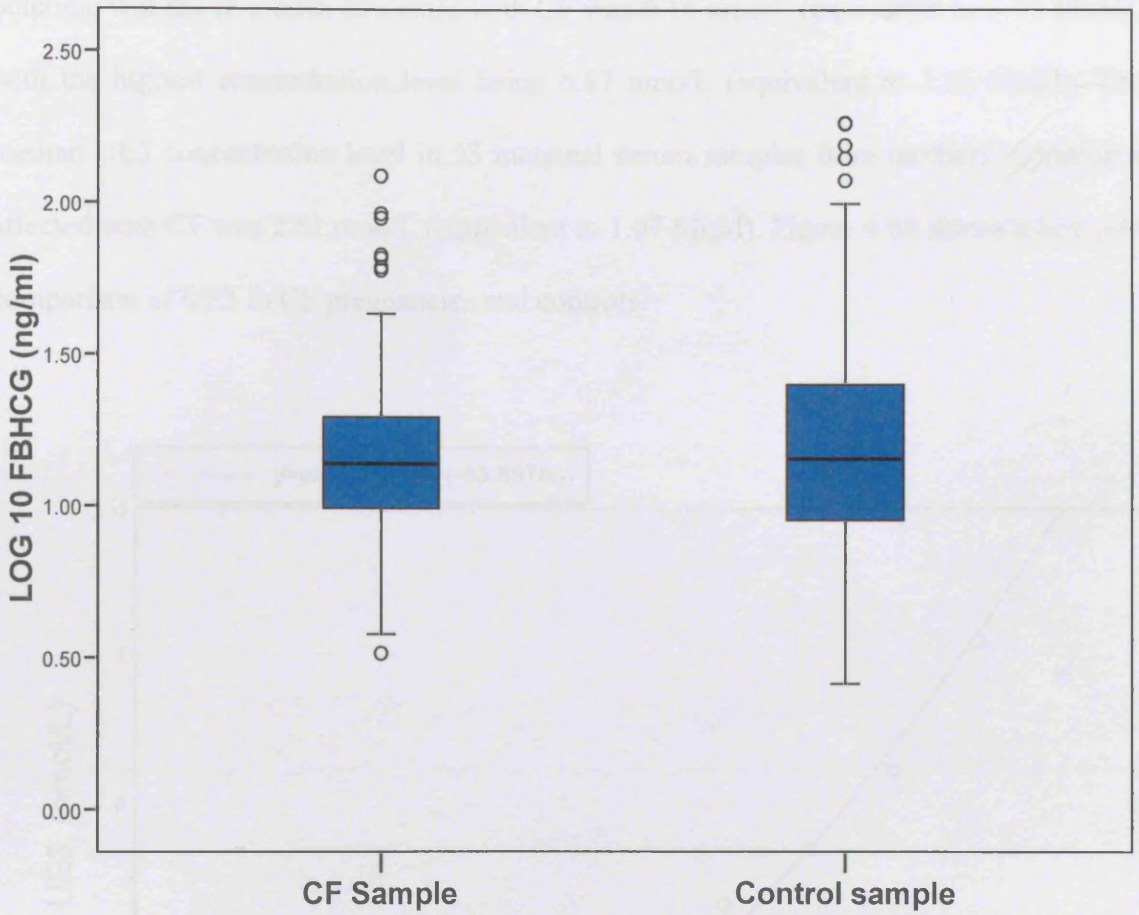

Figure 4-63: Box plot comparison of free β-hCG in 55 serum CF samples and 330 control serum samples. Circles indicate outlier free β-hCG values.

4.13.5 UE3 Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF

In 322 control pregnancy serum samples, the lowest UE3 was shown to be 0.26 nmo/L (equivalent to MoM 2.13), whereas the highest concentration level was 10.46 nmo/L (equivalent to 1.76 MoM). In these samples, the median UE3 concentration level was 2.48 nmo/L (equivalent to 1.01 MoM). Figure 4.64 shows the median UE3 concentration level in maternal serum control samples and table 4.30 presents median values at each weeks of gestation. Figure 4.65 shows UE3 (MoM) in samples from CF pregnancies.

The lowest UE3 concentration level in the 53 serum samples from pregnancies where the outcome was the live birth of a child with CF was 0.14 nmo/L (equivalent to 0.33 MoM), with the highest concentration level being 6.87 nmo/L (equivalent to 1.65 MoM). The median UE3 concentration level in 53 maternal serum samples from mothers of children affected with CF was 2.51 nmo/L (equivalent to 1.07 MoM). Figure 4.66 shows a box plot comparison of UE3 in CF pregnancies and controls.

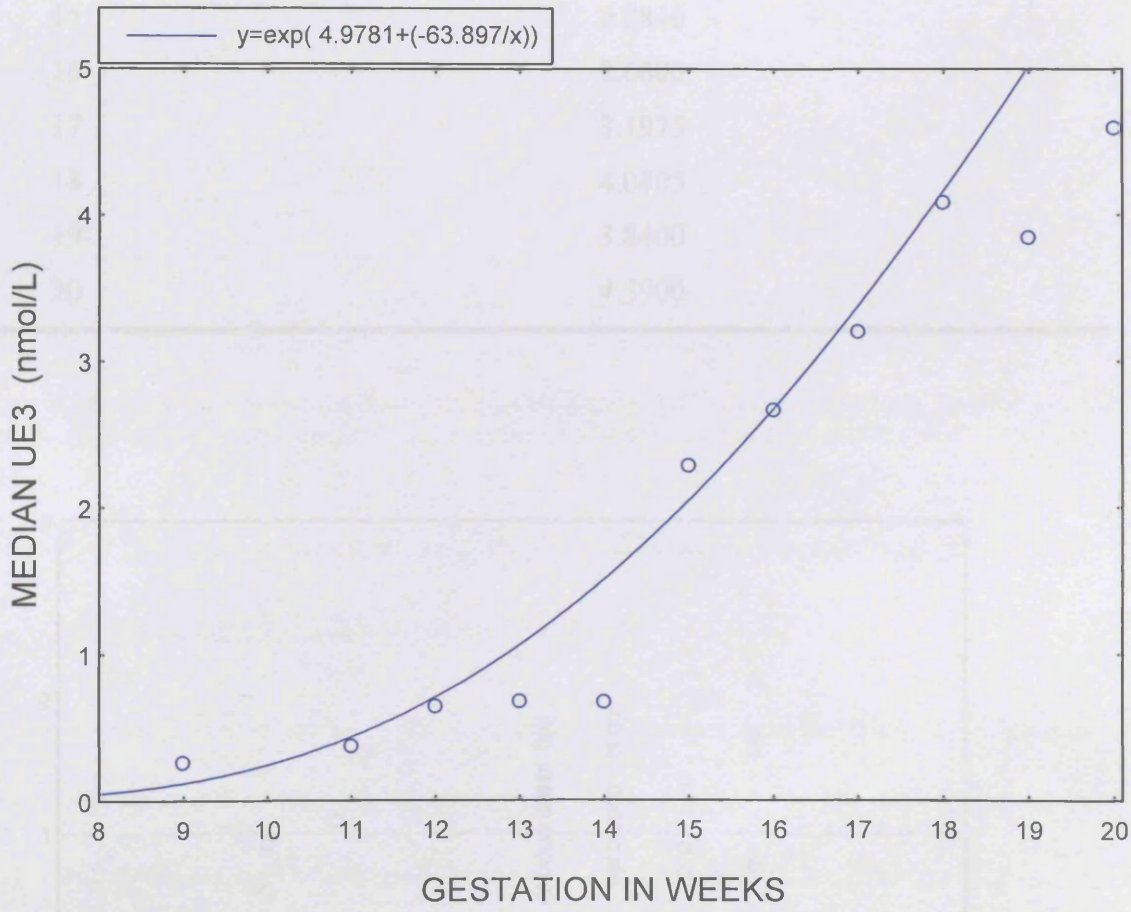

Figure 4-64: Median UE3 concentration level in maternal control serum samples.

Table 4-30: Median values at each weeks of gestation for UE3

Gestation	UE3 (nmol/L)
9	0.2550
11	0.3660
12	0.6370
13	0.6705
14	0.6660
15	2.2810
16	2.6600
17	3.1975
18	4.0805
19	3.8400
20	4.5900

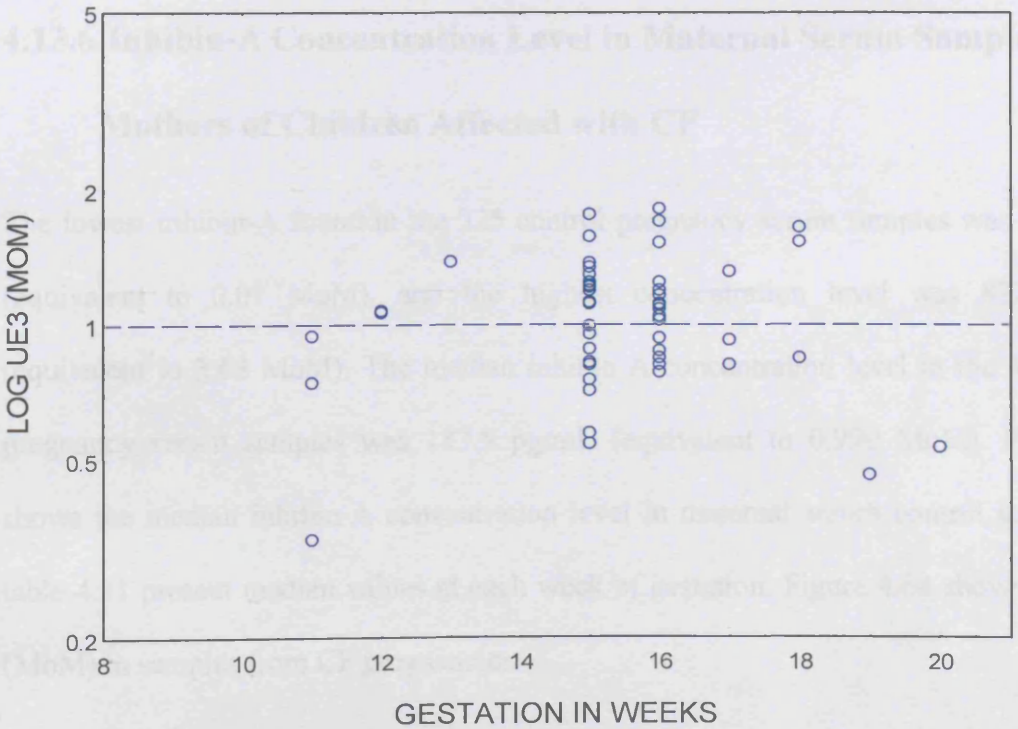

Figure 4-65: UE3 MoM in maternal serum samples from CF pregnancies.

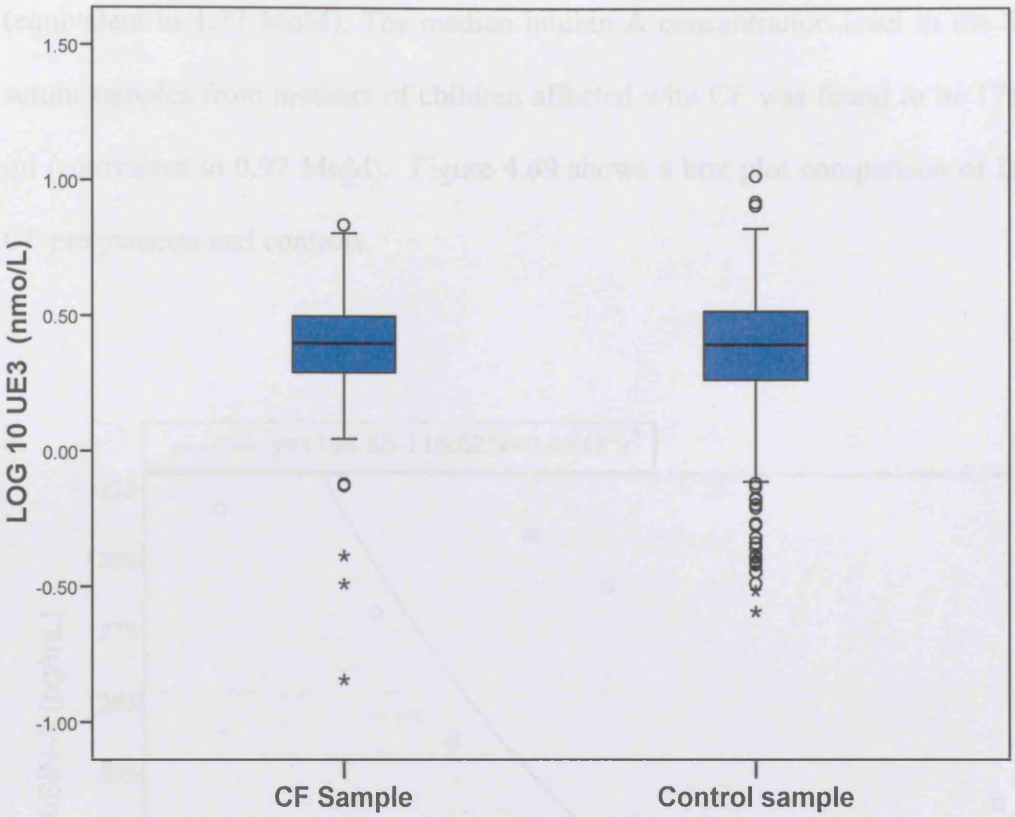

Figure 4-66: Box plot comparison of UE3 in 53 serum CF samples and 325 control serum sample. Asterisks indicate extreme UE3 values while circles indicate outlier UE3 values.

4.13.6 Inhibin-A Concentration Level in Maternal Serum Samples from Mothers of Children Affected with CF

The lowest inhibin-A found in the 325 control pregnancy serum samples was 1.3 pg/mL (equivalent to 0.01 MoM), and the highest concentration level was 822.0 pg/mL (equivalent to 3.68 MoM). The median inhibin-A concentration level in the 325 control pregnancy serum samples was 183.5 pg/mL (equivalent to 0.990 MoM). Figure 4.67 shows the median inhibin-A concentration level in maternal serum control samples and table 4.31 present median values at each week of gestation. Figure 4.68 shows inhibin-A (MoM) in samples from CF pregnancies.

The lowest inhibin-A concentration level found in the 53 serum sample of pregnancies where the outcome was the live birth of a child affected with CF was 60.6 pg/mL

(equivalent to 0.35 MoM) and the highest concentration level was 497.80 pg/mL (equivalent to 1.77 MoM). The median inhibin-A concentration level in the 53 maternal serum samples from mothers of children affected with CF was found to be 173.20 pg/mL ml (equivalent to 0.97 MoM). Figure 4.69 shows a box plot comparison of Inhibin-A in CF pregnancies and controls.

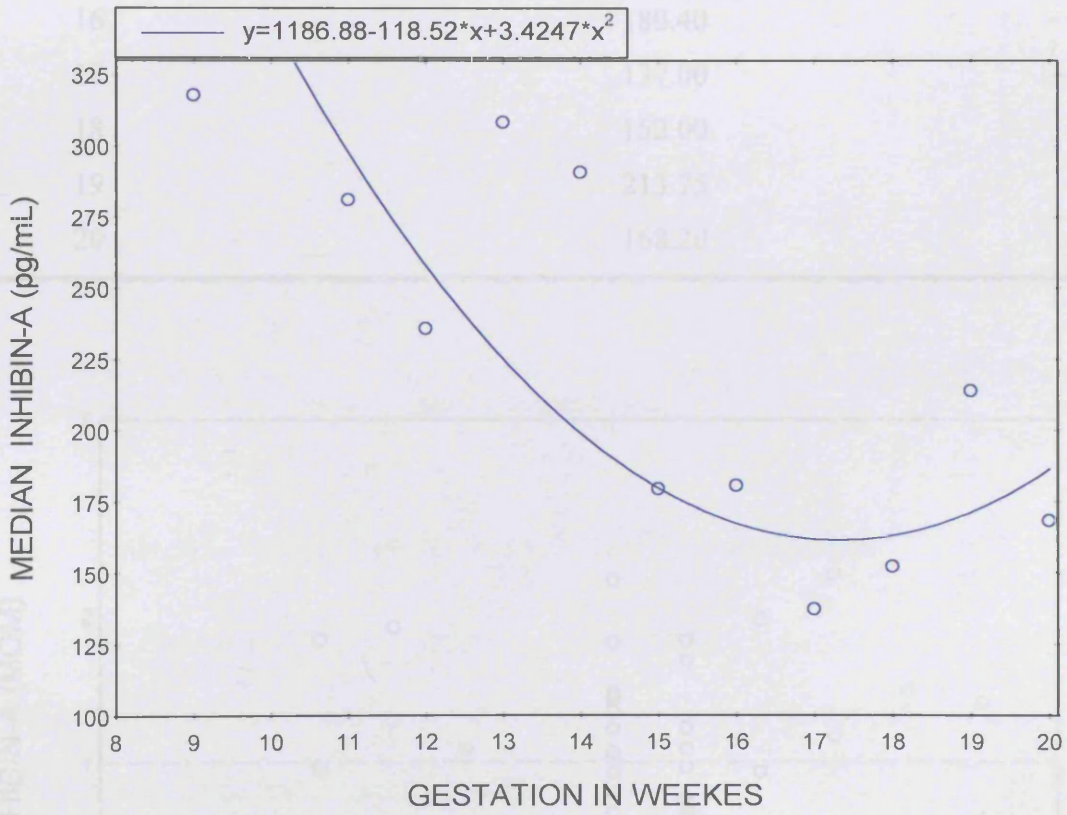

Figure 4-67: Median inhibin-A concentration level in maternal control serum samples.

Table 4-31: Median values at each weeks of gestation for inhibin-A.

Gestation	Inhibin-A (pgl/mL)
9	317.70
11	280.95
12	235.65
13	308.15
14	290.60
15	179.20
16	180.40
17	137.00
18	152.00
19	213.75
20	168.20

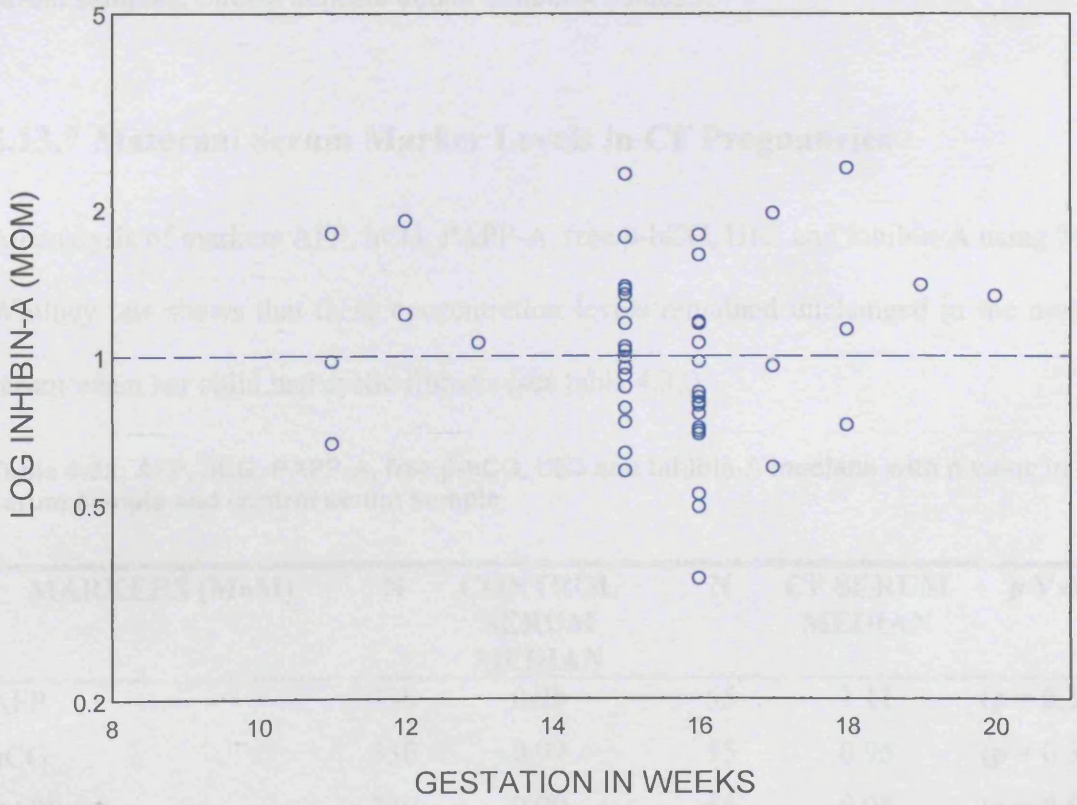

Figure 4-68: Inhibin-A in maternal serum samples and CF samples from pregnancies.

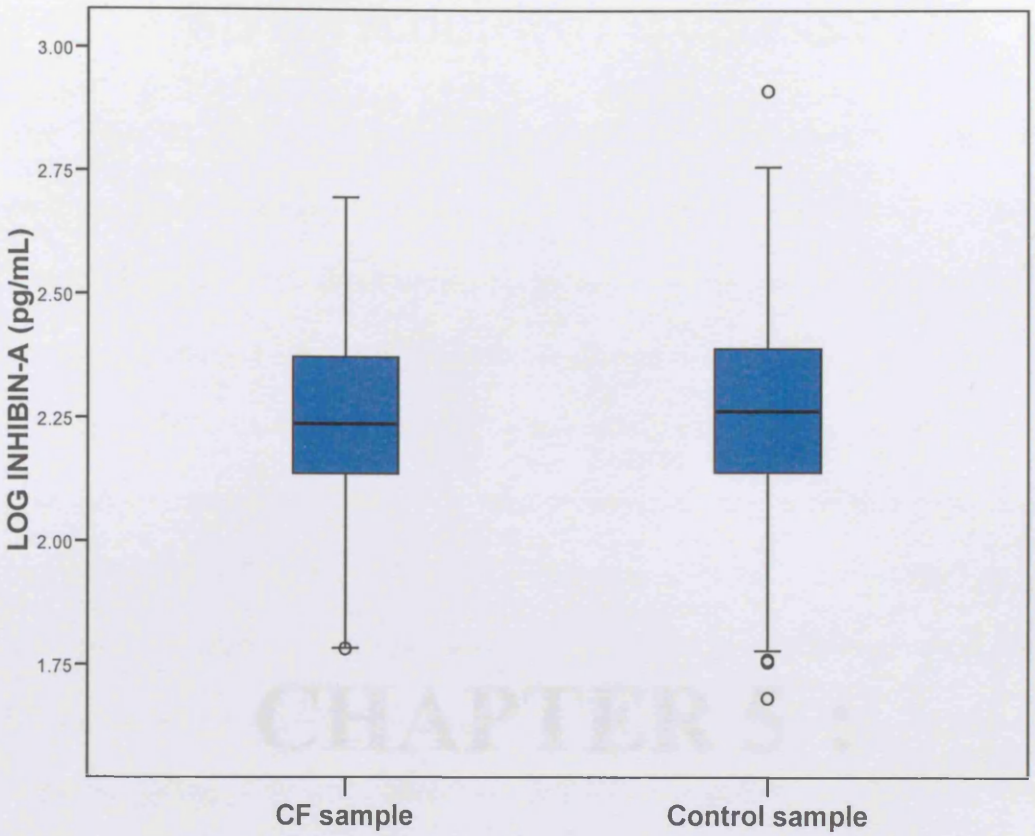

Figure 4-69: Box plot comparison of inhibin-A in 53 serum CF samples and 325 control serum samples. Circles indicate outlier inhibin-A values.

4.13.7 Maternal Serum Marker Levels in CF Pregnancies

An analysis of markers AFP, hCG, PAPP-A, free β-hCG, UE3 and inhibin-A using Mann-Whitney test shows that these concentration levels remained unchanged in the mother's serum when her child had cystic fibrosis (see table 4.32)

Table 4-32: AFP, hCG, PAPP-A, free β-hCG, UE3 and inhibin-A medians with p value in CF serum sample and control serum sample

MARKERS (MoM)	N	CONTROL SERUM MEDIAN	N	CF SERUM MEDIAN	*p* Value
AFP	330	0.98	55	1.11	(p = 0.335)
hCG	330	0.99	55	0.95	(p = 0.539)
PAPP-A	330	0.99	55	0.98	(p = 0.632)
Free β-hCG β-hCG	330	1.00	55	0.93	(p = 0.824)
UE3	322	1.01	52	1.07	(p =0.840)
Inhibin-A	322	0.99	52	0.97	(p = 0.650)

CHAPTER 5 :

DISCUSSION

5.1 NEWBORN BLOODSPOT MARKERS OF CF

The objective of prenatal and newborn screening is to identify babies with health problems. Such screening provides certain information concerning the health of babies. Prenatal testing is carried out during pregnancy from the early stages of gestation, while newborn testing, as the name suggests, is carried out just after birth. There are major differences between prenatal screening and newborn screening. Prenatal screening can give a diagnosis before the baby is born opening up the possibility of prevention of the birth of an affected child. Newborn screening aims to provide early detection of a disorder meaning that better medical intervention can be made. In Scotland, newborn screening for CF started in February 2003, based on the use of IRT to select infants for referral for DNA mutation testing. This study aimed to look for ways to make current IRT testing more precise by using an additional marker, PAP. The objective was to decrease the number of babies referred and carriers detected by DNA testing of screen positive babies and examine the cost implications of a change in protocol.

5.1.1 Current CF Screening

5.1.1.1 Workload and Cost

In Scotland between February 2003 and end of 2010 a total of 450,000 babies were screened for CF and of these, 3,122 were referred for DNA analysis because of an elevated IRT result (>70 ng/ml), a referral rate of 0.69%. This exceeds the 0.5% target based on using a 99.5th centile IRT cut-off and is due to a positive bias in the DELFIA IRT assay. While this reduces the likelihood of missing a CF case with relatively low IRT, it increases workload and costs.

Over the 8-year period that IRT screening has been in routine use, 198 babies have been detected with CF (incidence 1:2272) giving a positive predictive value of 6.3% for the screening protocol. The cost of DNA mutation analysis (31 or 29 mutation panel) of 3,122 screen positive babies is estimated at £62,440 equivalent to approximately £20/test or £7,805/year (personal communication of Dr A Cooke). In addition, over the same period, a total of 230 carriers were detected through DNA testing of screen positive babies. This gives a carrier rate within the screen positive group of about 1 in 12, which is approximately double the frequency in the general Scottish population. CF carriers are therefore relatively commonly picked up in the screen positive group. However, it is a requirement of the screening protocol that where a carrier is detected this is reported to the parents by the family doctor/practice nurse and a referral offered for genetic counselling. Although carriers are clinically unaffected, to be told that their baby is a carrier may cause anxiety in the parents and lead to counselling and cascade testing in the wider family. Thus, further significant costs arise in the programme due to the incidental detection of carriers.

Modifying the current protocol by changing the IRT cut-off to, for example the 99.7th centile (approx 90 ng/ml) and following-up only 0.3% of babies would reduce the number of DNA referrals to about 180/year in Scotland, which is about half the current rate. However, this change in cut-off would mean that CF cases with IRT concentrations between 70 ng/ml and 90 ng/ml would not be detected. An audit of the 198 CF cases detected over 8 years showed that there were 25 with an IRT value between 70 and 90 ng/ml, which would therefore have been missed if the 99.7th centile has been used as a cut-off.

Thus, the scope for modifying the current single marker protocol to reduce referrals for DNA testing and detection of carriers but maintain high detection of CF cases is limited. Additional markers of CF which can increase the specificity of screening are required.

5.1.1.2 Alternative Newborn Bloodspot Markers

In this study and elsewhere, a number of possible markers for CF screening have been examined.

Elevated sweat chloride is a strong indicator of CF but it is impractical to use as a screening test in newborn babies as the process of collecting the sweat is difficult and time consuming. Sweat testing has a valuable role as a diagnostic back-up test for DNA.

Faecal elastase is found in reduced concentration in the babies with pancreatic insufficiency. At day 3 in term infants the normal levels are expected and by two weeks of age in those born less than 28 weeks gestation; therefore the test should not be performed before this time (Davies, 2006) and the collection and analysis of stool samples is impractical for mass newborn population screening.

Direct testing of DNA extracted from dried bloodspots for a panel of CF mutations is potentially possible for newborn screening and would theoretically result in the detection of most CF cases. However, the presence of hundreds of rare mutations would require sequencing to identify the mutations in all CF cases and in addition, all carriers and many mutations associated with mild disease would be detected, leading to major counselling problems.

The aforementioned markers fail the Wilson and Jungner criteria for screening tests as they are too time consuming or expensive to be considered as suitable for newborn screening. The ideal marker is one that can be easily measured in large numbers of babies quickly and cheaply using the bloodspots already collected on the Guthrie card. In 2005, Sarles et al reported on the use of a second pancreatic marker, PAP, which could be measured in bloodspots and was found in elevated concentrations in babies with CF.

5.1.1.3 Pancreatitis Associated Protein (PAP)

Sarles et al (2005), in their studies in France, have shown that pancreatitis-associated protein (PAP) concentrations are also elevated in the blood of newborn babies affected with CF.

Sarles' studies started between November 2002 and December 2003, measuring IRT and PAP in all the newborn babies in 5 regional screening centres in France (n=204,749). They were all screened for CF through the French national screening programme, which was initiated in April, 2002. The routine screening strategy used is the following: a single IRT test at day 3 and then, for those found to have elevated IRT levels (defined as >50ng/mL up to February 2003 and >65 ng/mL subsequently), analysis of 20 CFTR mutations.

Babies in whom one or two mutations are found then undergo sweat testing. If no mutations are found, another blood spot is collected at day 21 to repeat the IRT and where IRT is >45 ng/mL, a sweat test is carried out. A diagnosis of CF is made if the sweat chloride results are ≥60 mEq/L. Babies whose first sweat test result is 40 to 59 mEq/L are followed up and checked at 3, 6 and 12 months.

ELISA was the choice for PAP analysis, (MucoPAP, DYNABIO S.A., Marseille, France). Blood spot samples of 3mm in diameter were punched from the screening cards and elution of dried blood was carried out in 150 μL of phosphate-buffered saline. Assays were carried out in duplicate, at the same time as IRT. Both assays were conducted locally and results sent to the main centre before diagnosis.

The Sarles protocol for CF screening is to test all newborns for PAP and IRT, after which two groups of newborns, those with IRT>50 ng/mL and with PAP >1.8 ng/mL, and those with IRT > 100 ng/mL and PAP>1.0 ng/mL are recalled for sweat testing. Sarles et al (2005) presented data which showed that this two-stage screening strategy would have the same performance as the IRT/DNA mutation strategy. However, this approach requires testing IRT and PAP in all newborns.

If such a policy was adopted in Scotland, this would require the measurement of PAP in addition to IRT in over 60,000 babies per year. The commercially available PAP ELISA (Dynabio S.A., Marseille, France) is priced at 423 Euros per 96-well plate (approximately £350) which is sufficient for analysis of 80 bloodspot specimens in singleton (the remainder of the plate wells are taken up by standards, QCs and blanks). This equates to about £4.38 per sample and would add a total of £271,560 to the annual cost of running the Scottish CF newborn screening programme.

Because of the high cost of this protocol, an alternative strategy has been explored in this study, testing PAP only in those babies who have an elevated IRT screening result. This greatly reduces costs (to £1533/year), although the practicalities of testing mean that only part of each plate can be used in the present ELISA format and this would increase costs above the theoretical minimum.

5.1.2 Marker Levels in Confirmed CF Cases

In this study, out of 3632 babies screened, 29 samples were positively identified as CF cases, of which 14 were girls and 16 boys. The median PAP concentration in controls was 0.19 ng/ml and in the 29 CF cases 1.91 ng/ml, equivalent to 10.0 MoM. This confirms that there is an association between elevated PAP and CF as reported by Sarles et al (2005). The corresponding median for IRT was 19.3 ng/ml in the control and 161.5 ng/ml in the CF cases, equivalent to 8.3 MoM. The lowest PAP level in the CF cases was 0.24 ng/ml (1.2 MoM). This PAP level therefore sets the lowest cut-off which can be used if the detection of CF cases is to be maintained when a PAP cut-off is included in the screening protocol along with IRT. A disadvantage of this approach therefore is that the detection rate of CF cannot be increased by incorporation of PAP as it is already set at a maximum by selecting babies with elevated IRT. However, if detection can be maintained and the screen positive rate reduced by developing a combined IRT/PAP protocol, this would improve the current screening programme.

The other commonly used newborn screening markers for phenylketonuria, phenylalanine (PHE) and tyrosine (TYR) were also examined for any association with CF. The median PHE concentration in controls was 56.2 μmol/L and in the 29 CF cases 54.0 μmol/L, equivalent to 0.96 Mom. The median TYR concentration in control was 93.8 μmol/L and in the 29 CF cases 59.0 μmol/L, equivalent to 0.62 MoM. Result showed, apart from PHE ($p = 0.890$) all markers show a statistically significant difference using the Mann-Whitney test. IRT ($p = <0.001$) and PAP ($p = <0.001$) levels were significantly elevated, while TYR levels were significantly reduced ($p = <0.001$) (see table 4.12). It is not clear what mechanism may be operating to cause this small reduction in TYR levels.

5.2 PREDICTIVE VALUE OF IRT AND PAP

5.2.1 Use of Cut-offs for IRT and PAP

In this study, if referral for DNA testing was based on an IRT level ≥99.5th centile and a PAP level ≥1.2 multiples of the control median, 25% fewer DNA analyses would be required and the number of carriers detected reduced from 32 to 24 for no loss of detection of CF cases (see table 4.21 and table 4.22).

This modest improvement in screening specificity falls short of the performance described by Sarles et al (2005) and suggests the strategy of testing PAP only in babies with an elevated IRT is less effective than testing all babies.

5.2.2 Use of Likelihood Ratio Method for IRT and PAP

Some of the disadvantages of using a fixed cut-off level for IRT and PAP can be overcome by using the likelihood ratio statistical method. The probability that a certain IRT or PAP level is associated with an infant affected by CF rather than one who is unaffected can be estimated from the overlapping control and CF distributions. For instance, it is 8.2 times more likely that an IRT concentration of 70.80 ng/ml ($\log_{10}$ 1.85) will be associated with a CF infant than an unaffected one. Similarly, a PAP level of 1.26 ng/ml ($\log_{10}$ 0.1) is 4.1 times more likely to be associated with an infant affected by CF than with an infant who is unaffected (see Figure 4.49 & Figure 4.51). Moreover, the product of the individual likelihood ratios can be calculated, thus combining the information from two markers due to their being only a low correlation between IRT and PAP levels in CF cases (Pearson correlation $p = 0.669$).

In 29 CF cases, the likelihood ratios for the two markers are multiplied together to give the overall risk for each affected CF baby (table 4.23). For babies affected by CF, the lowest

overall risk (not CF: CF) for both markers is 1:4.8 and the highest value is 1: > 50,000. If the case with the lowest likelihood ratio is excluded (and therefore lost to detection), the next lowest likelihood for a CF case is 1:36.99 (not CF: CF), which will be detected if a cut-off likelihood ratio of, say, 1:30 (not CF: CF) is chosen as the referral likelihood ratio for mutation testing. The effect of this on the number of referrals and carrier detection can be seen by reviewing the likelihood ratios in the elevated IRT group (n=314, Appendix A) and carrier group (n=32, table 4.24).

A summary of the results (table 4.25) shows the IRT and PAP risk in CF cases, CF carriers and those with increased IRT levels. The suggested combined risk cut-off for IRT and PAP of 1:30 (not CF: CF) for CF cases, CF carriers, and those with increased IRT levels would be an appropriate risk to choose in order to reduce the number of CF carriers from 32 to 16 (50%) and reduce further DNA analysis from 314 to 180 (43%), losing only a single CF case (Table 4.25).

5.3 Other PAP Studies

5.3.1 Study 1

In the south of Australia a study (Ranieri et al., 2009) was conducted to evaluate an IRT-PAP/DNA CF screening strategy. In this study, PAP was measured (MucoPAP, DYNABIO S.A., Marseille, France) with the adjustment of using europium labelled streptavidin and IRT (NeoIRT, Perkin-Elmer, Turku, Finland) on blood spots. A total 2,250 samples (cohort A) gave a normal population distribution with median, 95th and 99th percentile values for PAP of 0.14, 0.43 and 0.8 ng/mL, and for IRT of 17, 58 and 73 ng/mL respectively. These concentrations for PAP and IRT are similar to those described in the present study. PAP was also determined in newborns (n = 256) with an IRT ≥99th percentile and with (cohort C) or without (cohort B) one CFTR mutation. In cohorts B and

C, the median and 95th percentile values for IRT were 87.5, 177 ng/mL and 106, 499 ng/mL, and for PAP they were 0.16, 0.6 ng/mL and 0.27, 2.8 ng/mL respectively. The PAP values in cohort B were not found to differ significantly from the normal population (p=0.12, Kolmogorov-Smirnov test), while just 5% were found to have a PAP level ≥99th percentile. In cohort C, identified with an IRT ≥99th, one CFTR mutation and a normal sweat test, 35% had a PAP level ≥99th percentile. Therefore based on the use of the 99th centile as cut-off, the study results suggest that including PAP as described decreases by 65% the number of newborns requiring CFTR mutational analysis.

5.3.2 Study 2

In a published abstract in the *Journal of Cystic Fibrosis* (Vigano, et al., 2008), a multi-centre feasibility study was carried out comprising two sites in the USA, one in Germany and one in Italy with the aim of assessing the utility of the combined IRT-PAP CF protocol in comparison with the conventional IRT/CFTR strategy. IRT and PAP were measured in 18,080 newborns. IRT was measured in dried blood spots collected at age 3–5 days by a time-resolved fluoroimmunoassay (Perkin-Elmer Life Sciences, Turku, Finland). Based on the calculation of the percentiles of data obtained in the different centres, hypothetical cut-offs were set at IRT>50 ng/mL and PAP >1.8 ng/mL or IRT>100 ng/mL and PAP>1.0 ng/mL, the same as those chosen by Sarles et al (2005). The IRT/PAP strategy appeared to have good potential, as it was shown to have the ability to reduce the number of mutation analysis needed to 0.3%, which reduced both the cost of the screening and the number of carriers identified. However, this study was limited in that the sample size was fairly small and it did not permit the sensitivity in detecting mild CF forms to be evaluated.

5.3.3 Study 3

Another published study of PAP in the *Journal of Cystic Fibrosis* (Vernooij-van Langen, et al., 2009) was carried out in the Netherlands using two strategies in each sample. Immunoreactive trypsinogen (IRT) and pancreatitis-associated protein (PAP) were used in the first strategy. Where concentrations of IRT >50ng/ml and PAP >1.8ng/ml or IRT >100 ng/ml and PAP >1.0 ng/ml were found, the test was rated positive. Samples with IRT >50ng/ml were analysed for 36 CF-mutations in the second strategy. An extended gene-analysis (EGA) was carried out in samples with one CF-mutation and when two CF-mutations were identified the tests were rated positive.

72,874 newborns were screened and 127 tested positive with the IRT/PAP protocol. Of these, ten CF cases were identified. A further 11 CF-patients were identified from 18 tests with IRT-DNA-EGA. Eighty-four carriers and seven compound heterozygotes with R117H-7T as a second mutation were also revealed with the IRT-DNA-EGA. A retrospective analysis with both strategies was carried out in heel-prick samples of 18 known CF cases in order to assess the sensitivity. Thirteen cases were identified with the IRT-PAP and 18 with the IRT-DNA-EGA. Specificity and PPV for the IRT-PAP were 99.8% and 8.2%, and 99.99 % and 61% for the IRT-DNA-EGA. Based on the results of the screening programme and the retrospective analysis, sensitivity was 82% and 100%. It was concluded that improved test performance was obtained using the IRT-DNA-EGA strategy in comparison to current strategies, but that this was not the case for the IRT-PAP strategy.

5.3.4 Study 4

A recent PAP study in the south-west of Germany has been reported (Sommerburg et al., 2010). In this study, a prospective and sequential IRT/PAP approach was taken. IRT was

the first tier and PAP the second, and this approach was compared to a commonly used IRT/DNA protocol in a population-based newborn screening study conducted in the south-west of Germany. Prospective quantitation of PAP and genetic analysis for the presence of four mutations in the CFTR gene with the greatest prevalence in southwest Germany (F508del, R553X, G542X ,G551D) was carried out in all newborn babies with IRT> 99.0th percentile. If either PAP was ≥1.0 ng/mL and/or at least one CFTR mutation was detected, the newborn screening result was considered positive. Furthermore, IRT>99.9th percentile was also rated positive. Referral to a CF centre to test sweat Cl concentration followed a positive rating. Of 73,759 newborns tested, 98 (0.13%) were found to be positive with IRT/PAP and 56 (0.08%) with IRT/DNA. Of these 135 CF NBS-positive infants, 13 were diagnosed with CF after sweat testing, with similar detection rates for IRT/PAP and IRT/DNA. The PAP concentration of one of the 13 babies diagnosed with CF was <1.0 ng/mL.

It was concluded that sequential measurement of IRT/PAP offers good sensitivity and specificity while permitting reliable, cost-effective CF screening of newborn babies. It also avoids the need for genetic testing and its associated ethical issues.

5.4 PAP / IRT Screening Performance

Taken together, the results of the above studies and the present study show that PAP has a useful predictive value as a newborn screening marker of CF when combined with IRT. However the performance in each of the studies varies according to the protocol used and none matches the results reported by Sarles et al (2005), where sensitivity and specificity were high enough to allow diagnosis of CF cases in the screen positive group by sweat testing without the need for mutation testing, thus avoiding detection of carriers. However, this requires measuring PAP and IRT in all newborns at considerable cost. In the present

study, based on the measurement of PAP only in those babies with an elevated IRT, it has been shown that the referral rate for mutation testing can be reduced by 43% and carrier detection by 50% of that obtained by the use of IRT alone, using the likelihood ratio method. In practice, this would be a cost effective modification to the existing UK newborn screening programme, although the ELISA assay format would require modification (e.g. to the DELFIA fluoroimmunoassay method) to allow efficient testing of the small number of babies with elevated IRT detected each day.

5.5 Alternative Screening Strategies

5.5.1 Improving Specificity

Using likelihood ratios and the IRT/PAP protocol described in this study, results have been presented which show that this approach can reduce the number of referrals for DNA analysis by up to 43% for the cost of PAP testing in only about 0.69% of newborn babies. However, one CF case which was picked up by IRT screening would have been missed when PAP was also included in the screening protocol.

5.5.2 Improving Sensitivity

A possible alternative strategy which could increase detection rates whilst still minimising the number of screen positive babies would be to measure PAP in a greater proportion of newborns in the screened population. Instead of using the 99.5th centile as the IRT threshold for selecting babies for PAP testing, a larger follow-up using the 99th, 95th or even the 90th centile could be used. Following up 10% of elevated IRT results for PAP testing would increase workload to about 6,000 additional tests per year, but would still be more economic than testing all babies as described by Sarles et al (2005) and may detect additional CF cases which would have been missed using the 99.5th centile. Current

screening sensitivity of the routine IRT/DNA protocol is 96% (personal communication from M. Fitch).

A review of IRT levels in CF cases missed by the Scottish newborn screening programme from 2003-2010 (n=11) shows that IRT levels ranged from 17.6 ng/ml - 60.9 ng/ml. Thus only one additional case would have been detected by the standard protocol if a lower IRT cut-off of 60 ng/ml had been in use which, in turn, would have greatly increased the referral rate for DNA testing. A bloodspot sample was available from one missed case in the present study, which had an IRT level of 20.9 ng/ml (equivalent to 1.10 MoM) and was homozygous for the common deltaF508 mutation. PAP analysis produced a very high level of 13.5 ng/ml, equivalent to 71.05 MoM. Lowering the IRT threshold for PAP analysis to a manageable follow-up rate would be unlikely to improve the performance of the combined IRT/PAP screening strategy.

5.6 Disadvantage of the Sequential IRT / PAP Approach

Before the population can be screened using PAP, the babies must first be identified by the IRT assay. Initial IRT screening takes 24 hours from receipt of the Guthrie card to a result and elevated levels are confirmed by re-analysis of the same Guthrie card in duplicate, which takes a further 24 hours. Usually at that point bloodspots are sent to the molecular genetics laboratory for mutation testing. This step is however, delayed by a further 24 hours pending PAP analysis, adding a further day before mutation testing can be carried out and a diagnosis of CF confirmed or excluded and screen positive results referred to a CF specialist.

However, this delay is unlikely to have a negative effect on the success of treatment. Most CF infants are clinically well at birth and the NHS Quality Improvement Scotland standard for treatment is that 95% of positive cases have started treatment within 35 days of age and 100 % of positive CF cases have started treatment within 43 days of age unless

deliberately delayed for further testing, assessment or treatment. The additional time required to include PAP testing is therefore unlikely to delay treatment for babies with bloodspot samples taken around 5-8 days of age.

5.7 Factors Affecting Bloodspot Marker Measurements

5.7.1 Marker levels and Mutations in CF Cases

Sarles et al (2005) presented data which suggests that PAP levels may show lower levels in cases where the mutation types are associated with a milder CF phenyotype.

Of the 29 CF cases examined in this study, 13 were homozygous for the common mutation delta F508. Of the other 16 cases, two were confirmed by sweat test where the full mutations have not yet been identified. Of the remaining 14 cases, all were compound heterozygotes with 10 having delta F508/other mutation and four cases having other combinations: R556X/2789+5G>A, R117H/G542X, G551D/621+1G>T and Y569D/Y569D (table 4.11).

IRT, PAP, PHE, and TYR concentrations were compared between the two groups of mutation types (Delta F508 homozygous vs. other mutations). It was noted that both IRT and PAP medians were markedly lower in the "other" mutation group compared to the delta F508 homozygous group (IRT: 147.8 ng/ml vs. 187.7 ng/ml [figure 4.42]; PAP: 1.03 ng/ml vs. 2.54 ng/ml [figure 4.43]) with only two of the 10 CF cases with the lowest PAP results being homozygous for the delta F508 mutation.

Using the Mann-Whitney test, there were no statistically significant differences in median level between the two mutation groups for any markers (table 4.13). Studies on a larger

series of CF cases and mutation types should be carried out to explore this possibility further.

5.7.2 Marker Levels and Mutations in CF carriers

Delta F508 was detected in 22 of the CF carriers. The other mutations identified in the 32 CF carriers were the following: P67L (one sample), R560T (one sample), 1078delT (one sample), G551D (two samples) and R117H (Five samples) as shown in table 4.14. In contrast to the CF cases, the median concentration for each marker showed little difference between the two types of mutation. IRT, PAP, PHE and TYR concentrations were compared between the two groups of mutation types (ΔF508 vs. other mutation) using Mann-Whitney test. The result showed no significant differences in median level between the two mutation groups for any markers (table 4.16).

5.7.3 Age at bloodspot sampling

Variations in blood spot marker levels with day of sampling were investigated. In the control group (2876 samples) the regression analysis at age of sampling showed PAP levels to be significantly decreased ($p < 0.001$) with earlier age at sampling. No significant associations were found for IRT ($p = 0.959$), PHE ($p=0.276$) or TYR ($p = 0.249$) (Figures 4.9, 4.10, 4.11 and 4.12). In Scotland, the majority of bloodspot samples for newborn screening are collected at 5-8 days of age, with about 5% of samples being obtained later. In France and some other countries, bloodspot sampling is carried out earlier (24-72 hours). It is important therefore, that normal ranges and distribution parameters for each marker are established locally, as published data from centres which use earlier sampling will be unreliable.

5.7.4 Premature Babies

Marker studies were carried out in 182 infants already known to have been born prematurely (i.e., before 37 weeks of gestation). To discover any relationship between premature babies and levels of IRT, PAP, PHE and TYR concentrations a linear regression analysis (Figures 4.22, 4.23, 4.24, and 4.25) was carried out. All markers showed a downward trend with advancing gestation. This was statistically significant for IRT ($p < 0.001$), PAP ($p < 0.001$) and TYR ($p = 0.015$) but not statistically significant for PHE ($p = 0.226$). Therefore, when using IRT and PAP to screen for CF there will be a risk of false-positive results in premature babies unless higher IRT and PAP cut-offs are used.

5.7.5 Low Birth Weight Babies

Of 3632 blood spot samples, 189 were from infants known to have been born with low birth weight (less than 2500 grams). The relationship between low birth weight and levels of IRT, PAP, PHE and TYR concentration was examined using linear regression which showed that the only significant association was with PAP ($p=0.002$) where the level decreased with increasing birth weight. No significant changes were found for IRT ($p = 0.212$), PHE ($p = 0.180$) and TYR ($p = 0.436$) (Figures 4.38, 4.39, 4.40 and 4.41). Therefore, in any screening progamme for CF which uses PAP there will be a tendency to overestimate the risk in very low birth weight babies.

5.7.6 Ethnicity

To see if there was any difference in the IRT, PAP, PHE and TYR markers among all ethnic categories, the data was divided into two groups. Group 1 included all British/Irish (A) and any other white background (C). Group 2 included all other ethnic backgrounds (Table 4.9). The median was calculated for IRT, PAP, PHE and TYR in group 1 (A and C) and group 2 (all others). The result showed that the only statistically significant difference

between these groups was for TYR in the controls. There were too few individuals from 'other' ethnic groups in the CF and CF carrier sample groups for meaningful comparison (table 4.10).

It has previously been reported (Cheillan et al., 2005) that IRT levels are higher in Africans than in Europeans and therefore a higher IRT screen positive rate would be expected within that ethnic group. A further study from the USA (Giusti, 2008) raised the question of the need to correct for this difference in African-American infants. It is possible that PAP might be similarly elevated. Lack of any finding of higher IRT or PAP in the present Scottish study is probably due to the fact that only about 10% of the newborn screening population in Scotland are from non A & C groups and there are too few subjects in this study from these other ethnic groups for accurate medians to be established. Further studies are required in populations where there is a significant proportion of other ethnic groups.

5.8 Gestational Markers of CF

In section 4.9, results from the present study were presented which showed that in pregnancies where the outcome was the birth of a child with CF, birth weight was significantly reduced. This indicates that the patho-physiological effects of CF are present before birth. It has also been shown that the CFTR mutation is expressed in placenta (Faller et al., 1995; Mylona et al., 1996) and it is possible that these changes may be reflected in a change in placental function where the foetus has CF. The placenta synthesises and secretes a large number of hormones that are required for the maintenance of pregnancy. Many of these have been investigated in pregnancies with adverse outcomes and found to be altered in concentration. However, none have been investigated in CF pregnancies.

In the UK, as a routine part of antenatal care, a series of maternal serum markers are measured in pregnant women. During the first half of pregnancy, screening tests for Down syndrome are offered to all pregnant women. These tests are based on the analysis of a group of serum markers measured at 10-13 weeks or 15-20 weeks of gestation. The markers used are, at 10-13 weeks, free beta hCG and PAPP-A - both products of the syncytiotrophoblast of the placenta. In the second trimester the markers used are AFP, UE3, hCG or free beta hCG and inhibin A. AFP is synthesised in the foetal liver but reaches the maternal circulation by active transport across the placenta. UE3 is also partly of foetal origin, while hCG is a product of the syncytiotrophoblast. Inhibin A is also produced by the syncytiotrophoblast. If the pregnancy is affected by Down syndrome, a characteristic pattern of change in the maternal serum concentration of these markers can be used to estimate a probability that the foetus has Down syndrome. Where this likelihood is above a predetermined threshold, invasive prenatal diagnosis (CVS or amniocentesis) is offered.

In addition to their predictive value for Down syndrome, some of these prenatal screening markers also show changes in other adverse outcomes of pregnancy. For example, elevated AFP is reported to be associated with Sudden Infant Death Syndrome (Smith, et al., 2004) and spontaneous preterm birth (Smith, et al., 2006) , while lower levels of PAPP-A are associated with LBW, early delivery, preeclampsia and still birth (Smith, et al., 2002). Inhibin A levels have been reported as being increased in pregnancies with pre-eclampsia (Cuckle et al., 1998). As these markers are routinely tested in the majority of pregnancies, this offers an opportunity to test their predictive value for CF.

In the present study, the serum markers AFP, hCG, PAPP-A, Ffree β-hCG, UE3 and inhibin-A were analysed in 55 serum samples taken during pregnancies for which the outcome was the live birth of a child affected with CF. An additional total of 330

pregnancies of normal outcome were randomly selected as controls matched to the same gestational range. Both second-trimester (15 to 20 weeks) and first-trimester cases (10 to 13 weeks) were included.

5.8.1 AFP

In the 330 control pregnancy serum samples the median AFP concentration level was 27.1 U/mL (equivalent to 0.98 MoM). The median AFP concentration level in the 55 maternal serum samples from mothers of children affected with CF was 29.27 U/mL (equivalent to 1.11 MoM). Figure 4.53 shows the distribution of AFP (MoM) in samples from CF pregnancies. Although slightly elevated compared to controls, the concentration levels of AFP are not significantly different in the mother's serum when her child had cystic fibrosis ($p = 0.335$, Mann-Whitney test).

5.8.2 HCG

The median hCG concentration level in the 330 control serum samples was 27.51 U/mL (equivalent to 0.99 MoM). The median hCG concentration level in the 55 maternal serum samples from mothers of children affected with CF was 25.88 U/mL (equivalent to 0.95 MoM). The result presented in Figure 4.56 shows the distribution of hCG (MoM) in samples from CF pregnancies. This median is close to that in the controls and is not statistically significantly different from normal (Mann-Whitney test, $p = 0.539$)

5.8.3 PAPP-A

The median PAPP-A concentrations level in 330 control pregnancy samples was 6794.50 U/mL (equivalent to 0.99 MoM). The median PAPP-A concentration level in 55 maternal serum samples from mothers of children affected with CF was 7510.0 ng/ml (equivalent to 0.98 MoM). The results presented in figure 4.59 show the distribution of PAPP-A (MOM)

in samples from CF pregnancies. This median is close to that in the controls and is not statistically significantly different from normal (Mann-Whitney test p = 0.632).

5.8.4 FREE β-HCG

The median free β-hCG concentration levels in the 330 control pregnancy serum samples was 14.40 ng/ml (equivalent to 1.00 MoM), while in the 55 serum samples taken during pregnancies where the outcome was the live birth of a child with CF the median free β-hCG concentratios level was 13.80 ng/ml (equivalent to 0.93 MoM). Figure 4.62 shows the distribution of free β-hCG (MoM) in samples from CF pregnancies. This median is close to that in the controls and is not statistically significantly different from normal (Mann Whitney, p = 0.824).

5.8.5 UE3

In 322 control pregnancy serum samples, the median UE3 concentration level was 2.48 nmol/L (equivalent to 1.01 MoM). In the 52 serum samples from pregnancies where the outcome was the live birth of a child with CF, UE3 level was 2.51 nmol/L (equivalent to 1.07 MoM). The results presented in Figure 4.65 show the distribution of UE3 (MoM) in samples from CF pregnancies. This median is close to that in the controls and is not statistically significantly different from normal (Mann Whitney, p =0.840).

5.8.6 Inhibin-A

The median inhibin-A concentrations levels in the 325 control pregnancy serum samples was 183.5 pg/mL (equivalent to 0.99 MoM), while the median inhibin-A concentrations level in the 53 maternal serum samples from mothers of children affected with CF was found to be 173.20 pg/mL ml (equivalent to 0.97 MoM). The results presented in Figure 4.68 show the distribution of inhibin-A (MoM) in samples from CF pregnancies. This

median is close to that in the controls and is not statistically significantly different from normal (Mann Whitney, p = 0. 650).

5.9 Prenatal Screening Markers in CF Pregnancies

Although these markers show various changes in the case of low birth weight, prematurity, pre-eclampsia, Down syndrome, Edwards' syndrome, and chromosomal abnormalities (Smith et al 2002), no significant changes were found in this study for any of these markers in CF pregnancies. However some minor changes are evident and AFP in particular did show a marked concentration increase. Although not statistically significant, this suggests that further studies on a larger series of CF pregnancies may be worthwhile.

The case for routine prenatal population screening for CF has been considerably weakened in the UK with the introduction of newborn bloodspot screening and evidence from the Wisconsin study (Farrell, et al., 1997) that early identification of affected neonates combined with improved treatment regimes improves the long term outlook for affected children. However, identification of pregnancies at increased risk through serum marker screening would be a useful alert of the possibility of the birth of an affected child, allowing even earlier diagnosis and intervention. Further studies on an extended range of maternal serum markers would therefore be of interest.

CHAPTER 6 :

CONCLUSION

6.1 CONCLUSION

Newborn screening for CF is established in many countries. However, the clinical and cost effectiveness of newborn screening for CF has been questioned. The primary test involves IRT measurement and subsequent DNA analysis for infants with an elevated IRT concentration. Due to the relatively poor specificity of the IRT test, around 0.5% of the newborn population are referred for DNA testing and consequently a significant number of CF carriers are also identified. Alternative markers are required which can improve specificity.

In this study, PAP has been shown to be a potential marker in newborn screening for CF. An innovative strategy used in this study for newborn screening for CF is the likelihood ratio statistical method to combine IRT and PAP results and select those infants at highest risk for DNA testing. This approach reduces the number of carriers detected. By carrying out PAP testing only in those infants with an elevated IRT, the significant cost of whole population screening is avoided, and the cost of PAP testing is offset by the reduced number of DNA tests carried out.

The serum markers tested in this research were not found to be good predictors of CF. However, there are other markers such placental growth factors and ADAM12 that have not yet been tested, and there is a case for these to be tested in future research.

APPENDIX

APPENDIX A. Overall risk of CF from IRT and PAP levels in 314 increased IRT samples

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
1	71.00	.054	1.85	1.27	1.15	7.15	13.95	5.96	1.74	.08	1.10
2	72.21	.071	1.86	1.15	1.10	7.34	15.72	5.06	1.05	.09	1.38
3	73.52	.056	1.87	1.25	1.05	7.54	17.86	5.83	1.64	.08	1.43
4	75.76	.044	1.88	1.36	.96	7.88	22.13	6.65	2.35	.08	1.67
5	76.09	.029	1.88	1.53	.95	7.93	22.83	8.14	3.81	.07	1.71
6	71.82	.110	1.86	.96	1.12	7.28	15.13	3.82	.33	.11	1.72
7	76.58	.047	1.88	1.32	.93	8.01	23.91	6.38	2.11	.08	1.83
8	76.68	.053	1.88	1.28	.93	8.02	24.14	6.01	1.78	.08	1.90
9	75.25	.084	1.88	1.08	.98	7.80	21.09	4.59	.74	.10	2.00
10	71.17	.155	1.85	.81	1.14	7.18	14.19	2.99	.05	.15	2.13
11	71.06	.166	1.85	.78	1.15	7.16	14.04	2.82	.02	.16	2.25
12	77.00	.077	1.89	1.11	.92	8.07	24.88	4.83	.90	.09	2.26
13	78.91	.053	1.90	1.28	.85	8.36	29.70	6.04	1.81	.08	2.33
14	78.02	.072	1.89	1.14	.88	8.22	27.36	5.02	1.03	.09	2.41
15	77.27	.012	1.89	1.90	.91	8.11	25.51	11.75	8.05	.10	2.61
16	71.82	.183	1.86	.74	1.12	7.28	15.13	2.61	.00	.18	2.68
17	75.96	.118	1.88	.93	.95	7.91	22.56	3.66	.26	.12	2.68
18	81.58	.038	1.91	1.42	.76	8.76	37.88	7.16	2.82	.07	2.82
19	73.63	.176	1.87	.75	1.04	7.56	18.05	2.69	.01	.17	3.07
20	70.00	.246	1.85	.61	1.20	6.99	12.63	2.00	.07	.25	3.13
21	78.76	.103	1.90	.99	.86	8.34	29.30	4.00	.41	.11	3.17

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			*Overall risk*
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
22	76.23	.140	1.88	.85	.94	7.95	23.14	3.21	.10	.14	3.18
23	73.40	.190	1.87	.72	1.05	7.52	17.65	2.52	.00	.18	3.26
24	72.45	.213	1.86	.67	1.09	7.37	16.10	2.28	.01	.21	3.36
25	72.23	.229	1.86	.64	1.10	7.34	15.75	2.14	.03	.23	3.57
26	75.00	.181	1.88	.74	.99	7.77	20.59	2.63	.00	.17	3.60
27	75.71	.173	1.88	.76	.96	7.87	22.03	2.72	.01	.17	3.69
28	74.35	.198	1.87	.70	1.01	7.67	19.35	2.44	.00	.19	3.72
29	70.65	.278	1.85	.56	1.17	7.10	13.48	1.77	.15	.29	3.89
30	75.00	.202	1.88	.70	.99	7.77	20.59	2.40	.00	.20	4.05
31	75.41	.195	1.88	.71	.97	7.83	21.41	2.46	.00	.19	4.07
32	85.00	.056	1.93	1.25	.67	9.27	51.30	5.81	1.62	.08	4.11
33	77.44	.167	1.89	.78	.90	8.14	25.92	2.81	.02	.16	4.18
34	71.92	.276	1.86	.56	1.11	7.29	15.28	1.79	.14	.29	4.37
35	82.31	.104	1.92	.98	.74	8.87	40.44	3.98	.40	.11	4.39
36	71.39	.310	1.85	.51	1.14	7.21	14.50	1.58	.25	.33	4.85
37	74.23	.251	1.87	.60	1.02	7.65	19.13	1.96	.08	.25	4.86
38	76.82	.211	1.89	.68	.92	8.04	24.46	2.30	.01	.21	5.06
39	80.73	.152	1.91	.82	.79	8.63	35.08	3.03	.06	.15	5.18
40	70.11	.359	1.85	.44	1.19	7.01	12.77	1.34	.43	.41	5.26
41	84.58	.105	1.93	.98	.68	9.20	49.44	3.96	.39	.11	5.41
42	75.51	.253	1.88	.60	.97	7.84	21.61	1.95	.08	.26	5.53

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
43	71.00	.360	1.85	.44	1.15	7.15	13.95	1.34	.43	.41	5.75
44	89.93	.031	1.95	1.50	.54	9.99	78.26	7.87	3.53	.07	5.83
45	82.07	.152	1.91	.82	.75	8.83	39.58	3.02	.06	.15	5.84
46	77.31	.232	1.89	.63	.91	8.12	25.61	2.11	.04	.23	5.91
47	73.57	.308	1.87	.51	1.05	7.55	17.95	1.59	.24	.33	5.95
48	82.41	.153	1.92	.82	.74	8.88	40.80	3.01	.06	.15	6.06
49	80.16	.189	1.90	.72	.81	8.55	33.31	2.53	.00	.18	6.11
50	71.30	.371	1.85	.43	1.14	7.20	14.38	1.29	.47	.43	6.20
51	75.27	.285	1.88	.55	.98	7.81	21.13	1.73	.17	.30	6.30
52	81.45	.179	1.91	.75	.77	8.74	37.44	2.65	.00	.17	6.49
53	70.90	.401	1.85	.40	1.16	7.13	13.82	1.18	.58	.48	6.69
54	89.60	.070	1.95	1.15	.55	9.94	76.12	5.10	1.08	.09	6.64
55	86.23	.123	1.94	.91	.63	9.45	57.09	3.55	.21	.12	7.02
56	71.07	.417	1.85	.38	1.15	7.16	14.05	1.12	.65	.51	7.24
57	75.25	.314	1.88	.50	.98	7.80	21.09	1.56	.26	.34	7.18
58	85.74	.133	1.93	.88	.65	9.38	54.71	3.35	.15	.13	7.17
59	85.45	.139	1.93	.86	.65	9.33	53.35	3.24	.11	.14	7.27
60	83.89	.178	1.92	.75	.70	9.10	46.53	2.66	.01	.17	8.02
61	73.01	.404	1.86	.39	1.07	7.46	17.00	1.17	.60	.49	8.32
62	79.84	.263	1.90	.58	.82	8.50	32.35	1.87	.11	.27	8.70
63	86.63	.154	1.94	.81	.62	9.51	59.10	2.99	.05	.15	8.85

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
64	71.82	.462	1.86	.34	1.12	7.28	15.13	.98	.83	.61	9.16
65	78.54	.299	1.90	.52	.86	8.30	28.71	1.64	.21	.32	9.14
66	92.55	.081	1.97	1.09	.48	10.36	97.30	4.67	.79	.09	9.11
67	76.78	.340	1.89	.47	.92	8.04	24.37	1.43	.35	.38	9.26
68	75.83	.366	1.88	.44	.96	7.89	22.28	1.31	.45	.42	9.41
69	93.12	.080	1.97	1.10	.47	10.44	102.0	4.73	.83	.09	9.44
70	83.75	.215	1.92	.67	.70	9.08	45.96	2.27	.01	.21	9.69
71	88.30	.145	1.95	.84	.58	9.75	68.18	3.13	.08	.14	9.66
72	79.61	.292	1.90	.53	.83	8.46	31.68	1.69	.19	.31	9.76
73	75.72	.380	1.88	.42	.96	7.88	22.05	1.25	.51	.45	9.87
74	85.80	.192	1.93	.72	.64	9.38	55.00	2.50	.00	.19	10.25
75	87.15	.172	1.94	.76	.61	9.58	61.80	2.74	.01	.17	10.28
76	78.53	.330	1.90	.48	.86	8.30	28.68	1.48	.32	.36	10.45
77	81.79	.269	1.91	.57	.76	8.79	38.60	1.84	.12	.28	10.66
78	75.28	.415	1.88	.38	.98	7.81	21.15	1.12	.64	.51	10.82
79	82.85	.250	1.92	.60	.73	8.95	42.44	1.97	.08	.25	10.73
80	79.00	.328	1.90	.48	.85	8.37	29.95	1.49	.31	.36	10.83
81	76.06	.400	1.88	.40	.95	7.93	22.77	1.18	.58	.48	10.98
82	77.34	.369	1.89	.43	.90	8.12	25.68	1.30	.46	.43	10.99
83	78.76	.342	1.90	.47	.86	8.34	29.30	1.42	.36	.38	11.22
84	83.35	.252	1.92	.60	.71	9.02	44.36	1.96	.08	.25	11.29

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
85	85.02	.225	1.93	.65	.66	9.27	51.39	2.18	.03	.22	11.41
86	86.69	.198	1.94	.70	.62	9.52	59.40	2.43	.00	.19	11.45
87	76.85	.397	1.89	.40	.92	8.05	24.53	1.19	.57	.48	11.70
88	81.08	.306	1.91	.51	.78	8.68	36.21	1.60	.24	.33	11.90
89	75.49	.441	1.88	.36	.97	7.84	21.57	1.04	.75	.56	12.13
90	94.91	.093	1.98	1.03	.43	10.70	118.0	4.28	.56	.10	11.95
91	78.19	.378	1.89	.42	.87	8.25	27.79	1.26	.50	.44	12.34
92	76.55	.423	1.88	.37	.93	8.00	23.85	1.10	.68	.53	12.57
93	73.83	.503	1.87	.30	1.04	7.59	18.40	.87	1.00	.70	12.82
94	74.82	.478	1.87	.32	1.00	7.74	20.24	.93	.90	.64	12.95
95	72.90	.540	1.86	.27	1.07	7.44	16.82	.78	1.15	.78	13.18
96	82.66	.301	1.92	.52	.73	8.92	41.72	1.63	.22	.32	13.38
97	99.47	.058	2.00	1.24	.35	11.34	169.5	5.71	1.54	.08	13.70
98	91.40	.160	1.96	.80	.51	10.20	88.48	2.91	.04	.15	13.71
99	93.69	.131	1.97	.88	.46	10.53	106.8	3.39	.16	.13	13.83
100	79.68	.393	1.90	.41	.82	8.47	31.88	1.20	.56	.47	15.01
101	76.05	.489	1.88	.31	.95	7.93	22.75	.90	.95	.67	15.13
102	72.13	.615	1.86	.21	1.10	7.32	15.60	.63	1.46	.99	15.39
103	80.28	.387	1.90	.41	.81	8.57	33.67	1.23	.53	.46	15.48
104	101.59	.050	2.01	1.30	.31	11.63	199.8	6.18	1.93	.08	15.52
105	76.73	.483	1.88	.32	.93	8.03	24.25	.92	.92	.65	15.80

APPENDIX A (continued

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
106	82.56	.344	1.92	.46	.74	8.91	41.35	1.41	.37	.39	15.96
107	79.63	.414	1.90	.38	.83	8.47	31.73	1.13	.64	.51	16.18
108	96.33	.128	1.98	.89	.41	10.90	132.2	3.44	.18	.13	16.81
109	91.00	.207	1.96	.68	.52	10.14	85.58	2.34	.01	.20	17.35
110	84.58	.325	1.93	.49	.68	9.20	49.44	1.50	.30	.36	17.64
111	77.66	.499	1.89	.30	.89	8.17	26.46	.88	.99	.69	18.20
112	100.31	.090	2.00	1.05	.33	11.46	180.9	4.40	.63	.10	17.87
113	72.00	.693	1.86	.16	1.11	7.30	15.40	.51	1.78	1.23	18.90
114	87.76	.287	1.94	.54	.59	9.67	65.12	1.72	.17	.30	19.60
115	89.57	.256	1.95	.59	.55	9.93	75.92	1.92	.09	.26	19.77
116	90.00	.261	1.95	.58	.54	10.00	78.72	1.89	.10	.27	20.97
117	94.00	.198	1.97	.70	.45	10.57	109.6	2.43	.00	.19	21.13
118	102.67	.092	2.01	1.04	.30	11.78	217.0	4.33	.59	.10	21.73
119	102.73	.099	2.01	1.00	.30	11.79	218.0	4.11	.47	.11	22.96
120	107.66	.028	2.03	1.55	.23	12.46	315.6	8.28	3.96	.08	23.73
121	77.32	.597	1.89	.22	.90	8.12	25.63	.66	1.39	.94	23.99
122	95.29	.200	1.98	.70	.43	10.75	121.6	2.42	.00	.19	23.68
123	87.58	.351	1.94	.45	.60	9.65	64.12	1.38	.39	.40	25.50
124	83.36	.449	1.92	.35	.71	9.02	44.40	1.01	.78	.58	25.69
125	91.51	.278	1.96	.56	.50	10.21	89.29	1.77	.15	.29	25.82
126	85.87	.394	1.93	.40	.64	9.39	55.34	1.20	.56	.47	26.07

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
127	70.66	.897	1.85	.05	1.17	7.10	13.49	.29	2.56	2.03	27.32
128	85.31	.412	1.93	.39	.66	9.31	52.70	1.14	.63	.50	26.62
129	73.41	.782	1.87	.11	1.05	7.52	17.67	.40	2.13	1.55	27.30
130	88.86	.342	1.95	.47	.57	9.83	71.50	1.42	.36	.38	27.38
131	97.00	.201	1.99	.70	.39	10.99	139.5	2.40	.00	.20	27.37
132	72.95	.842	1.86	.07	1.07	7.45	16.90	.34	2.36	1.79	30.20
133	79.56	.601	1.90	.22	.83	8.46	31.53	.66	1.41	.95	29.85
134	72.26	.884	1.86	.05	1.10	7.34	15.80	.30	2.52	1.97	31.10
135	83.33	.500	1.92	.30	.71	9.02	44.28	.87	.99	.69	30.50
136	89.72	.353	1.95	.45	.55	9.96	76.89	1.37	.40	.40	30.79
137	81.32	.564	1.91	.25	.77	8.72	37.00	.73	1.26	.85	31.35
138	76.93	.723	1.89	.14	.92	8.06	24.71	.47	1.90	1.33	32.81
139	87.34	.420	1.94	.38	.60	9.61	62.82	1.11	.66	.52	32.66
140	99.54	.194	2.00	.71	.35	11.35	170.4	2.47	.00	.19	32.18
141	82.07	.562	1.91	.25	.75	8.83	39.58	.73	1.24	.84	33.26
142	90.11	.366	1.95	.44	.54	10.01	79.45	1.31	.45	.42	33.56
143	94.14	.294	1.97	.53	.45	10.59	110.8	1.67	.20	.31	34.49
144	80.06	.650	1.90	.19	.81	8.53	33.01	.57	1.61	1.09	36.01
145	105.14	.137	2.02	.86	.26	12.12	261.6	3.27	.12	.13	35.31
146	83.33	.555	1.92	.26	.71	9.02	44.28	.75	1.22	.82	36.47
147	81.31	.627	1.91	.20	.77	8.72	36.97	.61	1.51	1.02	37.78

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
148	93.26	.327	1.97	-.49	.47	10.46	103.1	1.49	.31	.36	37.06
149	104.00	.158	2.02	-.80	.28	11.97	240.1	2.93	.04	.15	36.81
150	73.87	.922	1.87	-.04	1.03	7.59	18.47	.27	2.66	2.15	39.64
151	77.00	.797	1.89	-.10	.92	8.07	24.88	.38	2.19	1.60	39.86
152	71.68	1.03	1.86	.01	1.12	7.25	14.92	.20	3.05	2.71	40.43
153	99.28	.232	2.00	-.64	.35	11.31	167.0	2.12	.04	.23	38.46
154	111.39	.080	2.05	-1.10	.18	12.96	414.2	4.72	.82	.09	38.45
155	96.46	.282	1.98	-.55	.40	10.92	133.6	1.75	.16	.29	39.30
156	85.90	.516	1.93	-.29	.64	9.40	55.48	.83	1.06	.73	40.38
157	92.43	.372	1.97	-.43	.48	10.35	96.35	1.29	.47	.43	41.65
158	83.44	.611	1.92	-.21	.71	9.04	44.72	.64	1.45	.97	43.60
159	86.36	.528	1.94	-.28	.63	9.47	57.73	.81	1.11	.76	43.65
160	112.07	.090	2.05	-1.05	.18	13.05	435.0	4.38	.62	.10	43.06
161	95.49	.323	1.98	-.49	.42	10.78	123.6	1.51	.29	.35	43.64
162	87.07	.520	1.94	-.28	.61	9.57	61.38	.82	1.07	.74	45.21
163	91.70	.421	1.96	-.38	.50	10.24	90.71	1.10	.67	.52	47.40
164	106.26	.170	2.03	-.77	.25	12.27	284.5	2.76	.02	.16	46.82
165	97.29	.308	1.99	-.51	.39	11.04	142.7	1.59	.24	.33	47.25
166	74.57	.000	1.87	.00	1.01	7.70	19.76	.22	2.94	2.53	50.09
167	109.59	.134	2.04	-.87	.20	12.72	363.6	3.33	.14	.13	48.02
168	93.53	.389	1.97	-.41	.46	10.50	105.4	1.22	.54	.46	48.87

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT					PAP			Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
169	87.32	.558	1.94	-.25	.60	9.61	62.71	.74	1.23	.83	52.00
170	81.17	.757	1.91	-.12	.78	8.70	36.50	.43	2.03	1.45	52.87
171	100.93	.274	2.00	-.56	.32	11.54	189.8	1.80	.14	.28	53.79
172	90.84	.488	1.96	-.31	.52	10.12	84.45	.91	.94	.66	55.82
173	81.87	.765	1.91	-.12	.76	8.80	38.88	.42	2.06	1.48	57.49
174	106.71	.200	2.03	-.70	.24	12.33	294.2	2.42	.00	.19	57.15
175	120.45	.028	2.08	-1.55	.10	14.15	780.8	8.29	3.98	.08	58.74
176	83.26	.743	1.92	-.13	.71	9.01	44.01	.45	1.98	1.40	61.58
177	80.48	.850	1.91	-.07	.80	8.60	34.29	.33	2.39	1.82	62.33
178	74.00	1.17	1.87	.07	1.03	7.61	18.71	.13	3.52	3.54	66.29
179	121.07	.056	2.08	-1.25	.10	14.23	814.4	5.83	1.64	.08	65.13
180	103.04	.285	2.01	-.54	.29	11.83	223.2	1.73	.17	.30	66.69
181	96.99	.400	1.99	-.40	.39	10.99	139.4	1.18	.58	.48	67.42
182	90.21	.567	1.96	-.25	.53	10.03	80.12	.72	1.27	.85	68.36
183	119.32	.012	2.08	-1.90	.11	14.00	722.9	11.75	8.05	.10	73.88
184	73.25	1.27	1.86	.10	1.06	7.50	17.40	.09	3.85	4.26	74.18
185	73.98	1.24	1.87	.09	1.03	7.61	18.67	.10	3.76	4.04	75.52
186	101.99	.324	2.01	-.49	.31	11.69	206.0	1.51	.30	.36	73.14
187	86.48	.713	1.94	-.15	.63	9.48	58.34	.48	1.86	1.29	75.52
188	96.50	.446	1.98	-.35	.40	10.92	134.0	1.02	.77	.57	76.63
189	80.10	.984	1.90	-.01	.81	8.54	33.13	.23	2.88	2.45	81.25

APPENDIX A (continued

Increased IRT	IRT (ng /ml)	PAP (ng /ml)	IRT $Log_{10\ IRT}$	$Log_{10\ PAP}$	PAP IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	Overall risk
190	114.56	.155	2.06	-.81	.15	13.38	519.3	2.98	.05	.15	77.89
191	82.50	.890	1.92	-.05	.74	8.90	41.13	.30	2.54	1.99	82.04
192	86.88	.745	1.94	-.13	.62	9.54	60.38	.44	1.98	1.41	84.91
193	122.64	.082	2.09	-1.09	.08	14.43	905.3	4.66	.79	.09	84.86
194	92.38	.590	1.97	-.23	.49	10.34	95.95	.68	1.36	.92	87.96
195	126.47	.042	2.10	-1.38	.06	14.91	1167	6.79	2.48	.08	87.83
196	102.58	.375	2.01	-.43	.30	11.77	215.5	1.27	.48	.44	94.45
197	97.82	.519	1.99	-.28	.38	11.11	148.9	.83	1.07	.73	109.37
198	90.00	.790	1.95	-.10	.54	10.00	78.72	.39	2.16	1.57	123.94
199	74.16	1.56	1.87	.19	1.02	7.64	19.00	.03	4.73	6.85	130.08
200	89.17	.846	1.95	-.07	.56	9.88	73.40	.34	2.37	1.80	132.26
201	81.73	1.16	1.91	.07	.76	8.78	38.39	.13	3.50	3.51	134.56
202	85.41	1.06	1.93	.02	.65	9.33	53.17	.18	3.15	2.86	152.17
203	87.78	.971	1.94	-.01	.59	9.67	65.23	.24	2.84	2.39	155.74
204	116.46	.253	2.07	-.60	.13	13.63	593.3	1.95	.08	.26	151.82
205	89.36	.919	1.95	-.04	.55	9.90	74.59	.27	2.65	2.13	158.93
206	96.42	.694	1.98	-.16	.40	10.91	133.2	.51	1.78	1.23	163.77
207	90.57	.897	1.96	-.05	.53	10.08	82.57	.29	2.56	2.03	167.42
208	84.07	1.18	1.92	.07	.69	9.13	47.28	.13	3.55	3.60	170.00
209	131.60	.090	2.12	-1.05	.04	15.55	1626	4.39	.62	.10	160.87
210	121.18	.203	2.08	-.69	.10	14.24	820.5	2.39	.00	.20	162.13

APPENDIX A (continued

Increased IRT	IRT	PAP	IRT		PAP						Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
211	127.40	.138	2.11	-.86	.06	15.03	1241	3.25	.11	.14	168.46
212	103.10	.543	2.01	-.27	.29	11.84	224.2	.77	1.17	.79	177.58
213	108.45	.425	2.04	-.37	.22	12.57	334.5	1.09	.68	.53	177.67
214	75.57	1.76	1.88	.25	.97	7.85	21.74	.01	5.31	9.21	200.31
215	121.60	.224	2.08	-.65	.09	14.29	844.1	2.18	.03	.22	186.65
216	102.95	.591	2.01	-.23	.29	11.82	221.7	.68	1.37	.92	203.74
217	83.87	1.35	1.92	.13	.70	9.10	46.45	.07	4.10	4.89	227.19
218	73.59	2.05	1.87	.31	1.04	7.55	17.98	.00	6.05	13.40	240.87
219	96.50	.833	1.98	-.08	.40	10.92	134.0	.35	2.32	1.75	234.27
220	135.39	.104	2.13	-.98	.02	16.01	2065	3.97	.40	.11	225.14
221	142.08	.041	2.15	-1.39	.01	16.82	3112	6.90	2.58	.07	233.30
222	129.10	.181	2.11	-.74	.05	15.24	1385	2.63	.00	.17	242.03
223	137.11	.102	2.14	-.99	.02	16.22	2298	4.04	.43	.11	246.13
224	122.00	.299	2.09	-.52	.09	14.35	867.2	1.64	.21	.32	276.04
225	104.75	.696	2.02	-.16	.27	12.07	254.1	.51	1.79	1.24	313.82
226	79.27	1.89	1.90	.28	.84	8.41	30.70	.00	5.64	10.90	334.77
227	133.84	.173	2.13	-.76	.03	15.83	1874	2.73	.01	.17	313.29
228	102.26	.798	2.01	-.10	.30	11.73	210.3	.38	2.19	1.61	337.62
229	148.71	.044	2.17	-1.35	.00	17.60	4607	6.60	2.30	.08	349.34
230	138.99	.144	2.14	-.84	.01	16.45	2580	3.15	.09	.14	363.31
231	138.74	.148	2.14	-.83	.01	16.42	2541	3.09	.07	.14	365.47

APPENDIX A (continued)

Increased IRT	IRT (ng /ml)	PAP (ng /ml)	IRT $Log_{10\ IRT}$	$Log_{10\ PAP}$	PAP IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	Overall risk
232	99.17	.973	2.00	-.01	.35	11.30	165.5	.24	2.84	2.39	396.23
233	143.48	.110	2.16	-.96	.00	16.98	3385	3.84	.33	.11	382.46
234	126.89	.307	2.10	-.51	.06	14.96	1200	1.60	.24	.33	395.13
235	131.31	.266	2.12	-.58	.04	15.51	1597	1.85	.12	.27	435.83
236	125.73	.355	2.10	-.45	.07	14.82	1112	1.36	.41	.40	449.82
237	151.88	.067	2.18	-1.17	.00	17.96	5532	5.23	1.18	.09	473.34
238	148.04	.110	2.17	-.96	.00	17.52	4431	3.82	.33	.11	502.39
239	84.40	1.96	1.93	.29	.68	9.18	48.67	.00	5.82	11.95	581.52
240	152.46	.085	2.18	-1.07	.00	18.03	5719	4.55	.72	.10	547.66
241	90.31	1.65	1.96	.22	.53	10.04	80.79	.02	5.01	7.89	637.15
242	129.37	.382	2.11	-.42	.05	15.27	1410	1.25	.51	.45	634.19
243	81.41	2.35	1.91	.37	.77	8.73	37.30	.01	6.76	19.02	709.54
244	94.34	1.50	1.97	.18	.44	10.62	112.6	.04	4.57	6.29	708.26
245	88.81	1.90	1.95	.28	.57	9.82	71.20	.00	5.67	11.06	787.83
246	78.06	2.81	1.89	.45	.88	8.23	27.46	.05	7.77	30.76	844.67
247	163.19	.021	2.21	-1.68	.01	19.24	10391	9.53	5.35	.08	836.51
248	112.13	.912	2.05	-.04	.18	13.06	436.9	.28	2.62	2.10	915.54
249	141.58	.289	2.15	-.54	.01	16.76	3020	1.70	.18	.30	919.07
250	156.06	.141	2.19	-.85	.00	18.44	7011	3.20	.10	.14	969.25
251	105.33	1.24	2.02	.09	.26	12.15	265.4	.10	3.75	4.04	1071.02
252	82.38	2.87	1.92	.46	.74	8.88	40.69	.06	7.89	32.63	1327.98

APPENDIX A (continued)

Increased IRT	IRT	PAP	IRT		PAP						Overall risk
	(ng /ml)	(ng /ml)	$Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	
253	134.21	.505	2.13	-.30	.03	15.87	1918	.86	1.01	.70	1345.53
254	154.56	.210	2.19	-.68	.00	18.27	6443	2.32	.01	.21	1321.57
255	124.00	.754	2.09	-.12	.08	14.60	991.5	.43	2.02	1.44	1428.01
256	116.67	1.06	2.07	.02	.13	13.66	602.0	.18	3.14	2.86	1721.30
257	121.20	.921	2.08	-.04	.09	14.24	821.6	.27	2.65	2.14	1758.33
258	140.99	.459	2.15	-.34	.01	16.69	2914	.98	.82	.60	1746.91
259	92.29	2.40	1.97	.38	.49	10.33	95.24	.02	6.89	20.21	1924.39
260	177.34	.016	2.25	-1.79	.06	20.77	21864	10.54	6.55	.09	1930.11
261	177.28	.074	2.25	-1.13	.06	20.76	21797	4.97	.99	.09	1937.73
262	99.38	2.04	2.00	.31	.35	11.33	168.3	.00	6.02	13.17	2216.27
263	134.40	.650	2.13	-.19	.03	15.89	1941	.57	1.60	1.09	2114.05
264	83.27	3.51	1.92	.54	.71	9.01	44.05	.15	9.10	57.30	2524.07
265	108.05	1.65	2.03	.22	.22	12.52	324.8	.02	4.99	7.83	2542.14
266	175.12	.145	2.24	-.84	.05	20.53	19515	3.13	.08	.14	2768.11
267	166.17	.238	2.22	-.62	.02	19.57	12202	2.06	.05	.24	2900.38
268	74.65	5.09	1.87	.71	1.00	7.71	19.91	.40	11.59	174.8	3481.08
269	143.59	.593	2.16	-.23	.00	17.00	3407	.67	1.37	.92	3148.87
270	152.18	.461	2.18	-.34	.00	18.00	5628	.98	.83	.60	3391.82
271	164.69	.300	2.22	-.52	.02	19.40	11269	1.64	.22	.32	3605.00
272	91.29	3.35	1.96	.52	.51	10.18	87.67	.12	8.81	50.15	4397.12
273	142.83	.707	2.15	-.15	.01	16.91	3255	.49	1.83	1.27	4146.49

APPENDIX A (continued)

Increased IRT	IRT (ng /ml)	PAP (ng /ml)	IRT Log_{10} IRT	Log_{10} PAP	IRT^{A}	IRT^{B}	IRT^{LR}	PAP^{A}	PAP^{B}	PAP^{LR}	Overall risk
274	164.68	.331	2.22	-.48	.02	19.40	11263.16	1.47	.32	.37	4117.44
275	86.88	3.97	1.94	.60	.62	9.54	60.38	.22	9.90	82.56	4984.99
276	75.00	5.71	1.88	.76	.99	7.77	20.59	.51	12.42	251.17	5171.51
277	97.02	2.99	1.99	.48	.39	11.00	139.70	.07	8.13	36.59	5111.49
278	88.00	4.03	1.94	.61	.59	9.71	66.46	.23	9.99	85.96	5713.25
279	145.01	.809	2.16	-.09	.00	17.16	3707.31	.37	2.23	1.65	6115.43
280	158.27	.537	2.20	-.27	.01	18.69	7932.03	.79	1.14	.78	6162.54
281	140.14	1.04	2.15	.02	.01	16.59	2767.38	.20	3.07	2.73	7557.85
282	121.70	2.04	2.09	.31	.09	14.31	849.83	.00	6.03	13.29	11295.18
283	170.82	.497	2.23	-.30	.03	20.07	15609.49	.88	.98	.68	10666.04
284	177.14	.447	2.25	-.35	.06	20.75	21642.38	1.02	.77	.57	12428.84
285	157.36	.904	2.20	-.04	.00	18.59	7540.36	.29	2.59	2.06	15514.93
286	148.66	1.19	2.17	.08	.00	17.59	4593.75	.12	3.60	3.71	17059.53
287	162.25	.822	2.21	-.09	.01	19.13	9873.41	.36	2.28	1.70	16795.46
288	114.58	3.04	2.06	.48	.15	13.38	519.99	.08	8.23	38.20	19865.67
289	124.77	2.33	2.10	.37	.07	14.70	1043.52	.01	6.73	18.74	19551.07
290	223.48	.093	2.35	-1.03	.32	25.33	187363.0	4.29	.56	.10	18937.58
291	204.25	.273	2.31	-.56	.19	23.50	80104.26	1.81	.13	.28	22600.79
292	211.73	.221	2.33	-.66	.24	24.22	112265.9	2.21	.02	.22	24439.58
293	150.59	1.39	2.18	.14	.00	17.81	5137.02	.06	4.24	5.26	27029.91
294	234.00	.086	2.37	-1.06	.40	26.29	291279.7	4.50	.69	.10	28171.97

APPENDIX A (continued)

Increased IRT	IRT (ng /ml)	PAP (ng /ml)	IRT $Log_{10\ IRT}$	$Log_{10\ PAP}$	IRT^{A}	IRT^{B}	IRT^{LR}	PAP PAP^{A}	PAP^{B}	PAP^{LR}	Overall risk
295	218.10	.205	2.34	-.69	.28	24.83	148587.60	2.37	.00	.20	29695.65
296	105.76	5.74	2.02	.76	.25	12.21	274.08	.51	12.45	255.21	>50,000
297	237.57	.226	2.38	-.65	.43	26.61	337151.93	2.17	.03	.22	>50,000
298	252.77	.142	2.40	-.85	.55	27.94	616952.90	3.18	.10	.14	>50,000
299	121.61	4.96	2.08	.70	.09	14.30	844.68	.38	11.41	161.60	>50,000
300	215.81	.635	2.33	-.20	.27	24.61	134442.93	.60	1.54	1.04	>50,000
301	232.87	.518	2.37	-.29	.39	26.19	278003.20	.83	1.06	.73	>50,000
302	155.63	2.94	2.19	.47	.00	18.39	6844.13	.07	8.04	34.94	>50,000
303	268.02	.205	2.43	-.69	.68	29.23	1100248.05	2.37	.00	.20	>50,000
304	204.52	1.54	2.31	.19	.19	23.53	81099.49	.03	4.68	6.65	>50,000
305	347.65	.052	2.54	-1.29	1.44	35.31	15720365.71	6.09	1.85	.08	>50,000
306	324.00	.194	2.51	-.71	1.21	33.61	7539099.75	2.47	.00	.19	>50,000
307	307.45	.488	2.49	-.31	1.05	32.37	4394092.99	.91	.94	.66	>50,000
308	335.53	.388	2.53	-.41	1.32	34.44	10842549.30	1.22	.54	.46	>50,000
309	94.45	28.5	1.98	1.46	.44	10.63	113.64	3.23	26.97	92998.9	>50,000
310	202.70	13.3	2.31	1.12	.18	23.35	74604.59	1.64	19.38	4623.24	>50,000
311	425.99	.960	2.63	-.02	2.22	40.45	139126953.8	.24	2.79	2.33	>50,000
312	280.08	5.55	2.45	.74	.79	30.22	1707631.15	.48	12.21	229.30	>50,000
313	653.18	.424	2.82	-.37	4.42	52.41	18324887866	1.09	.68	.53	>50,000
314	503.29	2.86	2.70	.46	2.99	44.93	890621506.0	.06	7.87	32.35	>50,000

REFERENCES

Ahmed, N., Corey, M., Forstner, G., Zielenski, J., Tsui, L.C., Ellis, L., Tullis, E. and Durie, P. (2003). 'Molecular consequences of cystic fibrosis transmembrane regulator (CFTR) gene mutations in the exocrine pancreas'. *Gut*, 52 (8):1159.

Aitken, D.A., Wallace, E.M., Crossley, J.A., Swanston, I.A., van Pareren, Y., van Maarle, M., Groome, N.P., Macri, J.N. and Connor, J.M. (1996). 'Dimeric inhibin A as a marker for Down syndrome in early pregnancy'. *New England Journal of Medicine*, 334 (19):1231-1236.

Albertini, A., Ghielmi, S. and Belloli, S. (1982). 'Structure, immunochemical properties and immunoassay of human chorionic gonadotropin'. *International Journal of Clinical & Laboratory Research*, 12 (1):289-298.

Balo, J. and Banga, I. (1950). 'The elastolytic activity of pancreatic extracts'. *Biochemical Journal*, 46 (4):384.

Beerman, H., Van Dorst, E.B.L., Kuenen-Boumeester, V. and Hogendoorn, P.C.W. (2009). 'Superior performance of liquid-based versus conventional cytology in a population-based cervical cancer screening program'. *Gynecologic oncology*, 112 (3):572-576.

Blau, N., van Spronsen, F.J. and Levy, H.L. (2010). 'Phenylketonuria'. *The Lancet*, 376 (9750):1417-1427.

Bogart, M.H., Pandian, M.R. and Jones, O.W. (1987). 'Abnormal maternal serum chorionic gonadotropin levels in pregnancies with foetal chromosome abnormalities'. *Prenatal Diagnosis*, 7 (9):623-630.

Bowling, F.G. and Brown, A.R.D. (1988). 'Newborn screening for cystic fibrosis using an enzyme linked immunoabsorbent assay (ELISA) technique'. *Clinica chimica acta*, 171 (2-3):257-261.

Braunstein, B., Kamdar, V., Rasor, J., Swaminathan, N. and Wade, M.E. (1979). 'Widespread distribution of a chorionic gonadotropin-like substance in normal human tissues'. *Journal of Clinical Endocrinology & Metabolism*, 49 (6):917.

Bronstein, M.N., Sokol, R.J., Abman, S.H., Chatfield, B.A., Hammond, K.B., Hambidge, K.M., Stall, C.D. and Accurso, F.J. (1992). 'Pancreatic insufficiency, growth, and nutrition in infants identified by newborn screening as having cystic fibrosis+'. *The Journal of Pediatrics*, 120 (4):533-540.

Chace, D.H., Kalas, T.A. and Naylor, E.W., (2003). 'Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns'. *Clinical Chemistry*, 49 (11):1797.

Cheillan, D., Vercherat, M., Chevalier-Porst, F., Charcosset, M., Rolland, M. and Dorche, C., (2005). 'False-positive results in neonatal screening for cystic fibrosis based on a three-stage protocol (IRT/DNA/IRT): Should we adjust IRT cut-off to ethnic origin?' *Journal of Inherited Metabolic Disease*, 28 (6):813-818.

Clark, B.E. and Thein, S.L., (2004). 'Molecular diagnosis of haemoglobin disorders'. *Clinical and Laboratory Haematology*, 26 (3):159-176.

Closa, D., Motoo, Y. and Iovanna, J.L., (2007). 'Pancreatitis-associated protein: from a lectin to an anti-inflammatory cytokine'. *World Journal of Gastroenterology*, 13 (2):170.

Crossley, J.R., Elliot, R.B. and Smith, P.A., (1979). 'Dried-blood spot screening for cystic fibrosis in the newborn'. *The Lancet*, 313 (8114):472-474.

Cuckle, H., (1996). 'Established markers in second trimester maternal serum'. *Early Human Development*, 47 (Supplement 1):S27-S29.

Cuckle, H., Sehmi, I. and Jones, R., (1998). 'Maternal serum inhibin-A can predict pre-eclampsia'. *BJOG: An International Journal of Obstetrics & Gynaecology*, 105 (10):1101-1103.

Cuckle, H., Wald, N. and Lindenbaum, R., (1984). 'Maternal serum alpha-fetoprotein measurement: a screening test for down syndrome'. *The Lancet*, 323 (8383):926-929.

Davies, J.C., (2006). 'New tests for cystic fibrosis'. *Paediatric Respiratory Reviews*, 7 (Supplement 1):S141-S143.

Davies, S.C., Cronin, E., Gill, M., Greengross, P., Hickman, M. and Normand, C. (2000). 'Screening for sickle cell disease and thalassaemia: a systematic review with supplementary research'. *Health technology assessment* (Winchester, England), 4 (3):i.

Dominguez-Munoz, J.E., Hieronymus, C., Sauerbruch, T. and Malfertheiner, P. (1995). 'Fecal elastase test: evaluation of a new noninvasive pancreatic function test'. *The American journal of gastroenterology*, 90 (10):1834-1837.

Faller, D.P., Egan, D.A. and Ryan, M.P. (1995). 'Evidence for location of the CFTR in human placental apical membrane vesicles'. *American Journal of Physiology-Cell Physiology*, 269 (1):C148.

Farrell, P.M., Kosorok, M.R., Laxova, A., Shen, G., Koscik, R.E., Bruns, W.T., Splaingard, M. and Mischler, E.H. (1997). 'Nutritional benefits of neonatal screening for cystic fibrosis'. *New England Journal of Medicine*, 337 (14):963-969.

Fauroux, B., Boulé, M., Lofaso, F., Zérah, F., Clément, A., Harf, A. and Isabey, D. (1999). 'Chest physiotherapy in cystic fibrosis: improved tolerance with nasal pressure support ventilation'. *Pediatrics*, 103 (3):e32.

Festini, F., Taccetti, G., Repetto, T., Reali, M.F., Campana, S., Mergni, G., Marianelli, L. and de Martino, M. (2005). 'Gestational and neonatal characteristics of children with cystic fibrosis: a cohort study'. *The Journal of pediatrics*, 147 (3):316-320.

Fialova, L. and Malbohan, I.M. (2002). 'Pregnancy-associated plasma protein A (PAPP-A): theoretical and clinical aspects'. *Bratislavské lekárske listy*, 103 (6):194-205.

Giusti, R., (2008). 'Elevated IRT levels in African-American infants: Implications for newborn screening in an ethnically diverse population'. *Pediatric Pulmonology*, 43 (7):638-641.

Grimes, D.A. and Schulz, K.F. (2002). 'Uses and abuses of screening tests'. *The Lancet*, 359 (9309):881-884.

Guthrie, R. and Susi, A. (1963). 'A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants'. *Pediatrics*, 32 (3):338.

Hannon, W.H. (1997). *Blood Collection on Filter Paper for Neonatal Screening Programs: Approved Standard*: NCCLS.

Hodson, M.E., Geddes, D.M. and Bush, A.A. (2007). *Cystic Fibrosis*. London: Hodder Arnold.

Hoffbrand, A.V. and Pettit, J.E. (1993). *Essential Haematology*: Blackwell Scientific.

Hsu, H.W., Zytkovicz, T.H., Comeau, A.M., Strauss, A.W., Marsden, D., Shih, V.E., Grady, G.F. and Eaton, R.B. (2008). 'Spectrum of medium-chain acyl-CoA dehydrogenase deficiency detected by newborn screening'. *Pediatrics*, 121 (5):e1108.

Hussa, R.O., (1982). 'Clinical Utility of Human Chorionic Gonadotropin and [alpha]-Subunit Measurements'. *Obstetrics & Gynecology*, 60 (1):1-2.

Jeannette, K., (2011). 'Newborn screening from an international perspectiveâ€"Different countries, different approaches'. *Clinical Biochemistry*, 44 (7):471-472.

Jorgensen, P.I. and Trolle, D. (1972). 'Low urinary oestriol excretion during pregnancy in women giving birth to infants with Down syndrome'. *The Lancet*, 300 (7781):782-784.

Keay, S.D., Vatish, M., Karteris, E., Hillhouse, E.W. and Randeva, H.S. (2004). 'Review: The role of hCG in reproductive medicine'. *BJOG: An International Journal of Obstetrics & Gynaecology*, 111 (11):1218-1228.

Keim, V., Rohr, G., Stöckert, H. and Haberich, F.J. (1984). 'An additional secretory protein in the rat pancreas'. *Digestion*, 29 (4):242-249.

Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.C. (1989). 'Identification of the cystic fibrosis gene: genetic analysis'. *Science*, 245 (4922):1073.

Klein, J., (2011). 'Newborn screening from an international perspective--Different countries, different approaches'. *Clinical Biochemistry*, 44 (7):471-472.

Kompare, M. and Rizzo, W.B., (2008). 'Mitochondrial fatty-acid oxidation disorders'. Elsevier, 140-149.

Kuhl, C.K., Kuhn, W. and Schild, H., (2005). 'Management of women at high risk for breast cancer: new imaging beyond mammography'. *The Breast*, 14 (6):480-486.

Lacey, J.M., Minutti, C.Z., Magera, M.J., Tauscher, A.L., Casetta, B., McCann, M., Lymp, J., Hahn, S.H., Rinaldo, P. and Matern, D., (2004). 'Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tandem mass spectrometry'. *Clinical Chemistry*, 50 (3):621.

Lafuste, P., Robert, B., Mondon, F., Danan, J.L., Rossi, B., Duc-Goiran, P., Mignot, T.M., Nunez, E.A., Benassayag, C. and Ferré, F. (2002). 'Alpha-fetoprotein Gene Expression in Early and Full-term Human Trophoblast'. *Placenta*, 23 (8-9):600-612.

Macri, J.N., Spencer, K., Aitken, D., Garver, K., Buchanan, P.D., Muller, F. and Boue, A., (1993). 'First-trimester free beta (hCG) screening for Down syndrome'. *Prenatal Diagnosis*, 13 (7):557-562.

Mallory, P.A. and Travis, J. (1975). 'Human pancreatic enzymes. Purification and characterization of a nonelastolytic enzyme, protease E, resembling elastase'. *Biochemistry*, 14 (4):722-730.

Mennie, M.E., Gilfillan, A., Compton, M., Curtis, L., Brock, D.J.H., Liston, W.A., Pullen, I.M. and Whyte, D.A. (1992). 'Prenatal screening for cystic fibrosis'. *The Lancet*, 340 (8813):214-216.

Merkatz, I.R., Nitowsky, H.M., Macri, J.N. and Johnson, W.E. (1984). 'An association between low maternal serum alpha-fetoprotein and foetal chromosomal abnormalities'. *American Journal of Obstetrics and Gynecology*, 148 (7):886.

Millington, D.S., Kodo, N., Norwood, D.L. and Roe, C.R. (1990). 'Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism'. *Journal of Inherited Metabolic Disease*, 13 (3):321-324.

Murray, A. and Clarke, A. (2002). 'The ethics of population screening'. *Current Paediatrics*, 12 (6):447.

Muttukrishna, S., Knight, P.G., Groome, N.P., Redman, C.W.G. and Ledger, W.L. (1997). 'Activin A and inhibin A as possible endocrine markers for pre-eclampsia'. *The Lancet*, 349 (9061):1285-1288.

Mylona, P., Glazier, J.D., Greenwood, S.L., Sides, M.K. and Sibley, C.P. (1996). 'Expression of the cystic fibrosis (CF) and multidrug resistance (MDR1) genes during development and differentiation in the human placenta'. *Molecular Human Reproduction*, 2 (9):693.

Nandhakumar, N. and Green, M.R. (2010). 'Interpretations: How to use faecal elastase testing'. *Archives of disease in Childhood -Education & Practice Edition*, 95 (4):119.

Newby, D., Dalgliesh, G., Lyall, F. and Aitken, D.A. (2005). 'Alphafetoprotein and alphafetoprotein receptor expression in the normal human placenta at term'. (Review) *Placenta*, 26 (2-3):190-200.

Paju, A. and Stenman, U.H. (2006). 'Biochemistry and clinical role of trypsinogens and pancreatic secretory trypsin inhibitor'. *Critical Reviews in Clinical Laboratory Sciences*, 43 (2):103-142.

Park, S.M. and Chatterjee, V.K.K. (2005). 'Genetics of congenital hypothyroidism'. *Journal of Medical Genetics*, 42 (5):379.

Proesmans, M., Vermeulen, F. and De Boeck, K. (2008). 'What's new in cystic fibrosis? From treating symptoms to correction of the basic defect'. *European Journal of Pediatrics*, 167 (8):839-849.

Rama, S. and Jagannadha Rao, A. (2003). 'Regulation of growth and function of the human placenta'. *Molecular and Cellular Biochemistry*, 253 (1):263-268.

Ranieri, E., Gerace R., Fletcher J., Krase M., Qstrup Jan and J-C., D., (2009). 'The effectivensss of a combined Immuno-Reactive Trypsin (IRT) - Pancreatitis Associated Protein (PAP) / DNA newborn screening strategy for cystic fibrosis (CF).' In: Gustvo Borrajo, V.C., Isabel Cristina Ibarra-Gonzalez,Gerader Lober,Marcela Vela-Amieva (ed). *7th international and latin american congress inborn errors of metabolism and neonatal screening*. Cancun mexico, 30.

Rastogi, M.V. and LaFranchi, S.H. (2010). 'Congenital hypothyroidism'. *Orphanet J Rare Dis*, 5 (1):17.

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N. and Chou, J.L. (1989). 'Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA'. *Science*, 245 (4922):1066.

Riordan, J.R., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., lannuzzi, MC, Collins, FS and Tsui, L.-C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA'. *Science*, 245:1066-1073.

Rodrigues, R., Gabetta, C.S., Pedro, K.P., Valdetaro, F., Fernandes, M.I.M., Magalhães, P.K.R., Januário, J.N. and Maciel, L.M.Z. (2008). 'Cystic fibrosis and neonatal screening (Fibrose cística ea triagem neonatal)'. *Cad. Saúde Pública*, 24 (Sup 4):S475-S484.

Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D. and Hidaka, N. (1989). 'Identification of the cystic fibrosis gene: chromosome walking and jumping'. *Science*, 245 (4922):1059.

Rose, S.R. and Brown, R.S., (2006). 'Update of newborn screening and therapy for congenital hypothyroidism'. *AAP Policy*, 117 (6):2290.

Ryynänen, M., Seppälä, M., Kuusela, P., Rapola, J., Aula, P., Seppä, A., Jokela, V. and Castren, O. (1983). 'Antenatal screening for congenital nephrosis in Finland by maternal serum α-fetoprotein'. *BJOG: An International Journal of Obstetrics & Gynaecology*, 90 (5):437-442.

Sarles, J., Berthézène, P., Le Louarn, C., Somma, C., Perini, J.-M., Catheline, M., Mirallié, S., Luzet, K., Roussey, M., Farriaux, J.-P., Berthelot, J. and Dagorn, J.-C. (2005). 'Combining Immunoreactive Trypsinogen and Pancreatitis-Associated Protein Assays, a Method of Newborn Screening for Cystic Fibrosis that Avoids DNA Analysis'. *The Journal of Pediatrics*, 147 (3):302-305.

Scriver, C. R., W. S. Sly and D. Valle (eds.) (1995). *The Metabolic and Molecular Bases of Inherited Disease.* New York: McGraw-Hill.

Seymour, C.A., Thomason, M.J., Chalmers, R.A., Addison, G.M., Bain, M.D., Cockburn, F., Littlejohns, P., Lord, J. and Wilcox, A.H. (1997). 'Newborn screening for inborn errors of metabolism: a systematic review'. *Health technology assessment* (Winchester, England), 1 (11):i.

Sinaasappel, M., Stern, M., Littlewood, J., Wolfe, S., Steinkamp, G., Heijerman, H.G.M., Robberecht, E. and Döring, G. (2002). 'Nutrition in patients with cystic fibrosis: a European Consensus'. *Journal of Cystic Fibrosis*, 1 (2):51-75.

Smith, G., Shah, I., White, I.R., Pell, J.P., Crossley, J.A. and Dobbie, R. (2006). 'Maternal and biochemical predictors of spontaneous preterm birth among nulliparous women: a systematic analysis in relation to the degree of prematurity'. *International Journal of Epidemiology*, 35 (5):1169.

Smith, G., Stenhouse, E.J., Crossley, J.A., Aitken, D.A., Cameron, A.D. and Connor, J.M. (2002). 'Early pregnancy levels of pregnancy-associated plasma protein a and the risk of intrauterine growth restriction, premature birth, preeclampsia, and stillbirth'. *Journal of Clinical Endocrinology & Metabolism*, 87 (4):1762.

Smith, G.C.S., Wood, A.M., Pell, J.P., White, I.R., Crossley, J.A. and Dobbie, R. (2004). 'Second-trimester maternal serum levels of alpha-fetoprotein and the subsequent risk of sudden infant death syndrome'. *New England Journal of Medicine*, 351 (10):978-986.

Soini, E. and Kojola, H. (1983). 'Time-resolved fluorometer for lanthanide chelates--a new generation of nonisotopic immunoassays'. *Clinical Chemistry*, 29 (1):65.

Sommerburg, O., Lindner, M., Muckenthaler, M., Kohlmueller, D., Leible, S., Feneberg, R., Kulozik, A., Mall, M. and Hoffmann, G. (2010). 'Initial evaluation of a biochemical cystic fibrosis newborn screening by sequential analysis of immunoreactive trypsinogen and pancreatitis-associated protein (IRT/PAP) as a strategy that does not involve DNA testing in a Northern European population'. *Journal of Inherited Metabolic Disease*, 33 (0):263-271.

Sziegoleit, A., (1984). 'A novel proteinase from human pancreas'. *Biochemical Journal*, 219 (3):735.

Sziegoleit, A., Krause, E., Klor, H.U., Kanacher, L. and Linder, D. (1989). 'Elastase 1 and chymotrypsin B in pancreatic juice and feces'. *Clinical Biochemistry*, 22 (2):85-89.

Tabor, A., Norgaard-Pedersen, B., Jacobsen, J., Trigg, M., Hitchens, J., Geier, M., Hutchinson, G., Susan Cowchock, F. and Ruch, D. (1984). 'Low maternal serum afp and down syndrome'. *The Lancet*, 324 (8395):161-162.

Tomasi Jr, T.B., (1977). 'Structure and function of alpha-fetoprotein'. *Annual Review of Medicine*, 28 (1):453-465.

van der Kamp, H.J. and Wit, J.M. (2004). 'Neonatal screening for congenital adrenal hyperplasia'. *European journal of Endocrinology*, 151 (Suppl 3):U71.

Van Lith, J.M.M., Pratt, J.J., Beekhuis, J.R. and Mantingh, A. (1992). 'Second-trimester maternal serum immunoreactive inhibin as a marker for foetal Down syndrome'. *Prenatal Diagnosis*, 12 (10):801-806.

Vernooij-van Langen, A.M., Loeber, J.G., Elvers, L.H., Triepels, R.H., Gille, J.J., van der Ploeg, C.P., Reijntjens, S., Dompeling, E. and Dankert-Roelse, J.E. (2009). 'Cystic fibrosis Heelprick among a newborn Population in the Netherlands: the CHOPIN study'. *Journal of Cystic Fibrosis*, 8 (Supplement 2):S9-S9.

Vigano, P., Corbetta, C., Mariani, T., Cattaneo, C., Bj¨orklund, C., Kasel, M., Karvonen, H., Hoffman, G.H., Laessig, R.H., Kopish, G.J., Eaton, R., Comeau, A., Stopsack, M., Hammermann, J., Nilson, N., Heine, C., Fenner, J. and Dagorn, J.C. (2008). 'Measurement of pancreatitis-associated protein in combination to immunoreactive trypsinogen for neonatal screening strategy of CF: a multicenter feasibility study'. *Journal of Cystic Fibrosis*, 7 (Supplement 3):S26-S26.

Wald, N.J., George, L., Smith, D., Densem, J.W., Petterson, K. (1996). 'Serum screening for Down syndrome between 8 and 14 weeks of pregnancy'. *BJOG: An International Journal of Obstetrics & Gynaecology*, 103 (5):407-412.

Wald, N.J. and Leck, I. (2000). *Antenatal and neonatal screening*. Oxford ; New York: Oxford University Pres.

Wang, S.S., Fernhoff, P.M., Harmon, W.H. and Khoury, M.J. (1999). 'Medium chain acyl-CoA dehydrogenase deficiency: human genome epidemiology review'. *Genetics in Medicine*, 1 (7):332.

Welsh, M.J. and Smith, A.E. (1993). 'Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis'. *Cell*, 73 (7):1251-1254.

Wilcken, B., (2007). 'Newborn screening for cystic fibrosis: Techniques and strategies'. *Journal of inherited metabolic disease*, 30 (4):537-543.

Wilkes, D.L., Schneiderman, J.E., Nguyen, T., Heale, L., Moola, F., Ratjen, F., Coates, A.L. and Wells, G.D. (2009). 'Exercise and physical activity in children with cystic fibrosis'. *Paediatric Respiratory Reviews*, 10 (3):105-109.

Wilson, J.M.G. and Jungner, G. (1968). 'Principles and practice of screening for disease; Principles and practice of screening for disease'. *Public Health Papers (WHO)* (34).