A review of newborn screening results and anthropometric measurements in infants diagnosed with cystic fibrosis in the West Midlands

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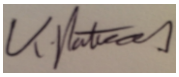
Signature of candidate  Date 29/07/2016

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ROLES AND RESPONSIBILITIES

Katie Patterson

In terms of data collection, I collected all of the NBS data from the West Midlands screening laboratory and all of the clinical data on the Stoke patients. I collected nutritional and biochemistry data for the Birmingham and shared care patients with the access provided by Dr Maya Desai and Carolyn Patchell. I collected microbiology data from BCH-only patients. This information for shared care patients was organised by Dr Desai who contacted the consultants at each of the district general hospitals to provide this information.

I performed the NBS data analysis alone. Dr Theocharis Kyriacou drove the data analysis and modelling for the nutritional parameters, although I was heavily involved in this.

Dr Francis Gilchrist

Dr Gilchrist was the project supervisor and oversaw the running of the whole process, providing guidance and suggestions throughout. He was involved in the development of the grader rating protocol and was an, 'expert grader' for patient graphs.

Dr Maya Desai

Dr Desai provided contact with the West Midlands Screening team and enabled access to data for BCH patients. She was responsible for organising microbiology data for shared care patients. Dr Desai was also involved in formulating the grader rating protocol.

Dr Theocharis Kyriacou

Dr Kyriacou performed the cluster analysis, classification and modelling on the clinical parameters. He provided many tutorials to educate me on these subjects. We worked together to formulate a way to best describe the nutritional data of each patient and the cohort as a whole, combining our medical and mathematical knowledge. The polynomial regression modelling with NARMAX is an equation that Dr Kyriacou developed and has published in previous work. This is the first time it has been used in medicine.

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Dr Carroll was involved in the development of the grader rating protocol. He was the second, 'expert grader' of patient graphs. He was also involved in discussion about data analysis and provided some helpful suggestions for this.

LIST OF ABBREVIATIONS

A

ABPA - Allergic bronchopulmonary aspergillosis

ASL - Airway surface liquid

ATP - Adenosine triphosphate

B

BAL - Bronchoscopic alveolar lavage

BCH - Birmingham Children's Hospital

BMI - Body mass index

BMIp – Body mass index percentile

BW - Birth weight

C

CF - Cystic fibrosis

CFRD - Cystic fibrosis related diabetes

CFTR - Cystic fibrosis transmembrane conductance regulator

CFSPID - Cystic fibrosis screen positive inconclusive diagnosis

Cl^- - Chloride

CLS - Clinical liaison service

CS - Computed score

D

DIOS - Distal intestinal obstruction syndrome

DNA - Deoxyribonucleic acid

E

ERR - Error reduction ratio

F

FE - Faecal elastase

FEV_1 - Forced expiratory volume in one second

FVC - Forced vital capacity

G

GCP - Good clinical practice

H

H_2O - Water

HBA_{1C} - Glycated haemoglobin

HCO_3^- - Bicarbonate

HAP - Height for age percentile

HRA - Health research authority

I

%IBW - Percentage of ideal body weight

ICSI - Intracytoplasmic sperm injection

IL-8 - Intraleukin-8

IRT - Immunoreactive trypsinogen

I.T - Information Technology

IUGR - Intrauterine growth restriction

K

K^+ - Potassium

Kg - Kilograms

M

MatLab - Matrix laboratory

MI - Meconium ileus

MDT - Multidisciplinary team

MRSA - Multi-resistant Staphylococcus Aureus

MSCS - Macroduct® sweat collection system

N

Na^+ - Sodium

NARMAX - Non-linear Auto-regressive Moving Average Model with eXogenous inputs

NBD - Nucleotide binding domain

NBS - Newborn screening

NGT - Nasogastric tube

NHS - National Health Service

NPD - Nasal potential difference

NPV - Negative predictive value

O

OGTT – Oral glucose tolerance test

P

PA - Pseudomonas Aeruginosa

PAP - Pancreatitis associated protein

PEG - Percutaneous gastrostomy

PERT - Pancreatic enzyme replacement therapy

PFT - Pulmonary function tests

PI - Pancreatic insufficiency

PKU - Phenylketonuria

PPV - Positive predictive value

PS - Pancreatic sufficiency

R

R&D - Research and development

RCT - Randomized controlled trial

RhDNase - Recombinant human deoxyribonuclease

RNA - Ribonucleic acid

RSH - Royal Shrewsbury Hospital

RSUH - Royal Stoke University Hospital

S

SA - Staphylococcus Aureus

SD - Standard deviation

SE - Standard error

U

UHNM - University of North Midlands

UK - United Kingdom

UKCFR - United Kingdom cystic fibrosis registry

USA - United States of America

W

WAP - Weight for age percentile

WHO - World Health Organisation

ABSTRACT

INTRODUCTION:

Newborn screening (NBS) for cystic fibrosis (CF) was implemented nationally in July 2007 and has reduced the age of diagnosis and produced better nutritional and survival outcomes.

AIM:

To assess demographic data of a screened cohort and their anthropometric and microbiology results from birth to 2 years. We undertook cluster analysis of the raw data and developed a model to predict weight and length z scores.

METHODS:

Data were collected from the West Midlands NBS Laboratory on babies screened between November 2007 and October 2014. A retrospective case notes review was performed on all confirmed cases.

RESULTS:

507,608 infants were screened. 200 had a positive CF NBS and 144 were subsequently diagnosed with CF (birth prevalence: 1/3525). In those with CF, 11.8% isolated *Staphylococcus aureus* within 2 years and 34.5% isolated *Pseudomonas aeruginosa*. The

median birth weight (BW) of the children with CF was 3.09kg with no difference between pancreatic sufficient/insufficient patients. The median time for infants to reach a z score of 0 for weight was 65 weeks and 90 weeks to achieve the same z score for length. Cluster analysis identified 2 distinct groups. We have developed robust models to predict weight and length z scores at 1 and 2 years of age using data available at first clinic visit.

CONCLUSION:

The birth prevalence of CF in the West Midlands is lower than the UK, which is likely to reflect its ethnic diversity. Babies with CF had a normal birth weight but lost weight by their first clinic visit. Infants with CF achieve normal weight before length. Our models to predict future length and weight have the potential to identify children at risk of poor growth in the first 2 years of life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 LITERATURE REVIEW

1.1 CYSTIC FIBROSIS

1.1.1 BACKGROUND

Cystic fibrosis is the most common life limiting disease in white populations. It is inherited in an autosomal recessive fashion with carriers expressing little or no symptomatology. The pathology is determined by malfunction of the exocrine glands and poor ion transport across epithelia. Ergo this systemic disease affects many organ systems but primarily results in respiratory complications and deteriorating pulmonary function. Research has led to the discovery of more than 2000 different mutations,[1] variable in phenotypical expression.

Cystic fibrosis has seen many advances over the last 80 years, since its discovery in 1938. The emergence of regional centres in 1950 and the involvement of multi-disciplinary teams (MDT's) have greatly improved care of such patients. Diagnostic tests have further evolved from sweat testing using pilocarpine electrophoresis to newborn screening on day 5-8 of life. Treatment advances have also been a major focus for researchers with improvements in nutrition and conventional pulmonary therapies followed more recently by genotype-specific treatments and the introduction of gene therapy. This research has proved vital in improving patients with cystic fibrosis quality of life and large increases in life expectancy have been seen.

1.1.2 THE HISTORY OF CYSTIC FIBROSIS

In comparison to many other common disorders of childhood, cystic fibrosis made a fairly late entry into medical pathophysiology, being introduced in the early twentieth century.

There were most likely numerous cases prior to this discovery, possibly dating back to the Middle Ages, however the interpretation and association between symptoms was not formally recognised or documented.

A chronological review of the literature on the history of cystic fibrosis brings the reader back to the Middle Ages, where a premature death was predicted for those children, whose skin tasted, "salty" when kissed.[2,3] Such broods were recognised as being, "bewitched," or "hexed."[2,3] Texts from the 17th century describe distinct symptoms and clinical signs now classically associated with cystic fibrosis, such as steatorrohoea and pancreatic insufficiency.[3] In 1905, Karl Landsteiner, a Viennese pathologist described the most pathognomonic feature of modern cystic fibrosis, meconium ileus (MI). It was he who linked this form of bowel obstruction with histological changes of the pancreas, namely an increase in intra lobular connective tissue and cellular infiltration resulting in dilation of the pancreatic ducts.

Although still nameless and poorly understood, it wasn't until the 1930's that cystic fibrosis was clearly described. The earliest account was in 1933 when Blackfan and Wolbach attempted to describe their findings under a report titled, 'Vitamin A deficiency in infants'.[4] It was this account that revealed the connection between pathology in the pancreas as well as suppurative lung disease, demonstrated by the autopsies of the thirteen infants in their series.[4] The authors concluded that this disease of the pancreas was the aetiology of vitamin A deficiency and failed to further investigate it.

In 1936 Guido Fanconi, a Swiss paediatrician who studied at the University of Zurich supplemented Blackfan and Wolbach's work. Regarded as one of the founders of modern

paediatrics, it is argued that Fanconi should be credited with what we now understand as cystic fibrosis.[4] The first English narrative of this theory however, was published in 1938 by an American pathologist, Dorothy Andersen who was based at The Babies and Children's Hospital of Columbia University in New York.[5] Her paper, 'Cystic fibrosis of the pancreas and its relation to celiac disease,'[6] confirmed cystic fibrosis as its own entity. This differed to what was previously believed about the relationship between cystic fibrosis and coeliac disease. Andersen further contributed to this field of study compiling the evidence to propose cystic fibrosis as a genetic condition and more specifically an autosomal recessive mutation.[7]

Shortly after this discovery, a devastating heat wave hit New York, which resulted in overwhelming hyperthermia and heat prostration in a number of Andersen's patients.[5] For those patients whose laboratory data were available, deranged electrolytes were a common denominator. The analysis showed low blood chloride (Cl^-) levels and high bicarbonate (HCO_3^-). This was fully reversed with rehydration therapy.[5] Captivated by this occurrence Paul di Sant'Agnese demonstrated the electrolyte imbalances in cystic fibrosis patients with particular reference to a marked elevation in Na^+, K^+, and Cl^- in sweat.[8] The authors proposed that the susceptibility of CF patients to dehydration was in fact due to increased salt loss from sweat glands.[5] It was this depiction that forms the basis of diagnosing cystic fibrosis, the sweat test, still the gold standard used today.[9] In the years that followed, research focused primarily on this idea of altered anion transport. Many reports concurred with di Sant'Agnese, with Fromter deducing the decreased permeability of Cl^- through sweat glands in cases of cystic fibrosis.[5,10]

1.1.3 EPIDEMIOLOGY

At present, there are 10,583 patients with cystic fibrosis living in the United Kingdom (UK) registered on the UK Cystic Fibrosis Registry (UKCFR),[11] with a carrier rate of one in 26.[12] The incidence of cystic fibrosis is one in every 2381 live births in the UK.[13] This has risen slightly since 1995 when it was analysed by Dodge et al[14] and documented to be one in 2415. Explanation of this could be increased immigration to the UK of ethnic groups with low incidences of CF. The prevalence, which is defined as; the number of cases of a disease in a population in a specified time period, has continued to slowly rise as the number of new cases (227) of CF outweigh the number of deaths (137).[11] This can be attributed to a number of different measures within CF care, namely; advanced knowledge on disease progression and a wider variety of treatment available. These factors together have also greatly improved survival rate in CF, with the median predicted age of survival, 40.1 years, which has improved from 29.0 years in 1989.[15]

1.1.4 GENETICS

Cystic fibrosis is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene, located on the long arm of chromosome seven, at position 31, is a membrane-spanning protein that plays a major role in the transport of Cl^- anions across epithelia of many different organ systems.[16] Dysfunction of this transport mechanism, results in the accumulation of viscous secretions, which eventually block and cause irreversible damage to epithelial tissues such as, the respiratory tract, pancreatic duct, biliary tree, genital tract and sweat glands.

At present there have been 2001 mutations identified.[1] The most common of these, found in approximately 70% of CF patients worldwide, is deletion of phenylalanine at position

508 (Phe508del).[17] To organise what seemed to be an ever expanding, rapid accumulation of data from the cystic fibrosis genetic analysis consortium, Tsui et al devised a classification system in which the various CF mutations are arranged.[18] This classification focused mainly on alterations within Nucleotide Binding Domains (NBD), as interruption of these was thought to affect biosynthesis and functioning of the CFTR. Later, Haardt et al hypothesized that the C-terminal tail also played a role in CFTR functioning. This novel strategy saw the development of class VI mutations.[19]

Classification of cystic fibrosis mutations and their functional effects[20]

- Class I: Protein synthesis defect; premature stop codon results in failure to synthesise full-length CFTR protein. Little or no functional CFTR.
- Class II: Folding defect; improper folding results in defective cellular assembly. Poor delivery of CFTR protein to cell surface. Little or no functional CFTR.
- Class III: Gating defect; CFTR protein reaches cell membrane but impaired opening of chloride channel. Some functional CFTR.
- Class IV: Narrow channel; structural defect that reduces the number of ions passing through resulting in a conductance defect. Function of CFTR is affected only at surface.
- Class V: Splicing defect; reduced quantity of functional CFTR due to errors in RNA splicing.
- Class VI: Truncation of c-terminus of CFTR; functional protein but unstable at apical cell surface.[19, 21]

This grouping of classes is important as it can help predict disease course and progression, although there are huge clinical variations in patients with the same genotypes. The greater functional alteration in the gene, the greater the phenotypic expression and an increased mortality rate.[17]

Mckone et al[17] decided to further group these patients by genetically stratifying them into high and low risk groups. They noticed that class I-III produced very low levels of functional CFTR and subsequently displayed a severe phenotype and higher mortality rate than those in the, 'low risk' stratification (class IV-V). The 'high risk,' genotype had a median survival of 36.3 years compared with the 'low risk,' genotype which had a median survival of 50 years.[17] It was also concluded that class I-III demonstrated an increased incidence of pseudomonas colonization, pancreatic insufficiency and higher sweat chloride levels. It is unfortunate to note that Phe508del is within this category.

It is interesting to learn that CFTR genotype can help to predict phenotype and pancreatic function. It must be emphasized however, that patients with the same genotype can display hugely variable outcomes depending on other factors such as environment, activity levels and adherence to treatment and physiotherapy.[16] This taxonomy is helpful clinically as one can appropriately prepare parents and carers about disease course. Certain genotypes such as R117H differ hugely from the matched Phe508del homozygotes and it is therefore likely that these patients will be pancreatic sufficient, have milder disease and normal or only mildly elevated sweat chloride levels.[16]

1.1.5 PATHOGENESIS

The CFTR is a chloride channel responsive to the phosphorylation of adenosine triphosphate (ATP). It is located systemically but most notably, within the lungs, sweat and pancreatic ducts, bowel and seminiferous tubules.[22] As previously described, the main roles of this protein are; regulation of sodium transport, acidification of intracellular organelles and maintenance of electrolyte balance. Malfunction of this system is normally a result of defective synthesis of the CFTR protein, altered channel function and intracellular trafficking.[22] This evidence effectively explains the high sweat chloride concentration and dehydrated viscous pancreatic secretions.

The pathophysiology underlying the pulmonary complications however, is less well understood. Four common theories had previously been hypothesized. One of these, the 'low volume hypothesis,' postulated that CFTR dysfunction leads to Na and H_2O hyperabsorption, resulting in dehydration of airway surface epithelia.[23] Inefficient clearance of the mucociliary surface of airway epithelium leads to copious amounts of adhesive mucus, ideal for the creation of, 'biofilms,' responsible for increased bacterial density and promotion of growth of specific organisms such as PA.[23,24]

Animal studies in recent years however have significantly contributed to enhancing our knowledge and understanding of the pathobiology of lung disease in CF. Studies performed on CF pigs, which exhibit the hallmark features of CF within months of birth,[25] have disproved the prevailing low-volume hypothesis. Demonstrating lack of CFTR-dependent changes in sodium absorptive flux; fluid absorption and depth of periciliary fluid in CF airway epithelia challenged this theory.[26,27,28] In a study measuring the Na^+ and K^+ concentration in airway surface liquid (ASL) in a CF pig and control, these

measurements did not differ, hence unable to explain defective killing of bacteria.[29] In addition, Chen et al deduced that defects in Cl^- and HCO_3^- transport exist but dysfunction of Na^+ transport within airway epithelia is not responsible for CF lung pathology.[30] Pezzulo et al concur with this statement concluding that dysfunction of CFTR protein or lack of CFTR protein leads to impaired bicarbonate transference within ASL.[29] This defect subsequently alters the pH of ASL, creating a more acidic environment. This environment leads to defective eradication of bacteria within the lung and inhibition of host antimicrobial response, rendering the CF lung more susceptible to infection.[25,29] This study effectively demonstrated enhanced bacterial killing in a more alkaline environment with particular destruction of *Staphylococcus Aureus* and *E-coli*.[29]

Another theory previously proposed was dysregulation of host inflammatory response. This hypothesis suggested an imbalance between pro-inflammatory and anti-inflammatory cytokines, with an over-whelming number of inflammatory mediators seen in cell cultures of uninfected tissue samples.[23,31] Recent studies however, have confirmed leucocyte count and inflammatory cytokines did not differ compared to controls.[25] In accordance with this, CF pigs had a low neutrophil and interleukin-8 (IL-8) count at birth, suggesting infection precedes inflammation of the lungs.[26]

1.1.6 CLINICAL MANIFESTATIONS

RESPIRATORY

Although the lungs of children with cystic fibrosis are macroscopically normal in appearance at birth, they quickly become infected and inflamed, with changes being

reported in babies as young as 16 weeks.[23,32] Cystic fibrosis subsequently presents as an obstructive airway disease with bronchiectasis being a pathological hallmark.[33]

A vast array of microbiological species exist in the airways of a patient with CF. This bacteriology is well documented and researched owing largely to the fact that expectorated sputum is available for culture.[34] Specific organisms associated with a decline in pulmonary function are: *Haemophilus influenza, Staphylococcus aureus, Methicillin-resistant staphylococcus aureus* (MRSA) *and Pseudomonas aeruginosa (Pa) and Burkholderia cepacia complex.*[23] *Pa* is a gram negative, aerobic bacterium and is arguably the most important pathogen within CF lungs. This opportunistic organism affects only those hosts in which defence mechanisms are impaired (e.g. bronchiectasis) and has little, if any effect on normal airway epithelium with adequate mucociliary clearance.[35] Within lungs damaged by cystic fibrosis there is impaired mucociliary clearance and increased binding of bacteria to airway epithelium. This allows bacteria the time to produce toxins and form hypoxic niches resulting in established infection.

Early *Pa* infection usually occurs with a sensitive organism with the non-mucoid phenotype. Infection with this species increases with age due to prolonged exposure.[36] If treated aggressively with a combination of oral and nebulized antibiotics, this can be eradicated in the majority of cases. If eradication is unsuccessful, *Pa* mutates into the mucoid phenotype, which is associated with biofilm formation and chronic infection. Patients chronically infected by this strain commonly exhibit a poorer prognosis, and higher mortality rate.[36] Acquisition of this mutation is also associated with poorer chest x-ray scores and lung function tests in addition to lower length and weight z scores.[37,38] In such instances management changes from eradication to suppression, normally with nebulized antimicrobial therapy such as tobramycin, colistin and aztreonam.[35,37,39] These

have been shown to improve lung function and reduce exacerbations. One study has suggested a role for planned intravenous antibiotics regardless of whether the patient is unwell or not but this is not widely practiced.[40]

As well as the direct effect of lower respiratory tract infection, some CF pathogens can have indirect effects. Infection with *Aspergillus Fumigatus* is frequently noted in CF patients. The most common pathology associated with this fungus is Allergic Bronchopulmonary Aspergillosis (ABPA). This is an allergic reaction with features of wheezing and bronchiectasis often requiring steroids for treatment.

The natural history of cystic fibrosis airway disease is one of recurrent lower respiratory infections, inflammation and progressive lung damage. This results in worsening obstruction and ultimately respiratory failure.[41] Some patients will experience other acute respiratory complications however, such as pneumothorax, massive hemoptysis, and pulmonary hypertension.[33,42]

GASTROINTESTINAL

In addition, CFTR dysfunction affects the pancreas, gastrointestinal tract and the hepatobiliary duct leading to significant comorbidities. Pancreatic insufficiency is a significant feature in patients with a class I-III genotype, leading to maldigestion and malabsorption of nutrients and fat soluble vitamins; A, D, E, K.[42] Pancreatic status can be determined by measurement of faecal elastase[43] or a serum test for tripsinogen in patients older than eight. This manifestation can effectively be managed through pancreatic enzyme replacement therapy (PERT) however, if untreated, patients present clinically with failure to thrive, stunted growth, steatorrhoea and a delay in puberty.

Another aspect of gastrointestinal complications within cystic fibrosis, are the group of intestinal obstruction syndromes; meconium ileus, distal intestinal obstruction syndrome (DIOS) and constipation, a collection of bowel symptoms differing in age onset and intestinal location. Meconium ileus, an accumulation of inspissated meconium within the distal bowel, presents within the neonatal period and is often the first clinical feature suggestive of a diagnosis of CF.[44] This condition has been reported to affect between 13-17% of neonates.[42,44] Thought to be unique to CF, the mechanism through which it presents, is a combination of, reduced motility and delayed transit time of intestinal contents and a build of viscous mucus.[44]

Distal intestinal obstruction syndrome is the complete or incomplete obstruction of the ileocecum with fecal matter.[42] There are a number of factors contributing to the development of this form of obstruction. These include; tissue hypertrophy, intestinal inflammation, non-adherence with PERT and defective water and chloride secretion into gut lumen due to dysfunction of the CFTR channel.[42] Symptoms classic of DIOS are; colicky abdominal pain, right iliac fossa mass, flatulence and anorexia. Although the lifetime prevalence is 8% in paediatric patients and 16% in adults,[44] the prognosis is good for patients with DIOS. Provided early intervention and aggressive treatment with either oral laxatives or intestinal lavage, the outcome is almost always successful.[44]

Constipation is more common within the CF population. Faecal impaction starts at the distal colon and extends proximally, differing from DIOS in terms of location.[42] The cause of constipation is thought to be due to decreased water secretion into the intestinal lumen. Inadequate fluid or fibre intake does not contribute to the development of constipation in

patients with CF.[44] Symptoms of constipation are often milder and longer standing and are easily managed with stool softeners and stimulant laxatives.

There are many hepatobiliary conditions associated with CF, the most concerning of which are, focal biliary cirrhosis, portal hypertension and liver cirrhosis.[42] The highest incidence of these are seen in the first ten years of life, with 41% of children documented to have elevated liver enzymes by 12 years old.[42] The aetiology behind this is an abnormal CFTR protein within the biliary system leading to impaired secretion of bile with decreased alkalinity.[45] These changes along with accumulation of toxic bile acids, damage the hepatocyte directly.[45]

ENDOCRINE

There are a few endocrine disorders associated with cystic fibrosis. One of which, is cystic fibrosis related diabetes (CFRD). This disorder has been reported in 50% of patients less than 30 years old, making it the most common complication of cystic fibrosis.[46,47] Recent data from the European registry found a 5% prevalence of CFRD in 10-14years and a 13% prevalence in 15-19years.[48] These epidemiological studies highlight the frequent occurrence of CFRD as well as the increasing likelihood of acquiring CFRD with age. CFRD shares features with diabetes mellitus type I and II, however it does display some unique characteristics. These are; increased energy expenditure, malnutrition, glucagon deficiency and gastrointestinal abnormalities such as delayed gastric emptying.

CFRD tends to occur in patients homozygous for Phe508del and those with pancreatic insufficiency.[49] The explanation for this lies in the fact that abnormal CFTR function causes obstructive damage to exocrine pancreas leading to progressive fibrosis and fatty

infiltration.[46] This in turn leads to disruption and dysfunction of pancreatic islet cells. It is widely accepted within the literature that CF patients with or without CFRD display a form of insulin insensitivity. This may be as a result of general poor health of the patient as insulin sensitivity is influenced by infection and inflammation.[46,50,51] Only patients who are pancreatic insufficient however display β-cell dysfunction,[52] leading to insulin deficiency. It is important to note that insulin insensitivity is not as important as insulin deficiency in the development of CFRD.[53]

Onset of CFRD is insidious and patients may be asymptomatic for many years. There are some recognizable clinical features common to this presentation; unexplained polyuria or polydipsia, failure to gain or maintain weight, poor growth velocity, delayed progression of puberty and an unexplained deterioration in pulmonary function.[54] Patients who develop such symptoms are at greater risk of poor prognosis and a rapid decline in weight loss and pulmonary function. This knowledge emphasises the importance of identifying patients prior to this development. Current UK practice includes screening patients annually from 12 years old[55] however many centres follow US recommendations which advise screening patients annually from the age of 10.[46] Monitoring is achieved through measurement of glycated-haemoglobin ($HBA_{1c)}$ or oral glucose tolerance test (OGTT), the gold standard monitoring test for CFRD in Europe.[52] A positive result is based on either a fasting hyperglycaemia of >7.0 mg/dl or 2hr blood glucose of >11.1mg/dl post glucose load.[46]
At present, insulin is the only recommended treatment for CFRD.[50] Further management focuses on achieving optimum nutritional status through a high fat, high calorie diet.[46,56]

As previously mentioned, CFRD is associated with worsening lung function, poorer nutritional status and decreased survival.[46,48,50] An article prospectively reviewing data

gathered from a Danish population found patients with CFRD had a median survival of 24 years compared with 34 years in non-diabetic controls.[48] Another prognostic indicator is development of microvascular complications. These do occur in CFRD however their presence is lower than in other forms of diabetes mellitus. Reported complications include; microalbuminuria, retinopathy, neuropathy, gastropathy.[57] In comparison, to date, there have been no reports of the common macrovascular complications of diabetes such as myocardial infarction, stroke and hypertension, despite the fact that these patients are living longer.

Bone disease has emerged as another endocrine complication in long term survivors of cystic fibrosis.[58] The pathogenesis for this is multifactorial owing to; poor nutritional status, low body mass index (BMI), vitamin D deficiency, delayed puberty and intermittent use of glucocorticoid therapy.[58] In particular osteopenia and osteoporosis have been highlighted, with bone resorption exceeding bone formation even in clinically well, stable patients.[23,58] Low bone density has also contributed to increased rates of fractures in patients with cystic fibrosis.[58] It has been proposed that supplements of vitamin D, K and calcium may prove vital in preventing this complication and a role for bisphosphonates in the adult population with low BMI has been suggested.[58] Bone disease is screened for after the age of 10 years.

REPRODUCTION

Abnormalities of the genito-urinary system also exist, demonstrated by the majority of men with cystic fibrosis displaying congenital bilateral absence of the vas deferens.[59] This is due to the sensitivity of the vas deferens to CFTR dysfunction. In fact, infertility and azoospermia (absence of motile, viable sperm in semen) can also be seen in men with only

one CFTR mutation and no other clinical manifestations of cystic fibrosis.[23,60] Fertility problems also arise in females with cystic fibrosis. Thickened cervical mucus and endometrial polyps arise de novo complicating conception.[61] In saying this, conception is possible but poses a greater risk to mother and baby. Complications of pregnancy include intrauterine growth restriction (IUGR) and prematurity.[61] It has also been suggested that women with chronic lung disease cannot cope with the physiological demands of pregnancy, adversely affecting their own health.

1.1.7 DIAGNOSIS

Although recent advances have implemented alternative diagnostic mechanisms, including newborn screening, measurement of sweat chloride concentration remains the gold standard analytical measure to confirm a diagnosis of CF.[62] This investigation involves stimulation of sweat using pilocarpine inotophoresis, usually on the lower arm or leg of infants.[62] Recommendations for reference values of sweat chloride have been extracted from guidelines from the Cystic Fibrosis Foundation.[62]

- $\leq$ 29mmol/L = Negative
- 30-59mmol/L = Borderline / indeterminate
- $\geq$ 60mmol/L = Confirmation of CF

Taking these values into consideration a sweat chloride level of >30mmol/L should be considered abnormal in an infant and should prompt further patient evaluation.[62] This is due to the fact that sweat chloride level increases with age in patients without cystic fibrosis.[23,63] Sweat testing is readily available in cystic fibrosis centres and is pain free. It has been proven that this diagnostic aid can be performed in patients as young as 48

hours.[63] For individuals not satisfying the diagnostic criteria but there is strong clinical suspicion, measurement of nasal transepithelial potential difference (NPD) is another diagnostic substitute. The downfall of this however is that it is labour intensive, technically difficult and not freely available across all cystic fibrosis centres.[64,65]

1.1.8 MANAGEMENT

As a complex, multi-system disease, it is recommended that care of cystic fibrosis patients be undertaken within a specialist cystic fibrosis centre. There are 25 specialist paediatric centres within the UK.[66] These centres involve a multidisciplinary team (MDT) including a specialist consultant paediatrician, a CF nurse specialist, physiotherapist, dietician, psychologist and social worker.[67,68]

RESPIRATORY

PHYSIOTHERAPY

Respiratory care is a vital aspect of CF management. Daily respiratory treatment involves regular chest physiotherapy. This is achieved through specific manoeuvres that promote airway clearance and relieve bronchial obstruction. The main methods employed are; postural drainage, percussion, clapping, flutter and acapella.[69] In addition, patients are encouraged to lead active lifestyles and exercise regularly to achieve the same objective.

ANTIBIOTICS

Daily treatment also comprises of medications. Antibiotics are used for prevention, and eradication of lower respiratory tract infections and clinicians have a low threshold for prescribing these. Prophylactic Flucloxacillin is recommended from diagnosis and given for the first two years in the UK to prevent infection with *Staphylococcus aureus.*[39] During an infective exacerbation, patients are treated aggressively with oral or intravenous

antibiotics. If the infective exacerbation is caused by a new infection, (such as *Pa*) additional nebulised antibiotics may be used in an attempt to eradicate the organism. If eradication is unsuccessful and the infection becomes chronic, the aim of treatment changes from elimination to suppression, usually with long-term nebulised antibiotics. Recent advances have seen the introduction of antibiotic delivery techniques such as, 'intelligent nebulisers' and 'dry-powder inhalers,' which generate high levels of drug within the airways with low toxicity and systemic effects.[67]

MUCOLYTICS

In conjunction with this, other treatment strategies involve the use of mucolytics and muco-active agents. Recombinant human deoxyribonuclease (rhDNase) has been shown to improve lung function and airway inflammation and reduce the number of exacerbations.[70] Hypertonic saline and inhaled Mannitol increase airway surface hydration and improve clearance of airways.

NUTRITIONAL

Optimising nutrition is another crucial aspect of CF care. Attainment of normal growth and nutritional status is a key outcome within specialist centres.[69] As patients with CF generally have increased energy expenditure and higher basal metabolic rate, a high fat, high calorie diet is advocated. Energy and nutritional supplements are also accessible but should not be used as a substitute.[71] If pancreatic insufficient, pancreatic enzyme replacement therapy (PERT) should be taken alongside meals. Administered in a capsule form, these digestive enzymes avoid inactivation by gastric acidity.[69] In patients failing to meet their target weights and oral intake is not sufficient, enteral nutrition is offered, either through a nasogastric tube (NGT) or a percutaneous endoscopic gastrostomy (PEG).[72]

NEWER MANAGEMENT STRATEGIES

Advances in research have offered newer therapeutic choices. Ivacaftor, a CFTR potentiator, is one of these. This directly targets the underlying CFTR protein defect, rehydrating the airway surface.[73,74] This drug is genotype specific, approved for patients with at least one G551D mutation and ≥ 6 years (approximately 4-5% of all CF patients). This exciting innovation has been shown to improve lung function; pulmonary exacerbation rate, weight gain and patient reported quality of life as well as dramatically decreasing sweat chloride levels.[73,74] There are cost implications however with Ivacaftor costing ≈ £182,000 annually.[75] Active research is looking into developing similar small molecule drugs to target the Phe508del genotype. The two groups being studied are, 'CFTR potentiators' and 'CFTR correctors.'[76]

In addition, the UK CF gene therapy consortium have worked to develop multi-dosed non viral gene therapy.[77] This disease has been a target for gene therapy since the CF gene was cloned in 1989.[78] This concept, in theory should work with the possibility of inserting one copy of normally functioning DNA into affected cells. Outcomes of trials however have been mixed. Alton et al, who published their research on the first non-viral gene therapy, reported evidence of, 'significant but modest' beneficial effects in lung function (FEV_1) compared with the placebo group.[79] Stabilisation of pulmonary function rather than improvement was documented.[79] Further trials within this sector are required before this concept is rolled out in clinical practice.

Management of cystic fibrosis requires significant commitment from the patient or caregiver. It is generally accepted that adherence to treatment is the most difficult aspect of co-ordinating care of CF patients.[67] This knowledge therefore reiterates the role of the

healthcare team in educating, supporting, advising and motivating patients and their families.

1.1.9 PROGNOSIS

Advances in treatments and diagnostics available and introduction of centralised care have extended the life expectancy of CF patients, with the current median survival at 37 years.[80,81] A UK model predicted a child born today with CF will typically live to be 50 years of age.[14] As mentioned above, this is dependent on other unchangeable intrinsic factors however such as genotype and gender. Aside from the first year of life, morbidity of females with CF is generally greater than that of males.[14] Furthermore, patients detected through newborn screening who receive expert management immediately from diagnosis, show improved benefits in clinical course and have an increased life expectancy compared to those diagnosed clinically.[82] The institution of very early treatment has been deemed, 'critical' for long term prognosis and survival outcomes.[82]

1.2 NEWBORN SCREENING FOR CYSTIC FIBROSIS

1.2.1 INTRODUCTION TO NEWBORN SCREENING

Newborn screening is a public health initiative enabling individuals with treatable, genetic conditions to be identified so that treatment can be instigated early, often prior to the onset of symptoms. This population specific public health program reduces infant morbidity and mortality through pre-symptomatic detection of disorders with dried blood specimens from neonates, analysed in central laboratories.[83] Screening for cystic fibrosis has been successfully adopted in many countries internationally with official institution of the scheme across England in 2007. In saying this, not all European countries have employed such advance.[84] CF screening is fully incorporated into the current blood spot regimen and based on the same laboratory populations. It relies on recognition of individuals with a high immunoreactive tripsinogen (IRT) level in the first week of life. This measure is not specific so a second 'tier-test' is required. Different NBS programs use different 2nd tier markers, which include; a second IRT, genetic (DNA) analysis or pancreatitis associated protein (PAP). Newborn screening offers a unique opportunity for clinicians to intervene at an earlier stage of disease, which subsequently translates to improved respiratory and nutritional outcomes. More analysis is required on the effect on quality of life and life expectancy.

1.2.2 HISTORY OF NEWBORN SCREENING

Neonatal screening of mass populations owes its tributes to Dr Guthrie, a New York oncologist who discovered a way to detect phenylalanine levels in children with phenylketonuria (PKU). He later developed a method of analysing dry blood spots collected on filter paper from heel pricks taken from newborns, later termed, 'Guthrie

cards.' These were used during the first statewide NBS program, implemented in Massachusetts in 1962[83] which screened for conditions such as congenital hypothyroidism, galactosemia, phenylketonuria and some haemaglobinopathies.[85]

The 1970's saw the introduction of NBS for CF in some areas. Two studies were originally published, one of which claimed an association with early diagnosis and improved clinical outcomes.[86] The other, studied pairs of siblings, both affected by CF, the older sibling diagnosed >1 year and the younger diagnosed <1 year. Results at seven years showed better prognosis, pulmonary function and fewer hospital admissions in the younger siblings, despite receiving similar treatment.[83,87] During this time, no universal screening program for CF existed and diagnosis relied on symptomatology. It had been postulated that a high protein content within meconium could be a useful measurement of detection, although this was never introduced.[88]

In 1979, Crossle and colleagues noted an elevated IRT in the blood of all patients with CF within the first few months of life, despite pancreatic status.[83, 105] This was a sensitive biomarker which overcame many of the drawbacks of previous screening methods and had the advantage of using Guthrie cards and could be added to other assays within centralized laboratories.[83] Screening using this determinant was first introduced in Australia and the USA in the early 1980's[84] but more information surrounding validity was required before recommendation for mass populaions.[83,85]

To provide more information, Farrell et al devised the 'Wisconsin Study.'[89] Their cohort was split in two with half receiving NBS results immediately and the other half having results withheld until constitutional diagnosis or the age of four years.[89,90] This sparked an

ethical debate but it was agreed the trial could be granted as the latter cohort still received the normal, 'standards of care.'[89] Even with publication of more positive outcomes, it wasn't until 2002 that the National Institute of Health deemed the program sufficient.[90] Northern Ireland benefited from the advance in 1989, Wales in 1996, Scotland in 2003 and England finally rolled out this system in July 2007.[84]

1.2.3 ETHICS

Population screening for genetic conditions requires judicious consideration and scrutiny before implementation, due to the potential harms and risks as well as the need to demonstrate benefits. Certain ethical principles such as beneficence and non-maleficence can be applied to NBS for CF. It is not in the patients best interests to be undiagnosed with a 5% mortality and adverse nutritional outcomes.[91] Likewise, guaranteeing all infants an equal opportunity for early diagnosis ensures care and prognosis of patients is unaffected by financial, demographic or geographical factors.[91]

Identification of carriers has posed controversy amongst screening this cohort. Such information can be harmful, by aggravating stigmatization[92] and beneficial, to parents who recognize the importance of this knowledge in future family planning.[93] Autonomy is an ethical theme applied here. Some people argue that by disclosing carrier status removes the child's privacy from family members[94] and the child's right to decide whether this information should be revealed. Furthermore, this information may lead to additional diagnostic tests, unnecessarily medicalizing infants. Similarly, the ability of NBS to identify infants with a 'mild' form of CF has been debated. Wilfond and Rothenberg argue that it is only ethical to include pancreatic insufficient mutations in the mutation panel, as

there is no supporting evidence to suggest that earlier diagnosis through NBS helps those with pancreatic sufficiency.[95]

1.2.4 CYSTIC FIBROSIS NEWBORN SCREENING

Following the work of Wilson and Jungner in 1968,[96] the World Health Organisation (WHO) agreed on ten criteria to ensure delivery of an effective screening program. It is important that these factors are considered when implementing a new scheme. Justification for the cystic fibrosis NBS program follows, with regards to these principles.

1. The condition should be an important one

This chronic disease, which affects a huge proportion of Caucasians in the UK, is life limiting as well as having a major affect on sufferers' quality of life, mostly physical functioning.[97,98] One must consider also, the impact this illness can have on family members, particularly psychological issues.[99]

2. There should be treatment for the condition

There are numerous treatment options available upon diagnosis, alluded to earlier. These are provided by the appropriate healthcare professional.

3. Facilities for diagnosis and treatment should be freely available

There are 25 specialist cystic fibrosis centres,[66] spread throughout the UK. Access to these is freely available for those who have a carrier status or positive screening result.

4. ***There should be a recognizable latent or early symptomatic stage of the disease***

There is a latent stage of CF in which patients are either asymptomatic or their symptomatology is misinterpreted by clinicians, hence the delay in diagnosis. There is an abundance of literature recognizing the benefits of diagnosis before the age of eight weeks.[100,101]

5. ***There should be a suitable test or examination***
6. ***The test should be acceptable to the population***

Blood spot cards are practical and suitable, being lightweight and easy to store and transport.[102] A small amount of blood is required (15-200µl),[102] meaning the patient isn't in pain for too long and little training is required for this procedure.

7. ***The natural history of the disease should be well understood***

The natural history and progression of CF is well understood but as the median age of survival increases,[80] and more patients survive to adulthood, new pressures are created, in terms of management, with treatments always changing.

8. ***There should be an agreed policy on whom to treat as patients***

All individuals with, 'classic CF,' or a genotype associated with a severe phenotype will be referred to a specialist centre for clinical evaluation and initiation of treatment. Individuals identified by NBS with a, 'mild' phenotype or 'equivocal' diagnosis are more difficult to treat as there are few guidelines on this. Although, attempts have been made to resolve this.[103]

9. ***The cost should be economically balanced in relation to medical care as a whole***

Newborn screening is cost effective. A detailed evaluation of this will appear later in this section.

10. ***Case finding should be a continuous process and not just a, "once and for all project"***

NBS for cystic fibrosis will be a continued process with permanent implementation.

1.2.5 GENERAL THEMES OF CF NEWBORN SCREENING PROTOCOLS

IRT as a biomarker has a high sensitivity (the ability of a test to correctly identify those with the disease) but a low specificity, (the ability of a test to correctly identify those without the disease) due to a false positive result in cases of; hypothyroidism, autonomic dysfunction, nephrogenic diabetes insipidus, adrenal insufficiency[23] and African-American ethnicity.[83,104] Interestingly, CF patients presenting with MI are the exception, with IRT values within the normal range.[65,104] Currently, all NBS protocols use blood levels of IRT as the first tier of testing[105] but to improve the specificity, a second tier test is required.[105] This comprises of either:

1) IRT-IRT, where a second blood sample is collected between days 14-21 as measuring IRT at this point is more specific[106,107]
2) IRT-DNA (deoxyribonucleic acid), which uses DNA mutation analysis to identify the most common CF mutations within that population
3) IRT-PAP (pancreatitis associated protein) – this improves specificity without having to perform extended gene analysis.

At present, there is no standardized screening protocol with an extensive number of CF NBS algorithms employed nationwide.[112] Explanation for this lies in the fact that protocols are designed according to resources, geography[107] healthcare provisions[106] and ethnic diversity of the population. For example, the selection of CFTR mutation panel should be based on data obtained from each region and consensus guidelines.[83] In saying this, there are international standards set by the European Neonatal Screening Working group, which each country should aim to meet.[108] These standards include; there should be an acceptable number of false positive results with a minimum positive predictive value of 0.3 (30%), the number of false negatives should be low and the protocol should demonstrate a 95% sensitivity and babies with a confirmed diagnosis should be seen by a specialist CF team within a maximum of 58 days after birth.[108]

In summary, use of one or a combination of these two-tiered tests is appropriate[104,106,109] however European Consensus guidelines consider IRT-DNA the optimal diagnostic strategy.[103] Although protocols vary dramatically, there are clear international standards, which should be met by all. To reiterate, irrespective of the protocol employed, a positive screen for CF requires judicious clinical assessment and referral for sweat testing and repeat gene analysis to confirm, and in some cases to refute this diagnosis.

1.2.6 UK SCREENING PROTOCOL

The UK CF screening program was rolled out nationally in 2007. On average 800,000 neonates are screened each year with approximately 300 positively screened babies.[110] Screening takes place within several central laboratories. The West Midlands laboratory is one of 16 newborn screening labs across the UK. It is responsible for the analysis of all

genetic conditions screened for within the heel-prick test. This regional lab covers a huge geographical area, *(see figure 1.1)* screening over 70,000 babies each year.

Figure 1.1 West Midlands Screening Laboratory geographical areas

UNIQUE FEATURES OF THE UK NBS PROTOCOL

The aims of the UK national screening program for cystic fibrosis include: maximizing diagnosis of CFTR defects, minimizing second heel pricks, minimizing the identification of CF carriers and preventing diagnostic delay.[111] These aims are achieved through four unique features of the UK NBS protocol, as described below.

1. A high IRT-1 cut off

Data from laboratories reveal a significant variation in IRT levels within populations. Therefore, IRT cut off levels should be calculated specifically according to that population. It is important that these levels are set correctly; if the cut off level is too low this will result in a disproportionate increase in the number of carrier individuals identified.[111] The first step in the screening algorithm is analysis of the first IRT assay. The UK NBS protocol has set an extremely high cut-off (99.9th percentile) for the first IRT blood sample,

compared to other European countries. If the IRT-1 is <99.9th percentile, CF is not suspected and no further testing is required. If however the assay is ≥ 99.9th percentile, the sample is re-assayed in duplicate and mutation analysis is performed. The advantage of this feature is that the protocol is effective in detecting those who truly have the disease. As a result of this, the PPV will increase. This is at the expensive of decreasing the sensitivity. Of the few missed cases in England, (i.e. 'affected, not detected') the majority result from having an IRT-1 below the cut off.

2. The limited 4-panel gene analysis

The second tier of the UK NBS protocol is DNA analysis. Inclusion of mutation analysis improves the performance of the program and removes the need for a second blood sample to be taken, ensuring rapid diagnosis.[105] In the UK, initially only a 4-panel DNA analysis, is performed. This considers the most common alleles within the English population: Phe508del, Gly551Asp, Gly542*, (621+1G>T).[112] If two mutations are detected, CF is highly suspected. If one mutation is detected, further gene analysis is performed with a 29 or 31 mutation panel. Amazingly, this initial panel detects up to 90% of patients with at least one CFTR-disease causing mutation. Including DNA analysis within the protocol improves the timeliness of diagnosis and the PPV but will increase the numbers of inconclusive diagnosis and carriers, which is considered undesirable for the NBS programme. Repeat genetic analysis should always follow a positive screening result.

3. Second IRT level on day 21

The third tier of the UK NBS protocol, contributing to this programs uniqueness, is a second blood sample for IRT taken on day 21 of life. IRT level decreases with age in infants without CF but remains high in children with CF, making it more specific. There

has been no evidence to suggest that IRT testing in week 3 is more sensitive than that in week 1. This IRT/IRT characteristic also improves PPV by reducing the number of infants referred for a sweat test.[105]

4. The 'Safety net'

A final element contributing to the originality of the UK NBS protocol is its' safety net strategy. This strategy, which involves further testing the IRT-1 sample, irrespective of the extended gene analysis results, has been adopted to avoid missed cases. The main advantage of this strategy is that it compensates for discrimination against ethnic populations with less common CFTR mutations, not included on the extended gene panel.[105] This is particularly important in multicultural cities in England such as Birmingham and London. The disadvantage is the referral of a large number of children for further diagnostic tests and assessment, in return for detection of very few infants with CF. These features are demonstrated below:

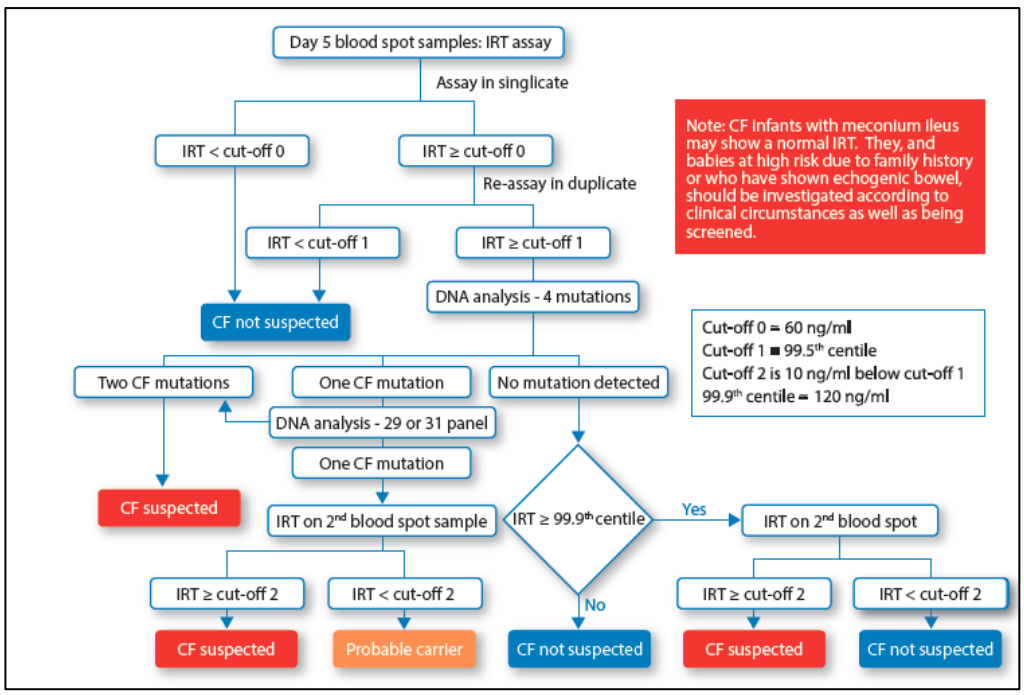


Figure 1.2 Algorithm for UK newborn screening program[111]

SAMPLE COLLECTION:

Parents are offered an information leaflet throughout pregnancy outlining the risks and benefits of the national screening program. At birth, verbal and written consent is obtained. A midwife or health visitor collects the blood sample on day five of life. These are collected onto blood spot cards, which are deposited within 24 hours to screening laboratories throughout the UK.[110,111]

FOLLOW UP:

Families of individuals, who have been identified as carriers, are referred to a genetic counsellor or specialist CF nurse. This is important to decrease parental anxiety and offer information relating to planning future pregnancies. The Clinical Liaison Service (CLS) will communicate with regional cystic fibrosis centres about positive-screened babies. Parents of these infants are telephoned and seen by a healthcare professional within 24 hours. A clinic appointment in CF clinic is arranged within the following five days for thorough clinical evaluation. This is essential as initiation of treatment is recommended within 28 days for those with two mutations and 35 days for those with one CFTR mutation.[110] This is imperative considering the benefits of newborn screening are dependent on diagnosis before two months of age.[100] Infants referred to specialist CF centres are offered a sweat test, as this remains the gold standard.

1.2.7 ADVANTAGES OF NBS

There is clear evidence regarding the benefits of newborn screening for cystic fibrosis. Beneficial nutritional outcomes have been documented and described; the evidence for respiratory benefits is less obvious.

NUTRITIONAL

Optimum nutritional status in early life and an increase in BMI corresponds to decreased risk of death.[113] It is also associated with a reduction in colonization of *Pa*.[113, 114] Data obtained from the cystic fibrosis foundation patient registry show that approximately 30% of individuals have a weight below the 3rd percentile.[15, 111] The Wisconsin study, which randomly allocated patients to be screened at birth against those who were diagnosed by conventional means, showed that the control group (standard diagnosis) displayed much greater declines in length and weight z scores in the first six months of life.[113] After six months the length and weight z scores improved but did not reach levels comparable to the screened cohort.[113] A study conducted in the Netherlands replicated these results, showing length, weight and BMI z scores to be better in the screened group, these reached significance at transfer to adult care.[113]

RESPIRATORY

Although diagnosis of CF through NBS can lead to improved nutritional and anthropometrical outcomes, there has been uncertainty about pulmonary outcomes and demonstration of this has been challenging. This is possibly due to the fact that there have been a number of treatment advances and improvement of pulmonary function is multifactorial. The Wisconsin study, which used a unique blinding method found no difference in the pulmonary function tests (PFT), spirometry (FEV_1/FVC) or chest x-ray scores of the newborn-screened group compared with the control. This however may have been influenced by the increased colonization of *Pa* in the screened cohort as mixed clinics were a source of cross infection between patients.[114] In contrast, Dijk et al who compared a screened cohort with a group of unscreened children in the years prior, reported improved

spirometry values with increasing FEV_1/FVC in the screened group.[115] This conflicting evidence emphasises the need for more studies evaluating this outcome.

Pseudomonas acquisition is associated with increasing mortality. Diagnosis through NBS offers opportunity for increased surveillance and rapid eradication to prevent the adverse effects of *Pa*.[116] In one study, 25% of patients diagnosed by conventional means were reported to be colonized with *Pa* at initial clinic visit,[83] whereas patients diagnosed through NBS have delayed bacterial invasion and a lower rate of first time infection with *Pseudomonas*.[115,117] In addition, the number of children with 'chronic' *Pseudomonas* (>50% positive *Pa* cultures in the preceding 12 months)[118] but defined as, 'three successive positive sputum cultures' for the purpose of this study, was 77% in the screened population compared to 100% in the unscreened patients.[115] This was a statistically significant difference. Similarly, infants diagnosed through NBS in Australia were less likely to have positive bacterial cultures obtained by suction or bronchoscopic lavage (BAL).[115,119]

SURVIVAL

Many studies concur about the survival and prognostic benefit, however the age at which this is significant is contended. Dankert-Roelse et al found a survival rate of 94% at the age of 11 years in a screened group compared with 65% of subjects in a non-screened cohort.[82] A study in New South Wales agreed with this finding but said that a survival advantage does not become apparent until adult years (*reflected in figure 1.3*).[115] An Australian cohort study comparing 57 screened children to 60 unscreened infants noted a statistically significant difference in survival at both 10 and 15 years.[119,120] Furthermore, four studies (one Randomized Control Trial and three observational) found CF-related childhood mortality to be 5-10% lower in screened cohorts.[120]

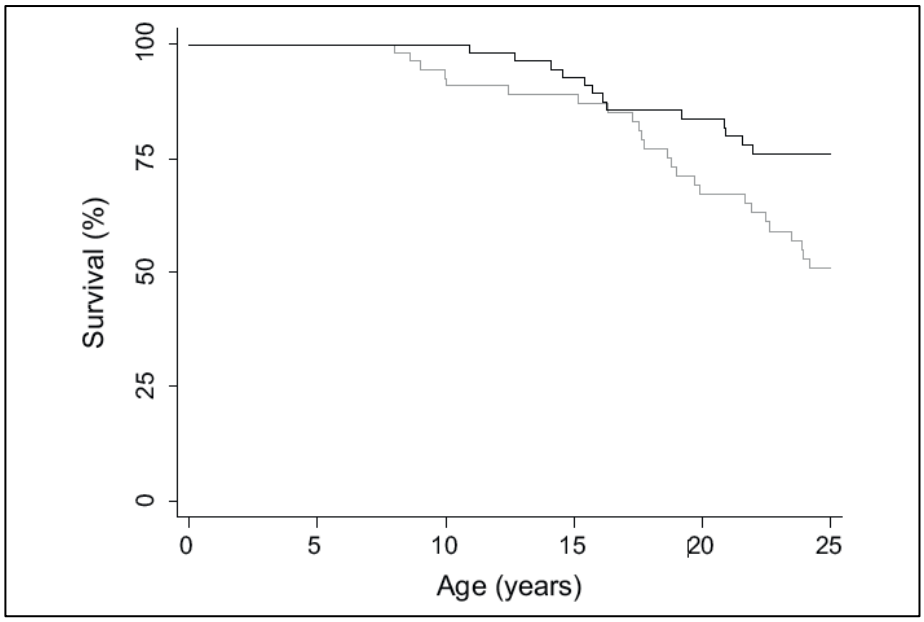


Figure 1.3: Kaplan-Mayer analysis showing superior survival in a screened cohort (darker line) at 25 years compared with an unscreened cohort (lighter line) in New South Wales. Figure taken from Dijk et al [115] with permission.

COST EFFECTIVE ANALYSIS

With the current health economy in financial crisis, facing limited resources, newborn screening programs undergo thorough financial evaluation and cost-benefit analysis prior to introduction. Lee et al [121] contrasted the annual cost of newborn screening with traditional methods. The conclusion revealed a two-tiered (IRT/DNA) screening test to be significantly cheaper ($2.47pp) than by sweat testing (range $36-$310pp). They also revealed this fee to be similar to the cost of accepted genetic tests such as PKU.[121] In a recent study, analyzing the cost of four different two-tiered strategies, the cost effectiveness ratio varied from €23,600- €23,900 per life year gained. Of the four, IRT-PAP was the most economic.[122] In addition to the costs saved by diagnosis alone, other economical concepts have focused on the potential savings after diagnosis. Patients diagnosed through newborn screening have improved clinical outcomes, reduced treatment needs,[123] less time spent in hospital[84] and fewer inpatient admissions.[124] Granted that two-tiered testing is cheaper than sweat testing, many UK CF centres still use pilcarpine

inotrophoresis to confirm diagnosis. Furthermore, the cost of providing families of carriers with genetic counseling has not been fully considered. In summary, from a public health perspective, the cost of NBS for CF is economically justifiable. With further research into the sensitivity, specificity and efficacy of NBS, the need for sweat testing may lessen. Similarly, as technology advances, one might expect the cost of genetic analysis and IRT measurement to decrease, leading to significant financial advantages.

OTHER

There are several other advantages of NBS. The Wisconsin group demonstrated a lower head circumference in children diagnosed by conventional means at 10 years old compared with the screened group.[125] It was speculated that this correlated with poorer cognitive function as vitamin E levels were found to be low and this is associated with stunted cognitive development.[83] There are also benefits to the parents and families of those affected with CF. Knowledge provided by NBS can assist parents in future reproductive decision making and may ultimately reduce the number of children born with CF. A better relationship between families and the CF team has also been suggested as there is more time for education and explanation rather than symptoms presenting acutely. A Dutch study performed found parents in the 'pre-diagnostic' period (first parental concern to definitive diagnosis) had fewer negative feelings and greater confidence in the medical profession than those parents whose children were diagnosed after three months.[126]

1.2.8 DISADVANTAGES OF NBS

Although there are many benefits of newborn screening, a number of disadvantages have been recognised which contributed to the delay in implementation of this scheme across England.

ACQUISITION OF PA

As newborn screening attempts to diagnose children at an earlier stage in their lives, they attend CF clinic early on. Despite cross-infection protocols this creates a potential for cross infection between patients. The Wisconsin group found a disproportionately higher number of children infected with *Pa* compared with the unscreened population.[115,127] This was during the period when children attended mixed clinics, not segregated according to what bacteria they grow and this factor, in hindsight probably contributed significantly to this discovery.[84]

DIAGNOSIS OF ATYPICAL CASES

Incorporation of DNA analysis into the two-tiered screening strategy has resulted in detection of cases that may never have been clinically diagnosed, at least not until adulthood. In cases of equivocal diagnosis, there is little guidance on management. Long-term outcomes are unknown and there is unnecessary medicalization of the family.[106] Patients undergo superfluous diagnostic tests and investigations and are exposed to microbiological harm by attending CF clinic.

DETECTION OF CARRIERS

An unwanted outcome of newborn screening is the detection of carriers – those children identified as positive by NBS but have only one dysfunctional CFTR gene. There are a number of difficulties that arise with the detection of carriers; parental anxiety, induced depression, reduced parental bonding and treatment of the child as 'sick'.[128] Some parents require thorough explanation of carrier status and struggle with understanding this concept. They believe that the child still has the potential to acquire CF, needs treatment and may become ill as a result of being a carrier. These negative emotional responses from parents[129] require genetic counseling which is time consuming and costly.[130] In

consideration of this, the attitudes and feelings of parents of CF carriers has been explored by Parsons et al who discarded the view that carrier detection had a negative impact on parental bonding and documented parents thoughts on knowing CF status as 'useful information,' relevant to their child's future life.[131,132] In contrast, there is no immediate benefit to the child knowing this carrier status and it can be considered an ethical violation of the child's right not to know.[93]

PARENTAL BONDING

Parents are more vulnerable to depression when their first child is diagnosed in the first few months of life.[133] This has the potential to affect parent-baby relationship and bonding. Many parents experience high levels of stress whilst awaiting additional confirmatory tests[93] however reports conclude that the difference between parents of screened cases and those who have been diagnosed clinically is proportionate.[84,132]

FALSE NEGATIVES

False negatives are children reported as normal but actually have the disease, in essence, 'affected, not-detected.' Examples of reasons for false negatives include: meconium ileus, blood transfusion, viral gastroenteritis and prematurity.[111] Newborn screening algorithms for CF routinely result in false negatives. This may be because individuals with CF have IRT values below the cut off or they have been missed at the DNA analysis stage.[134] The impact of this includes false reassurance for parents, delay in diagnosis and treatment and the reluctance of clinicians to suggest this diagnosis due to the newborn screening result.

RISK OF ETHNIC DISCRIMINATION

As the mutation panel in NBS is specific for that population, there is an increased risk of false negatives in patients from a non-western origin. Similarly, as the mutation spectrum

is largely unknown in diverse ethnic populations, patients must undergo sweat testing for confirmation of results.

1.2.9 CYSTIC FIBROSIS SCREEN POSITIVE, INCONCLUSIVE DIAGNOSIS

Once a child has a positive CF NBS the diagnosis is usually confirmed or refuted using a sweat test. This is not always the case and in some children the diagnosis remains unclear: CF Screen Positive, Inconclusive Diagnosis (CFSPID). Such children normally fall into two groups:

- **Group A**: Those with a normal sweat chloride but with two CFTR mutations, one of which has unclear phenotypic consequences
- **Group B**: Those with an intermediate sweat chloride (30-59mmol/L^{-1}) with one or no CFTR mutations.

These children have no definitive diagnosis of CF but have a number of risk factors for developing some of the features. There is very limited information on the outcomes; some may have few, if any clinical features. Currently, diverse practice exists on how to care for these children. To combat this confusion and negative impact of screening, a UK group devised a study on how to adequately categorize and manage infants with inconclusive results following newborn screening.[135] Clear guidance and recommendations are required for CF teams in this situation as it is important not to over-medicalize these children but also not to miss those infants that may develop significant disease.

1.3 LITERATURE REVIEW SUMMARY

Cystic fibrosis is the most common genetic, life-limiting disease within the UK. CF results from poor ion transport across epithelia resulting in viscous secretions. It is a multi-system disease, affecting the respiratory and intestinal systems most severely. Screening for CF has been practiced widely for many years but was not introduced nationally, across the UK until July 2007. This diagnostic advance has allowed paediatricians to practice preventative medicine rather than waiting for children to present with clinical symptoms. By then, permanent damage may have occurred. NBS programs for cystic fibrosis produce clear benefits in terms of nutritional outcomes. The proof of respiratory benefits and improved lung function has been harder to demonstrate, but the evidence for this is slowly increasing.

2.0 STUDY METHODOLOGY

This chapter provides an overview and description of the chronological tasks completed in order to fulfill the study aims. A discussion of the aims, ethical considerations, literature search strategy, study design, data collection and data analysis techniques will follow.

2.1 AIMS

The aims of this study were:

1. To review the CF newborn screening results from the West Midlands NBS Lab from 1st November 2007 to 31st October 2014
2. Identify all children diagnosed with CF in this time period
3. Collect background clinical data including: faecal elastase, sweat chloride, IRT, genotype, ethnicity, birth weight, date of screening, date of first clinic visit
4. Review patients notes and collect all the available anthropometric data from birth to 2 years
5. Review microbiology data, documenting the date patients first isolated *Staphylococcus aureus/ Pseudomonas aeruginosa*
6. Analyse these data by clustering and classification
7. Develop a model to predict weight and length z scores of these children at age 1 and 2

2.2 ETHICAL CONSIDERATIONS

For this type of study, no official ethical approval was required, as it was deemed, 'service evaluation,' an examination of how standard care is delivered and not generalizable to the whole population. This was confirmed by completion of the Health Research Authority (HRA) tool questionnaire (Appendix A). Parental consent was not required as data were

pooled anonymously. However, approval was obtained from the Research and Development (R&D) and clinical audit departments at both sites, University Hospital of North Midlands (UHNM) and Birmingham Children's Hospital (BCH). In addition, the ethics of newborn screening was appraised within the literature review and Good Clinical Practice (GCP) training was completed prior to starting the project (Appendix B). Finally, the author obtained an honorary contract at BCH to allow access to the necessary data.

2.3 LITERATURE SEARCH STRATEGY

To acquire a broad understanding of cystic fibrosis as a disease entity and newborn screening as a diagnostic test, a review of the literature was undertaken. This was conducted informally as the project did not take the form of a systematic review.

Core medical databases such as EBSCO, Medline and PubMed were used to identify relevant papers. Key articles were suggested by the project supervisor, particularly a paper from South East London, which formed the basis to this project.[136] To perform the search, keywords and phrases were identified and entered into the MeSH tool, which suggests different words for the same terminology, i.e. 'newborn screening,' and 'neonatal screening.' These keywords were merged using the appropriate Boolean tool, illustrated below (*figure 2.1).* The search included UK and international literature and did not exclude research on animal subjects. Other sources of information were Google Scholar, Cystic Fibrosis Trust website and the CF foundation website for relevant guidelines.

'Cystic fibrosis' AND 'newborn screening' OR 'neonatal screen*' AND 'efficacy' OR 'treatment outcome'

Figure 2.1 Search operation

2.4 STUDY DESIGN

The study design was a database review and retrospective cohort study. This type of study is performed post-hoc with the cohort assembled at a similar time point or early in the development of a common disease and followed thereafter. Newborns with a diagnosis of CF were assembled at screening and anthropometric data for the following two years was analyzed, retrospectively. Although the data for this project were collected within a four-month period, the data obtained includes measures that were taken in the past. A retrospective cohort study is disadvantageous in this respect, as it is reliant on other people's data collection and accurate record keeping. This study design was most appropriate however, given the short time frame in which to complete the service evaluation and lack of funding. Retrospective cohort studies are also useful in measuring a number of variables or multiple outcomes.[137] As is typical of all observational studies, only association, not causation can be inferred from the results, as confounding factors cannot be controlled, making this a limitation of this type of study design.

2.5 STUDY POPULATION

The study population for this project included all babies who had undergone newborn screening within this region between 1st November 2007 and 31st October 2014. These dates were chosen as NBS for CF was made available to the West Midlands laboratory in November 2007 and 31st October was chosen as the cut off date to allow for collection of clinical data for each confirmed case of CF (defined by; identification of two abnormal CFTR genes or one gene and a positive sweat test) or CFSPID for two years post-diagnosis. CFSPID children were included because this criterion was not available at the time of their diagnosis' henceforth they are all registered on the CF Trust registry and monitored in the same way as other patients. Patients were excluded if they moved out of

area or died within the first two years of life. Patients with pre-existing co-morbidities were not excluded. As this cohort study was population based i.e. all patients diagnosed with CF within the region were included, the cohort members should be representative of the population of all patients with CF, minimizing selection bias.

2.6 DATA COLLECTION

Data were gathered across three centres: Royal Stoke University Hospital (RSUH) Royal Shrewsbury Hospital (RSH) and Birmingham Children's Hospital (BCH). There were two main aspects to the data collection: collection of the NBS data and collection of clinical data on all patients with confirmed CF.

2.6.1 NBS DATA

The NBS data were collected initially. Information on the number of screened babies within the specified dates was provided by the West Midlands laboratory team, as were the numbers of CF carriers. Two datasets were then reviewed, crosschecked and analysed. One of which was an automatic database, created via a business objects query, which collects data from the West Midlands screening lab information management system, 'Omni Lab' (integrated software solutions). These results automatically come across from the analytical instruments for each patient sample. The other was a manually created database, for which the laboratory team is responsible for inputting relevant information. This holds data on all positively screened babies. The datasets contained all patient demographics and some biochemistry results. False positives had not been omitted from these datasets but were highlighted. These patients, identified as screen positive but with no clinically confirmed diagnosis were counted and removed from the main database. Discrepancies in the databases were noted and subsequently checked with the paediatric respiratory

consultants at both centres to confirm whether or not these patients were currently under the care of either team. Data cleaning involved amalgamating information from both spreadsheets and removing information irrelevant to this study. Ethnicity was originally presented in code format. The West Midlands Laboratory ethnic code guideline was used to replace letters with more descriptive nominal data. This can be viewed below:

A	BRITISH
B	IRISH
C	ANY OTHER WHITE
D	WHITE AND BLACK CARIBBEAN
E	WHITE AND BLACK AFICAN
F	WHITE AND ASIAN
G	ANY OTHER MIXED
H	INDIAN
J	PAKISTANI
K	BANGLADESHI
L	ANY OTHER ASIAN
M	CARIBBEAN
N	AFRICAN
P	ANY OTHER BLACK
R	CHINESE
S	ANY OTHER ETHNIC CATEGORY
Z	NOT STATED

Table 2.1 The West Midlands Laboratory ethnic codes guideline

2.6.2 ANONYMISING PATIENT DATA

Once the master spreadsheet was complete after cleaning, each patient name was replaced with a unique study number. This was to ensure patient information remained anonymous. A separate spreadsheet with the unique study number and patient's name only was then created, to refer back to. Each spreadsheet was stored on a password-protected encrypted memory stick. Any discussion surrounding this data, between the healthcare professionals involved, was shared between, 'nhs.net' email accounts.

2.6.3 CLINICAL DATA

As a smaller CF centre and the author's base site, collection of the clinical data was initiated at RSUH to see which anthropometric and microbiological information could be obtained within a short timeframe. A list of patients under the care of RSUH/ RSH was created. The medical notes of these patients were gathered from the medical records room within the CF centre. Within the clinical notes, a blue record sheet of all patient's lengths and weights from birth to present was available. This made collection of anthropometric data relatively simple. Every length and weight for each patient within the first two years of life was recorded. Information such as birth weight, genotype and date of diagnosis were crosschecked with the medical records to ensure these figures correlated correctly with the information from the NBS laboratory. The microbiology and biochemistry data were collected from RSUH I.T. software, 'iCM.' Sweat chloride results and faecal elastase measurements were found within the biochemistry results section. The microbiology results were viewed in, 'trend' view, allowing documentation of the date the patients' first isolated *Staphylococcus aureus* and *Pseudomonas aeruginosa*, had they isolated it at all. The microbiology data were collected for the first two years of life.

Information on six patients was collected from Royal Shrewsbury Hospital. Dr Martin Rees, consultant paediatrician, kindly facilitated this by requesting the medical records. The anthropometric, microbiology and biochemistry data were obtained from the clinical notes. Microbiology and biochemistry information was obtained from the 'results' section of the medical records.

Nutritional data from BCH were obtained from the dietetics department as it was presumed this would be a faster method of collection. Carolyn Patchell, CF dietician, facilitated this. The information collected was adequate for pancreatic insufficient patients, who see dietetics often. There was minimal information on pancreatic sufficient patients or shared care patients, who are seen more regularly in their local district general hospitals; Wolverhampton, Coventry, Hereford, Royal Hallamshire, and Heartlands. A third of the patients fell within this category. The medical records of these patients were requested and lengths and weights for the first two years of life were obtained from the 'correspondence' section of the notes. Biochemistry and Microbiology results for BCH-only patients were gathered from BCH computer software program. To collect these results on shared care patients, Dr Maya Desai, consultant respiratory paediatrician at BCH provided the CF consultants from the hospitals listed above, with a list of patient names and requested they record the date of patient's first isolation of *Sa* and *Pa* within 2 years.

2.7 DATA ANALYSIS AND STATISTICAL DESIGN

2.7.1 ANALYSIS OF NBS DATA

Primary data analysis involved generating descriptive statistics for the newborn screening information. The positive predictive value (PPV) and negative predictive value (NPV) of the screening test were calculated. The birth prevalence, number of false positives and false negatives over this time was also evaluated. Detailed information on these missed cases was analysed. The frequency of patients diagnosed each year will be presented in a table, in addition to gender ratios. Demographics of patient's meeting the criteria to be classified as, 'CFSPID,' will be presented although we did not exclude this group in the subsequent data analysis as they are reviewed and assessed exactly the same as those patients with 'classical CF,' in our centres. The median/mean (depending on whether or not the variable was normally distributed) was calculated for:

- Age at screening
- Age at diagnosis
- Birth weight
- Sweat chloride
- IRT
- Faecal elastase

Descriptive statistics were chosen to explain the information gathered about newborn screening so that we could easily illustrate the main findings of our first study objective through tables and graphs.

2.7.2 ANALYSIS OF MICROBIOLOGY DATA

Descriptive statistics form the basis for interpretation of microbiology results. The numbers of patients isolating *Sa* and *Pa* in years one and two have been calculated in addition to the median age of isolation. A Kaplan-Meier analysis graph was used to demonstrate the percentage of patients free from each bacterium over the two-year period. A second Kaplan-Meier graph compares isolation rates at each centre.

2.7.3 REPRESENTATION OF ANTHROPOMETRIC DATA

The second aspect of data analysis involved converting the raw lengths (cm) and weights (kg) into a more clinically relevant figure. Z-scores have been recommended by the World Health Organisation (WHO) to represent a child's anthropometry.[138] They are useful as a standardized measure, being comparable across age, sex and other variables. This conversion was appropriate for this study as the nutritional data collected from each child varied in terms of time point e.g. patients did not have a recording of these measures at exactly the same age. The 'WHO Anthropometric z-score calculator, version 3.2.2, January 2011' was downloaded from: http://www.who.int/childgrowth/software/en/. This can be visualized in *figure 2.2*. The child's DOB, gender, length and weight for each clinic date were manually entered. All lengths were measured recumbent. The outputs of the z-score calculator were; length z score, weight z score, length-for-weight z-score and BMI.

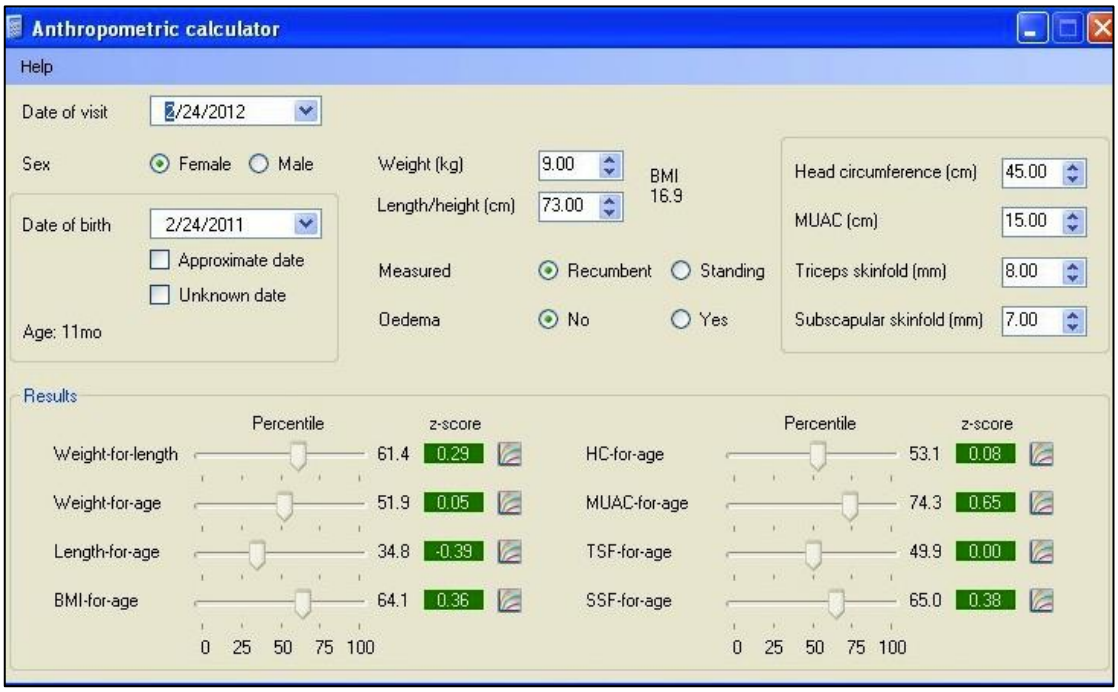


Figure 2.2 Screenshot showing World Health Organisation (WHO) anthropometric calculator

Using these calculated z scores; straight-marked scatter graphs were drawn of each patient's weight and length over the 24-month period. With age in weeks (x-axis) and z score (y-axis), *see figures 2.3-2.4.* The axis range for each graph was consistent, to allow easy comparison between patients.

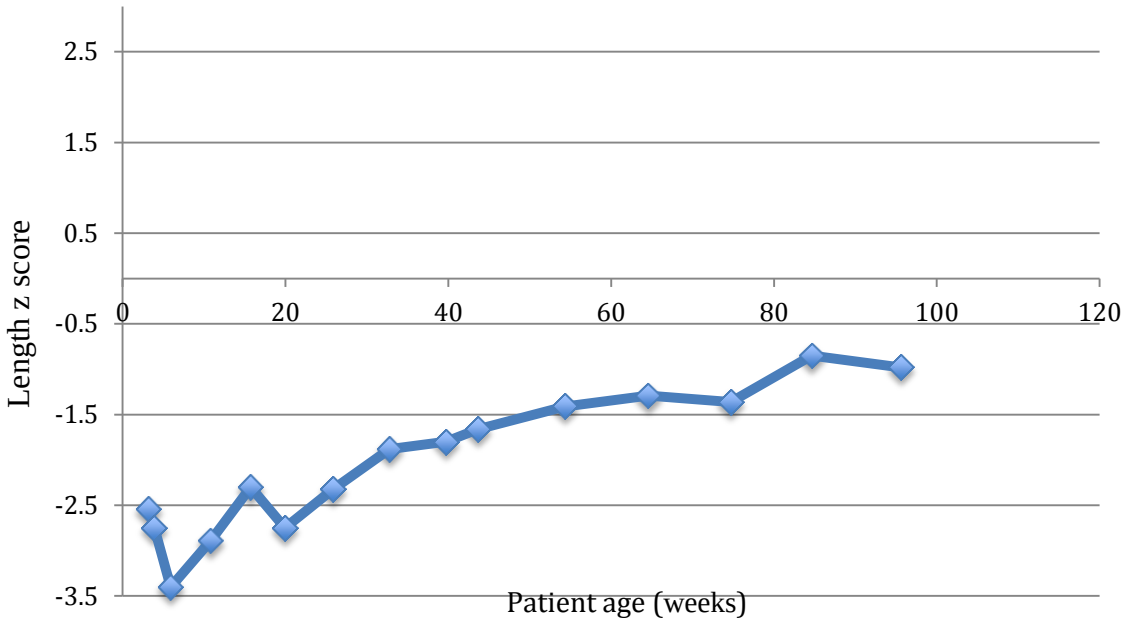


Figure 2.3 Straight-marked scatter graph showing patient S1 length z scores over two years

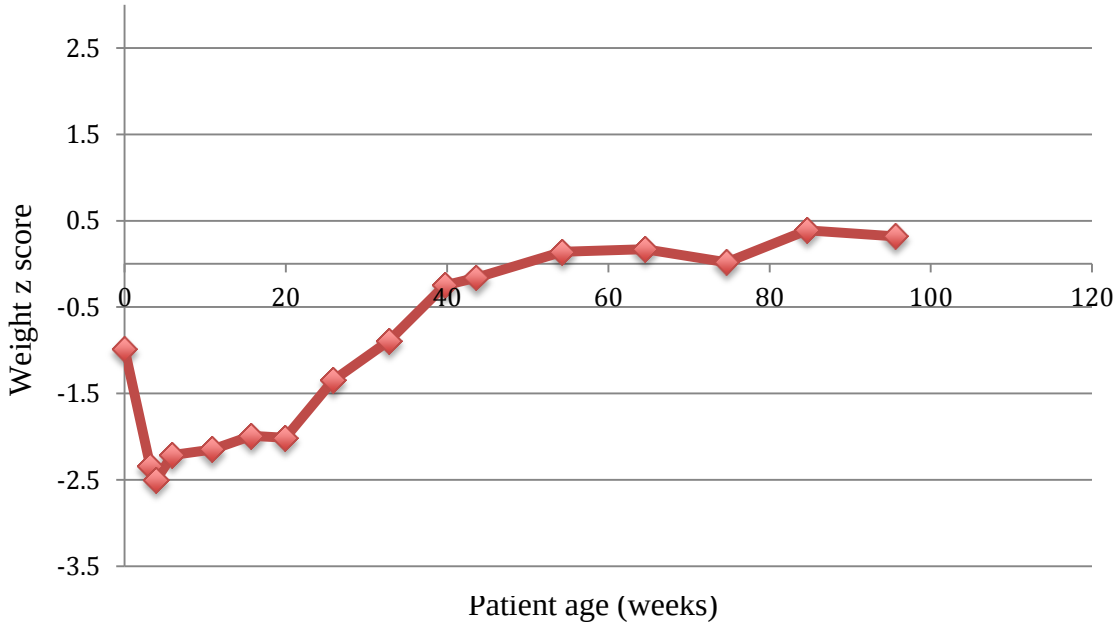


Figure 2.4 Straight-marked scatter graph showing patient S1 weight z scores over two years

These graphs assisted us in fulfilling several objectives. The deflection gradient from birth weight to first clinic visit was recorded. We anticipated an initial decline in weight during this period as newborns often lose up to 10% of their body weight within the first week of life. We aimed to discern whether or not there was a significant difference of weight loss during this untreated disease period between pancreatic sufficient and insufficient babies. The mean weight loss z score was calculated for the PI/PS groups separately. An unpaired T-test was then selected to inform us whether or not this difference was significant. A line of best fit was fitted to the graphs, which started from first clinic visit. The time (in weeks) for which this line intercepted the y-axis at a z score of -2, -1 and 0 was recorded to inform us how long it took infants to reach these markers.

The graphs also helped with creating a mathematical model that uses information collected from birth to the first clinic visit (the model input) to predict a child's nutritional outcome in the first two years of life (the model output). Such outcome will be in terms of the child's weight and length variation. The collected data from the children considered for this study were used to estimate the parameters of such a predictive model. However, the weight and length time points collected for each child in the study contain variation beyond the mean trend that we are interested in (i.e. the one that is influenced by having CF). For example, a transient illness (unrelated to CF) that could have affected a child's weight or length at some point during their first two years of life may manifest as a drop in their weight or stunted growth. The counter-argument could also be true in that a fluctuation in length/weight may indeed be due to CF and should not be ignored.

2.7.4 EVALUATION OF ANTHROPOMETRIC DATA

We needed to produce parameterized descriptions of the weight/length data for each child that would serve as sufficient predictive indicators for clinicians in the future. It would be these short representations of the weight/length data (instead of the sets of individual time points) that would be used to create the models that would provide estimates for paediatricians in the future.

As discussed above, we initially considered fitting a straight line on the timeline data for weight/length for each patient and using the parameters of that line (i.e. slope and intercept) as the representation for each data set. However, as the example in *figure 2.5* shows, in a great number of cases a straight line fit did not seem to capture adequately the general trend of the data and it was masking too much, the variations that we wished to detect. Other curve fittings (such as logarithmic or power) did not work well universally for all weight/length data. In addition, it would have been more difficult to use the parameters associated with such curves in order to describe the weight/length trajectory of a child.

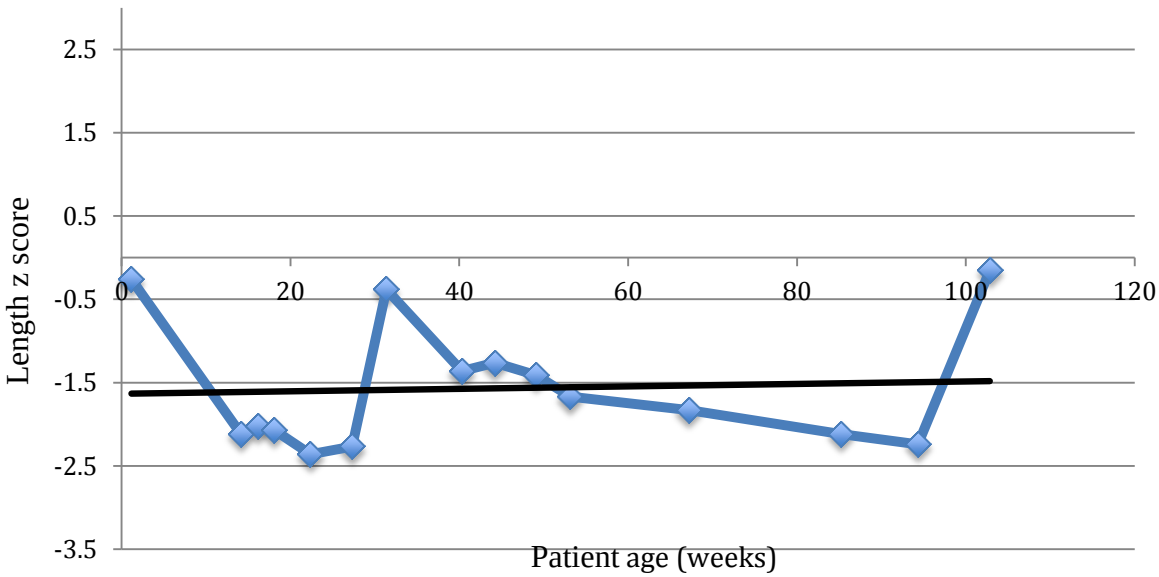


Figure 2.5 Straight-line marked scatter graph with trend line showing marked variation in Z score for length over two years

After consulting with CF clinicians in order to understand better what they would like to be able to predict for a child diagnosed with CF at birth, we formulated a grader rating protocol (discussed in the following section). As will be demonstrated in the results section, this protocol did not sufficiently serve its purpose. For this reason, we decided to use *two* different descriptors of the timeline data:

1. Use experts in the field to provide their opinion about the weight/length trajectory of each child used in the study in terms of a grade/rating. A grade was obtained for each year (of the two for which data was recorded) and for each attribute per child i.e. 8 grades per child that we called *rater scores*.
2. Calculate *computed scores (CS)* for the same periods for each attribute per child by simply finding the mean of the z score values in that period.

The methods for obtaining the above are described in more detail below.

2.7.5 RATER SCORES AS AN EVALUATION METHOD

CF clinicians used their expertise to grade each patient graph on their nutritional picture from 0-2years. A graph scoring system was devised to enable this. Two meetings were held with the investigator and three experts to create a grading system protocol. A 4-point scale was seen as preferable to eliminate mean bias. Raters consisted of two consultant paediatricians with a specialist interest in CF. Both raters were experienced in the diagnosis, treatment and management of CF patients. Neither rater had used this grading system before. The grading system protocol can be viewed below:

0 = Poor growth/weight gain
1 = Some growth/weight gain but significantly below the optimum
2 = Some growth/weight gain but slightly below
3 = Good growth/weight gain

Figure 2.6 Grading system protocol

The raters were provided with a PowerPoint presentation containing all patient graphs for length and weight. Raters graded each graph from 0-12 months and 12-24 months to see if patients' nutritional progression varied between the first and second years of life. The graphs provided, hid from view either the first or second 12 months (depending on what was being graded) so as not to influence the rater's score *(*see *figure 2.7).* The raters did not have access to any patient data or demographics. Grading was performed at a separate location and at differing times to disable conferring. Rater's recorded their grading on paper. This information was subsequently entered onto the master database.

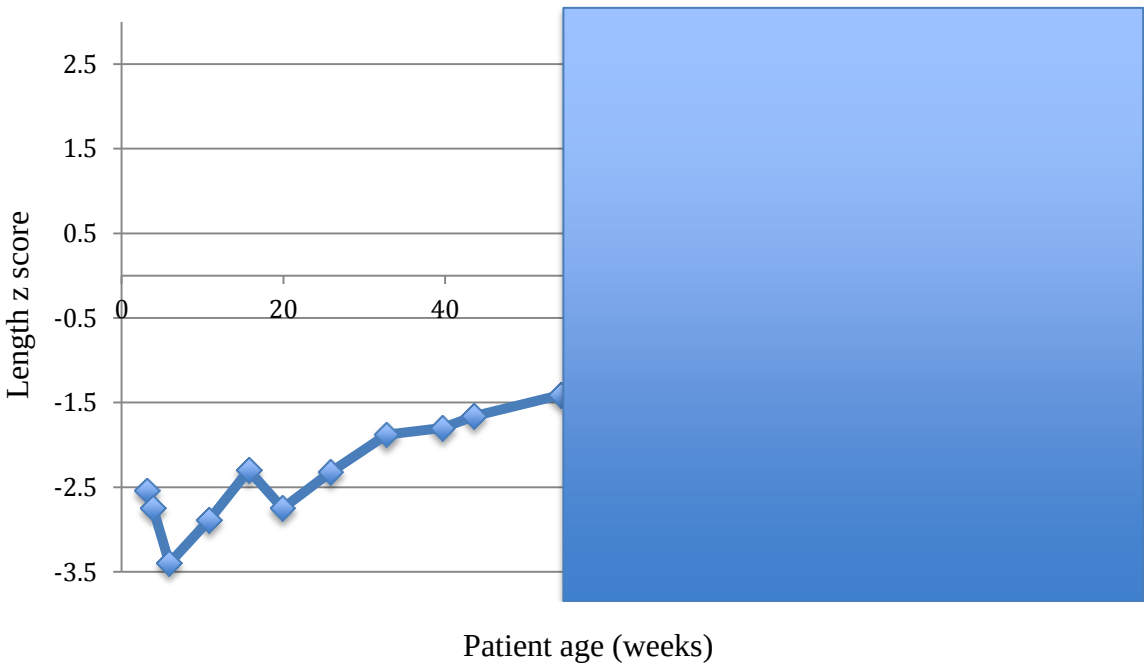


Figure 2.7 Example of straight-marked scatter graph provided to rater showing length z scores for the first 12 months of life

Reproducibility is a fundamental requirement of a measurement and can be evaluated using reliability and/or agreement statistics. To pilot this protocol, the inter-rater reproducibility was tested. Kappa statistic was calculated to check the inter-rater agreement for the first 15 patients. The kappa statistic was 0.79 (good/very good) with 88% agreement. The protocol was therefore deemed suitable as a measurement of nutritional progression for the whole dataset. For the complete dataset, weighted kappa, (standard error) was 0.75, (0.02) with a 95% confidence interval (CI) of 0.71-0.81 (good/very good) and 84% rater agreement. Kappa statistic is the preferred statistical method of inter-rater reliability as Chi-square (χ^2) is a measure of association rather than agreement and Pearson's correlation coefficient takes no account of chance. A weighted kappa is preferable as it gives weights to disagreement and shows the magnitude of discrepancy.

Once the agreement between the raters was deemed acceptable the median of the ratings given by each rater was calculated for each of the four metrics (i.e. length in the first year, length in the second year, weight in the first year and weight in the second year). These median values will be henceforth referred to as *expert ratings*.

2.7.6 COMPUTED SCORES AS AN EVALUATION METHOD

Due to the need for a more objective assessment of the nutritional picture of each child, we devised *computed scores*. In order to produce the computed scores the mean of the individual z score values for each of the periods corresponding to those that the expert raters graded for, were computed. This resulted in four values that corresponded to:

1. Mean length trajectory during the first year
2. Mean length trajectory during the second year

3. Mean weight trajectory during the first year
4. Mean weight trajectory during the second year

2.7.7 CLUSTER ANALYSIS AND K-MEANS ALGORITHM

Cluster analysis was initially performed on the data in order to see if there were any distinguishable groups of children amongst our sample and if so, what the features/attributes would be of these groups. This was also done in order to check the validity of our sample by comparing our findings with existing categorizations in the literature.

The k-means clustering algorithm [139] was used for this step because of its simplicity and effectiveness in most clustering applications. K-means clustering aims to partition a number of observations into *k* clusters in which each observation belongs to the cluster with the nearest mean. This is the mean that is serving as a prototype of the cluster. As the algorithm computes distances between observations it can only work with numerical attributes. However, an acceptable workaround for ordinal or nominal attributes is to use them to generate single-digit binary numerical attributes that indicate whether the presence or otherwise of a value in the original attribute and then use the generated numerical attributes instead of the ordinal/nominal one. For example for the attribute "ethnicity" the first column (which contained the original information) was replaced by the 6 columns following it for the purposes of applying the k-means algorithm:

Ethnicity	**Caucasian**	**Other White**	**Pakistani**	**Other Mixed**	**Indian**	**WhiteAndBlackCarribean**
Caucasian	1	0	0	0	0	0
Caucasian	1	0	0	0	0	0
Other White	0	1	0	0	0	0
Caucasian	1	0	0	0	0	0
Pakistani	0	0	1	0	0	0
Caucasian	1	0	0	0	0	0
Pakistani	0	0	1	0	0	0
Caucasian	1	0	0	0	0	0
Other Mixed	0	0	0	1	0	0
Pakistani	0	0	1	0	0	0
Indian	0	0	0	0	1	0
Caucasian	1	0	0	0	0	0
WhiteAndBlackCarribean	0	0	0	0	0	1
…	…	…	…	…	…	…

Table 2.2 Table showing how nominal attributes (ethnicity) were incorporated into cluster analysis through transferring data into a numerical format

All attributes (excluding rater scores and computed scores) were used for clustering, though patients with missing values in any of the attributes had to be discarded from the data. This left only 79 patients with full datasets. In light of the fact the majority of missing data values were sweat chloride results, we used the John Hopkins CFTR2 website,[140] which can generate sweat chloride results based on specific genotype combinations, determined by average results from thousands of patients in the registry. Unfortunately this feature is not available for calculation of faecal elastase. This meant the total dataset included 114 samples out of a possible 139.

Another constraint of the k-means algorithm is that the number of clusters (k) that it calculates needs to be specified. Furthermore, the algorithm always produces the best possible cluster means (or centroids) but this does not mean that there is a good separation between them. In order to find the number of clusters that are separated the best, a metric was used called the silhouette value, which is a measure of the cluster separation.[141] Following clustering, the silhouette value of each sample is a measure of how similar it is to its own cluster (cohesion) compared to other clusters (separation). The silhouette value ranges between -1 and 1. A high value indicates that the sample is well matched to its own cluster and poorly matched to neighboring clusters. The mean of all silhouette values provides a global measure of a clustering attempt (i.e. with a specific value for k – the number of clusters).

Within our data set, the mean silhouette value for two clusters was 0.78, three clusters 0.48 and four clusters, 0.41. Below, is a diagrammatic representation of the silhouette value, with each line representing each patient/observation.

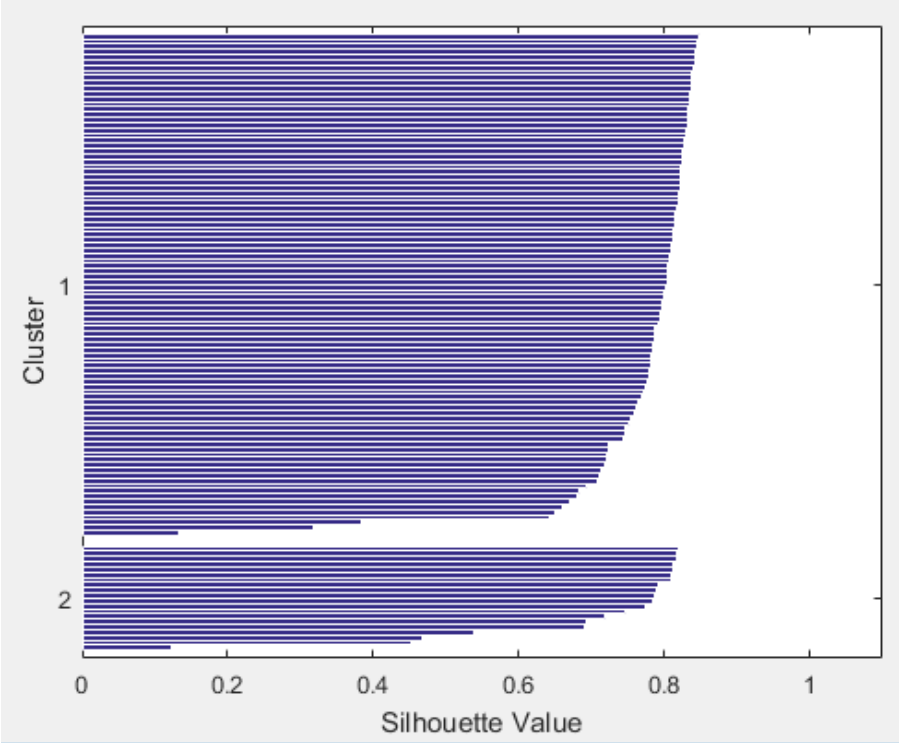


Figure 2.8 Diagram showing silhouette value of 0.78 when data set is split into two clusters

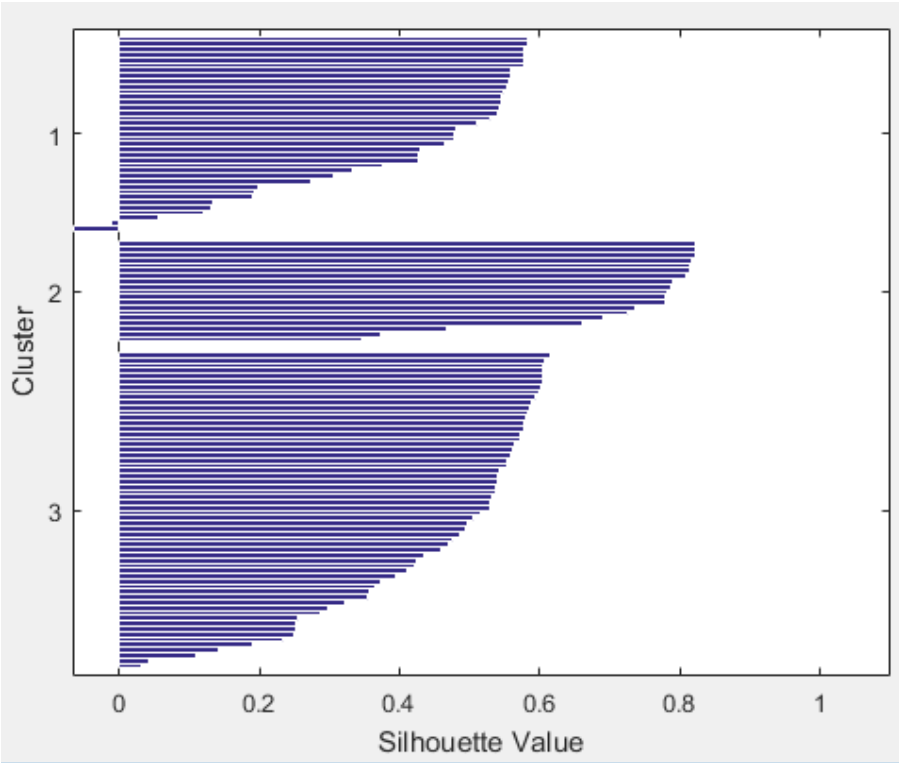


Figure 2.9 Diagram showing silhouette value of 0.48 when data set is split into three clusters. (The separation here is not clear, with one patient negative and a lower overall silhouette value)

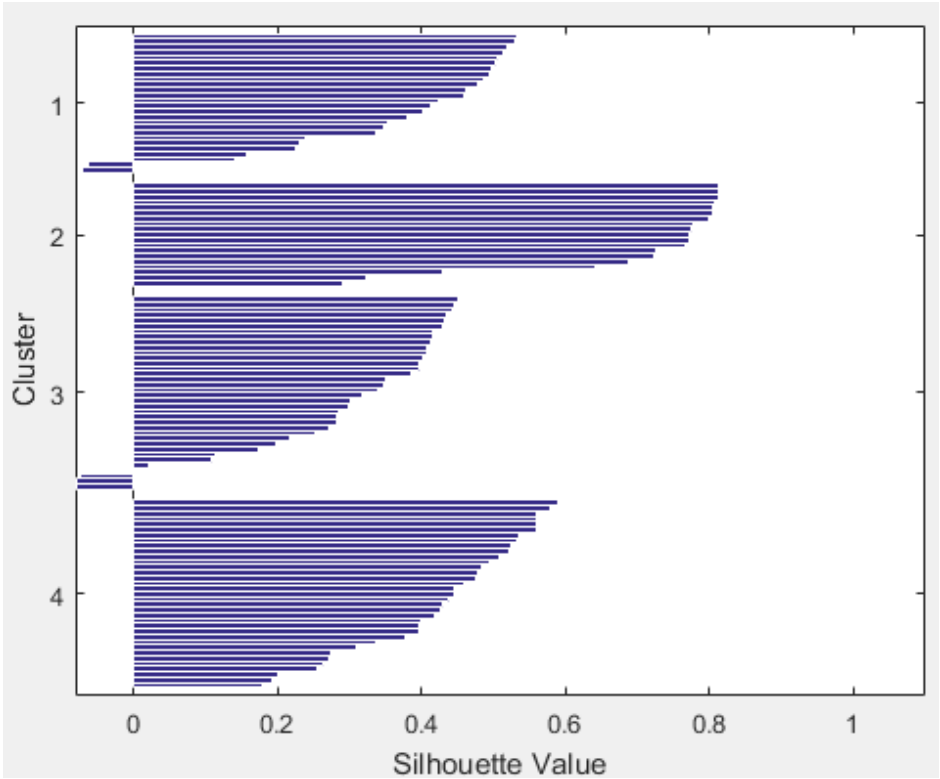


Figure 2.10 Diagram showing silhouette value, 0.41 when data set is split into four clusters (Here, the separations are more unclear and there is a lower overall silhouette value)

We therefore concluded that the best separation for our data could be achieved with two clusters. The script was run through Matlab to produce two distinct clusters. This software provided us with the number of patients in each cluster and the mean figure for each variable/input including nominal and interval data. This will be presented in the results section.

2.7.8 CLASSIFICATION OF DATA

Clustering indicated that our data sample was representative of the population of children born with CF and therefore we proceeded with attempting to find suitable classification models that would be able to predict the weight and length z scores of a child diagnosed by newborn screening.

Several classification algorithms were tried (using all attributes used for clustering excluding *Sa/Pa* isolation as this information would not be present at initial clinic visit) in order to produce the best possible predictive model in terms of success rate but also simplicity bearing in mind that it would need to be easily implemented and accessible to clinicians in the future. Due to the relatively small number of samples available for model estimation, all modelling attempts were validated using the 5-fold cross validation method[142] and any error rating reported in this thesis reflects that. The best performing (and coincidentally among the simplest) classification algorithms on our data was a decision-tree classification method.[143] The classification models produced will be presented in a number of ways: the model script (words describing the model), a decision tree, and confusion matrices. In total, eight models were produced, four using the *expert ratings* as the outcome and four using the *computed scores* as the outcome. Explanation of these will be provided in the results section.

2.7.9 POLYNOMIAL REGRESSION MODELLING

The majority of modelling algorithms produce models that are, 'opaque' in the sense that they provide no clear insight as to *how* the independent variables (or input attributes – here the patients' attributes at first clinic visit) influence the dependent variables (or output attributes – here the weight/length z scores). In the case of decision trees for example a tree does not incorporate a reason as to why a split occurs at a certain value for a particular attribute. Such models are suitable for predicting, estimating or simulating but they do not allow any further investigation of the underlying problem – here, the main contributors and the magnitude of their influence on the weight/length z scores of CF patients.

Polynomial regression modelling[144] was used in order to estimate the parameters of polynomial models that could be used to calculate the weight/length z scores of patients. This would fulfill the same role as the decision trees mentioned earlier but there are two fundamental differences:

1. Polynomial models can only use numeric attributes and so all ordinal and nominal attributes (ethnicity, genotype) had to be discarded for this modelling approach but,
2. Polynomial models are by their nature, 'transparent' and therefore can allow quantitative insights as to how the input attributes influence the output ones.

Both modelling methods are useful and complement one another. Decision trees can be thought of as more useful in the field for prognosis whereas polynomial models can lead to further research into the factors that influence the nutritional progression of CF patients.

2.8 THE NARMAX MODEL

The regression modelling methodology used to produce the models here is called NARMAX (Nonlinear Auto Regressive Moving Average Model with eXogenous inputs).[145–147] This methodology estimates the parameters of a complex non-linear polynomial that can be used to predict the output values given the input values. A significant advantage of this regression method over others is that during the model parameter estimation process it rejects any polynomial terms that are not significant to the calculation of the output thus leading to the simplest possible polynomial equation that represents the problem. This is done by computing the significance of each polynomial term expressed in terms of an *Error Reduction Ratio (ERR).* The ERR is an indication of the reduction in the model's prediction error that occurs when the model term considered is

introduced in the model. The value of the ERR is therefore proportional to the significance of the term it corresponds to. The ERR's of the terms of the final model are one of the first indications of how significant the different attributes are (or combinations of them) to the calculation of the output.

3.0 RESULTS

The study results can be found in this section. An overview of the newborn screening results will be provided in addition to the results of the biochemistry, microbiology and anthropometric clinical parameters of patients with cystic fibrosis and our mathematical models to predict nutritional parameters of babies with CF in their early years.

3.1 NEWBORN SCREENING

Over the seven-year period, 507,608 babies were screened within the West Midlands region. Of these, 200 were screen positive. Of the screen positive babies, 139 were true positives (clinically confirmed diagnosis through a sweat test or identification of two gene mutations) and 5 met the criteria for CFSPID, giving a total of 144 true positives. The remainder, (56) were false positives. 108 patients were cared for in Birmingham Children's Hospital (BCH) and 36 in University Hospital of North Midlands (UHNM). 25 (17%) patients presented with MI. Clinical data were unavailable for 5 patients: two patients died before the age of two years from additional co-morbidities, two patients moved out of area and one patient's notes could not be accessed within the data collection period. Newborn screening data and demographics were available for these patients however, so they will be included in the results of newborn screening only. Figure 3.1 denotes a visual representation of these results:

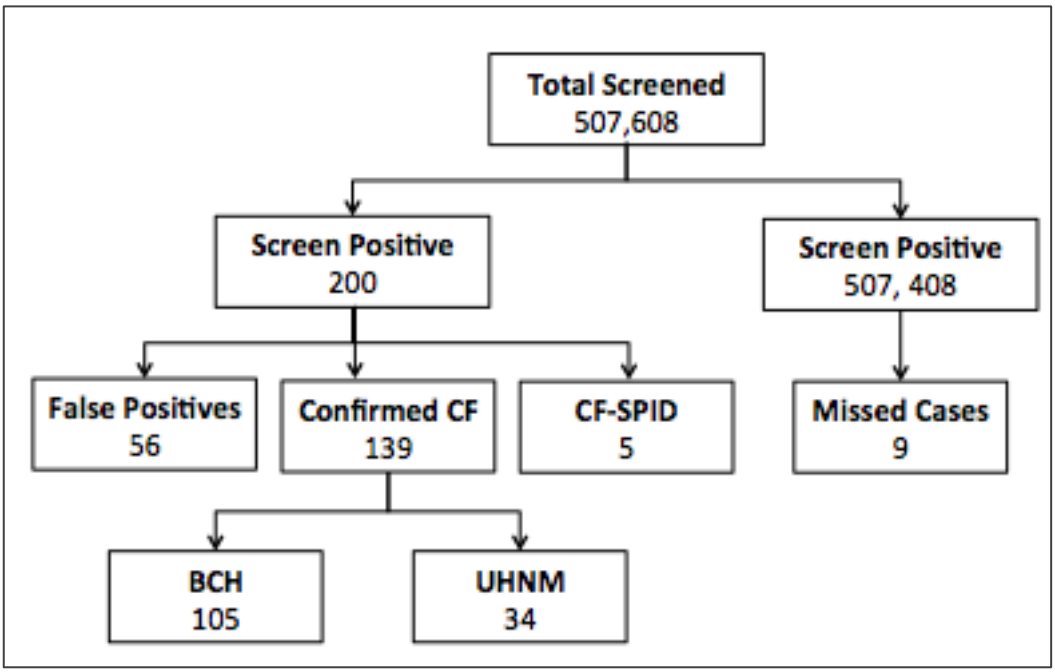


Figure 3.1: Flow diagram showing the numbers and outcomes of babies screened in the West Midlands

Year of Diagnosis	Number of patients diagnosed	Male: Female
2007-2008	10	6:4
2008-2009	16	7:9
2009-2010	24	13:11
2010-2011	20	9:11
2011-2012	21	11:10
2012-2013	21	13:8
2013-2014	20	8:12
2014-2015	12	6:6
TOTAL	**144**	**73:71**

Table 3.1: Number of patients diagnosed each financial year within the West Midlands Region and the male to female ratio

The birth prevalence of our cohort was 1/3525 with a positive predictive value (PPV) of the screening test of 72% and a negative predictive value (NPV) of 99.9%. The specificity of this screening test for our cohort was 99.9% and sensitivity of 94%. In total, 101 carriers were identified. To date there have been 9 false negatives, 'affected not detected,' although not all show features of classic CF. All information available on missed cases are presented in table 3.2.

Table 3.2: Additional information of the 9 patients missed by NBS within our study period

Patient	Age at presentation(weeks)	Gene1	Gene2	MI	PI/PS	Sweat chloride	Additional comments
1	6.7	Phe508 del	p.Gln1291His	No	PS	48	Mild phenotype Symptomatic
2	343 (6.5 years)	Phe508 del	c.709C >G	No	PS	67	Classic CF
3	?	Phe508 del	R117H	Yes	PS	75	Moved out of region
4	?	Phe50 8del	R117H	No	PS	49	Indeterminate diagnosis Testing due to sibling with CF Indeterminate diagnosis
5	?	Phe508 del	Phe508 del	Yes	PI	Not performe d	Classic CF
6	12.3	Phe508 del	Arg347 His	No	PS	69	Admitted with respiratory symptoms
7	13.4	Phe508 del	621+1 G>T	No	PI	Insuffici ent sweat test	Classic CF. Diagnosed on CF genetics, presented with FTT and hypoalbuminaeami a
8	2.1	Phe508 del	R117H	No	PS	32	Indeterminate diagnosis. Parents known to be carriers, cord blood sample, IRT 40
9	56	Phe508 del	Phe508 del	No	PI	Insuffici ent	Insufficient sweat test. Inpatient admission for failure to thrive

The mean age of screening was 7.5 days. The median age of diagnosis for our cohort was 22 days. Of all the true positive cases of CF, 135/144 (93.75%) were Caucasian and 9/144 (6.25%) were Asian, Afro-Caribbean or other white. 53% of patients were homozygous for Phe508del whilst 42% were heterozygous for Phe508del. The median IRT was 159mcg/L and 17/144 (11.8%) of patients required a second IRT with a median IRT of 104mcg/L.

According to the definition of CFSPID described earlier within the literature review,[135] five patients met one of these two criteria. Their disease features are presented in table 3.3:

Patient	**Gene 1**	**Gene 2**	**Sweat chloride**	**IRT**	**Faecal elastase**
1	Phe508del	R117H	29	68	442
2	Phe508del	R117H	22	89	484
3	Phe508del	R117H	27	121	500
4	Phe508del	Asp1152His	28	81	450
5	Phe508del	R117H	22	69	437

Table 3.3: Demographics of patients meeting criteria for, CFSPID

3.2 CLINICAL PARAMETERS

3.2.1 BIOCHEMISTRY

The following clinical parameters will be based on a cohort of 139 due to the 5 patients who had to be excluded, as mentioned above.

Sweat chloride results were documented in 113/139 (81.3%) patients. Of the 113 patients who had sufficient sweat chloride samples, the median sweat chloride was 94mmol/L. 17/113 (15%) patients had sweat chloride values below 59mmol/L (none of whom met the criteria for CFSPID as they had two disease causing mutations) and 5/113 (4.4%) below 30mmol/L, all of whom had CFSPID, as shown in the previous section.

Faecal elastase measurements were obtained from 116/139 (83.4%) patients. Of these patients the median faecal elastase concentration (FE1) was 15μg/g. Pancreatic insufficiency was evident in 95/116 (82%) patients with the remainder being pancreatic sufficient. Of those who were PS, 8 patients had a FE1 of >500 μg/g.

3.2.2 MICROBIOLOGY

Over the first year of life, 15/139 (10.7%) patients had their first isolation of *Staphylococcus aureus* (*Sa*). In the second year of life, one other patient isolated this bacterium, totaling 16/139 (11.5%) patients with their first isolation of *Sa* within 2 years. The median age of isolation was 11.2 weeks (2.9 months). In contrast, in the first year, 21/139 (15%) patients isolated *Pseudomonas aeruginosa* (*Pa*) for the first time and a total of 48/139 (34.5%) had isolated this bacterium within two years. The median age of first

isolation was 61 weeks (1.2 years). The Kaplan-Meier analysis below demonstrates percentage of children free from each species over two years.

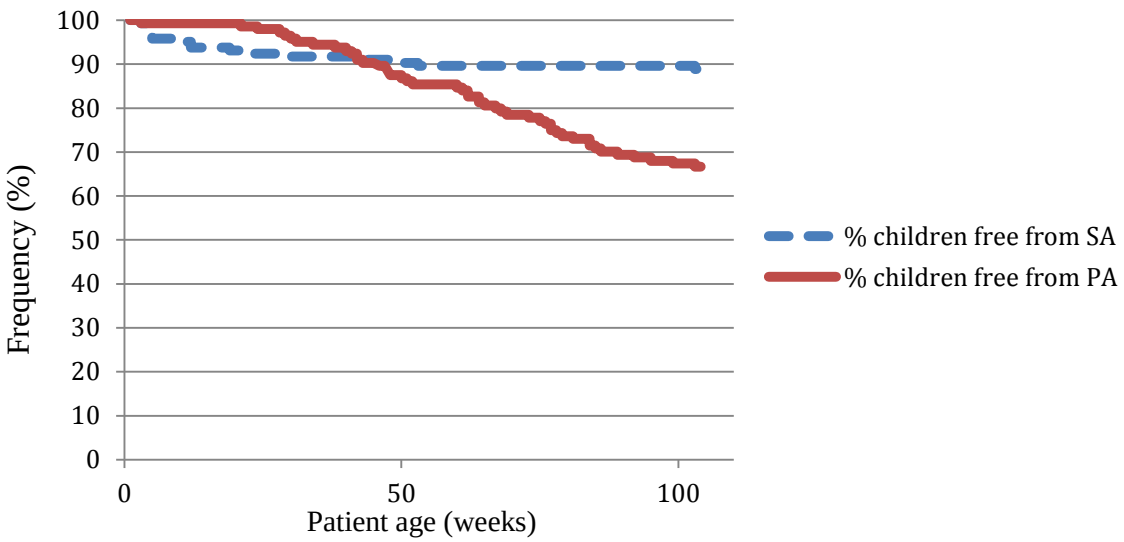


Figure 3.2: Kaplan-Meier graph showing percentage of CF patients free from isolation of SA and PA

The second Kaplan-Meier graph below, considers the difference in percentage of patients isolating each bacterium between the two tertiary centres, BCH and UHNM. Within the first two years, 9% of patients from Centre 2 isolated *Sa* compared to 18% of children in Centre 1. In comparison, lower rates of first acquisition of *Pa* were demonstrated at centre 1 with only 21% of patients isolating this species within two years compared to 37% of centre 2 patients.

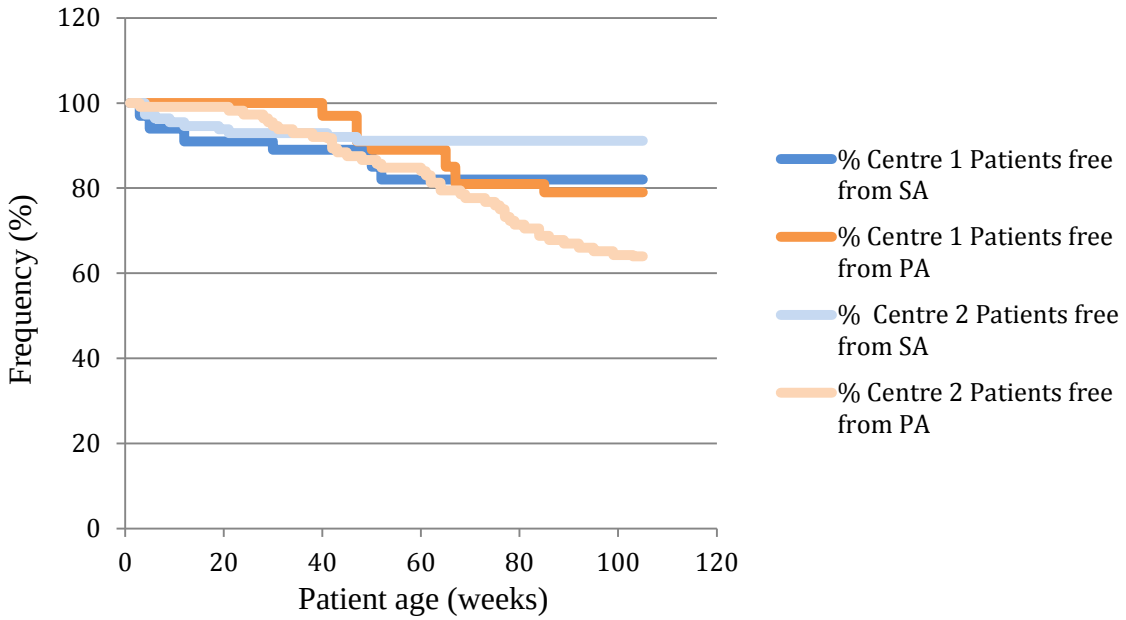


Figure 3.3: A Kaplan-Meier analysis showing isolation of both species between the two tertiary CF centres involved in the study

3.2.3 NUTRITION

The mean birth weight of the whole cohort was 3.09kg. Having categorized patients by pancreatic sufficiency according to accepted reference ranges, i.e. FE1 <200ug/g = PI and FE1 ≥200 µg/g = PS, an unpaired T-test was performed to determine whether or not there was a significant difference between birth weight z score and rate of change in z score from birth to first clinic visit, between these groups. There was no significant difference in BW z score ($p = 0.29$) but there was a significant difference in the rate of weight loss/gain ($p = 0.007$). These results are presented below:

	Mean birth weight z score (SD)	**Mean weight loss z score from birth to first clinic (SD)**
Pancreatic sufficient	-0.36 (1.08)	-0.33 (0.33)
Pancreatic insufficient	-0.05 (1.17)	-0.1 (0.32)

Table 3.4: Difference in birth weight z scores and rate of weight loss from birth to first clinic between pancreatic sufficient and pancreatic insufficient patients

By fitting a straight line on timeline data for weight/length z scores of each patient and using the parameters of the line (i.e. slope and intercept) as the representation for each data set, we noticed four subgroups of patients:

1) Patients with a BW z score ≥0 who continue to improve
2) Patients with a BW z score ≥0 who fail to maintain this z score
3) Patients with a BW z score <0 who demonstrate an improvement in z score
4) Patients with a BW z score <0 who fail to improve in z score

The group we were particularly interested in was group 3, as these patients tend to be those with pancreatic exocrine dysfunction who exhibit greater nutritional progression following management intervention. We aimed to discern how long it would take these patients to reach their potential z scores. In this group, the median time to achieve a weight z score of -2, -1 and 0 was 18, 33, 65 weeks respectively and to achieve the same z scores for length was 30, 51, 90 weeks, respectively. This calculation was also performed for patients in group 4 however the results were negative, suggesting this group fail to reach these z scores within 2 years.

In all, 19/139 (13.6%) of infants failed to regain their birth weight z score within the follow up period. This has previously been identified as a way of defining nutritional failure.[148] The features of these children were examined to look for any common traits but none were found. This can be found in appendix C.

3.3 MODELLING

3.3.1 CLUSTER ANALYSIS

Clustering using the simple k-means algorithm revealed that best separation of the data is achieved with two clusters (mean silhouette value = 0.78). The centroids of the two clusters are shown below (*Table 3.5*). Cluster 1 contained 20 of the 114 patients and the remaining 94 patients belonged to cluster 2.

Attribute	**Cluster 1 (20)**	**Cluster 2 (94)**
BW Z score	0.005	-0.355
Rate of change: BW z score to 1st clinic z score	-0.105	-0.317
FE1	467	15
Sweat Chloride	42	99
IRT	85.5	172
Gene 1: Phe508del	1	1
Gene 2: Phe508del	0	1
Gene 2: R117H	1	0
Gestation	39.5	39
Meconium Ileus	0	0
Isolation of SA	0	0
Isolation of PA	0	0

Table 3.5: Centroid values of the two cluster groups

(N.B. The remaining genotypes were not used by the clustering algorithm to differentiate between the two clusters and so, have been omitted from the above table.)

The two cluster centroids above are a representation of their cluster members but they provide no information about the boundaries of the clusters or which attribute(s) can be used to classify a new sample into either cluster. For this reason we have applied a classification algorithm to the now clustered data.

3.3.2 CLASSIFICATION ANALYSIS

For this step, all attributes (as listed above) used for clustering were used as *predictors* and the class value was used as the *response* (or *outcome*). As mentioned in the methods, a simple tree-based classification method was used because it performed best in comparison to other methods that we tried but it has also revealed a simple to interpret model. The following model classifies the data with 100% accuracy:

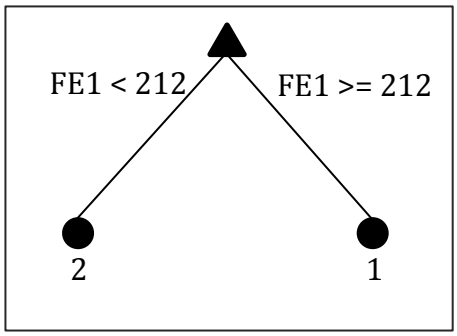


Figure 3.4: Simple tree diagram showing classification model 1

Classification/decision trees can be also described by a, 'script' or set of classification rules and confusion matrices. For the tree shown above a single classification rule can be used to describe it as follows:

"If (FE1 < 212) then (class=2) elseif (FE1≥212) then (class = 1)"

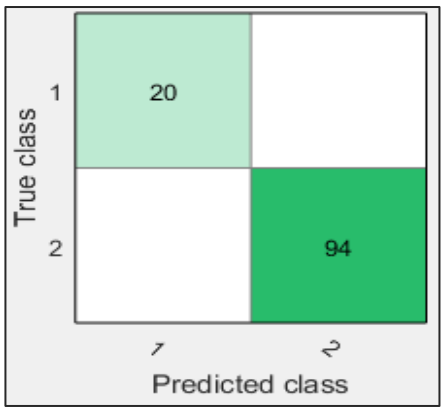


Figure 3.5: Confusion matrix representing classification model 1

A confusion matrix of a model summarizes its accuracy by indicating the number of samples in the data set that are correctly or wrongly classified for each class. In this example, as the matrix shows, all samples are correctly classified with this model (ideally we want all patient samples to be within the green diagonal, as this shows that the model prediction correctly meets the true class in which the patient falls).

This model suggests that faecal elastase alone can be used to classify all the patient samples to one of the two classes that clustering has produced. The boundary value for faecal elastase is 212 μg/g.

To further investigate the validity of our data we repeated the classification, excluding FE1 from the set of predictors. The motivation for this was to evaluate which/how other predictors could be used in order to classify the data and whether these findings corroborate with existing literature. The decision tree and confusion matrix obtained in this step are presented below:

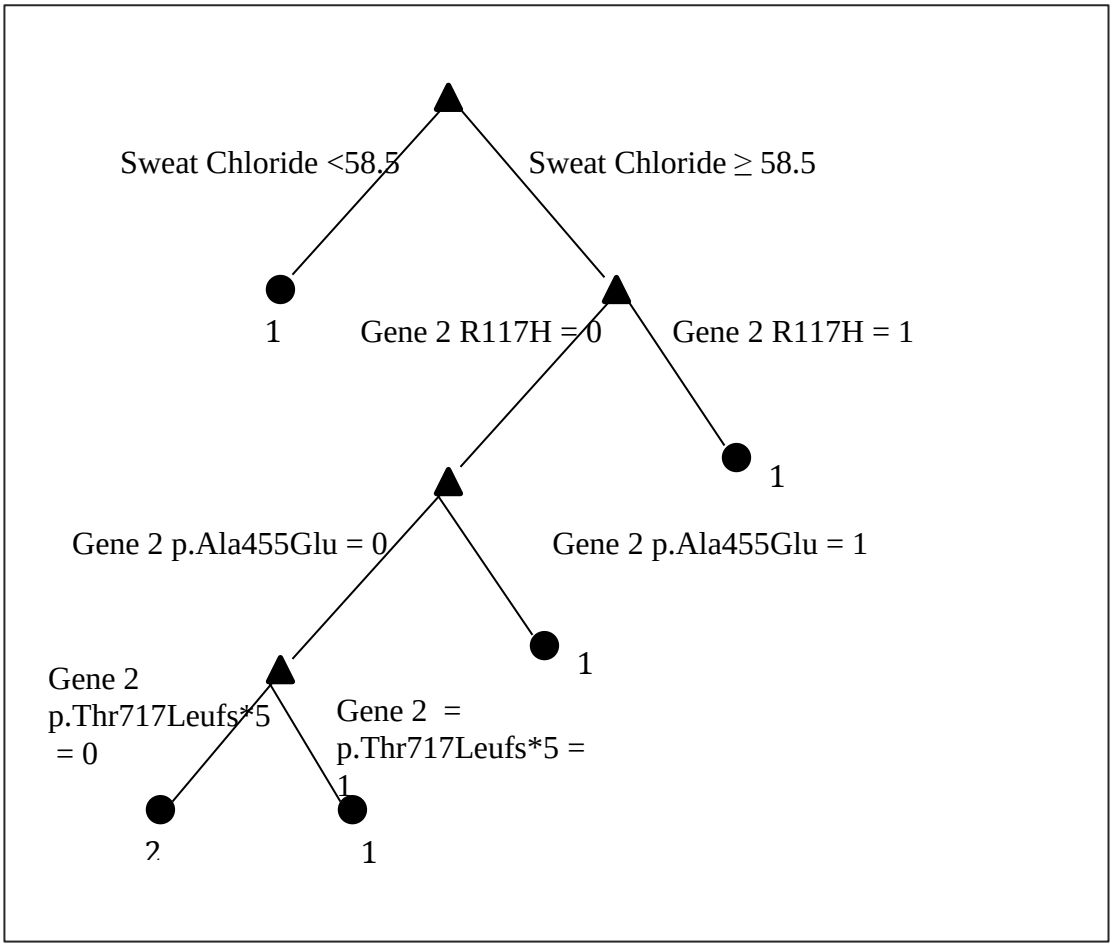


Figure 3.6 Simple tree diagram showing classification model 2

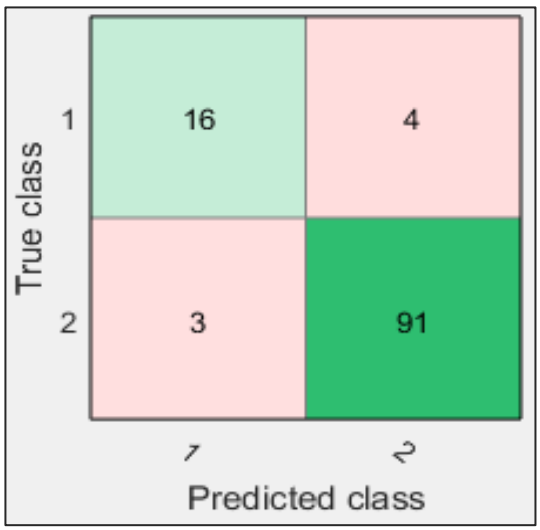


Figure 3.7 Confusion matrix representing model 2

In this case the model is 93.9% accurate showing that the removal of FE has decreased the prediction accuracy that can be obtained with the remaining attributes. Here, patients in whom the model incorrectly classifies, lie within the pink boxes. For example, you can see that 4 children were misclassified in that following this model the patients are predicted to be in class 2 but their true class, according to the cluster analysis is class 1.

We then attempted to produce further models without faecal elastase and sweat chloride but the prediction accuracy of the resulting models was too poor hence not worth reporting here. This indicates that there is little information beyond the attributes considered so far that can be used to predict the class that a sample belongs to.

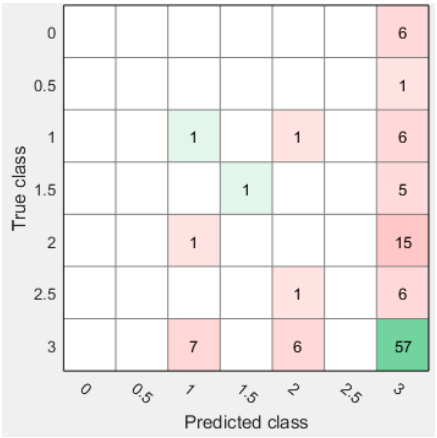
3.3.3 CLASSIFICATION MODELS USED TO DETERMINE NUTRITIONAL OUTCOMES IN FIRST TWO YEARS

Having checked the validation of our data sample the next objective was to create models that can be used as prediction tools of weight and length for children with CF. The aim is to use data available at the first clinic visit to estimate the weight/length z scores in the first 2 years of life. Weight and length prediction is summarized/represented by the raters' scores and the computed scores (as described in the previous chapter). Each of these values will now form the response/outcome for every model produced.

The following four confusion matrices correspond to the raters' scores for weight/length for the first and second year of life. The values beneath each, show the accuracy of the model in predicting the correct score and in brackets the accuracy of the model if the error of prediction is widened by one value. In other words, the value in the brackets describes

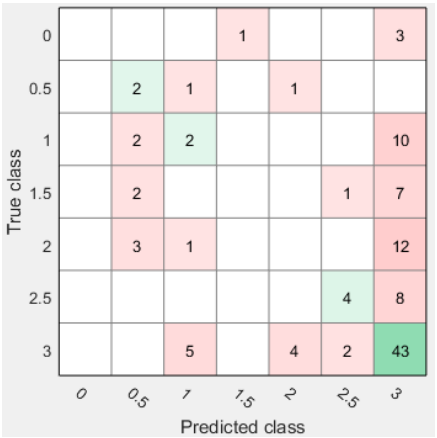
the error of prediction if the predicted class is still considered correct if it is within half a grade away from the actual grade.

Length Year 1



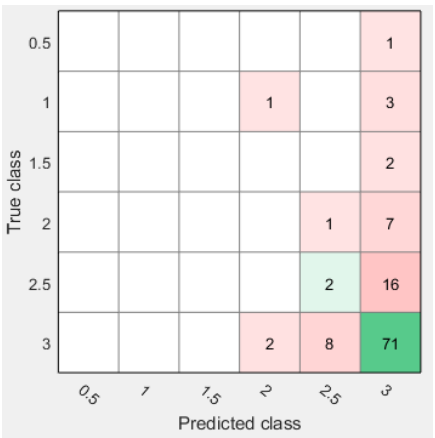
51.8% (57.9%)

Length Year 2



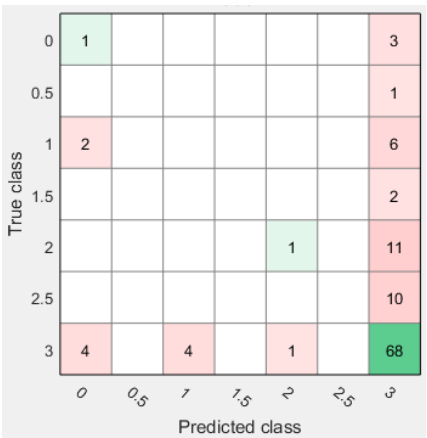
44.7% (56.1%)

Weight Year 1



64.0% (86.0%)

Weight Year 2



61.4% (70.2%)

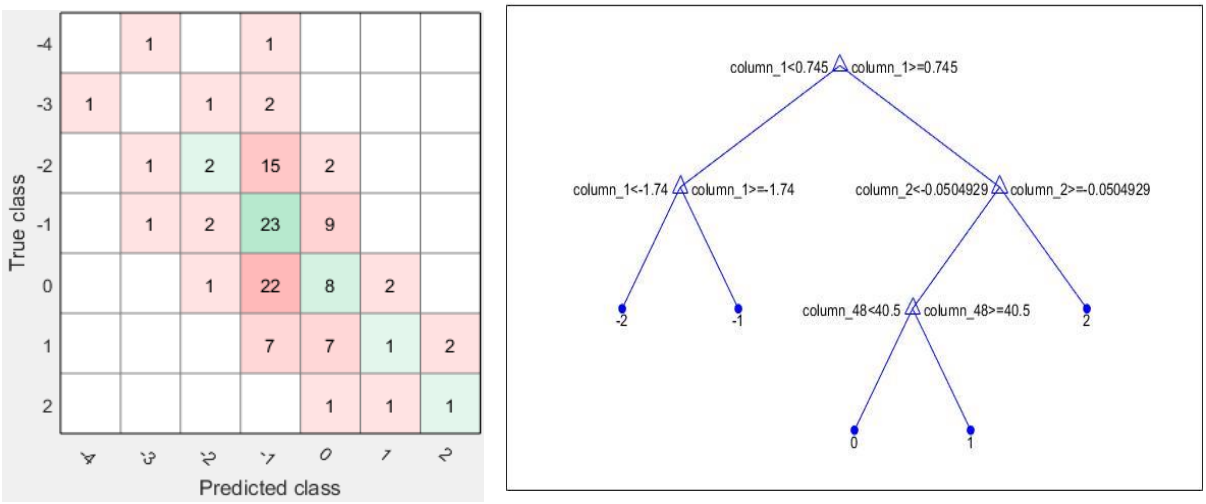
Figure 3.8: Four confusion matrices showing the model outcome and accuracy from rater's scores as a measure of prediction

In the above matrices, the predicted class is a descriptive term, corresponding to the median rater scores from the grader rating protocol. As you can see from Figure 3.8, in each of the four models, the graders' rated most patients as a, '3' according to the grader

rating protocol. This meant that there was little variation in terms of predicted class hence the rater scores did not provide a helpful assessment in terms of the children's nutritional progression. Furthermore, there are many samples, which lie outside the green diagonal (represented by pink boxes). In most instances these outliers actually are the furthest away from the diagonal possible, hence demonstrating low prediction accuracy. For the above reasons, we agreed that the grader ratings should no longer feature in the further data analysis, however the model scripts and tree diagrams for the above matrices are attached as an appendix (appendix D).

The next section will diagrammatically describe the four classification models based on the computed scores as a predictor outcome measure. Here, the confusion matrices and corresponding tree diagrams will be shown. When comparing the two tables the reader should note that the meaning of the classes here is different to the classes produced by rater scores. In the case of computed scores the class is a numerical value derived by rounding the mean of the z scores of the length/weight time points. It should also be noted here that due to the relatively small number of samples in the data, all models were validated using the 5-fold cross validation method and even though the models shown in the tree diagrams have been created using all the data at the end (as normal practice dictates) the error rates and corresponding confusion matrices were obtained from the validation process. This is why in some cases the possible output values of the confusion matrix (predicted values) do not correspond to all the possible outputs in the decision tree.

Length Year 1

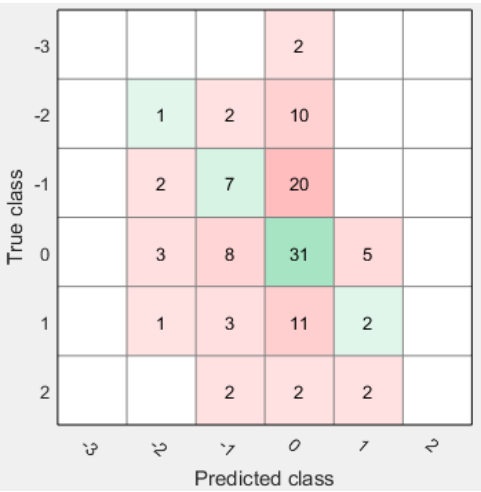


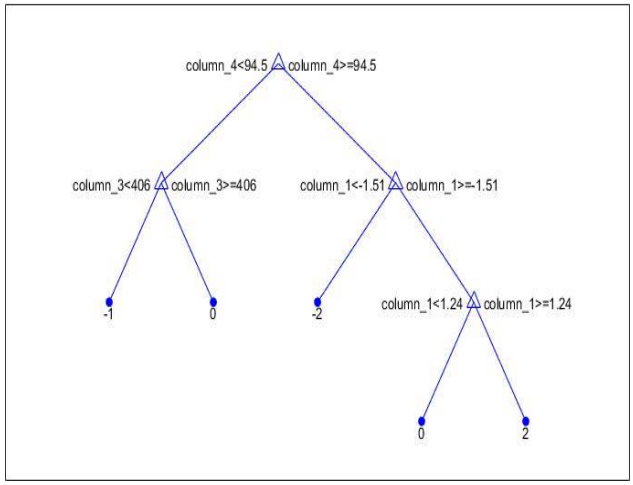
30.7% (86.8%)

Figure 3.9: Confusion matrix and simple tree diagram representing the model for prediction of a child's length z score in the first year of life

In the above tree diagram the model to predict a length z score for the child at age 1, dictates a split (as represented by a triangle) at a birth weight z score of 0.75. The second split (column 1) is a birth weight z score of -1.74, dictating whether a patient will fall into class, '-1' or '-2' (as represented by a circle). Column 2 relates to the rate of change in weight z score between birth weight and first clinic visit and column 48 corresponds to gestation age. Here, the confusion matrix demonstrated a tight fit around the green diagonal suggesting this model can be used with 86.8% accuracy.

Length Year 2



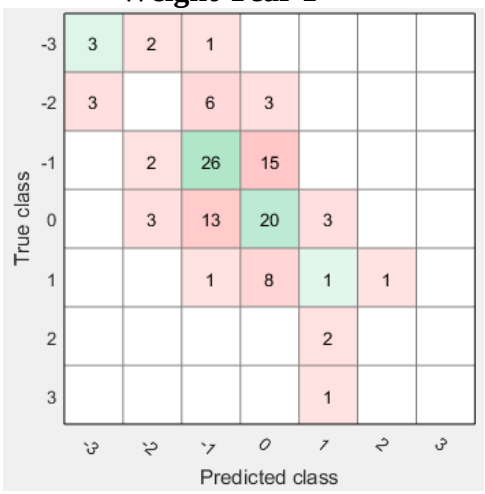


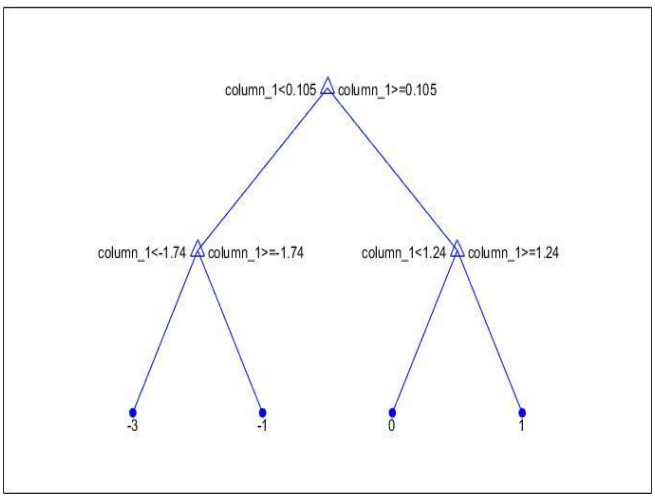
36% (79.8%)

Figure 3.10: Confusion matrix and simple tree diagram representing the model for prediction of a child's length z score in the second year of life

In the model predicting length z score in year 2, the first split occurs at column 4 (sweat chloride) 94.5mmol/L. The subsequent split determining whether a patient falls into class, '-1' or '0' is reliant on a faecal elastase measurement above or below 406µg/g. The remaining branches are again, determined by a child's birth weight z score (column 1). Note here, that the model predicting length in year 2 is less accurate than the first model.

Weight Year 1





43.9 (92.1%)

Figure 3.11: Confusion matrix and simple tree diagram representing the model for prediction of a child's weight z score in the first year of life

The above model, depicting prediction of a child's weight in year 1 exhibits the highest prediction accuracy of all models. The only attribute required to form this model is birth weight z score (column 1).

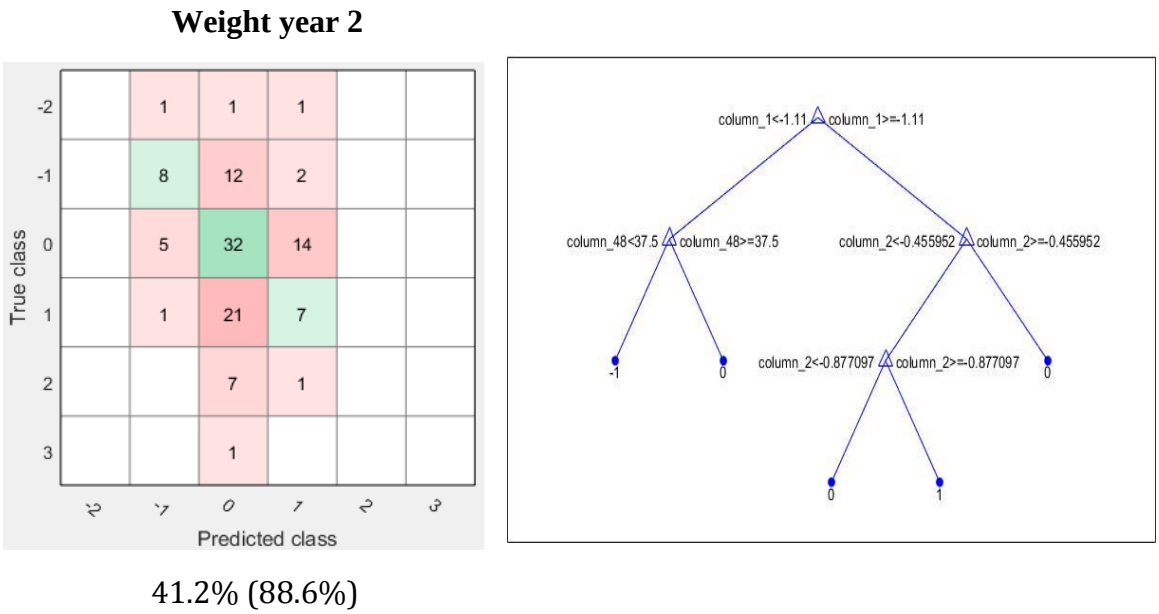


Figure 3.12: Confusion matrix and simple tree diagram representing the model for prediction of a child's weight z score in the second year of life

The final model, shown above uses three attributes to determine class: column 1, 2 and 48 which correspond to birth weight z score, difference in z score from birth to first clinic and gestation, respectively. This model also demonstrates a high accuracy rate.

3.3.4 PROGNOIS ESTIMATION BY REGRESSION MODELLING

To further enhance our understanding of how the nutritional parameters (i.e. the z scores) are influenced by the initial data (obtained at birth and first clinic visit) of a child, we have used regression modelling in the hope of being able to determine not just *which* are the most important factors that influence the prognosis estimates but also *how* these factors affect prognosis.

Regression models of the computed scores (CS) only, are shown here because these were by far the most accurate. The table below lists the four regression models that correspond to the computed scores for weight/length for the first and second year of life. The second column shows the model equation to the right and the ERR of each term in the model to the left. Note that the constant terms do not have an ERR associated with them. The rightmost column shows the mean absolute z score error and standard deviation (SD) giving an idea of the degree of accuracy of these models. The hope is that these specific regression models (formulas) can be used by clinicians to calculate an infant's predicted z scores for weight and length in year 1 and 2, given information available at first clinic visit.

Output	**Model (ERR and term)**		**Mean absolute error (SD)**
CS length Year 1		+0.1367064100	0.7 (0.5)
	47.548504	+0.7016365828 * BW z score	
	7.870393	+1.2320975748 * BW_fc z score	
	0.485969	-0.0011104836 * IRT	
CS length Year 2		+0.8628669492	0.85 (0.58)
	22.324245	+0.4393428847 * BW z score	
	3.841531	+0.9529351351 * BW_fc z score	
	0.459736	-0.0001892367 * FE1	
	0.234628	+0.0060498375 * Sweat chloride	
	1.844230	-0.0020951020 * IRT	
	0.105220	-0.0253269628 * Gestation	
CS weight Year 1		+2.0934251271	0.51 (0.39)
	57.473588	+0.7612702922 * BW z score	
	11.468433	+1.5310133254 * BW_fc z score	
	0.657965	-0.0010027649 * FE1	
	0.056306	-0.0025337148 * Sweat chloride	
	0.098044	-0.0005162566 * IRT	
	0.232643	-0.0403565267 * Gestation	
CS weight Year 2		+3.4021272875	0.67 (0.51)
	19.545299	+0.4334043128 * BW z score	
	3.429956	+0.8737986493 * BW_fc z score	
	0.584699	-0.0008647250 * FE1	
	1.322280	-0.0016680936 * IRT	
	0.777011	-0.0633390224 * Gestation	

Table 3.6 Regression model calculations for use in predicting length and weight z scores at years 1 and 2 for infants diagnosed by newborn screening

As before, the regression modelling reiterates that birth weight z score is the biggest predictor of these nutritional estimates as the ERR for BW is highest in all models. As you can see from table 3.6, the most accurate regression model, with the lowest mean absolute error, is weight z score for year 1.

All regression models above were created using 80% (91 patient samples) of the data and were validated using the remaining 20% (23 patient samples). The error values shown were derived from the validation step in each case. As an example, figure 3.13 below shows the actual (blue line) and model predicted values (red line) in a plot for both the training set (top graph) and validation set (bottom graph) in the case of the model for the computed score for weight profile in first year of life. Each point on the graph marks an observation/patient. It is important to note that there is no significance of these points being adjoined. The x-axis corresponds to the number of patients used in that plot and the y-axis is the predicted computed scores (mean z score). The schematics of the remaining models can be viewed in Appendix E.

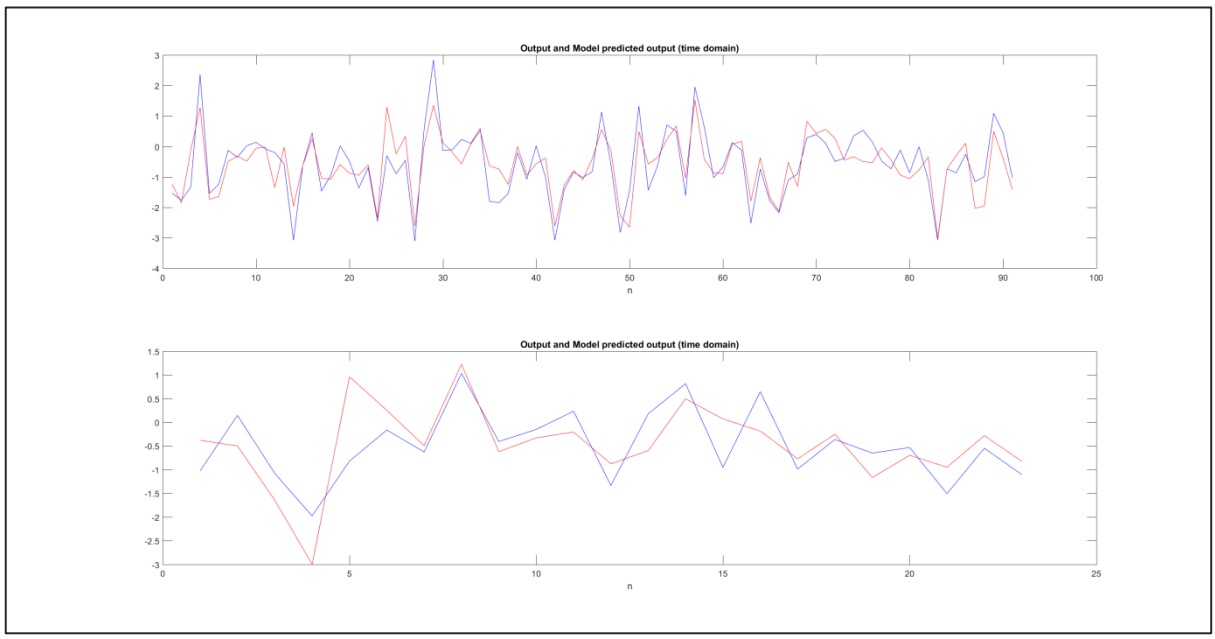


Figure 3.13: Schematic representation of the actual values and the model predicted values for weight z score in at age 1.

4.0 DISCUSSION

INTRODUCTION

This chapter will focus on analysing and discussing the results of our service evaluation in relation to current literature, relating it back to the study aims.

4.1 REVIEW OF THE WEST MIDLANDS NEWBORN SCREENING DATA

Overall, 507,608 babies were screened over the seven year period. On average, the West Midlands screening laboratory screened more than 72,000 babies each year. The birth prevalence of 1/3525 was lower than the expected figure for the whole of the UK, which is 1/2381.[13] This is probably due to the ethnic diversity within the region, however it is likely there are other reasons for this. It could be argued, with better genetic counseling programs, higher rates of antenatal detection[149,150] and new assisted conception methods such as Intracytoplasmic Sperm Injection (ICSI), the prevalence of CF may gradually be decreasing. The male to female ratio in this study of 73 males: 71 females, confirms previous knowledge that CF affects males and females equally. The median age of screening was 7.5 days, which is slightly higher than recommended practice which aims to collect all blood samples on day 5 of life (as this will improve timeliness of diagnosis), except in exceptional circumstances this can be performed between day 5-8 of life.[151] The median age of diagnosis for our cohort was 22 days, which has significantly reduced from 2.4 years since the introduction of newborn screening across the UK. This figure is very similar to the group from South East London who reported a median age to diagnosis of 20.5 days.[136] The European standards of care suggest that the majority of infants should be seen by the specialist CF team within 35 days of birth and no later than 58 days.[108] These targets were met for each of the 139 patients. Excluding the missed cases, all patients were

diagnosed within 8 weeks, which is within the window of opportunity to create a better prognosis.[100]

In keeping with the aims of the CF newborn screening protocol, the rates of carrier detection were low with only 101 carriers being identified. This suggests that the West Midlands screening laboratory have correctly identified an appropriate IRT cut off level, specific to this population. This group is offered genetic counseling with a geneticist. Although diagnosis of CF is not suspected, parents are informed that this cannot be ruled out and families are offered the gold standard diagnostic sweat test for confirmation (this is not currently practiced in England, but other parts of the UK such as Wales). Parents are also encouraged to be vigilant in looking out for symptoms suggestive of CF. In addition, the 3-tiered; IRT-DNA-IRT UK NBS protocol seems optimum, demonstrating a specificity of 99.9% and a high sensitivity of 94%. It is important to note that the figure for specificity is temporary and will change as more false negative cases present. Although the figure for sensitivity doesn't quite meet European standards (95%), the reason for this is because we classified 9 patients as false negatives when in fact only 3 of these children who had been missed by the NBS protocol had, 'classic CF'. Had we excluded the cases of equivocal diagnosis, our reported sensitivity would have been 98%. This suggests that the West Midlands, UK CF NBS is performing extremely well. Out of the total number of positively screened babies, there were 56/200 (28%) false positives. This is likely to be due to the fact that IRT as a biomarker is not specific to CF and can be raised in other circumstances such as prematurity, hypothyroidism, adrenal insufficiency and autonomic dysfunction.[23] Analysis of medical records of these false positive patients did not form part of this study however it would be useful to examine why these patients had falsely elevated IRT's. The PPV of 72% and NPV of 99.9% are high, suggesting the performance of the

UK NBS program far exceeds the expectations set by the European neonatal screening working group, which state that NBS programs should aim for a minimum PPV of 0.3 (30%).[108] To date, this is the highest reported PPV for CF NBS in Europe.[152]

Of the 144 babies with a diagnosis of CF or CFSPID in our region, the median IRT was 159mcg/L. 17/144 (11.8%) patients required a second blood spot between days 21-28 for a further IRT level. The median of this was 104mcg/L. While the IRT-DNA 31-mutation panel, extended gene analysis strategy picks up 95% of positive CF children in the Caucasian population, it only identifies 65% of ethnic minorities.[105,153] The 2nd IRT measurement is an important element in the UK protocol whereby children with CF who do not have CF mutations within the 31 mutation panel are detected. This element is particularly important in the multicultural area of the West Midlands.

4.1.1 CYSTIC FIBROSIS SCREEN POSITIVE, INCONCLUSIVE DIAGNOSIS

There were 5 patients meeting the criteria for CFSPID, that is those with a normal sweat test and identification of two CFTR mutations or, those with an indeterminate sweat test with one or no mutations.[135] In this study, all patients satisfied the former criteria. Although it is important not to burden patients and families by over-medicalization, the introduction of NBS has accentuated the survival benefits of early intervention in all patients with suspected CF. Furthermore, early education and knowledge about this condition and its implications may help a patients' understanding, should they develop symptoms. As the advantages of identifying these children seem to outweigh the disadvantages, the centres involved in this study, review and assess this group of children in the same way as those who exhibit the pathological hallmark features of CF. For this

reason and the fact that they are placed on the CF Trust registry, we included this sample in our further evaluation.

4.1.2 FALSE NEGATIVES

Over the seven-year period, there were nine missed cases (false negatives). Of these only 3 had mutations usually associated with severe disease and are clear cases of false negative CF NBS. The remaining 6 cases have mutations associated with mild disease or no disease at all. As the aim of CF NBS is to detect children with severe disease it could be argued that these 6 cases are not false negatives. These children were detected by a variety of methods, for unique reasons and diagnosed at various ages. Each patient was a Phe508del heterozygote and two patients had meconium ileus. Previous literature has stated that IRT is an unreliable biomarker in cases of MI in the first week of life, though IRT may be abnormal in the following weeks.[111] In saying this, 25/144 (17%) patients in this cohort had MI, all of whom had an abnormal IRT. The incidence of meconium ileus for our cohort is similar to that reported in other studies worldwide which report an incidence of between 10%-20% of all CF neonates.[154,155] This data confirms that all babies with MI should be rapidly investigated and clinicians should maintain a high level of suspicion when such a patient presents. The number of missed cases (1.28 per year) was lower than the predicted figure of three false negatives per year[136] however it is likely that there are more missed cases that are yet to present to either of the tertiary centres within our region. In turn this will influence the calculated birth prevalence.

4.2 BIOCHEMISTRY PARAMETERS

Out of the 139 patients of whom we had access to clinical data, 113 (81.3%) had sufficient sweat chloride samples with a median sweat chloride of 94mmol/L. Of these, 17/113 (15%) patients had a borderline result of between 30-59mmol/L and 5/113 (4.4%) had a negative sweat test, ≤29mmol/L. The most common, pathological explanation for a false negative sweat test result is oedema or hypoproteinaemia, sometimes seen in infants with pancreatic insufficiency prior to starting PERT.[150] Treatments such as corticosteroids also affect sweat chloride concentration.[150] Unfortunately, this specific information was not collected on these patients with a negative or borderline sweat test so no conclusions can be drawn about this. As sweat chloride level changes with age and severity of mutation, the borderline concentration range of 30-59mmol/L is considered significant. As mentioned previously, for patients with a sweat chloride of ≤29mmol/L, 4/5 expressed the R117H (p.Arg117His) genotype, which is already known to have a lower sweat chloride level (mean = 59mmol/L according to the CFTR2 John Hopkins website).[140]

Despite the national protocol, 26 (18.7%) patients had no recording of a sweat chloride level. This could be due to either not performing the test or an unsatisfactory sample. Insufficient samples can be caused by a number of factors including weight, age, skin condition, collection system and hydration status.[63,150,156] In a study performed in Colorado, shortly after the introduction of newborn screening in this area, adequate sweat sampling was reported in 99.2% of patients, although these patients had a mean age of 6.2 weeks.[157] Although successful samples have been reported in infants as young as 48 hours,[63] other studies have warned that sweat testing may only be achieved in 74% of patients <6 weeks old.[63] Taking this into consideration, accounting for the median age at diagnosis/first clinic visit of our cohort, when sweat testing occurs, the number of patients

with an adequate sample is good, especially in comparison to other UK centres.[136] We do need to look closer however, at why these patients did not have a sweat chloride recording. If the reason is due to collection difficulty or lack of sweating in young infants we may need to consider methods other than the traditional Gibson and Cooke equipment, for example the Macroduct® sweat collection system (MSCS) which requires only 15µl of sweat for a sufficient sample in comparison to >75mg.[158] Worryingly, this does mean that there are a small proportion of patients in our study, whose diagnosis was made solely on NBS or genetic mutation analysis, presumably from the patient's initial blood sample.

Faecal elastase measurements were successfully obtained in 116/139 (83.4%) patients in our cohort. As this diagnostic assay is linear, with a lower limit of <15 µg/g and an upper limit of >500g µg/g[159] we were not able to document specific values above or below this range. With this in mind, the median faecal elastase for the whole cohort was <15 µg/g. Out of the 116 babies who had their faecal elastase measured, 95 (82%) were pancreatic insufficient, 18% were pancreatic sufficient (median FE 476µg/g) with 8 patients' results being >500 µg/g, a result similar to a healthy, non-CF infant. Similarly to the normal sweat chloride results, 6/8 of these patients were R117H heterozygotes with the other two patients having c.3718_2477C>T and p.Ala455Glu mutations. This confirms previous knowledge that genotype has a direct correlation with pancreatic function and R117H heterozygotes do exhibit a milder clinical phenotype.[160] The number of pancreatic insufficient patients was slightly lower than expected as current literature anticipates 85-90% of CF patients have pancreatic insufficiency.[161] It is likely that the neonates, who had no FE recording, were pancreatic insufficient and were started on PERT anyway but this information was not collected. On the other hand, each year more genetic mutations are being identified with increasing numbers of 'mild' cases of CF and increasing numbers of

patients falling into the CFSPID category. It is possible therefore, that the number of CF patients with normal pancreatic function is increasing. It is also important to note that FE concentrations can change over time and a patient who was once pancreatic sufficient may later exhibit pancreatic exocrine dysfunction.[162]

4.3 STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA ISOLATION

Microbiology results of all 139 patients were reviewed over the 2-year study period. These included samples from cough swabs, sputum samples and bronchoalveolar lavage (BAL). Although all patients within the cohort were placed on prophylactic antibiotic treatment (e.g. Flucloxacillin), in keeping with national recommendations,[39] within the first year, 15/139 (10.7%) patients isolated *Sa* and 16/139 (11.5%) in the second year of life. *Staphylococcus aureus* is usually the first bacteria to colonize the respiratory tract of infants with CF but the source of this is unknown. Potential reservoirs include the natural environment (e.g. soil and water), the environment in health care settings (e.g. sinks), contaminated equipment (e.g., nebulizers), other contaminated objects, and other CF patients.[163] The median age of first isolation of *Sa* in our cohort was 11.18 weeks (2.8 months) which was similar to another UK study, which reported 50% of patients to have acquired *Sa* within the first 3 years of life with a median age of isolation of 2.9 months.[136]

In contrast to *Sa*, *Pseudomonas aeruginosa* (Pa) is a more aggressive pathogen associated with an accelerated decline in lung function and a worse prognosis. A group in the US using data from Wisconsin, who performed a prospective study looking at isolation of non-mucoid and mucoid strains of *Pa* in screened infants, showed isolation of non-mucoid *Pa* in 29% of patients at 6 months.[36] Although we did not define the phenotype of *Pa* in our

cohort, only 3/139 (2%) of patients in this study had first isolation of this bacterium at the same stage. Within the first year more of our cohort isolated *Pa*, 21/139 (15%) compared to *Sa*. One patient even isolated this bacterium as young as 2.85 weeks. Potential sources of *Pa* infection include acquisition from the environment or transmission between CF patients or siblings.[164] Other studies suggest that the time to acquisition of *Pa* is shorter in babies diagnosed by newborn screening compared to those diagnosed by conventional methods,[165] as very young patients are attending the same clinics as older patients, causing problems with cross infection. Early clinical exposure and social interactions may contribute to the higher rate of *Pa* infection in CF infants.[165] In saying this, only 48/139 (34.5%) of patients had first isolation of *Pa* within the first two years of life with a median age of 1.18 years, similar to the age of isolation of patients within the South East London study, although there was an isolation rate of 47% here in the first two years.[136] When comparing age at first isolation and percentage of children isolating these bacteria between the group in South East London, it is important to note that this region routinely performed bronchoscopic lavage compared to the two centres within our region which obtained most samples through conventional methods.

Comparing first isolation of bacteria between the two tertiary centres, one centre had a higher isolation rate of *P.aeruginosa* than the other. The reason for this is unknown and needs to be further investigated. One cause could be different infection control measures, although standards for these are internationally set. A higher incidence of *Pa* in centre 2 may actually show that this centre is better at detecting microbia by performing more respiratory cultures, for example. No conclusions can be drawn about these results, due to the number of confounders not adjusted for in this study. Interestingly, the centre with higher rates of *Pa*, demonstrated a lower acquisition rates of *Sa*.

One study found that female gender was associated with earlier risk of acquisition of Pseudomonas aeruginosa[166] but our data verified no difference in gender with 22 females and 24 males isolating *Pa* within the first 2 years of life. This lack of difference is probably due to the small numbers within our study.

4.4 EVALUATION OF Z SCORES AS AN ANTRHOPOMETRIC MEASURE

There is extreme variation nationally in terms of measurement and documentation of an infant's anthropometric measures, an outcome relevant to all paediatric sub specialties. Infancy is a time of rapid growth, with healthy children doubling their birth weight at 4 months and tripling it by 1 year of age.[167] This is a time where CF patients are particularly vulnerable due to the increased stress and metabolic demand on their bodies. In addition, malnutrition and pancreatic insufficiency are usually the first symptoms to manifest in cystic fibrosis so accurate anthropometric measurements are important as many management decisions are based upon them.

Currently within the centres involved in our study, raw data for lengths and weights are documented in the medical records and dietetic notes and transferred onto traditional length, weight and BMI percentile charts. This study highlighted that weights were performed more regularly than lengths as we had between 10-15 weight measurements per patient in comparison to 5-12 lengths per patient. It was also noticed that very rarely, if at all, a child's head circumference was measured and documented which was a disappointing finding considering head circumference is a reliable indictor of nutritional status and cognitive function.[83] For this reason, collection of head circumference data did not form part of our study.

Z scores have a number of advantages compared to percentile charts, which are not specific enough to highlight children failing to achieve their optimum growth parameters. Z scores are calculated based on a reference population, they are transferable across age and gender and they can be analysed as a continuous variable in studies.[168] As patients attended the outpatient clinics at different ages, z scores were deemed the most appropriate method of transferring raw length and weight data into a standardized measure that could be used to compare patients. How a z score is calculated can be viewed below.[169] As mentioned previously, BMI percentile is not a validated measure of anthropometry in children <2 years old although it is recommended in older children and young adults with CF.[170, 171]

$$\frac{\text{Standards mean value} - \text{Value of subject}}{\text{Standard deviation (SD) of standard}}$$

Figure 4.1 Z score calculation

This work has emphasised the need for a standardized protocol when assessing anthropometric measurements of children with CF, as standards of care define optimal service provision necessary to deliver best outcomes possible for patients. Anecdotal evidence obtained from dieticians at Great Ormond Street Children's Hospital and hospital's affiliated with King's College London, which have implemented z score usage into current practice, has been very optimistic. Nutritional parameters in CF children are an extremely responsive measure to treatment intervention and beneficial for clinical research in this age group, provided they can be accurately measured.

4.5 IDENTIFICATION OF THE 'AT RISK' OF MALNUTRTION POPULATION

Over the last decade several studies have reported improved outcomes when cases of malnutrition are detected early and treated appropriately.[172] It is therefore crucial that a method of identifying patients, in the, 'at risk' of poor nutrition category is established to allow for timely intervention. Defining the, 'at risk' of malnutrition population within CF children has proven an arduous task however, with no accepted and validated agreement amongst nations. In addition, there are many different forms of anthropometric indices used to identify the, 'at risk' of nutritional failure group, including, body mass index percentiles (BMIp), height-for-age percentile (HAP), weight-for-age percentile (WAP) and percent ideal body weight (%IBW).[171, 171]

A German study comparing anthropometric indices in CF children concluded that BMIp was the most accurate predictor of nutritional failure in CF infants compared to other, more conventional methods,[173] although BMIp is not constant across the paediatric age range and has not been validated for use in CF children.[173,174] The advantage of %IBW as a nutritional measure is that it incorporates weight and length for age in the same measure, with the ability to identify stunting and wasting[173,175] and has been recommended by the Cystic Fibrosis Foundation consensus report on nutrition.[72] On the other hand, calculation of this index is complex with a polynomial equation and wide inter-rater assessments have been observed.[173,176] The benefits of using HAP and WAP include that they are population specific and standardized, as mentioned in the previous section.

The second problem arises when trying to define, 'malnutrition' or 'nutritional failure' in children with CF. The Wisconsin neonatal screening group defined a group of patients as, 'non responders,' which were those who failed to achieve a weight comparable to their

BW z score within two years of diagnosis.[148] In contrast the Cystic Fibrosis Foundation registry classify insufficient growth as having a length and weight <5th percentile for age, a length <90% of reference median and weight <80% of reference median.[148] The WHO working group define malnutrition as having a HAP or WAP <5th percentile[177] and other studies have described their own reference ranges for nutritional failure such as %IBW <90% and BMI <15th percentile.[173]

Low weight and length z scores do not necessarily reflect the, 'at risk' of under nutrition population as this may be influenced by genetic factors and parental height. In addition, not all patients in the, 'at risk' category will have nutritional insufficiency.

As there is no validated method of assessing children's nutritional prognosis we agreed that expertise was required to help identify these patients who necessitated closer clinical evaluation. This was the justification behind development of the grader rating protocol to assess nutrition. An integral part of the design of this protocol was to check the consistency of ratings between graders. We achieved a sufficient weighted kappa value and high rater agreement despite the raters being blinded to one another's scores and having no access to clinical data. Unfortunately, the protocol was an unsatisfactory measure of nutritional prognosis and a poor indicator of identifying, 'at risk' patients as the expert raters graded the majority of patients in the, '2' or '3' category, rendering this method poor at distinguishing between patients. A possible cause for this is that raters did not know the variation in patient graphs. Had they known, they would be better informed as to how to assess which patients are doing better and which are performing poorly. In retrospect there are two other techniques that could be applied to prevent this happening in future work:

1) A larger (e.g. 5-point scale) to differentiate patients more precisely
2) Use of 'queue sort' method[178]

The latter method is a method used in computer science, which stacks data on priority and then grades these accordingly. For application in this sense, this technique would involve graders sorting patient graphs from worst performing to best performing (or vice versa) and then assigning a grade to these patients.

Our second attempt at creating a measure able to identify suboptimal nutrition, was creation of, 'computed scores,' which were the mean of all of the patients' z scores for years 1 and 2 for length and weight. Using these computed scores as a classification output and as an outcome measure within our polynomial regression model, we inferred that this method sufficiently predicted patients failing to reach their nutritional potential.

In summary, there are many ways of measuring and assessing a child's anthropometry although there isn't a validated method in CF babies. In addition, defining what is meant by, 'malnutrition' or 'nutritional failure' is a controversial topic but reference values for these criteria should probably be region or nation specific as there are so many environmental factors contributing to this outcome.

4.6 BASIC NUTRITIONAL PARAMETERS

Many studies have suggested that birth weight of cystic fibrosis patients is subnormal and significantly lower than the non-CF population, implying that CF is prenatal in origin and observable at birth.[179, 180, 181] Others have failed to confirm this conclusion and argue birth weights of CF children tend to be similar, or slightly lower than the healthy population but

a large proportion fall below the 50th percentile by the time of diagnosis.[162,148] This latter statement supports our findings as the mean birth weight of our cohort was 3.09 kg, falling within the lower end of normal range for all children in England and Wales.[182] In support of the observation that CF does not manifest until birth, our data confirmed no significant difference in the birth weight of pancreatic sufficient, mean (SD) -0.05 (1.17) versus pancreatic insufficient, mean (SD) -0.36 (1.08) groups, suggesting that pancreatic function, has little if any effect on birth weight z score. In comparison, a significant difference was noted in these two groups when considering weight gain (or loss) from birth to first clinic visit (this is a period of untreated CF). Even within the first three weeks of life the pancreatic insufficient group demonstrated difficulty in gaining weight. A similar study which compared pancreatic sufficient and insufficient neonates' weight gain from birth to first clinic visit also reported less weight gain in pancreatic insufficient patients from birth to diagnosis (13.4±3.4 vs 22.3±4.0 gm/day; p=0.05).[162]

Although all patients in our study were diagnosed by newborn screening and placed on the appropriate treatment, 19/139 (13.7%) patients did not achieve catch up weight gain comparable to their birth weight z score over two years, supporting a finding from the Wisconsin newborn screening group who defined a group of, 'non-responders,' despite receiving appropriate dietician input, fat soluble vitamins and PERT.[148] Having looked further into the demographics of this subgroup, no common features or patterns were noted. This suggests there are multiple factors influencing the nutritional status of an infant with CF such as: genotype, breast feeding, nutritional supplementation, diet, calorie intake, eating behaviour, activity levels and severity of lung disease, although socioeconomic and environmental factors probably impact this the most. Conversely, the majority (79.9%) of patients did exhibit catch up weight z score comparable to birth weight z score within the

two-year follow up period. Literature suggests that patients, who demonstrate rapid catch up, tend to maintain stable or improved length and weight z scores in the following years.[148] Our study results also suggest that the length of time for patients weight catch up is markedly more accelerated than length catch up, which concurs with the findings from Farrell et al that report, catch up linear length may take up to four years in a child diagnosed in infancy.[183,184]

4.7 EVALUATION OF CLUSTER ANALYSIS

Initial cluster analysis segregated all of our data into two distinct groups. Throughout the duration of the data analysis and modelling we did not make any inferences about these groups or label them specifically as a 'good prognosis' group and a 'poor prognosis' group. It is extremely difficult however to ignore these separations with knowledge about the disease features. The 20 patients in group 1 exhibit features we would normally consider to be present in those with, 'mild' CF, and group 2 (94 patients) with a more severe disease type. It is interesting to note that all of the attributes in cluster 1 are better besides the final attribute, that is, weight z score in year 2, which is lower than cluster 2. This makes sense physiologically as the, 'poorer' performing group would be exhibiting, 'catch-up' weight gain at this stage. The same cannot be said for length z score in year 2, yet again confirming that length catch up is less likely to occur within the first 2 years.

As 95% of our cohort were either homozygous or heterozygous for the most common mutation, phe508del, it was not surprising that this was present for gene 1 in both clusters. The mutations segregating the groups for gene 2 however, were Phe508del (cluster 2) and R117H (cluster 1). It has long been recognised that the variable clinical course of patients

with CF can be attributed to genotype.[185] Numerous studies have reported the occurrence of pancreatic insufficiency, poorer lung function and more severe disease in patients homozygous for Phe508del,[185] and conversely normal pancreatic exocrine function, lower sweat chloride levels and a milder clinical phenotype in patients expressing R117H.[186] It is important not to delve too much into these highlighted gene mutations due to our very small sample size.

Contrary to what we were anticipating, isolation of *Staphylococcus aureus* or *Pseudomonas aeruginosa* were not used by the clustering algorithm (k-means) in order to distinguish between the two clusters. This is probably explained by rapid detection and eradication of bacteria, as we did not collect information on patients chronically colonized. It may also be true that early acquisition of these species does affect morbidity and mortality of CF patients[187] but this does not become apparent until later years.

4.8 THE ROLE OF CLASSIFICATION IN VALIDATING THE DATA

The cluster analysis and initial classification validate our data as the emerging attributes and their boundary values broadly agree with currently accepted reference ranges.

Using all attributes as predictors, the classification model that was produced, with 100% accuracy marked FE1 concentration as the best predictor of class, with a split at 212 μg/g. The established cut off levels and reference ranges for FE1 concentration were initially studied by Löser et al who investigated patients with various degrees of pancreatic insufficiency. They found that using a cut off of <200g μg/g the test demonstrated a 93% sensitivity and specificity.[188, 186] In comparison, a multidimensional European study found the best cut off was 184 μg/g but recommended a range of 160-200μg/g for each nation to

establish their own cut off, as different countries have different distributions of pancreatic function.[161,189] The methods of how these studies established appropriate reference values are not easily accessible. This information reiterates that knowledge of an infant's pancreatic phenotype is useful clinically as a prognosticator.[185]

Having removed faecal elastase to establish whether any other attributes were important in determining class, sweat chloride appeared as the second best predictor for this purpose, with a cut off value of 58.5mmol/L. Again, this is remarkably similar to the current established cut off of 60mmol/L,[64,189,190] further emphasising our patient sample is representative of the CF population. Furthermore, our cluster analysis agrees with literature that sweat chloride levels directly correspond to genotype.[138, 185] Classification model 2 demonstrated 93.9% accuracy. Although this tree diagram displays several branches, it is important to maintain a level of suspicion about these, as our cohort was small. Many more patients would be required to study the effect of specific gene mutations in terms of classification.

Following the classification step of the data analysis, we can maintain that we have found more accurate, evidence-based boundaries for faecal elastase and sweat chloride, at least for this specific cohort. Determining "nation-specific" cut off levels (as recommended by the European group) is helpful and clinically important. It would be useful if further work followed, using our "method" with more samples to produce more accurate, established reference ranges.

4.9 CLASSIFICATION MODELS AS PREDICTION TOOLS

As discussed in section 4.5, raters' scoring for the classification prediction models was unhelpful due to the lack of spread in grading's, perhaps because of a weakness in the grader rating protocol. On the other hand, the computed scores demonstrated a high accuracy rate, in terms of the error rate (especially the one in brackets) and the confusion matrices are a useful visual interpretation of this. Of all the inputs, the variables featured in the classification models include: BW z score, rate of weight change in z score from birth weight to first clinic visit, sweat chloride, faecal elastase and gestation. Most of these feature once, however birth weight z score appears to be a core differentiator between classes. This finding suggests that birth weight z score is the most helpful determinant of an infants nutritional progression over the course of two years. It is important to emphasise that causality cannot be inferred from these results and we are not suggesting a low birth weight z score results in poor nutritional outcomes, but the factors affecting birth weight z score i.e. maternal health and maternal nutrition, have a potential to influence this outcome. Although this information is interesting, paediatricians generally have no input during the antenatal phase (unless a child is picked up in this period through identification of echogenic bowel or genetic analysis) meaning we cannot establish any intervention to affect the birth weight of a child. This new knowledge however provides us with a means of detecting the, 'at risk' of malnutrition group, something that is currently very difficult. We can henceforth be more aggressive with treatment at an earlier stage and have a lower threshold for clinical intervention in patients who's BW z score is low.

Higher rates of accuracy were evident in models for year 1 compared with year 2 for both weight and length. The reason for this is probably due to the fact there are many more contributors to these nutritional parameters (not adjusted for in this study) that will

influence a child's growth, for example socioeconomic and environmental factors, the effect of which become more apparent in the second year of life. In addition, these patients will have established treatment and close clinical supervision during year 1 which will influence their nutritional progression in year 2. We have also demonstrated higher prediction accuracy in weight models compared with length classification models. The reasons for this are similar to those above, in that other factors (in this instance, parental height) do not form part of the model estimate. Whether or not the accuracy rates of these classification models are acceptable for use in clinical practice is an uncertainty, as this is a novel approach and so estimating the efficacy of this prediction tool is not easily achieved.

4.10 USE OF NARMAX POLYNOMIAL FUNCTION AS A PREDICTOR OF NUTRITIONAL PROGNOSIS

There were many benefits of using the NARMAX model in this study to complement our classification analysis. Where classification was able to predict which attributes bear an effect on a child's prognostic class, the polynomial regression model gives a measure of the weighting each of these attributes has on the final outcome. This is expressed in terms of the ERR value. The higher the ERR, the more influence that variable has on the model equation hence if this variable is removed, the degree of inaccuracy rises (this only applies to linear models). Furthermore, this polynomial function rejects attributes, which have little impact on the outcome, providing us with the simplest possible equation. It was reassuring to see that the variables impacting the classification and polynomial regression models i.e. BW z score, z score from BW to first clinic, IRT, sweat chloride, FE and gestation, correspond with one another. Other similarities include the fact that BW z score

appears in each of the model equations and prediction of weight z score in year 1 has the highest accuracy in both methods.

In practice, the classification model may be less helpful as clinicians will have to have access to pictures of the simple tree diagrams and using the child's attributes, follow the branches to reach a predicted z score, which will not be specific to them. In comparison, the regression model is more user-friendly and time efficient as this will involve storing the four model equations on an excel spreadsheet, inputting the data of a child at first clinic visit and a specific z score (+/- SD) will be produced. This is an extremely exciting innovation and could potentially be a very important tool for paediatricians in the future as the calculated z score range can be used for identification of patients at risk of nutritional failure and guide clinicians as to subsequent management strategies.

5.0 STUDY LIMITATIONS

Although this research satisfied the study aims, there are several limitations, which may have had a potential impact on the research findings and the degree to which they can be trusted.

The first limitation lies within the study design. Due to time constraints the study generated was retrospective in design meaning the nutritional information used to create our prediction models was reliant on other people's collection of data. As a result of this, data were not collected in a rigorous fashion therefore some patients had fewer lengths/weights recorded than others and there was no validated method of measurement of nutritional indexes applied to all patients. This design also made controlling for confounding variables impossible hence why only correlation, not causality can be inferred from our findings. This type of design was most appropriate however given the lack of time and funding. In addition, as the outcome of interest (i.e. nutritional prognosis of NBS infants) was not the original reason for data collection, there is limited bias in this type of cohort study. It was difficult to minimize these obstacles however we attempted to overcome this limitation by applying the use of z scores, which adjust for time, rather than using raw length and weight data. The only way to eliminate all confounding variables in future research is by performing a prospective cohort study whereby additional data is collected, in this case for example, calorie intake.

Another drawback of this study was the statistical model constraints. As the mathematical program used could not incorporate nominal attributes or patients with missing data in any of the columns, our dataset was reduced from 139 patients to 79. Although this end figure

is 57% of all confirmed cases of CF within the West Midlands in the 7-year study period, the data set is still relatively small and may therefore not be a true representation of the CF population in this region. Additionally as this research is region specific, these results may not be generalized to the UK population. Nevertheless this statistical design was chosen as it has allowed us to create something novel and innovative. In order to surmount this problem, we used the John Hopkins CFTR2 website to provide information about average sweat chloride levels for certain genotype combinations and we inserted these values into the dataset (as most missing values were sweat chloride results). As there were only two patients with missing values for genotype or ethnicity, we agreed that losing two patient samples was justified in order to include two additional nominal attributes for the classification step of modelling. This brought our final dataset to 114 out of a potential 139 (82%), on which the models were based. Although the patient sample is small, the accuracy of our models was high resulting in 4 validated models for use in clinical practice. To overcome this limitation in future research, knowledge about the computing programs prior to conducting the study would be useful to ensure that nothing done in the data collection step will impede the data analysis. However, as this study was retrospective in design this problem was impossible to alleviate.

Another potential drawback of the data analysis methodology is its relevance to clinical practice. As clinicians are likely to be unfamiliar with this type of analysis, and modelling is rarely applied to medical research, the readability and interpretability of these study results may be compromised. It must be emphasised that these classification groups are key to informing clinicians about different disease groups. To reduce this limitation we created schematics and visual representations of our modelling results to provide the reader with easy-to-interpret diagrams.

A potential pitfall of this study is the lack of control group in whom to compare our findings. Although this was intentional in design, a control group would have offered us the opportunity of seeing directly the effects newborn screening and earlier diagnosis has on the nutritional prognosis of CF children. For example, we have reported that 19/139 CF infants failed to regain their BW z score within two years. To strengthen this finding it would be useful to know what proportion of healthy children fail to regain their BW z score. We chose not to have a control group however as there are ethical implications for this. As screening for CF is now standard care within the UK, withholding this diagnostic advance would be unethical. Another way of comparing screened and unscreened infants in CF is by collecting data from patients diagnosed prior to screening. The problem with this is that many infants were not diagnosed until the first or second year of life prior to NBS so data would be insufficient. Secondly, even within the last decade there have been many treatment advances within CF, which we could not control for. In an attempt to combat this limitation we split our cohort into pancreatic sufficient and insufficient groups using the pancreatic sufficient group as a control. In retrospect another way we could have split the cohort is by categorizing our patients into a, 'CFSPID' group, something we failed to endeavor due to the small number of samples in this category.

In retrospect, including the 5 patients categorized as, CFSPID, in our modelling analysis was a limitation. Our reasons for this are plausible (i.e. they are reviewed as regularly as other CF patients in our region and their data are stored on the CF national database) however recent literature recommends that these patients should not be reviewed or treated in the same way as children with classic CF. They should be seen between 6-12months after birth and annually thereafter and they do not even warrant annual respiratory cultures.[135] Although sufficient data were available for these patients, it is not appropriate

to compare children with an inconclusive diagnosis with children who have ‘classic’ CF and very distinct phenotypical consequences. Inclusion of these children may have slightly altered our modelling results and so this group will be excluded in future work and we plan to remodel these data for any subsequent publication.

6.0 CONCLUSION

6.1 NEWBORN SCREENING

Our study has confirmed that the West Midlands screening laboratory is satisfying the aims of the UK newborn screening program[109] by; identifying the majority of true positive cases, preventing diagnostic delay, minimizing the number of children requiring a second IRT and identifying low numbers of carriers. This is a result of appropriate calibration of mass spectrometers and defining suitable IRT cut off levels, specific for this region. In saying this, there were a small number of children, which were missed by this protocol. It is therefore crucial that the reasons for this are investigated in order to minimize this in the future and alter the screening protocol as necessary. As the NBS protocol performed well in a multicultural region such as the West Midlands, it will be interesting for other regions to perform similar reviews of newborn screening data to measure performance of this algorithm, considering the extended mutation analysis tier of the protocol is based on the most common mutations in a Caucasian population.

The age at which children with CF are now being detected has significantly reduced since the introduction of newborn screening and the nutritional benefits of this are already clear. However, the respiratory benefits of newborn screening may not become apparent until the first screened cohort reach adolescence or early adulthood, when lung function traditionally starts to deteriorate.

In summary, the UK NBS protocol performs well compared to other European studies, expressing a very high PPV and achieving European standards.[108] It is further evidence of the exciting advances in the care and management of patients with cystic fibrosis. In

comparison to the US, although the evidence of benefits of NBS as a diagnostic tool are plentiful it must be emphasised that the screening protocol is not intended to replace the gold standard diagnostic sweat test in the UK.

6.2 BIOCHEMISTRY PARAMETERS

This study has enabled us to thoroughly evaluate the biochemistry results of specific genotypes and reflect on our centres' performance in collection of such measures. Disappointingly, it has highlighted that a significant proportion of infants did not have a sweat test performed or accurately documented. Prior to this review, this issue had not been cogitated as patients were receiving appropriate care through diagnosis by NBS. This has consequently informed our practice and we are now being more observant about such cases.

Our cluster analysis confirmed the correlation between high sweat chloride, low faecal elastase and severe genotypes and conversely, a low sweat chloride and high FE1 in milder genotypes. The classification aspect of modelling informed us of what FE1 (212μg/g) and sweat chloride (58.5mmol/L) reference ranges successfully partitioned our cohort, confirming our data were representative of the CF population as a whole as these values are comparable to accepted ranges. This information has also allowed us reflect on where these reference ranges originated and whether they are specific enough for use within the UK population.

6.3 COLONIZATION OF BACTERIA IN RESPIRATORY TRACT

As S.*aureus* and P.*aeruginosa* are associated with a decline in lung function[37, 38] in CF patients, we chose to evaluate the first date of isolation of these two species. In addition, as

the research in terms of microbiology data within newborn-screened patients are limited, we hoped to offer some insight into how such infants are affected.

Despite being on prophylactic treatment, 12% of patients had first isolation of *Sa* within the first two years of life. Although this figure is lower than other studies evaluating the same measure, prophylaxis is failing in some children. Given that there is a significant treatment burden to families and patients on prophylaxis and the evidence demonstrating higher rates of *Pa* acquisition in infants on *Sa* prophylaxis,[190] as well as the cost implications, the need for further research to prove its benefits and justify the usage is stressed.

Overall, we report that more than a third of our cohort had their first isolation of *P.Aeruginosa* within two years of diagnosis, though we did not distinguish between mucoid and non-mucoid types. Interestingly, the number of positive *Pa* samples was greater than positive *Sa* samples, suggesting that infection control policies are not as effective as anticipated and clinical segregation needs to be more stringent. The median age of isolation for *Sa* was less than 3 months and *Pa*, 61 weeks, earlier than the age that many patients would have even been diagnosed with CF by conventional means. The effect of early detection and eradication of these species in CF children and the delay or prevention of bronchiectasis will be interesting to study in future research.

We conclude that it is common for CF infants to colonize one or both of these species in their first years. Our modelling results suggest that isolation of these bacteria in early life does not influence the height or weight z scores in years one or two. It is important that

these microorganisms are detected and eradicated rapidly and that more work is done evaluating their influence on pulmonary function.

6.4 NUTRITIONAL OUTCOMES OF NEWBORN SCREENED INFANTS

This study has created a platform in which nutritional parameters of newborn-screened infants with CF in the West Midlands can be evaluated. This is extremely important, as poor clinical outcomes are associated with malnutrition in CF infants.[184] As expected, the BW of our cohort was normal, but slightly lower than the UK average and we observed no difference between PI/PS groups suggesting CF is not prenatal in origin.

We conclude that z scores are the most reliable and accurate measure of assessing the an nutritional indexes of an infant with CF and these should be used more frequently in clinical practice. The paediatric consensus report recommends that infants identified with poor growth should be seen more frequently, every 2-4 weeks,[184] however identifying the patients with poor nutrition remains problematic. We have deduced that clinician observation (eye-balling growth charts) has its limitations especially when trying to classify the nutritional parameters. As BW z score featured as the most important determinant of nutritional progression in our models, a baby with a low birth weight may be considered, 'at risk' of malnutrition. Computed scores were also a beneficial and accurate determinant of nutritional progression in our cohort and so, have formed the foundations for which our models were created. This finding is in keeping with a study looking at morbidity and mortality of young children with CF, which concluded that a strong predictor of nutritional progression was a low baseline weight percentile.[191]

Due to not having a control group, we are unable to infer that newborn screening has contributed to better height and weight z scores than an unscreened group. However, the majority of patients in this cohort reached a weight and length z score of 0 within two years as well as regaining their BW z score. We have also observed that infants display a faster rate of weight catch up compared with length.

In order to fully evaluate the nutritional benefits of newborn screening it is vital that similar research is performed in a prospective manner, to assess the time point at which these individuals reach their height and weight potential and what other factors influence this.

6.5 THE NARMAX MODEL - A PREDICTOR OF INFANT LENGTH AND WEIGHT Z SCORES

Previous attempts have been made to assess severity of CF and devise prognostic tools for measure of lung disease such as the Brasfield score and the Shwachman-Kulczycki (SK) severity indicator, however these scoring systems are subjective with potential for inter-rater differences.[192] To our knowledge, this novel approach is the first to attempt prediction of nutritional parameters of infants with cystic fibrosis identified by newborn screening, using unique and specific demographic data relevant to that patient. This innovative tool will allow paediatricians to intensify medical therapy should the patient be deemed, 'at risk' and consider new approaches to treatment. For patients identified with a good length and weight z score from birth, this will provide reassurance to parents and families of these children and should encourage them to adhere to treatment and engage appropriately with the cystic fibrosis team.

6.6 SUMMARY OF CONCLUSIONS

This study presents a seven-year service evaluation of all babies screened within the West Midlands from 1st November 2007 to the 31st October 2014. This is the second study of its kind, evaluating the relatively new CF NBS program within the United Kingdom. It is however the first to demonstrate the clinical outcomes, with particular focus on nutritional data and the first to attempt to create a nutritional prognostic indictor, through mathematical modelling.

Due to treatment advances, multi-professional involvement and care at specialist centres, the life expectancy of CF patients continues to increase. With continual improvement of clinical outcomes, the need for clinicians to predict prognosis for these patients at an individual and group level, arises. It has long been known that prevention and early intervention have been shown to be the most successful in terms of combating nutritional failure.[184] By introducing a novel approach for prediction estimation, there is potential to decrease mortality in young patients, through earlier detection and intervention of patients in, 'at risk' groups. Our four NARMAX models provide prediction estimation calculations of weight and length z scores at age one and two. These enable us to:

1. Identify patients at risk of under nutrition hence making us more aware of the need to follow closely the nutritional progression of these patients. We can also be more aggressive with treatment earlier on and have a lower threshold for interventions such as enteral feeding and nutritional supplementation.

2. In patients whose prediction estimations are encouraging, this will provide reassurance to parents of newborns and provide a general target for clinicians and parents to work toward, together.

In moving forward, we aim to transfer to an era of preventative medicine, rather than curative. This review has provided clinicians with a suitable tool to identify struggling patients early on so they can intervene at an appropriate time point. In the near future, we hope to incorporate these algorithms into routine assessment of newly diagnosed CF patients within the West Midlands and evaluate the validity of it in clinical practice.

7.0 RECOMMENDATIONS FOR UK CLINICAL PRACTICE

Given our experience in this study the following recommendations would benefit future retrospective studies analysing NBS and nutritional outcomes. We plan to implement these at the two tertiary CF centres that have been involved in this study.

Particular recommendations include:

- Ensure sweat tests are always undertaken. There is a continual need for a sweat test to confirm diagnosis.
- Ensure repeat genetic testing is performed on children with a positive NBS.
- To encourage the recording of birth length and birth head circumference.
- To ensure that length and head circumference are recorded at each CF clinic in the first 2 years.
- Consider reporting anthropometric data as z scores in future research plans.
- Use our model to estimate future weight and length to identify children at high risk of poor nutritional outcomes.
- Ensure UK NBS program continues to function well
- Clinicians should have a very low threshold for performing/rechecking sweat tests
- Infants categorized as CFSPID, should be managed as per internationally agreed recommendations.

8.0 FUTURE RESEARCH RECOMMENDATIONS

This work has highlighted several avenues that could be explored further where evidence is lacking. To improve care of newborn screened infants with CF, high quality research in the following areas should be considered:

1) Extend our study to gain anthropometric, microbiology and lung function data on these children as they get older.
2) Use our model prospectively in order to determine its validity in clinical practice.
3) A longer, prospective cohort study to evaluate additional factors affecting nutritional prognosis. Other nutritional parameters that would be useful to include would be; maternal and paternal birth weight, calorie consumption, adherence to PERT, activity levels, number of chest infections/hospital admissions and social environment.
4) A longitudinal prospective study examining the effect of breast feeding/formula feeding on the nutritional parameters of infants in early life.
5) A study to determine a standardized protocol for how to assess and document nutritional indices in CF infants.
6) A study to define the term, 'malnutrition' in CF children to enable us to identify infants failing to reach their target nutritional indices.
7) A retrospective analysis evaluating the reasons for misdiagnosis in the, 'affected, not detected' group.

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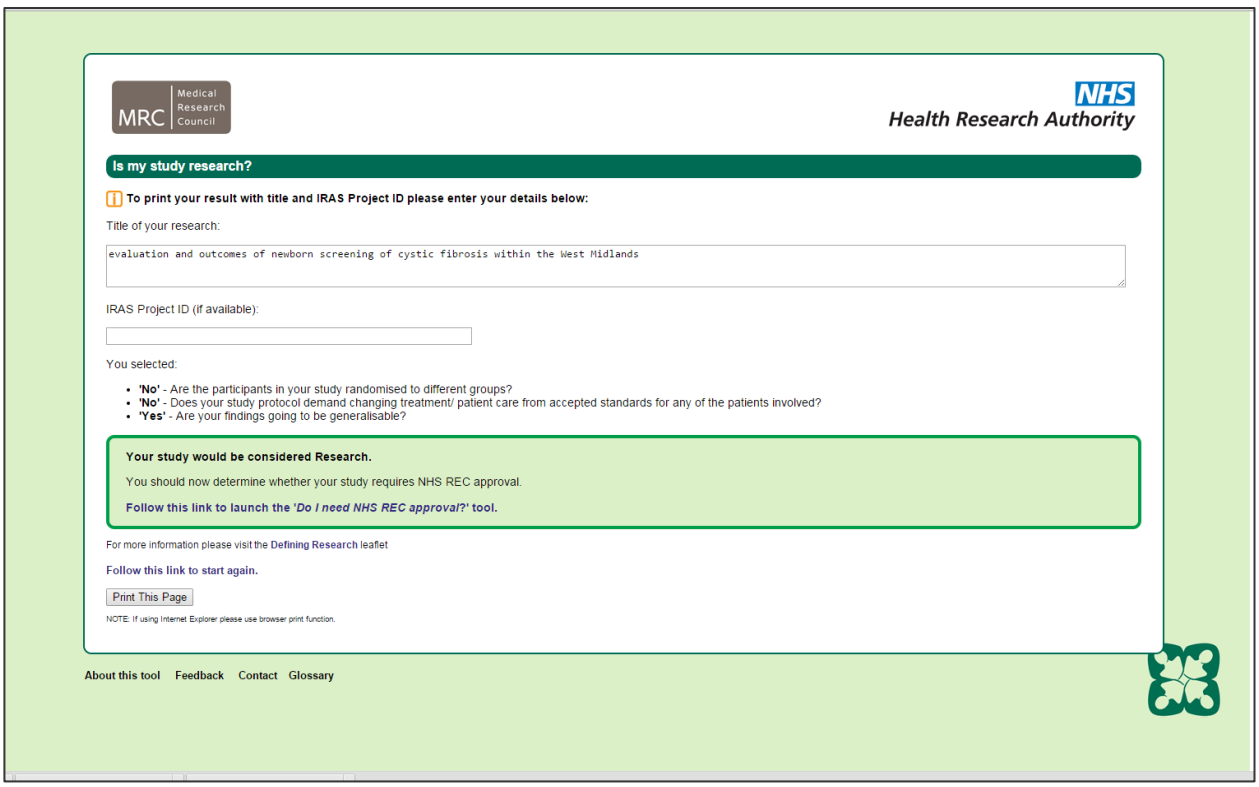
APPENDIX A

http://www.hra-decisiontools.org.uk/research/



APPENDIX B

NHS
National Institute for Health Research
Clinical Research Network

CERTIFICATE of ACHIEVEMENT

This is to certify that

katie patterson

has completed the course

Introduction to Good Clinical Practice eLearning (Secondary Care)

September 2, 2015

Modules completed:

Introduction to Research in the NHS
Good Clinical Practice and Standards in Research
Study Set Up and Responsibilities
The Process of Informed Consent
Data Collection and Documentation
Safety Reporting

This course is worth 4 CPD credits

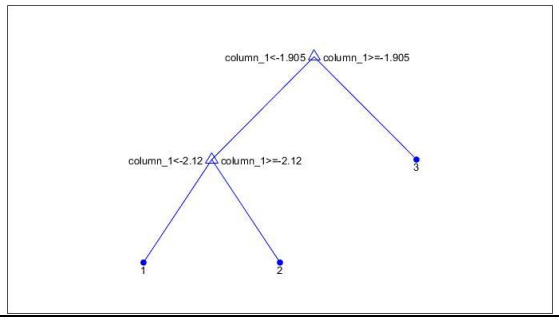
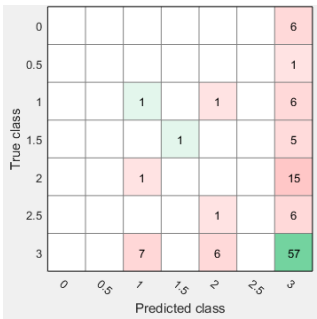
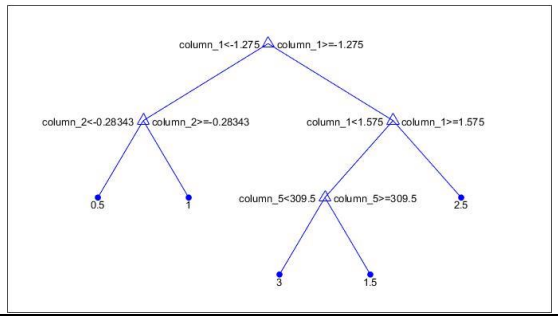
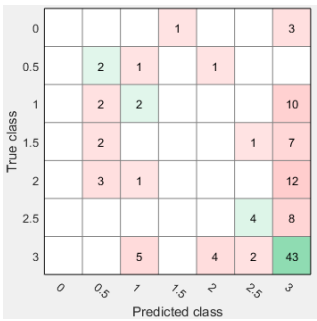
CPD
The CPD Certification Service

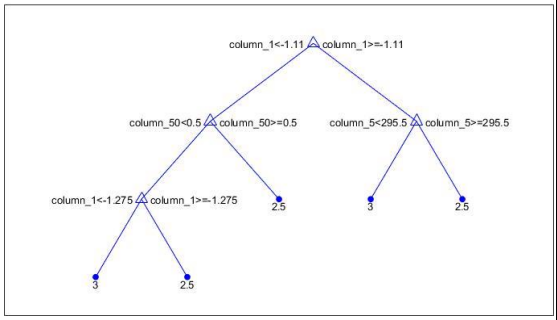
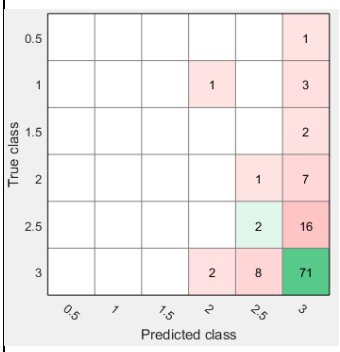
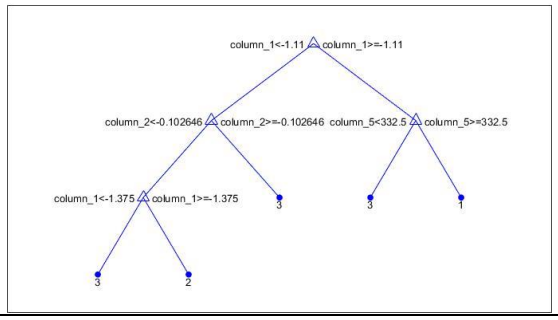
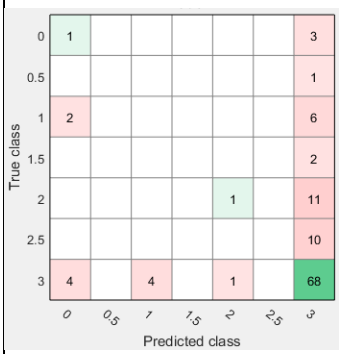
National Institute for Health Research
Clinical Research Network

Patient number	Gender	Gene 1	Gene 2	BW z score	Gestation	FE1	MI
S27	M	Phe508del	Phe508del	0.31	41	<15	Y
S28	F	Phe508del	c.2184delA	0.69	38	<15	Y
S34	F	Phe508del	Phe508del	1.16	39	25	Y
B5	M	Phe508del	c.489+1G>T	0.31	41	<15	N
B14	M	Phe508del	Phe508del	-0.26	40	<15	N
B26	F	Phe508del	Phe508del	0.1	38	<15	N
B28	M	Phe508del	Phe508del	1.6	42	20	N
B37	F	Phe508del	Phe508del	-0.29	?	<15	N
B42	M	Phe508del	thr1122LysfsX12	-0.82	?	?	N
B44	M	Phe508del	Phe508del	1.3	?	<15	N
B54	F	Phe508del	Phe508del	1.22	39	<15	N
B55	F	Phe508del	p.Glu60*	1.14	40	<15	N
B63	F	Phe508del	c.1585-1G>A	0.25	40	<15	N
B75	M	Phe508del	p.Lys1177SerfsX15	-0.37	38	?	N
B82	F	Phe508del	Phe508del	0.44	39	25	N
B98	M	Phe508del	Phe508del	-0.26	39	<15	N
B99	F	Phe508del	Phe316Leufs*12	2.38	42	<15	N
B105	F	Phe508del	Phe508del	1.55	40	<15	N
B108	M	Phe508del	C.3495delG	0.31	39	22	N

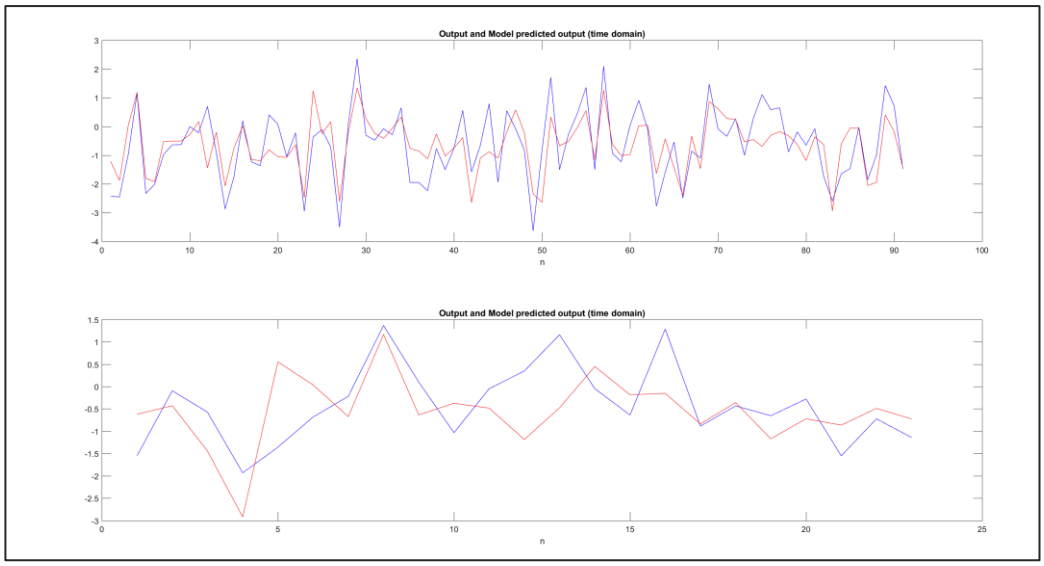
APPENDIX C: *Demographics of the group who failed to regain their BW z score within 2 years*

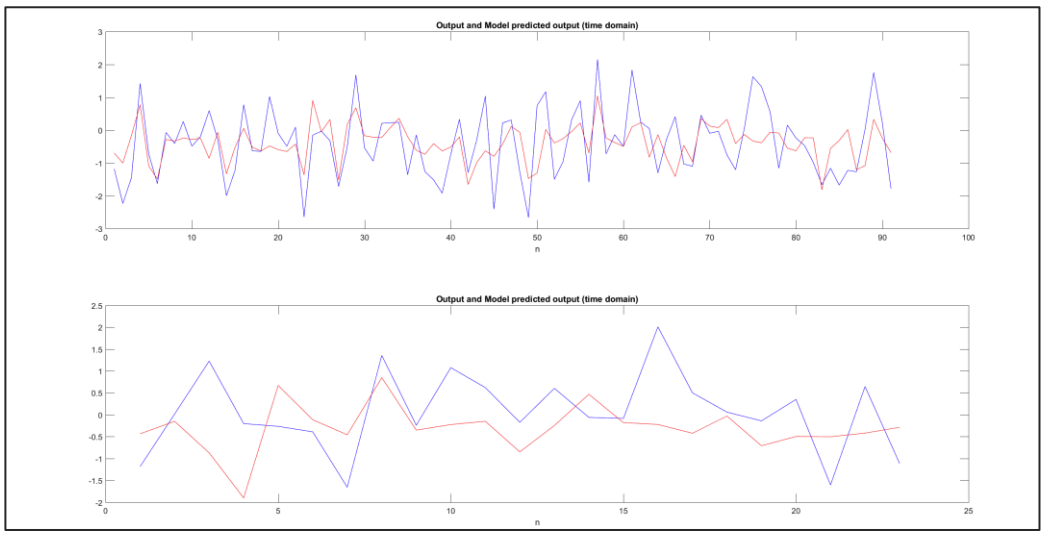
APPENDIX D

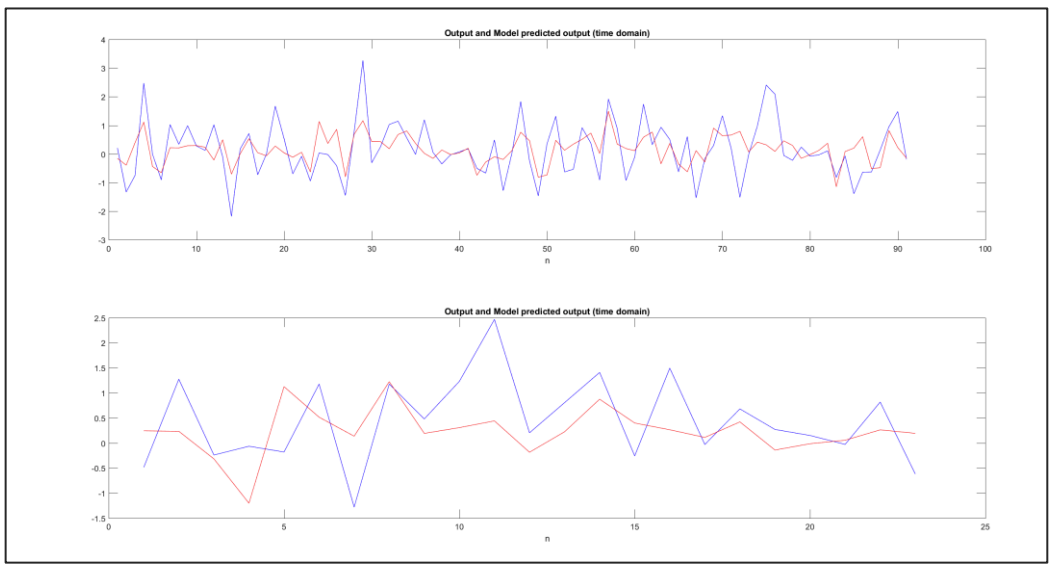
Ouput	Model	Error	Confusion matrix
Raters' score for length profile in first year of life.	1 if BW z score <-1.905 then node 2 elseif BW z score >=-1.905 then node 3 else 3 2 if BW z score <-2.12 then node 4 elseif BW z score >=-2.12 then node 5 else 1 3 class = 3 4 class = 1 5 class = 2 	51.8% (57.9%)	
Raters' score for length profile in second year of life.	1 if BW z score <-1.275 then node 2 elseif BW z score >=-1.275 then node 3 else 3 2 if BW_fc z score<-0.28343 then node 4 elseif BW_fc z score >=-0.28343 then node 5 else 1 3 if BW z score <1.575 then node 6 elseif BW z score >=1.575 then node 7 else 3 4 class = 0.5 5 class = 1 6 if IRT <309.5 then node 8 elseif IRT >=309.5 then node 9 else 3 7 class = 2.5 8 class = 3 9 class = 1.5 	44.7% (56.1%)	

Raters' score for weight profile in first year of life.	1 if BW z score <-1.11 then node 2 elseif BW z score >=-1.11 then node 3 else 3 2 if MI= 0 then node 4 elseif MI =1 then node 5 else 3 3 if IRT<295.5 then node 6 elseif IRT>=295.5 then node 7 else 3 4 if BW z score <-1.275 then node 8 elseif BW z score >=-1.275 then node 9 else 3 5 class = 2.5 6 class = 3 7 class = 2.5 8 class = 3 9 class = 2.5 	64.0% (86.0%)	
Raters' score for weight profile in second year of life.	1 if BW z score <-1.11 then node 2 elseif BW z score >=-1.11 then node 3 else 3 2 if BW_fc z score<-0.102646 then node 4 elseif BW_fc z score >=-0.102646 then node 5 else 3 3 if IRT<332.5 then node 6 elseif IRT>=332.5 then node 7 else 3 4 if BW z score <-1.375 then node 8 elseif BW z score >=-1.375 then node 9 else 2 5 class = 3 6 class = 3 7 class = 1 8 class = 3 9 class = 2 	61.4% (70.2%)	

APPENDIX E







Regression model schematics for length year 1, length year 2 and weight year 2, respectively)