# Dissecting mechanisms involved in the aetiological overlap between Type 2 diabetes and cancer

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by

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## Memorandum

The work in this thesis is the original work of the author. Experiments were undertaken at the Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, under the supervision of Professor Mark McCarthy and Dr Anna Gloyn. Funding was provided by the Medical Research Council.

The work on immunoblot analysis of components of the PI3K-AKT pathway in adipose and muscle tissue samples was performed by Simon Rudge and Qifeng Zhang as part of a collaboration with Professor Michael Wakelam's group at the Babraham Institute in Cambridge. The work on *PTEN* sequencing in control subjects and analysis of common variation at the *PTEN* locus was performed by Martijn van de Bunt, a DPhil student in the Gloyn/McCarthy lab.

The work on *ANRIL* transcript profiling in human islets, and development of an siRNA targeting *ANRIL* for gene knockdown was performed with Dr Theo Kyriakou as part of a collaboration with Professor Hugh Watkin's group at the Wellcome Trust Centre for Human Genetics.

I hereby state that no part of this thesis has been submitted for any other degree at this or another university. This thesis is approximately 69, 000 words.

# Dissecting mechanisms involved in the aetiological overlap between Type 2 diabetes and cancer

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In candidature for the degree of Doctor of Philosophy Hilary term 2013

## Abstract

The advent of large scale genome wide association studies (GWAS) have uncovered a plethora of proposed susceptibility loci, most previously not connected to T2D pathogenesis. One of the most intriguing GWAS discoveries is the frequency with which T2D susceptibility loci map close to genes with roles in cell-cycle regulation. This previously unsuspected link between genes known for being dysregulated in cancer and T2D pathogenesis is supported by epidemiological evidence linking T2D and cancer. The aims of this thesis were to gain insight into T2D pathogenesis by studying specific examples involved in the complex interplay between T2D and cancer.

I investigated two loci already known for their roles in cancer pathogenesis: the tumour suppressor *PTEN* locus on chromosome 10 and the *CDKN2A* and *CDKN2B* locus at chromosome 9p21 (Chr9p21). PTEN is known to negatively regulate the phosphatidylinositol-3-kinase/AKT (PI3K-AKT) pathway which mediates many of the metabolic downstream effects of insulin signalling. Rodent data supports a role for PTEN in insulin action: tissue-specific *Pten* knockout and *Pten* haploinsufficient mice display improved glucose tolerance and insulin sensitivity. I recruited individuals with a rare cancer predisposition syndrome, Cowden syndrome, due to *PTEN* mutations and assessed their glucose homeostasis. Using oral glucose tolerance tests, euglycaemic hyperinsulinaemic clamp studies and adipose/muscle tissue biopsies, I have demonstrated that these *PTEN* haploinsufficient individuals display heightened insulin sensitivity and that this is via amplified signalling through the PI3K-AKT pathway.

The Chr9p21 T2D susceptibility loci are proximal to the tumour suppressors *CDKN2A* and *CDKN2B.* A noncoding RNA, ANRIL, is transcribed close to the regions of T2D association and a hypothesised mechanism for the T2D association is that the T2D variants affect *ANRIL* expression leading to altered *CDKN2A/CDKN2B* expression. Through a series of molecular studies I have demonstrated that *ANRIL* may have regulatory effects upon *CDKN2A/CDKN2B* expression in human islets. Although there is some rodent evidence suggesting a role for *CDKN2A* in regulation of beta-cell mass and glucose homeostasis, there is no direct evidence linking CDKN2A/B to glucose homeostasis in humans. I recruited individuals with rare *CDKN2A* mutations and performed oral and intravenous glucose tolerance tests to assess beta-cell function. The sample size precluded definitive conclusions but interesting trends were observed and further studies are planned. Collectively, the data presented in this thesis illustrate some mechanisms that may contribute to the aetiological overlap between T2D and cancer.

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## Table of Contents

## List of Abbreviations

| | |
|---|---|
| **ADP** | adenosine diphosphate |
| **AMPK** | AMP-activated protein kinase |
| **ANRIL** | antisense non-coding RNA in the INK4 locus |
| **ATP** | Adenosine-5'-triphosphate |
| **AUC** | area under curve |
| **B2M** | beta-2-microglobulin |
| **BAT** | brown adipose tissue |
| **BMI** | body mass index |
| **BSA** | Bovine serum albumin |
| **CAD** | coronary artery disease |
| **CDK** | cyclin-dependent kinase |
| **CDKN2A** | cyclin-dependent kinase inhibitor 2A |
| **CDKN2B** | cyclin-dependent kinase inhibitor 2B |
| **cDNA** | complementary DNA |
| **ChiP-Seq** | chromatin immunoprecipitation sequencing |
| **Chr** | chromosome |
| **CMRL** | Connaught Medical Research Laboratories |
| **CS** | Cowden syndrome |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DI** | disposition index |
| **DNA** | deoxyribonucleic acid |
| **DPP-4** | dipeptidyl-peptidase-4 |
| **DXA** | dual X-ray absorptiometry |
| **ERK** | extracellular signal-regulated kinase |

| | |
|---|---|
| **FACS** | fluorescence-activated cell sorted |
| **FAMMM** | familial atypical multiple mole melanoma syndrome |
| **FDG-PET** | fluorodeoxyglucose-positron emission topography |
| **Fox01** | forkhead box protein 01 |
| **G6P** | glucose 6 phosphate |
| **GAPDH** | glyceraldehyde 3-phosphate dehydrogenase |
| **GCK** | glucokinase |
| **GFP** | green fluorescent protein |
| **GKRP** | glucokinase regulatory protein |
| **GLP-1** | glucagon-like peptide-1 |
| **GLUT 1/2/3/4** | glucose transporter 1/2/3/4 |
| **GS** | Glycogen synthase |
| **GSK3** | glycogen synthase kinase 3 |
| **GUSB** | beta glucuronidase |
| **GWAS** | genome wide association studies |
| **HBSS** | Hank's buffered salt solution |
| **HEK** | human embryonic kidney |
| **HKGs** | house keeping genes |
| **HNF1A** | hepatocyte nuclear factor 1-alpha |
| **HNF1B** | hepatocyte nuclear factor 1 homeobox B |
| **HNF4A** | hepatocyte nuclear factor 4-alpha |
| **HPLC-MS** | high-performance liquid chromatography-mass spectrometry |
| **HPRT1** | hypoxanthine-guanine phosphoribosylytransferase 1 |
| **HsCRP** | highly sensitive C-reactive protein |
| **HUVECs** | human umbilical vein endothelial cells |
| **IDF** | International Diabetes federation |

| | |
|---|---|
| **IGF-1** | insulin like growth factor 1 |
| **IGFBP** | IGF binding protein |
| **IGT** | impaired glucose tolerance |
| **IL-6** | interleukin 6 |
| **InsI** | insulinogenic index |
| **IPF1** | insulin promoter factor 1 |
| **IRS1** | insulin receptor substrate 1 |
| **IVGTT** | intravenous glucose tolerance test |
| **$K_{ATP}$ channels** | ATP-sensitive potassium channels |
| **KRB** | Krebs-Ringer buffer |
| **LKB1** | liver kinase B1 |
| **MDM2** | mouse double minute 2 homolog |
| **MODY** | maturity-onset diabetes of the young |
| **MRI** | magnetic resonance imaging |
| **mRNA** | messenger RNA |
| **mTOR** | mammalian target of rapamycin |
| **ncRNA** | non-coding RNA |
| **NEFAs** | non-esterified fatty acids |
| **NeuroD1** | neurogenic differentiation factor 1 |
| **NPH insulin** | neutral protamine Hagedorn insulin |
| **OBB** | Oxford Biobank |
| **OCDEM** | Oxford Centre for Diabetes, Endocrinology and Metabolism |
| **OGTT** | oral glucose tolerance test |
| **OR** | odds ratio |
| **OXCIT** | Oxford Consortium for Islet Transplantation |
| **PAI-I** | plasminogen activator inhibitor-1 |

| | |
|---|---|
| **PBS** | phosphate buffered saline |
| **PCR** | polymerase chain reaction |
| **PET** | positron emission tomography |
| **PGK1** | phosphoglycerate kinase 1 |
| **PI3K-AKT** | phosphatidylinositol-3-kinase AKT (or protein kinase B) |
| **PIP2** | phosphatidylinositol biphosphate |
| **PIP3** | phosphatidylinositol triphosphate |
| **PNDM** | permanent neonatal diabetes |
| **PPAR** | peroxisome proliferator-activated receptor |
| **PPIA** | peptidylprolyl isomerise A |
| **PRCs** | polycomb repressive complexes |
| **PTEN** | phosphatase and tensin homolog |
| **QRT-PCR** | quantitative reverse transcription PCR |
| **RACE** | rapid amplification of cDNA ends |
| **RB1** | retinoblastoma 1 |
| **RCT** | randomised control trial |
| **RIA** | radioimmunoassay |
| **RNA** | ribonucleic acid |
| **RPMI** | Roswell Park Memorial Institute |
| **SD** | standard deviation |
| **SEM** | standard error of the mean |
| **SHBG** | sex hormone binding globulin |
| **shRNA** | short hairpin RNA |
| **siRNA** | small interfering RNA |
| **SNPs** | single nucleotide polymorphisms |
| **STAT** | signal transducer and activator of transcription protein |

| | |
|---|---|
| **T1D** | type 1 diabetes |
| **T2D** | type 2 diabetes |
| **TGF-β1** | transforming growth factor beta-1 |
| **TSC2** | tumour suppressor tuberous sclerosis complex 2 |
| **TZDs** | thiazolidinediones |
| **UBC** | ubiquitin C |
| **VSMCs** | vascular smooth muscle cells |
| **WHO** | World health organisation |

# Chapter 1

## Introduction

# Chapter 1

## 1.1 The growing epidemic of type 2 diabetes

Type 2 diabetes (T2D) is a burgeoning public health problem; by 2030, the number of adults diagnosed with T2D worldwide is expected to rise from the current count of 366 million to 552 million (WHO 2011; www.who.int/**diabetes**/facts/en/; IDF 2012 www.**idf**.org/**diabetes**atlas/5e/Update**2012**). In America, diabetes prevalence in 2007 was 10.7% of those aged above 20 years, with an estimated 1.6 million new cases per year and T2D the most common form, accounting for ~95% prevalent cases (www.cdc.gov/**DIABETES**/pubs/factsheet11.htm[u). In the UK there are currently around 3.8 million people living with diabetes, and, by 2035, this is expected to increase to 6.3 million (Hex et al. 2012). Care for those with T2D presently consumes around 10% of the NHS budget with the cost of direct patient care (including treatment, intervention and complications) for those living with diabetes estimated at £9.8 billion (£1 billion for Type 1 diabetes and £8.8 billion for Type 2 diabetes) (Hex et al. 2012). The T2D problem is global, with prevalence in the developing world predicted to double between 2000 and 2030 (WHO 2011).

## 1.2 The pathogenesis of T2D is incompletely understood

For a disease reaching epidemic proportions, reducing in age at diagnosis and affecting all populations, still relatively little is known of its pathophysiology. In 1951 Lawrence and Bornstein measured the amount of insulin in the blood and noted that older and obese patients with diabetes have insulin, but those who were young have none (Bronstein and Lawrence 1951). They were amongst the first to recognise the separate entities of type 1 diabetes (T1D) and T2D. Since then it is well recognised that insulin resistance is an early feature of the pathogenesis of T2D

and more prevalent in this diabetes subtype than T1D. Insulin resistance is generally defined as a reduced ability of a given concentration of insulin to lower blood glucose. One common model for the pathophysiology of T2D involves a prodrome of insulin resistance necessitating high insulin secretion: subsequently a period of impaired glucose tolerance ensues when beta-cells begin to dysfunction and can lead to development of T2D. The 'normal' beta-cell can adapt to changes in insulin resistance: a decrease in insulin action is accompanied by an increase in insulin secretion and vice versa (Figure 1.1) (Stumvoll et al. 2005). In people with normal glucose tolerance, a hyperbolic relationship exists between beta-cell function and insulin sensitivity (Turner et al. 1979; Kahn et al. 1993). Deviation from this hyperbola occurs in impaired glucose tolerance and T2D when beta-cell function is inadequately low for the degree of insulin resistance (Kahn et al. 1993; Kahn 2003; Stumvoll et al. 2005).

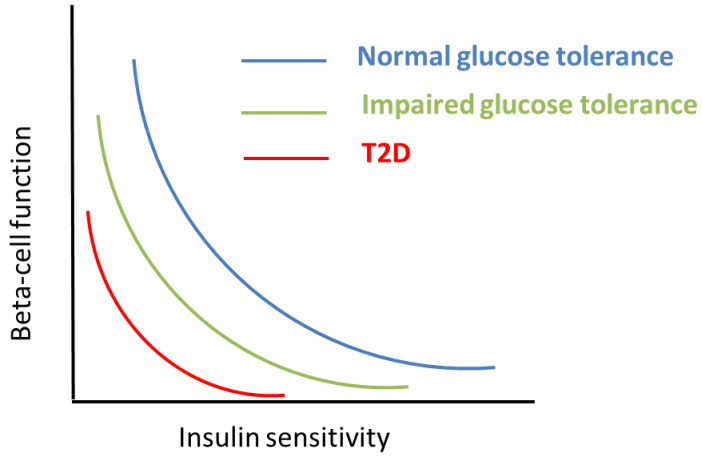



**Figure 1.1** Adapted from Stumvoll et al 2005 (Stumvoll et al. 2005). Hyperbolic relationship between insulin sensitivity and beta-cell function in people with normal glucose tolerance. Deviation from this hyperbola occurs initially in impaired glucose tolerance (IGT) when there is insulin resistance with beta-cell compensation and then T2D when insulin resistance occurs without beat-cell compensation.

Therefore both insulin resistance *and* beta-cell dysfunction appear important for T2D development, although T2D can occur without insulin resistance compared to beta-cell dysfunction which is a critical factor in T2D development.

### 1.3 Possible mechanisms underlying insulin resistance and beta-cell failure in T2D

Several mechanisms have been proposed to contribute to the development of insulin resistance including inflammatory cytokines, non-esterified fatty acids (NEFAs), adipokines, mitochondrial dysfunction and changes in gastrointestinal microorganisms (Stumvoll et al. 2005; Johnson and Olefsky 2013). Proposed pathological mechanisms leading to progressive beta-cell dysfunction include glucotoxocity, lipotoxicity and amyloid formation (Lorenzo et al. 1994; Stumvoll et al. 2005; Poitout and Robertson 2008; Giacca et al. 2011), although it is acknowledged that a potential role for loss of beta-cell mass (or inadequate beta-cell mass initially) may also co-exist with the beta-cell dysfunction (Sakuraba et al. 2002; Butler et al. 2003; Yoon et al. 2003; Meier et al. 2008; Giannini and Caprio 2013).

The study of severe insulin resistance syndromes has also given insights into the pathogenesis of more common insulin resistance and T2D (Parker et al. 2011; Semple et al. 2011). For instance, increased hepatic glucose production is recognised in T2D and insulin resistance (Lin and Accili 2011) but is also a feature of insulin receptoropathies and lipodystrophy syndromes suggesting a hepatic defect in the insulin activated PI3K-AKT pathway that would normally inhibit Fox01 (Forkhead box protein 01) activation (Figure 1.2), thus leading to increased hepatic gluconeogenesis (Stears et al. 2012).

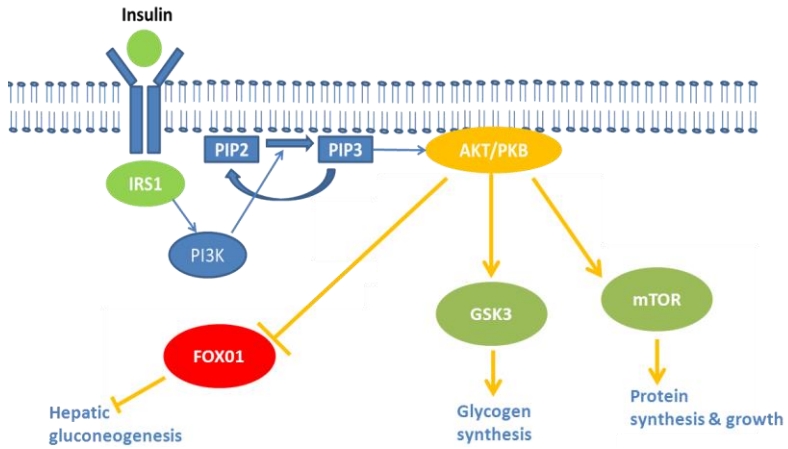



**Figure 1.2** Schematic of insulin stimulation of PI3K-AKT pathway and subsequent inhibition of FOX01 which would otherwise go on to suppress hepatic gluconeogenesis. FOX01, Forkhead box protein 01; GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin.

Clinical and biochemical overlap between lipodystrophy syndromes and insulin resistance has also added weight to the 'lipid overflow' hypothesis that describes lipid-induced insulin resistance (Kim et al. 2000; Virtue and Vidal-Puig 2010). Briefly, a chronically increased positive energy balance exceeds a presumed fixed capacity of adipose tissue to expand, leading to adipocyte failure: the ensuing oxidative stress and inflammatory infiltrate leads to dysfunctional lipolytic regulation, adipose tissue function and subsequent lipotoxicity in other peripheral tissues (Neuschwander-Tetri 2010). This is then believed to lead to the deposition of 'ectopic fat' around skeletal muscle, liver and pancreas (amongst other organs) compromising their normal metabolic function (Savage et al. 2007; Erion and Shulman 2010; Samuel et al. 2010). The potential contribution of mitochondrial dysfunction to this process of ectopic fat accumulation and subsequent insulin resistance is also recognised (Morino et al. 2006), although the possible bidirectionality of this relationship has also been highlighted by studies in patients with extreme insulin resistance (Sleigh et al. 2011).

### 1.3.1 The role of beta-cell dysfunction in diabetes pathogenesis

Although insulin resistance is recognised in many patients with T2D, a degree of beta-cell dysfunction is present in *all* individuals with the condition, highlighting the fundamental role of the beta-cell in maintaining normoglycaemia. As with the monogenic insulin resistance syndromes, monogenic causes of beta-cell dysfunction have emphasised the importance of various components in normal beta-cell function in addition to underlining the role of the beta-cell in diabetes pathogenesis. Early pedigree-based linkage analysis studies and candidate gene screening revealed several genes containing rare causal mutations responsible for monogenic forms of diabetes including maturity-onset diabetes of the young (MODY) and permanent neonatal diabetes (PNDM). Although monogenic forms of diabetes are rare, accounting for at most only ~2% diabetes, their discovery has given valuable insight not only into diabetes pathogenesis but also directed targeted treatment to specific diabetes subtypes (Thanabalasingham and Owen 2011). For example, the

discovery of activating mutations in the gene encoding the Kir6.2 subunit of the ATP-sensitive potassium channels ($K_{ATP}$ channels) in pancreatic beta-cells, as a cause of permanent neonatal diabetes, highlighted the importance of this channel in beta-cell function but also demonstrated the specific benefit of sulphonylureas in this group of patients (Gloyn et al. 2004; Pearson et al. 2006). In fact the importance of the $K_{ATP}$ channel in diabetes is demonstrated by the fact that heterozygous activating mutations in *KCNJ11* or *ABCC8* (encoding the Kir6.2 and SUR subunits of the $K_{ATP}$ channel respectively) cause diabetes with a varying spectrum of severity depending on the mutation (Gloyn et al. 2004; Gloyn et al. 2005; Babenko et al. 2006)

MODY can be broadly divided into glucokinase MODY and transcription factor MODY (which includes mutations in HNF-4alpha, HNF-1alpha, IPF-1, HNF-1beta and NeuroD1) depending on the mutated component. The role of these mutations in causing monogenic forms of diabetes demonstrates the importance of these factors within the beta-cell (Figure 1.3). The clinical relevance of recognising the distinct monogenic forms of diabetes is also demonstrated by the fact that diagnosis of specific subtypes has impact on therapeutic options and prognosis for patients (Thanabalasingham and Owen 2011).

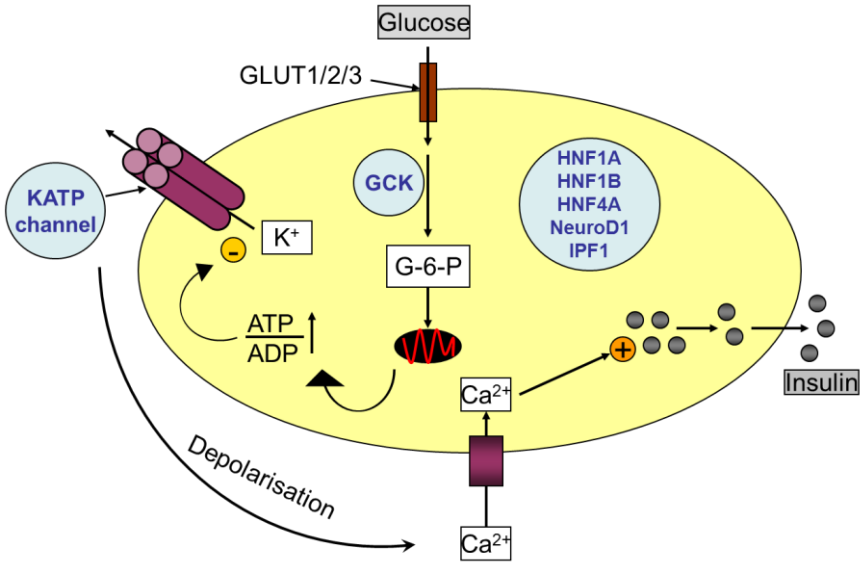



**Figure 1.3** A schematic of glucose stimulated insulin secretion in the pancreatic beta-cell. Adapted from A Pal and AL Gloyn : Systems biology of regulated exocytosis in pancreatic beta-cells: Genetically programmed defects in beta-cell function. Springer Verlag 2011. Glucose enters the cell through the GLUT transporter and is phospohorylated by glucokinase to glucose -6-phosphate , the main substrate of glycolysis. Glucose metabolism increases ATP levels within the cell which closes the KATP channel leading to membrane depolarization. Subsequently the voltage-gated calcium channels open, whereby calcium entry triggers insulin exocytosis, which requires transcription of the insulin gene via the pancreatic transcription factors. The genes implicated in monogenic diabetes and crucial for this mechanism of insulin release are in blue and highlighted in blue circles.

In summary, the study of rarer forms of insulin resistance and beta-cell dysfunction have demonstrated the importance of various components in diabetes pathogenesis, not least with respect to the implication of genes also harbouring common variants contributing to genetic predisposition to common T2D (Gloyn et al. 2003; Gudmundsson et al. 2007; Winckler et al. 2007). By analogy, the clinical heterogeneity of common T2D may also, in principle, be concealing subtypes of disease that could be revealed by deeper inspection of data from genome-wide association studies (GWAS). The genetics of common T2D has illuminated several factors that contribute to beta-cell dysfunction leading to T2D development, and has subsequently given novel insights into the pathogenesis of T2D (discussed in the following section). A complete appreciation of all the pathophysiological mechanisms at play in the development of common T2D is important ultimately to identify novel pathways for therapeutics, help in stratification of patients with T2D, individualisation of treatment and also in biomarker discovery to specify distinct subtypes of disease. The functional follow-up of genetic variants implicated in T2D-risk by GWAS is challenging, and one of the focusses of this thesis is to assess the functional implications of specific T2D-risk variants and investigate the mechanisms by which these variants impart their risk.

### 1.4 The study of common risk variants and novel insights into T2D pathogenesis

The discovery of less penetrant but more common risk variants for T2D has come with the advent of large scale GWAS which have the advantage of not being confined to suspected biological candidates in T2D pathogenesis as they follow the 'biologically agnostic' approach of seeking out any common variation that appears to associate with disease. The first round of GWAS in 2007 confirmed known loci at *PPARG* (Altshuler et al. 2000), *KCNJ11* (Gloyn et al. 2003) and near *TCF7L2* (first identified ahead of the GWAS era by detailed association analysis of a previously-demonstrated linkage signal on chromosome 10q (Grant et al. 2006)), in addition to revealing novel loci including signals near *CDKAL1*, *HHEX*, *SLC30A8*, *IGF2BP2* and *CDKN2A* (Saxena et al. 2007; Scott et al. 2007; Sladek et al.

2007; Steinthorsdottir et al. 2007; Zeggini et al. 2007). The incremental aggregation of individual GWAS studies into better-powered meta-analyses has driven the total number of common variant signals for T2D over 60 (Kooner et al. 2008; Dupuis et al. 2010; Voight et al. 2010; Cho et al. 2012; Morris et al. 2012; Palmer et al. 2012). Therefore there is much potential for gaining fresh insight into T2D pathogenesis in the follow-up of these novel T2D risk loci.

One of the insights gained from GWAS for T2D pertains to the relative contributions of insulin resistance and beta-cell dysfunction to diabetes pathogenesis: there has been a preponderance of T2D signals being driven primarily by beta-cell dysfunction as a number of variants show associations with parameters of insulin secretion and beta-cell function (Grarup et al. 2007; Pascoe et al. 2007; Staiger et al. 2007; Florez 2008; Palmer et al. 2008; Voight et al. 2010). A minority of identified variants point to constitutive defects involving the tissues of insulin action such as fat, muscle and liver (these include the loci near *PPARG, KLF14, IRS1* and *ADAMTS9*) (Altshuler et al. 2000; Rung et al. 2009; Voight et al. 2010; Morris et al. 2012) but in the cases of the loci near *FTO* and *MC4R*, the effect on insulin resistance is secondary to raised BMI (Frayling et al. 2007; Freathy et al. 2008; Morris et al. 2012).

The most rapid progress in gaining pathological insight from novel T2D loci has come when the association signal has been localised to a coding variant, defining the transcript of interest and providing better opportunities for functional validation. An example is the causal coding variant identified in *SLC30A8* which encodes the zinc transporter, ZnT8 (Nicolson et al. 2009; Wijesekara et al. 2010). The variant protein has been shown to display reduced zinc transport activity and these findings reveal the key role of zinc transport in insulin processing and secretion in the beta-cell (Nicolson et al. 2009; Wijesekara et al. 2010).

Another example of successful T2D coding variant follow up is the P446L variant in *GCKR* which encodes glucokinase regulatory protein (GKRP). This variant, which associates with fasting glucose and triglyceride levels (Saxena et al. 2007), has been shown to increase hepatic glucokinase activity via an effect of fructose -6- phosphate (at physiological levels) upon P446L-GKRP activity (Beer et al. 2009): this provides a plausible biological mechanism underlying the association of this variant with fasting glucose levels and also raised triglycerides given the enhanced glucokinase activity could lead to an increase in substrates used for lipogenesis (Beer et al. 2009). This is also an example of the importance of studying such putative mutational mechanisms in human model systems as far as possible, as introduction of the P446L variant into a rodent system produced no subsequent effects on rat GKRP despite a high degree of homology between proteins (Rees et al. 2012).

Despite the challenges faced in functional evaluation of non-coding variants (i.e. linking T2D risk variants to coding genes, and therefore underlying mechanisms, which may be greater than 100Kb away), further insights into T2D pathogenesis have come from the study of non-coding variants. For example, the follow-up of non-coding variants near *MTNR1B* and *TCF7L2* demonstrate the range of approaches employed to define underlying biological mechanisms. The T2D risk variants mapping close to *MTNR1B* (encoding the melatonin receptor 1B) link coordinated regulation of glucose homeostasis and circadian rhythm with T2D risk (Bouatia-Naji et al. 2009; Prokopenko et al. 2009). In contrast to *SLC30A8*, at this locus the common T2D-risk variants detected by GWAS map to non-coding sequence, and the assignment of the effect to *MTNR1B* (rather than one of the other nearby transcripts) comes from a combination of biological candidacy, functional analysis and resequencing studies, which have shown that rare coding variants within the *MTNR1B* gene are enriched in T2D cases over controls (Bonnefond et al. 2012).

Further insights into pathogenesis (although some of these remain speculative) came via the T2D-risk variants that were found to map intronic to *TCF7L2*, a gene for which there was no prior claim to biological candidacy. Definitive proof that *TCF7L2* is the gene via which the related risk variants exert their effect, remains elusive, exemplifying the challenges in connecting non-coding variants to their downstream effects. The combination of genetic fine-mapping and chromatin immunoprecipitation sequencing (ChiP-Seq) studies, has demonstrated that rs7903146, the most plausible candidate for the causal variant, influences the local chromatin state in human islets, though this could not be tied to *TCF7L2* expression (Gaulton et al. 2010). Furthermore, it has been shown that the TCF7L2 transcription factor, induces expression of a number of genes required for insulin secretion as well as beta-cell proliferation (Rulifson et al. 2007; Loder et al. 2008). If *TCF7L2* is indeed the culprit, it implicates the Wnt signalling pathway, recognised for its role in developmental processes, in T2D pathogenesis.

The functional follow up of the *KLF14* locus, which is an imprinted region with maternal specific attributed T2D risk (Voight et al. 2010), demonstrates the use of cis- and trans- expression quantitative trait loci (eQTLs) in discerning mechanisms of function (Kong et al. 2009; Small et al. 2011; Morris et al. 2012). Differential tissue *KLF14* expression had been shown to be associated with T2D-associated SNPs, the strongest T2D-association occurring with adipose tissue expression (Voight et al. 2010), and as this gene encodes a transcription factor, association of the T2D variant with expression of >16000 genes was tested for in 776 healthy female twins (Small et al. 2011). This showed significant associations for ten genes, of which several associated with metabolic phenotypes, and revealed KLF14 as a potential master transcriptional regulator (Small et al. 2011).

A final example of newly implicated pathways in T2D pathogenesis, and one of the over-arching themes of this thesis, is the frequency with which novel T2D-risk loci have mapped close to, or within cell-cycle genes (Voight et al. 2010; Morris et al. 2012). Pathway analyses attempt to map T2D associated transcripts to known pathways (Sun 2012): protein-protein interaction analyses use a variety of computational approaches based on sequence homology, gene co-expression and phylogenetic profiles developed for the genome-wide inference of interactions between pairs of proteins (Zhang et al. 2012). By combining data from several T2D GWAS in meta-analyses, pathway and protein-protein interaction analyses have demonstrated an enrichment for genes involved in cell-cycle regulation (Voight et al. 2010; Morris et al. 2012). These highlighted regulators of the G1 phase of mitosis in particular, with most implicated genes being cyclin-dependent kinase (CDK) inhibitors (*CDKN2A* and *CDKN2B*, *CDKN1C* and *CDKN2C*) and cyclins that activate CDKs (*CCNE2*, *CCND2*, *CCNA2*) (Morris et al. 2012). Many of these genes are strongly implicated in beta-cell mass regulation as they control *CDK4* or *CDK6*, which are known to be potent regulators of beta-cell replication (Rane et al. 1999; Fiaschi-Taesch et al. 2010).

This observed link between cell-cycle genes and T2D susceptibility loci is particularly intriguing, holding potential for previously unsuspected links to cancer pathogenesis and linking the two very common conditions of diabetes and cancer. Deciphering this genetic link is as challenging as the follow-up of other novel T2D risk loci given that most of the loci linked to cell-cycle genes are in non-coding regions of the genome. Another factor limiting biological inference and clinical translation, at loci linked to cell-cycle genes and generally, has been the modest effect sizes of most identified susceptibility loci (OR ~1.05 -1.35) (Frayling et al. 2007; Voight et al. 2010; Morris et al. 2012).

### 1.5 T2D-susceptibility loci linked to genes involved in cell-cycle regulation and cancer

As aforesaid, T2D-susceptiblity loci are enriched for cell-cycle genes (Voight et al. 2010; Morris et al. 2012), many of which are known to be expressed in islets (Zeggini et al. 2007): this raises the possibility that the T2D-predisposing effects of these variants are related to variation in beta-cell proliferation and subsequent beta cell mass. In the first tranche of susceptibility loci identified in 2007, risk loci were identified at the *CDKAL1* locus on chromosome 6 (Saxena et al. 2007; Scott et al. 2007; Sladek et al. 2007; Zeggini et al. 2007) and expression of *CDKAL1* mRNA was demonstrated in human pancreatic islets and skeletal muscle (Zeggini et al. 2007). The *CDKAL1* gene encodes a 65 kDa protein– cyclin-dependent kinase 5 regulatory subunit associated protein 1-like 1 (CDKAL1) of unknown function. It does share significant protein domain and amino acid homology with CDK5 regulatory subunit associated protein 1 (CDK5RAP1) which is known to be an inhibitor of CDK5 activation (Ching et al. 2002). CDK5 is thought to be involved in the regulation of beta-cell function via p35/CDK5 complexes that down-regulate insulin expression (Wei et al. 2005; Ubeda et al. 2006). In mice, *Cdkal1* deletion was associated with impaired insulin secretion and glucose homeostasis (Okamura et al. 2012) consistent with data in humans showing *CDKAL1* variants to associate with reduced first-phase insulin secretion (Groenewoud et al. 2008). However, the proposed role of *CDKAL1* as a cell-cycle regulator has been somewhat misleading as it subsequently transpired that this gene encodes a methylthiotransferase (Arragain et al. 2010; Wei et al. 2011), localises to the endoplasmic reticulum in mice and is probably required for insulin processing in the beta-cell (Wei and Tomizawa 2011).

Another example of T2D-risk loci linked to cell-cycle genes are the loci identified at *CDC123/CAMK1D* (Shu et al. 2010; Imamura et al. 2011). *CDC123* encodes a protein involved in promoting cell-cycling through its interaction with eIF2 (Bieganowski et al. 2004) and *CAMK1D* encodes a member of the

Ca2+/calmodulin dependent protein kinase 1 subfamily of serine threonine kinases involved in human granulocyte apoptosis (Verploegen et al. 2005).

The evidence for the 'cell cycle' link is probably most compelling at chromosome 9p21 (Chr9p21), and is the subject of investigations presented in Chapters 4 and 5. The T2D-association signals map in the vicinity of the genes encoding the cyclin dependent kinase inhibitors, CDKN2A (cyclin-dependent kinase inhibitor 2A, also known as p16 or p14 $^{ARF}$ in an alternate reading frame) and CDKN2B (cyclin-dependent kinase inhibitor 2B, also known as p15) (Figure 1.4). These are well recognised tumour suppressor genes: germline *CDKN2A* mutations are a cause of familial melanoma syndromes and somatic *CDKN2A* or *CDKN2B* mutations are amongst the most commonly mutated genes in cancer (Hussussian et al. 1994; Kim and Sharpless 2006).

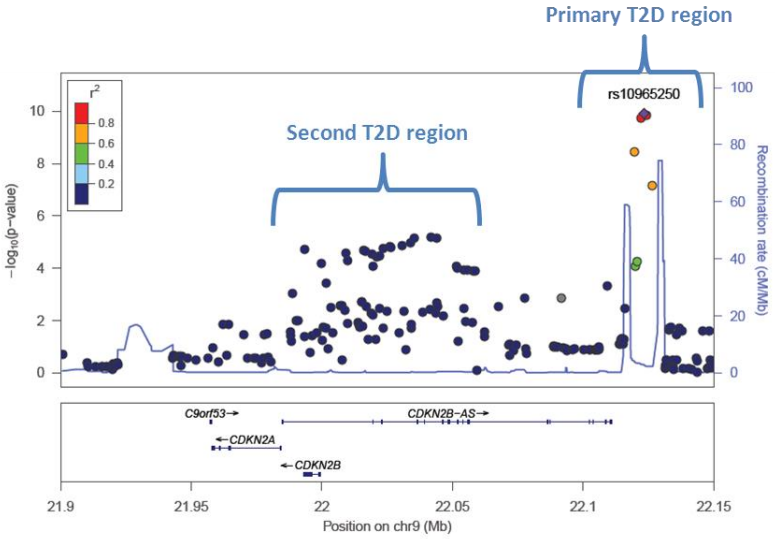



**Figure 1.4** Adapted from Pal and McCarthy (Pal and McCarthy 2013). Regional plot showing position of SNPs in Mb on chromosome 9 with location of the most proximal known genes in the region (x axis): strength of association of the SNPs with T2D shown on y axis.

Rodent data are consistent with a role in glucose homeostasis: overexpression of *Cdkn2a* and knockout of *Cdk4*, the main biochemical target of the cyclin dependent kinase inhibitor encoded by *Cdkn2a* and *Cdkn2b* (Figure 1.5), lead to islet hypoplasia and aT2D-like phenotype (Rane et al. 1999; Krishnamurthy et al. 2006).

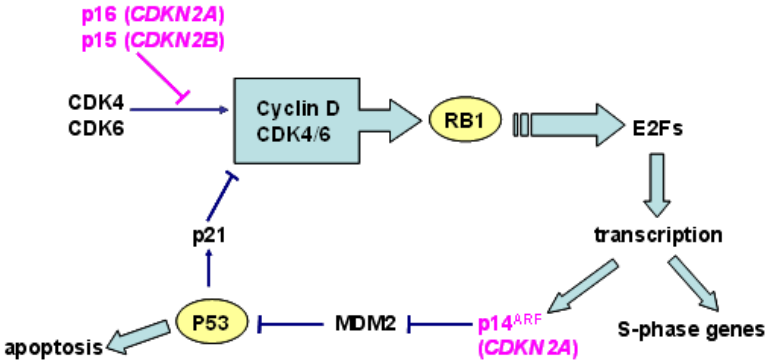



**Figure 1.5** Products of *CDKN2A* and *CDKN2B*, p16 and p15 respectively, suppress cell cycling by inhibiting actions of cyclin dependent kinase 4 and 6 (CDK4 and CDK6) which would otherwise go on to activate the retinoblastoma (RB1) pathway. RB1 would normally activate the E2F transcription factors leading to transcription of the S-phase genes in addition to *CDKN2A* in its alternate reading frame, giving rise to p14ARF which inhibits MDM2, an important negative regulator of the tumour suppressor p53.

The mechanism by which the T2D risk variants at chromosome 9p21 (which also harbours an independent association signal for cardiovascular disease) mediate *CDKN2A/B* expression is as yet unclear: however a non-coding RNA, ANRIL (also known as CDKN2B-AS) transcribed close to the regions of T2D association (Figure 1.4), and antisense to *CDKN2B*, is likely to be involved (discussed in more detail in **Chapter 4**) (Pasmant et al. 2007; Folkersen et al. 2009; Visel et al. 2010; Holdt and Teupser 2012). Investigations of the 'cell-cycle link' at Chr9p21 will be addressed in Chapters 4 and 5 of this thesis where more background details of this locus will be discussed.

Finally, one of the strongest cases for genetic links between T2D and cancer comes from the identification of a common variant in *HNF1B* which influences *both* T2D risk and prostate cancer risk (Gudmundsson et al. 2007; Winckler et al. 2007; Machiela et al. 2012). Gudmundsson and colleagues initially identified a variant in intron 2 of *HNF1B* that was associated with an *increased* risk of prostate cancer (OR 1.22) and went on to seek an association with T2D risk given the knowledge that rare *HNF1B* mutations are a cause of MODY (Horikawa et al. 1997; Gudmundsson et al. 2007). This group showed that the risk allele for the same variant for prostate cancer was associated with *protection* from T2D (OR 0.91) (Gudmundsson et al. 2007). Epidemiological studies, although prone to numerous biases and confounding factors as discussed in the following section, have generally shown an *inverse* relationship between T2D and prostate cancer risk (Gong et al.

2006; Kasper and Giovannucci 2006; Calton et al. 2007) that would be consistent with the divergence of risk attributed by the *HNF1B* variant.

In summary, these genetic links between cancer and diabetes add an interesting aspect to the pre-existing epidemiological data (discussed below in **section 1.6**) that linked these two major health conditions and hint at possible alternative underlying mechanisms relevant to T2D pathogenesis which will be discussed in section 1.9.

### 1.6 Strong epidemiological links exist between cancer and T2D

Before discussing possible biological mechanisms underlying a T2D and cancer/cell-cycle link, the epidemiological literature linking these two conditions will be discussed to emphasise the connections that were recognised between these conditions prior to the genetic links suggested by GWAS for T2D. An epidemiological link between diabetes and cancer has been recognised since 1932 (Wilson 1932), but over the last 10 years substantially more epidemiological data has amassed suggesting that people with diabetes are at significantly higher risk for many forms of cancer, and that the two conditions are diagnosed within the same individual more frequently than would be expected by chance (Giovannucci et al. 2010). More recently observational evidence also suggests that some anti-diabetes therapies are associated with an altered risk of cancer (Yang et al. 2004; Evans et al. 2005; Currie et al. 2009; Libby et al. 2009; Smith and Gale 2009).

Numerous epidemiological studies indicate an increased risk of cancer in people with T2D: however the strength of the association depends on the specific cancer site (Vigneri et al. 2009). Generally the observed increases in cancer risk have been reported in Asian and Western populations (Noto et al. 2010). The strongest relationships have been demonstrated for liver (El-Serag et al. 2006),

pancreatic (Huxley et al. 2005) and endometrial cancers (Friberg et al. 2007) where relative risk conferred by having diabetes is quoted as twofold or higher. The risks of breast (Larsson et al. 2007), colorectal (Larsson et al. 2005), bladder (Larsson et al. 2006), non-Hodgkin lymphoma (Mitri et al. 2008) and kidney (Larsson and Wolk 2011) cancers are deemed 20 – 40% higher in people with T2D than those included without T2D. Although these studies collectively imply the direction of any causation between T2D and cancer to be that the former condition increases the risk of the latter disease, this is not actually known and it remains difficult to draw any firm conclusions on causation between these two conditions from epidemiological evidence given the biases and confounding factors to which these studies are vulnerable (discussed in the following section).

Interestingly, prostate cancer is about 10 – 20% *less* likely in men with T2D and is the only cancer where having diabetes apparently reduces risk (Kasper and Giovannucci 2006; Giovannucci et al. 2010). For other cancers, there are fewer studies, but there appears to be no increased incidence of lung or ovarian cancer in T2D (Weiderpass et al. 2002; Hall et al. 2005). Data for patients with T1D and cancer risk is conflicting. Analyses of T1D cohorts, compared to the general population, suggested increased risked in some cancers such as ovarian cancer (Swerdlow et al. 2005) but this is not consistent across all studies (Zendehdel et al. 2003; Shu et al. 2010).

In addition to analysing the T2D – cancer incidence/prevalence association, cancer outcomes have also been analysed with respect to T2D. It is possibly more difficult to link diabetes and cancer mortality or prognosis given the potential confounding variables (e.g. cancer treatment modalities, host factors, cancer subtypes). Several meta-analysis studies have addressed this specific question (Barone et al. 2008; Snyder et al. 2010; Stein et al. 2010; Peairs et al. 2011). Collectively these show that diabetes is associated with an increased mortality compared with normoglycaemic individuals across all cancer types but particularly in relation to endometrial, breast and colorectal cancer (Barone et al. 2008).

Finally, the epidemiological evidence is concentrated upon analysing cancer risk in patients with diabetes. There is sparse data on the corollary of that, i.e. the incidence of diabetes in patients with cancer. Although there is some data on the mortality and outcomes of cancer in patients with diabetes (discussed in **section 1.7** below), there are few studies directly looking at the prevalence of diabetes and hyperglycaemia amongst cancer patients (DL 2012), which again limits interpretation of *direction* of any causation between diabetes and cancer incidence.

### 1.7 The epidemiological links between cancer and T2D may have confounding factors

Important considerations must be taken into account when interpreting the mainly observational data to evaluate the impact of having diabetes on cancer risk. Potential confounders include for example the idea of 'reverse causality' whereby the cancer itself may lead to the onset of diabetes (Giovannucci et al. 2010; Johnson et al. 2012). For pancreatic cancer the association is complicated by the fact that the cancer itself can cause diabetes (although a positive association remains when restricted to cases where the diabetes diagnosis precedes the pancreatic cancer diagnosis by 5 years (Giovannucci et al. 2010)): diabetes-related factors such as steatosis, non-alcoholic fatty liver disease and cirrhosis may also enhance susceptibility to liver cancer.

Another potential difficulty in interpreting the epidemiological data is the heterogeneity of cancer itself and how biological features differ according to tissue of origin: cancer risk also varies widely according to age, sex and geographical location. Studies will differ in the cancer screening modalities and patients themselves will have had different cancer treatments that may have variable impacts on glycaemia (e.g. steroids or chemoradiation which can induce hyperglycaemia (Nguyen et al. 2009)). Common risk factors such as age, sex, being overweight or obese, physical inactivity, diet, alcohol and smoking are also potential confounders in assessing risk of cancer incidence in T2D

(Giovannucci et al. 2010; Johnson et al. 2012). Duration of diabetes is also a factor and the spectrum of agents used to treat it will vary widely and treatments may well have an impact on the cancer risk themselves (see section 1.9). Randomised control trials would be the gold standard for trying to dissect out clear conclusions on the relationship between diabetes and cancer incidence but are difficult to use when investigating relatively rare adverse events. Therefore observational studies currently provide the best available evidence on the links between cancer, diabetes and diabetes therapies (**section 1.8**) but the data must be interpreted within the recognised limitations of these studies (Johnson and Gale 2010; Johnson et al. 2012).

### 1.8 The links between anti-diabetes therapies and cancer-risk

There is also more controversial evidence for a proposed link between T2D and cancer with reported associations between cancer risk and anti-diabetes therapies such as insulin, sulphonylureas and the insulin sensitisers, metformin and the thiazolidinediones (Smith and Gale 2009; Giovannucci et al. 2010). Whilst much of these data are also observational from epidemiological studies, some molecular connections have also been described, particularly with regard to metformin (Zhou et al. 2001; Kalender et al. 2010). It is helpful to consider these associations because if anti-diabetes therapies truly affect cancer risk, this again suggests a possibly causal link between the two conditions, particularly where this can be crystallised to molecular 'cross-talk' which may illuminate aspects of T2D or cancer pathogenesis.

#### 1.8.1 Insulin treatment and cancer risk

Exogenous insulin is needed to treat all people with T1D , and its use in people with T2D has increased markedly with between 40 – 80% considered for insulin treatment (due to progressive beta-cell dysfunction over time) to achieve more stringent glycaemic targets (Nathan et al. 2009). Subcutaneous insulin results in significantly higher levels of systemic insulin than with endogenous

insulin, thereby possibly increasing the risk of cancer associated with hyperinsulinaemia (Call et al. 2010). Given the widespread use of insulin, this is highly controversial and definitive evidence linking insulin therapy to increased cancer risk is still awaited.

In particular, insulin analogues such as insulin glargine have been implicated, possibly due to its higher binding affinity for the IGF-1 receptor (Shukla et al. 2009) (the insulin and IGF-1 axis and their relationship to cancer is discussed in **section 1.9**). A series of observational studies have examined a possible association between insulin use and risk of cancer in T2D and results have not been consistent. A German study suggested an association with dose response for those on higher doses of insulin glargine having increased cancer risk (Hemkens et al. 2009), although it should be noted that adjustment for insulin dose complicated the analysis, and in fact the incidence of neoplasms was higher in the group treated with human insulin rather than those on analogues, but the doses of human insulin used were much larger. Importantly there was also no correction for BMI in the dose adjusted analysis (Hemkens et al. 2009). In a UK led study, Currie et al found that insulin use was particularly associated with higher risk of colorectal and pancreatic cancers but had no association with breast or prostate cancer risk compared with metformin monotherapy (Currie et al. 2009). Swedish and Scottish studies did not find an association between insulin glargine use and cancer risk compared to other insulins (Colhoun 2009; Jonasson et al. 2009), and in complete contrast a Chinese study found *reduced* cancer risk among patients with T2D on insulin (Yang et al. 2010). A recent US study found a 30% increased prevalence of cancer in T2D patients on insulin compared to those not currently on insulin (Li et al. 2013), despite adjustment for duration of diabetes. Rarely do any of these epidemiological studies account for all potential confounding factors (including age, BMI, gender, insulin dose, duration of diabetes and glycaemic control) in their analyses. In addition length of follow up in many of the studies is limited and does not permit sufficient numbers of

cancer cases within the period observed to come to definitive conclusions regarding specific tumour types.

In contrast to these observational studies, randomized control trials (RCTs) of reasonably long duration (>5 yrs) therapy with insulin glargine versus other forms of insulin did not find an excess in cancer risk or mortality (Rosenstock et al. 2009). Of note, however, was the relatively small number of patients in this study (1000) and thus a very small number of cancer endpoints (57 cancer cases in the glargine arm versus 62 in the NPH insulin arm). The much larger and more recent RCT, the ORIGIN (Outcome Reduction with an Initial Glargine Intervention) trial (comparing glargine versus placebo in patients with impaired fasting glucose or newly diagnosed T2D) included around 12,000 patients who were followed up for 6-7 years (Gerstein et al. 2012). It also found no increased cancer risk in insulin glargine-treated patients, although it should be noted that was with the use of low dose glargine (0.4IU/kg), early in the disease process. Another cardiovascular outcome RCT in T2D, ACCORD (Action to Control Cardiovascular risk in T2D), demonstrated no specific adverse safety signal with insulin therapy (Gerstein et al. 2008). However, of note, these latter studies were designed and powered for cardiovascular outcomes and the former RCT for retinopathy. The numbers included in such RCTS are not specifically powered to detect cancer outcomes and so number of cancer cases over the duration of such trials is likely too small to provide definitive evidence regarding cancer incidence.

Whilst plausible that sulphonylurea treatment may be linked to increased cancer risk given the higher circulating insulin levels (see **section 1.9.1**), it is difficult to disentangle the possibly negative effect of sulphonylurea treatment from the possibly protective effect of metformin (see section **1.8.2**), as this is the most common comparator drug in these studies (Giovannucci et al. 2010). Most

current studies have a very small number of cancer cases and therefore limited power to investigate associations with specific cancer sites (Currie et al. 2009). Likewise there is little data on the glucagon-like peptide-1 (GLP-1) analogues and dipeptidyl-peptidase-4 (DPP-4) inhibitors which have probably not been in use long enough or in sufficient numbers of patients to assess any effect on cancer risk. There are limited data from animal studies: in rodents, liraglutide (a GLP-1 analogue) has been suggested to increase risk of medullary cancer, and sitagliptin (a DPP-4 inhibitor) possibly to lead to pancreatic ductal hyperplasia (Butler 2009; Butler et al. 2010).

In summary, currently available data on insulin treatment and cancer risk in T2D are inconclusive with studies so far finding positive, insignificant or negative associations. The observational studies have the limitations already alluded to (see **section 1.7**) with the main confounder that insulin is more often prescribed in patients with longer duration of T2D and is more frequently used in those with comorbidity preventing the use of other medications. It is acknowledged that these studies can rarely fully account for the potential confounders of body mass, insulin dose, degree of glycaemic control, age and other patient characteristics which will surely also affect cancer risk (Giovannucci et al. 2010).

### 1.8.2 Insulin sensitisers and cancer risk

Metformin is the most commonly used treatment in T2D (Nathan et al. 2009) and although its mechanism of action is not fully understood, it exerts its glucose lowering affect through reducing hepatic gluconeogenesis (Shaw et al. 2005). Observational studies have suggested a protective effect of metformin on cancer outcomes (Evans et al. 2005; Currie et al. 2009; Libby et al. 2009; Zhang et al. 2013) although another recent meta-analysis of RCTs comparing metformin with other anti-diabetes therapies showed no overall protective effects of metformin on mortality or cancer risk (Stevens et al. 2012). However limitations of this latter study included short follow-up (with

regard to cancer mortality outcomes), heterogeneity of groups and incomplete cancer data (Stevens et al. 2012). Supportive in vitro evidence has accumulated showing that metformin can inhibit cancer cell growth and proliferation, and cause partial cell cycle arrest in cancer cell lines with a dose-dependent effect (Zakikhani et al. 2006; Alimova et al. 2009; Cantrell et al. 2010).

The effects of metformin on the AMP-activated protein kinase (AMPK) signalling pathways are thought to contribute (Zakikhani et al. 2006) to its anti-cancer effects. Metformin is an AMPK activator which leads to inhibition of mammalian target of rapamycin (mTOR) through phosphorylaton and subsequent activation of the tumour suppressor tuberous sclerosis complex 2 (TSC2) (Zhou et al. 2001) (Figure 1.6).

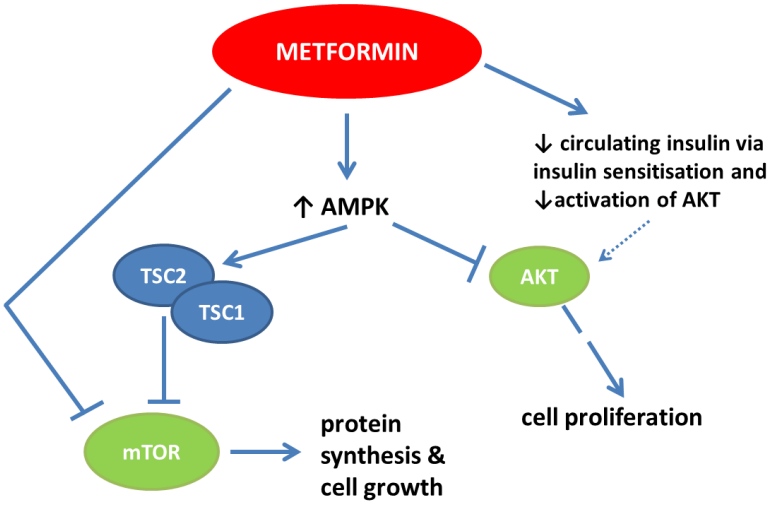



**Figure 1.6** Schematic of metformin's anticancer effects via inhibition of the mTOR and AKT pathways of cellular growth and proliferation.

The mTOR is a key integrator of growth factor and nutrient signals as well as a critical regulator of the PI3K/PKB/AKT pathway, one of the most frequently disrupted signalling pathways in cancer (Markman et al. 2010). Metformin may have additional anticancer properties independent of AMPK and TSC2, related to the inhibition of Rag GTPase-mediated activation of mTOR (Kalender et al. 2010). Other possible anticancer mechanisms could be suppressing tyrosine kinase receptors such as HER1 and HER2 (Vazquez-Martin et al. 2009), anti-inflammatory effects (Grenader et al. 2009), antioxidant effects (Kourelis and Siegel 2012) and killing of cancer stem cells (Vazquez-Martin et al.

2011). The mechanism of metformin's anti-cancer effect is also thought to be via insulin lowering (Figure 1.6) as metformin-induced inhibition of in vivo experimental cancers is associated with a reduction in insulin levels and activation of insulin receptors in neoplastic tissue (Algire et al. 2010).

The other main group of insulin sensitising drugs are the thiazolidinediones (TZDs) which are peroxisome proliferator-activated receptor (PPAR)γ agonists. In vitro studies have shown that these agents can inhibit growth and induce apoptosis and cell differentiation (Ohta et al. 2001): however rodent studies suggest PPARγ agonists may facilitate tumourigenesis (Rubenstrunk et al. 2007). The weight of observational studies in humans suggests overall there is no increased cancer risk associated with TZD use in T2D, but a recognised modest increase in risk of bladder cancer with pioglitazone therapy in particular (Lewis et al. 2011; Bosetti et al. 2013).

## 1.9 Potential underlying mechanisms for cancer diabetes overlap

As already discussed (**section 1.6**), the link between cancer and T2D may not be a causal association but simply due to common predisposing risk factors thus linking the two conditions via indirect effects. However several plausible direct biological mechanisms have been proposed which could also account for the link. Most currently propose that the high insulin/ high glucose/ chronic inflammatory setting in T2D is an especially hospitable environment for cancer growth. Two other proposed mechanisms (**section 1.9.4** and **1.9.5** on common signalling pathways and effects on beta-cell mass) perhaps hold more potential for novel insights into T2D pathogenesis and why variants that have an effect on cancer growth may also influence T2D risk.

### 1.9.1 High insulin , Insulin-like growth factor – 1 (IGF-1) and other hormones that may promote cancer growth

One of the most intuitive and traditionally held explanations for the observed epidemiological link between cancer and T2D is that the insulin resistance and subsequent hyperinsulinaemia that characterizes the early stages of T2D, provides a hormonal environment that promotes tumour cell growth (Pollak 2008). This theory is supported by a meta-analysis demonstrating an association between high serum insulin or c-peptide levels with a significantly increased risk of certain cancers (Pisani 2008). Increased endogenous insulin levels have also been associated with a worse prognosis in breast cancer (Goodwin 2008).

Insulin itself is a growth factor and promotes cell growth directly via insulin receptors (Law et al. 2008) and indirectly via IGF-1 receptors (Hellawell et al. 2002; Frasca et al. 2008). IGF-1 has more potent mitogenic and anti-apoptotic activity than insulin (Weinstein et al. 2009), and insulin leads to higher circulating levels of active IGF-1 by reducing hepatic production of IGF binding protein (IGFBP) – 1 and IGFBP-2 (Powell et al. 1991; Ooi et al. 1992; Renehan et al. 2006). Numerous cancer cell lines express insulin and IGF-1 receptors (Frasca et al. 1999; Vella et al. 2002) which activate multiple downstream pathways (including many involved in cell proliferation and protection against apoptosis) mainly via the insulin receptor substrate (IRS) family of kinases on binding of insulin and IGF-1 (Giovannucci 2001; Mardilovich et al. 2009).

Hyperinsulinaemia may also affect cancer growth indirectly via an effect on hormones other than IGF-1. Increased insulin levels lead to reduced hepatic production of sex hormone binding globulin (SHBG) which leads to increased oestrogen and stimulation of oestogen-dependent tumours (Calle and Kaaks 2004). Insulin resistance is also associated with a higher production of NEFA (as discussed in **section 1.2**), interleukin-6, plasminogen activator inhibitor-1 and tumour necrosis factor α, all of which may have cancer promoting effects (van Kruijsdijk et al. 2009). Finally, insulin or insulin

resistance may have an indirect effect on levels of the adipokine, adiponectin: adiponectin is associated with insulin sensitivity and insulin-sensitive obese individuals have been shown to have increased circulating adiponectin levels compared to insulin-resistant obese individuals (Kloting et al. 2010). A number of epidemiological studies have shown strong inverse relationships between cancer and adiponectin levels (Mantzoros et al. 2004; Wei et al. 2005; Barb et al. 2007) and this hormone has also been shown to have an antiproliferative effect in breast cancer cell lines (Dos Santos et al. 2008).

However, there is an inherent flaw in the theory that hyperinsulinaemia, secondary to insulin resistance, promotes cancer. If the hyperinsulineamia is a response to insulin resistance, then insulin action in peripheral tissues may be reduced, not increased. Thus although insulin (and IGF-1) are recognised growth factors, perhaps these properties are attenuated in insulin *resistant* tissues so that insulin may not have the same growth promoting effect. Similarly, in hepatic insulin resistance insulin may *not* stimulate higher IGF-1 levels via reduced hepatic IGFBP production: although IGFBP-1 levels are suppressed in impaired glucose tolerance and in early T2D, they have been observed to increase after 10 years of T2D, possibly via hepatic insulin resistance (Lewitt et al. 2008). Theoretically the same argument could be applied to the proposed hormonal sequelae of hyperinsulinaemia detailed above: when hyperinsulinaemia is secondary to insulin resistance the higher circulating insulin levels may not invoke the hormonal changes one would expect with an increased insulin effect.

One way to settle this issue would be to advocate differential tissue insulin sensitivity. This principle has been proposed, for example, in polycystic ovarian syndrome (PCOS) to explain how mechanisms invoking increased insulin action (e.g. insulin-induced hyperandrogenism) occur in the face of insulin

resistance (Poretsky 1991): reproductive tissues such as the ovaries and pituitary gland have been shown to display insulin sensitivity, whilst muscle, liver and adipose tissue also show differential insulin resistance (Ciaraldi et al. 2009; Wu et al. 2012). If one applies the same principles of differential insulin sensitivity to cancer in insulin resistant hyperinsulinaemic states, one could make a case for hyperinsulinaemia promoting cancer growth in tissues that retain insulin sensitivity. This theory could explain why increased incidence of particular tumour types have been associated with T2D (as discussed in **section 1.6**) (Vigneri 2009). Another observation one might expect if hyperinsulinaemia did promote cancer growth would be a more evident increase in cancer risk associated with T1D: T1D combines increased circulating (exogenous) insulin levels with no insulin resistance. However, as discussed in section 1.6, no consistent relationship between cancer and T1D has been demonstrated.

### 1.9.2 The 'Warburg effect' and hyperglycaemia as a mechanism linking T2D and cancer

A second possible underlying mechanism for the complex interactions between diabetes, diabetes treatments and cancer, is the effect of glucose itself. The so-called 'Warburg hypothesis' emphasises the dependence of many cancers on glycolysis for energy, creating a high requirement for glucose as glycolytic ATP generation requires significantly more glucose than oxidative phosphorylation (Vander Heiden et al. 2009). Indeed the increased glucose consumption by cancers is the principle underlying FDG-PET (fluorodeoxyglucose-positron emission topography) imaging of cancers which detects tissues with high rates of glucose uptake (Bourguet et al. 2003).

This 'hyperglycaemia hypothesis' is supported by observational studies demonstrating a relationship between elevated blood glucose and cancer incidence or mortality (Jee et al. 2005; Seshasai et al. 2011). However, it is difficult to ascertain if it is actually glucose mediating this association, or if it is a surrogate for another causative factor such as hyperinsulinaemia. Generally, high glucose-requiring

cancer cells are able to satisfy their requirements under normoglycaemic conditions (Pollak 2009) and studies investigating dose response relationships between glucose concentrations and tumour growth show that increased levels do increase proliferation, but the effect plateaus around a glucose concentration of 5mmol/l (Tannock and Kopelyan 1986). In principle, although one might assume hyperglycaemia would confer a relative growth advantage to cancer, evidence from large RCTs of intensified glucose control for T2D does not support the hypothesis: lowering blood glucose does not *reduce* the risk of cancer in T2D (Johnson and Bowker 2011). Of note, this may well be achieved with more intensive insulin regimes, with theoretical growth promoting effects of insulin possibly negating the effects of low glucose. Given the heterogeneity of cancers, hyperglycaemia may confer a growth advantage for a subset of tumours, but accumulated evidence to date is more consistent with the activation of the insulin and IGF-1 axis being a more important variable than glucose in determining tumour development.

### 1.9.3 Chronic inflammation in T2D

One of the indirect effects of insulin, T2D and/or obesity is the augmentation of other pathways such as inflammatory pathways which may have an impact on tumour development. As already briefly discussed, adipose tissue is an active endocrine organ releasing free fatty acids, IL-6, monocyte chemoattractant protein, plasminogen activator inhibitor-1 (PAI-I), adiponectin, leptin and tumour necrosis factor-α (van Kruijsdijk et al. 2009). The theoretical risk of a pro-inflammatory state being favourable for cancer growth is borne out by some molecules in particular: expression of PAI-1 has been linked to worse prognosis in breast cancer (Ulisse et al. 2009) and IL-6 activation of signal transducer and activator of transcription protein (STAT) signalling is known to facilitate cancer cell proliferation, survival and invasion whilst suppressing host anti-tumour immune defences (Yu et al. 2009). A dietary effect mediated via inflammatory changes also supports the 'pro-inflammatory hypothesis' for linking diabetes and cancer growth (Park et al. 2010).

However the arguments against chronic inflammation being an underlying mechanism for links between T2D and cancer would include the fact that some inflammatory factors are also known to *suppress* tumour growth (Coussens and Werb 2002). Also, where cancer incidence has been linked more strongly to inflammatory conditions, for example with colorectal cancer in inflammatory bowel disease (Ekbom et al. 1990), this has been a local tissue-specific effect. In T2D chronic (low-grade) inflammation is particularly recognised in adipose tissue and pancreatic islets, as well as muscle and liver (Donath and Shoelson 2011), and yet a preponderance for cancers affecting these tissues is not particularly recognised, apart from perhaps, pancreatic and liver cancer.

### 1.9.4 Common signalling pathways may link T2D and cancer

A more direct, plausible explanation for the links between diabetes and cancer may be the existence of common signalling pathways. This has already been discussed with reference to metformin (**section 1.8.2**) which clearly has a role in metabolic pathways but also appears to have an effect on integral cell-cycle pathways via its activation of AMPK, and provides an underlying mechanism for observed epidemiological links between metformin treatment and cancer risk.

As discussed earlier (**section 1.9.1**) insulin itself, (and IGF-1) is able to activate metabolic and mitogenic pathways via IRS-1 and IRS-2 which activate the PI3K-AKT pathway and the Ras/extracellular signal-regulated kinase (ERK) pathways (Cohen 2006). The Ras/ERK pathway has mainly mitogenic activity via activation of gene transcription needed for proliferation and/or cell differentiation (Meloche and Pouyssegur 2007). The PI3K-AKT pathway (Figure 1.8) has roles in cell growth, proliferation, apoptosis, cell migration and cytoskeletal rearrangement (Vivanco and Sawyers 2002), as well as mediating many of the metabolic downstream effects of insulin (Cohen 2006).

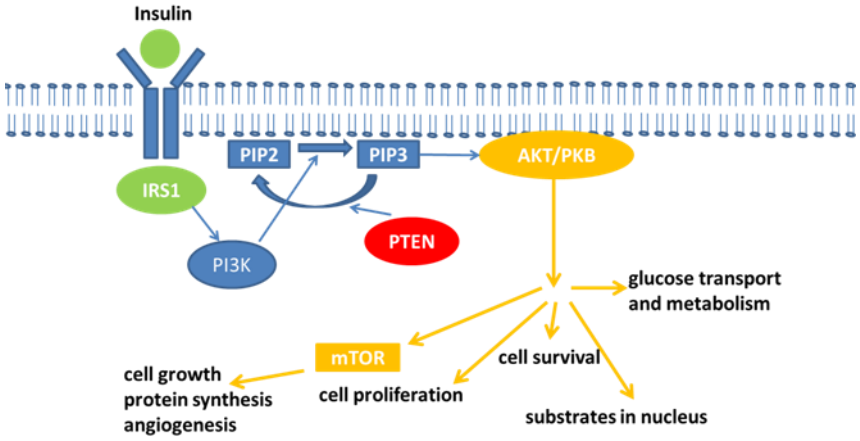



**Figure 1.8** Insulin signalling via the PI3K/AKT pathway. PI3K is activated by IRS1 (amongst other cell receptor and ligands) and phosphorylates phosphatidylinositol 4,5-biphosphate ($PIP_2$) to phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) which subsequently recruits AKT to the plasma membrane where it is in turn phosphorylated and activated. Activated AKT has multiple downstream effects in the cell needed for cellular growth and metabolism. PTEN negatively regulates this pathway by dephosphorylating $PIP_3$ to $PIP_2$.

The PI3K-AKT pathway is one of the most clear-cut examples of the existence of pathways common to cell growth and metabolism and potentially provides a more direct mechanism linking diabetes and cancer. The PI3K-AKT pathway and PTEN, which negatively regulates this pathway (Figure 1.8), will be discussed in more detail in Chapter 3.

### 1.9.5 Beta-cell mass and rates of cell proliferation

Finally, perhaps the simplest explanation for the mechanistic link between T2D and cancer is an effect via cell cycling and proliferation on functioning beta-cell mass: pro-proliferative states give rise to cancer but may also impact on functional beta-cell mass and therefore diabetes risk if this pancreatic islet tissue is affected. Particularly with regard to the GWAS disclosure of cell cycle genes as candidates in T2D risk/pathogenesis, it is plausible that whilst a risk allele may promote cell cycling and predispose to cancer it could simultaneously promote increased functional beta-cell mass thereby reducing risk of T2D. Conversely if a risk allele has its effect through reduced cell cycling this would protect against cancer, but if this subsequently reduced functional beta-cell mass it may predispose to T2D. This divergent effect would also explain the mechanism by which alleles

such as that of the *HNF1B* variant, have a divergent effect on T2D and prostate cancer risk i.e. increasing the risk of one condition whilst reducing the risk of another (Gudmundsson et al. 2007; Thomas et al. 2008; Machiela et al. 2012). These effects may be tissue specific such that the influence of given cell-cycle components (whether up or down-regulated) may differ by tissue, therefore predisposing to quite specific local cancers such as prostate cancer, and also tissue-specific metabolic effects.

**1.9.5.i Evidence for altered beta-cell mass in T2D**

If altered beta-cell mass due to genetic variants affecting cell proliferation rates is to be considered as a mechanism linking cancer and diabetes, two assumptions must be acknowledged: firstly one must accept that altered beta-cell mass has a role in T2D pathogenesis, and secondly, that beta-cell mass is a fluid entity, vulnerable to change over time which may impact on beta-cell function and capacity for insulin secretion.

The bulk of evidence for altered beta-cell mass in T2D comes from autopsy data: although there is wide variation, most studies have shown some reduction in beta-cell mass (between 0 and 65%) in pancreatic specimens from individuals with T2D compared to non-diabetic pancreatic specimens (Rahier et al. 1983; Kloppel et al. 1985; Sakuraba et al. 2002; Butler et al. 2003; Yoon et al. 2003). There is also increased beta-cell apoptosis noted in these pancreases compared to controls, suggesting this beta-cell loss may be progressive unless there is some mechanism of regeneration (Butler et al. 2003; Marchetti et al. 2004). The major limitation of these studies is the quality of autopsy data to quantify beta-cell mass: the pancreas is one of the first organs to undergo autolysis (Shimizu et al. 1990); there be changes in beta-cell mass during the illness leading to death; frequently the whole pancreas has not been dissected at autopsy; there is often limited clinical data available; there are no means to retrospectively measure insulin secretion and sensitivity to relate

this to the beta-cell mass quantified. Although there are promising results for use of magnetic resonance imaging (MRI) to quantify pancreatic beta-cell mass in the future (Antkowiak et al. 2012; Antkowiak et al. 2013), currently, a technique to accurately measure beta-cell mass in living humans remains elusive. Therefore it is useful to consider the autopsy data as evidence for the role of declining beta-cell mass in T2D.

Animal data generally support the notion of reduced beta-cell mass in diabetic states but most studies have been performed in rodents aged less than one year and in contrast to humans, young rodents have a high capacity for pancreas regeneration after partial pancreatectomy and a higher beta-cell turnover as adults than in humans, thus limiting direct extrapolation from animal models (Matveyenko and Butler 2008). In addition, there are important differences in cell-cycle regulators between rodent and human beta-cells (discussed in the next **section 1.9.5.ii**) which also limits direct conclusions from rodent data regarding beta-cell mass.

In non-diabetic humans there is a wide variation in beta-cell mass as measured from autopsy data (Meier et al. 2008). Meier and colleagues used abdominal computer tomographies in children aged 4 weeks through to 20 years in conjunction with autopsy data from children of similar age range: they demonstrated rapid expansion of beta-cell mass in childhood, primarily through beta-cell replication and concluded that the adult beta-cell mass is almost achieved by 5 years of age (Meier et al. 2008). From these autopsy data, although having the aforesaid limitations, the wide range in beta-cell mass in adults becomes evident after childhood suggesting that the rate of growth in the early phase may determine the ultimate beta-cell mass in adulthood (Meier et al. 2008). Rodent data suggests adult beta-cell mass is partly dependent upon the number of embryonic progenitor cells (Stanger et al. 2007) and it is likely that in humans also, beta-cell mass in adulthood will be

partly determined by the islet progenitor cell pool and the status of the embryo during the period of rapid expansion of differentiated endocrine cells during fetal development (Matveyenko and Butler 2008). However, it is also evident from rodent studies that different factors are involved in increasing and maintaining beta-cell mass during embryonic development compared to in adulthood: in the embryo beta-cell differentiation occurs from multipotent pancreatic progenitor cells with neogenesis and proliferation occurring through to weaning, and in adults a balance between apoptosis and replication appears to determine beta-cell mass (Bonner-Weir et al. 2010; Gunasekaran et al. 2012). These data again have the caveats of being from rodent models, and data in humans is mainly derived from autopsy specimens.

Autopsy data has indicated some fluidity in beta-cell mass in adulthood: in pregnancy an increased beta-cell mass has been observed, although in contrast to rodent data, there was no observed change in beta-cell size, replication or apoptosis, and the increased beta-cells appeared to be in increased number of smaller islets (Butler et al. 2010). Rodent data has also previously indicated adaptive increases in beta-cell mass to pregnancy (Parsons et al. 1995) and also obesity (Bock et al. 2003) with beta-cell replication as the main mechanism (Dor et al. 2004). Of note the increased beta-cell mass associated with obesity is much smaller and variable in humans (Butler et al. 2007; Rahier et al. 2008).

The theory of beta-cell mass plasticity, with increases and decreases in number during adulthood (Bonner-Weir 2001; Butler et al. 2003; Dor et al. 2004; Ritzel et al. 2006), has generally replaced the previously held view that beta-cell mass is determined in embryogenesis and early life, with little change thereafter (Rahier et al. 1983; Clark et al. 1988; Clark et al. 2001). Given the association of some T2D susceptibility loci with cell cycle genes (**section 1.5**), the theory that aberrant production

of beta-cells in utero/later life or changes in cell turnover/regeneration are implicated in T2D pathogenesis, is an attractive hypothesis. However, as described above, much of the evidence for this theory is based upon autopsy data and young rodent models whose beta-cell turnover is higher than in older rodents or adult humans (Butler et al. 2003; Teta et al. 2005; Butler et al. 2007). Studies assessing beta-cell longevity in humans suggest that, unlike in rodents, beta-cells are long-lived with very little evidence of significant islet cell turnover in response to the insulin demand required in the pathogenesis of T2D (Cnop et al. 2010; Perl et al. 2010). The fact that some patients undergoing bariatric surgery appear to have had their T2D 'cured' is perhaps evidence that beta-cell mass is adequate for insulin requirements but beta-cell *function* is reversibly compromised in T2D, and that this function can be regained (Pories et al. 1987; Rubino et al. 2006; Taylor 2008; Kashyap et al. 2010).

A possible way to resolve the issue of beta-cell mass in T2D pathogenesis is to consider the effect of senescence on beta-cells. Evidence for longevity of beta-cells is also supported by the observation that there are age-related changes in cell-cycle proteins in islet cells (Cnop et al. 2010; Cnop et al. 2011; Kohler et al. 2011). Kohler and colleagues demonstrated higher replication in prenatal human beta-cells compared to adult beta-cells and significantly higher expression of the cell-cycle proteins p16 and p27 along with cyclin D3 in the adult human beta-cell compared to those obtained from prenatal specimens (Kohler et al. 2011). Thus it was postulated that the age-dependent increase in expression of cell-cycle inhibitors, p16 and p27, may contribute to the observed restriction of beta-cell replication with ageing (Kohler et al. 2011). This is consistent with data from mice overexpressing, or deficient for p16: transgenic mice that overexpressed p16 showed reduced islet proliferation similar to that observed in older mice, and p16 deficient mice showed increased islet proliferation in older (but not younger) mice (Krishnamurthy et al. 2006). Therefore a hypothesis for the mechanism underlying T2D susceptibility loci associated with cell cycle genes could be that T2D-

associated variants attenuate the age-related expression of cell cycle proteins and subsequent beta-cell replication. This is relevant to the studies presented in Chapters 4 and 5 of this thesis where the possible roles of *CDKN2A* (encoding p16) and *CDKN2B* (encoding p15) will be discussed.

### 1.9.5.ii Regulation of beta-cell proliferation in humans

The hypothesis proposed in the previous section relies upon beta-cell proliferation being a dynamic entity. An appreciation of 'normal' beta-cell proliferation/replication regulation is therefore desirable. Although beta-cell replication in rodents has been well delineated and also reproducibly stimulated in vitro and in vivo, it has been challenging to reproduce the same results in human beta-cells (Nir et al. 2007; Fiaschi-Taesch et al. 2009; Fiaschi-Taesch et al. 2010). Stewart and colleagues have previously proposed a model for human beta-cell replication (Figure 1.9) based upon immunoblot studies for each component protein performed on extracts of isolated human islets from multiple donors (Fiaschi-Taesch et al. 2009) and focusses upon the G1/S checkpoint in the cell-cycle which represents a critical checkpoint in most cell types.

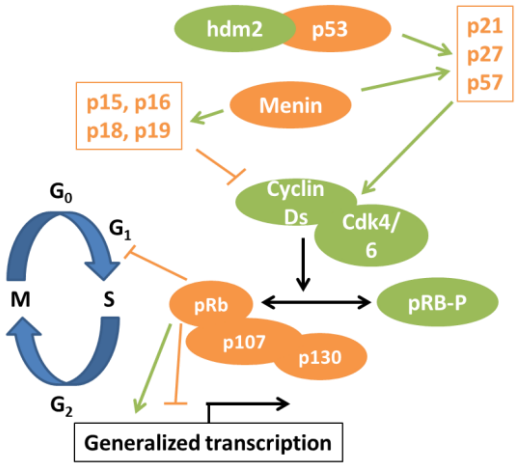



**Figure 1.9** Simplified schematic of regulatory components of the G1/S checkpoint proteome in human islets. Adapted from Fiaschi-Taesch et al 2009 and based upon their studies of immunoblots of each protein performed on multiple human islet preparations. Proteins in green stimulate progression of the cell-cycle, those in orange are cell-cycle inhibitors.

Recently the same group has published an immunocytochemical "atlas" of G1/S control molecules in the human beta cell confirming that the majority of the molecules in the above model are present in human beta-cells, but interestingly most of the critical regulatory molecules (including the cyclins and CDKs) are located in the cytoplasm rather than the nucleus (which only contained the cell-cycle

inhibitors pRb, p57 and p21) (Fiaschi-Taesch et al. 2013). Cyclin D3 and cdk6 were also used to stimulate beta-cell proliferation and were observed to translocate into the nucleus: proliferation occurred where cell-cycle inhibitors p15, p18 and p19 remained cytoplasmic, but did not take place if nuclear cell-cycle inhibitors were present (Fiaschi-Taesch et al. 2013).

This increased appreciation of beta-cell cycle control in humans is important for future insights into T2D pathogenesis, to help understand, for instance, which components may be vulnerable by their relation to associated T2D susceptibility loci. For example, the above model includes p15 and p16 (cyclin dependent kinase inhibitors) encoded by *CDKN2B* and *CDKN2A* which are the most proximal coding genes to the T2D susceptibility loci at Chr9p21, and the basis of investigations presented in Chapters 4 and 5 of this thesis.

## 1.10 Thesis aims

The aims of my thesis were to gain insight into T2D pathogenesis by studying specific examples involved in the complex interplay between T2D and cancer, particularly using *human* models of disease. I aimed to investigate two loci already known for their notable roles in cancer pathogenesis: the tumour suppressor *PTEN* locus on chromosome 10 (**Chapter 3**) and the *CDKN2A* and *CDKN2B* locus at Chr9p21 (**Chapters 4 and 5**).

### 1.10.1 The tumour suppressor gene *PTEN* on chromosome 10

*PTEN* is a tumour suppressor gene encoding a phosphatase that inhibits the PI3K-AKT pathway which has multiple roles within the cell including cellular growth and metabolic processes (Figure 1.8) (Vivanco and Sawyers 2002; Manning and Cantley 2007). For the work presented in this thesis, it was hypothesised that studies of the *PTEN* locus would give evidence supporting common signalling pathways as a mechanism underlying the T2D and cancer link. I aimed to recruit individuals with

rare, germline *PTEN* mutations and perform a series of physiological studies to define any differences in glucose homeostasis in these cases compared to control subjects.

### 1.10.2 The *CDKN2A* and *CDKN2B* locus at Chr9p21

It was hypothesised that studies of the T2D-associated loci at Chr9p21, would give supportive evidence for beta-cell proliferation effects being a mechanism underlying an association between T2D and cancer: *CDKN2A* and *CDKN2B* encode cell-cycle inhibitors and are the most proximal coding genes to the T2D-associated signals at Chr9p21.

Using a series of human physiological and cell molecular studies, I aimed to investigate the purported role of the *CDKN2A/CDKN2B* loci in the aetiological overlap between cancer and T2D, and in so doing, be able to add to the potential mechanisms underlying the epidemiological links between these two common health conditions.

# Chapter 2

General Methods

# Chapter 2

## 2.1 Subjects studied

### 2.1.1 Patients with Cowden syndrome due to *PTEN* mutations

Patients with Cowden syndrome (CS) due to *PTEN* mutations were identified through a collaborative group of UK Clinical Geneticists formed for the purposes of this study. This comprised Dr Lisa Walker and Dr Helen Linden (Oxford), Dr Trevor Cole, Dr Eamonn Maher, Dr Nicola Cooper and Dr Carole McKeown (Birmingham), Dr Louise Izatt (Guys and St Thomas's), Dr Katharine Lachlan (Southampton), and Professor Mary Porteous (Edinburgh). Diagnosis of CS due to *PTEN* mutation was made by the referring clinician to the study and was based upon clinical diagnostic criteria for CS (at least two major criteria – macrocephaly, thyroid carcinoma, breast carcinoma, endometrial carcinoma; or one major and three minor criteria – other thyroid lesions, mental retardation, gastrointestinal hamartomas, lipomas, fibrocystic disease of the breast, uterine fibroids, fibromas, genitourinary tumours or malformations; or four minor criteria) (Eng 2000) in addition to *PTEN* mutations confirmed by a certified Molecular Genetics diagnostic laboratory. Further details of specific inclusion criteria are given in **Chapter 3**.

Having been informed of the research study (by their Clinical Geneticist), if the patient expressed an interest in taking part, they were sent an information pack (containing full details of the study, its rationale, methodology and what would be required of participants, and a reply slip to be returned to the research team if the patient wished to take part in the study. On receipt of this, the patient was contacted to arrange a study visit and answer any further queries. All participants attended OCDEM or received a home visit by the research team. All participants received a letter after their study visits, acknowledging their part in the study and giving results of any baseline blood tests

deemed relevant to general health by the research team (e.g. fasting blood glucose, lipid profile). For further details, see **Chapter 3**.

### 2.1.2 Patients with familial melanoma due to *CDKN2A* mutations

Patients with familial melanoma due to *CDKN2A* mutations were recruited via a collaboration set up with Professor Tim Bishop and Professor Julia Newton-Bishop (ICRF, Leeds) for the purposes of this study. These collaborators have access to over 55 pedigrees characterised by melanoma predisposition and *CDKN2A* mutations. Diagnosis of melanoma was based upon clinical and histopathological criteria and was made by the referring Oncologist. Current ethical guidance for this project does not allow carrier individuals within families segregating severe *CDKN2A* mutations to be identified. We therefore surveyed all members of selected families, and re-identified mutation carriers after collection. That is, we were not permitted to recruit specific family members by genotype and thus invited individuals to take part in the study who were most likely to have familial melanoma due to *CDKN2A* mutation: subjects were invited if there was presence of multiple family members with melanoma with at least one member having early age of onset (age <45 years) and/or multiple primary tumours. This ensured a large group of invited subjects with a high likelihood of carrying a mutation in *CDKN2A.* However, as multiple family members were invited within each selected pedigree we were also able to obtain familial controls for the study who were not mutation carriers given we had access to genotype post participation in the study.

All identified subjects were sent an information pack detailing the study and a reply slip which was returned if they were interested in taking part. On receipt of this, subjects and interested family members were contacted to arrange joint study visits to OCDEM, or where this was not possible the research team conducted home visits. For further details see **Chapter 5**.

### 2.1.3 Ethics

An ethics application to enable these studies in mutation carriers and control subjects was made and approved in 2008 by the Oxfordshire Research Ethics Committee B (REC reference 08/H0605/130). All participants gave full informed written consent before participating in any part of the study.

### 2.1.4 Control subjects (familial controls and Oxford Biobank)

Healthy control subjects were recruited for the physiological studies on subjects with CS due to *PTEN* mutations and also the physiological studies on subjects with familial melanoma due to *CDKN2A* mutations. For the latter study, familial controls had already been recruited, given genotype became available after study participation. Where additional control subjects were required, these were recruited from the Oxford Biobank (OBB) via collaboration with Professor Fredrik Karpe (OCDEM). The OBB is a randomised population based cohort of 30 – 50 year old healthy men and women living in Oxfordshire, who have undergone detailed examination of metabolic parameters (BMI, BP, waist and hip circumference, skin fold thickness measurements, body fat bioimpedance measurements) at a screening visit, donated DNA and given informed consent to be re-approached for a recruit by genotype or phenotype in physiological studies. Therefore, where individuals of particular gender/age/BMI/activity were required as matched controls for either *PTEN* or *CDKN2A* mutation carriers, the OBB database was surveyed and appropriate subjects were selected and invited to take part in the study. For further details see **Chapters 3 and 5**.

## 2.2 Clinical assessment

All cases fulfilled inclusion criteria: age 18-80 years; cancer free for at least 2 years and not taking any medication (e.g. steroids) that may interfere with glucose tolerance. If subjects were unable to come to Oxford for study participation, the research team (myself and a research nurse) conducted

home visits. All participants in the physiological studies (CS subjects – **Chapter 3**; Familial melanoma subjects - **Chapter 5**; and Control subjects) underwent baseline clinical assessment (**section 2.2.1**) and one or several of the physiological studies or procedures described in **2.2.2 - 2.2.5** depending on which part of the study they were participating in.

### 2.2.1 Baseline clinical characteristics and anthropometry

A complete history and full clinical examination was performed on all study participants according to a standard protocol created for the study. Activity level was assessed and graded as 'low', 'medium' or 'high' according to broadly defined groups in Table 1.

| Activity Grade | Definition |
|---|---|
| Low | Rarely takes exercise |
| Medium | Vigorous exercise* 1-2 times a week/on feet much of day/walk or cycle to work > 1 mile/gardening or golf 3 or more times a week |
| High | Vigorous exercise3 or more times a week |

**Table 1** Activity grade definitions allocated to study subjects * Vigorous exercise – sufficient to raise pulse

Presence of any physical features typical of CS in subjects with this diagnosis (e.g. skin tags, lipomas, macroglossia, papillomas, haemangiomas, macrocephaly) was noted. Presence of any melanoma related lesions or scars from previous surgery was noted in those with a history of familial melanoma.

Basic anthropometric measures were taken: two systolic and diastolic blood pressures taken at least ten minutes apart; height (metres); weight (kilogrammes); waist circumference (measured midway between the uppermost border of the iliac crest and the lower border of the costal margin) and hip circumference (measured around the widest portion of the buttocks). Body mass index (BMI) was calculated according to BMI = weight (kg)/height ($m^2$).

Body fat was measured by bioimpedance using a Bodystat 500 (Bodystat, Douglas, UK). In a subset of CS subjects and their matched control subjects, more detailed anthropometry including skin fold thickness measurements were taken. This is discussed in **Chapter 3**.

### 2.2.2 HOMA analysis

HOMA methodology was utilised as a basal, rather than dynamic, measure of insulin sensitivity (HOMA IR) and beta cell function (HOMA B) (Matthews et al. 1985). HOMA IR and HOMA B were calculated using the computer model available via the University of Oxford Diabetes Trials Unit website (http://www.dtu.ox.ac.uk/homacalculator/download.php.) where both formulae incorporate fasting insulin and fasting glucose levels (see Chapter 3 Table 3.3 for more detail).

### 2.2.3 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) is a dynamic test of glucose clearance from the blood, and is used clinically to assess an individual's glucose tolerance depending on fasting glucose and 2 hour glucose levels using WHO criteria (http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf). Experimentally it can be used to derive surrogate measures of insulin sensitivity and/or beta cell function as the plasma glucose and insulin responses during this test reflect the ability of the pancreatic beta-cells to secrete insulin and the sensitivity of the insulin target tissues (Reaven et al. 1993; Stumvoll et al. 2000) – see **Chapter 3** and **Chapter 5** for more details.

Subjects were asked to undergo an overnight fast for at least 12 hours and took only water on the morning of the test. A 23 gauge cannula was placed in an antecubital vein (to avoid the need for multiple venepunctures during the test) and baseline fasting blood samples taken for subsequent analysis of glucose and serum and plasma hormones. Subjects were then asked to drink a standard

75g glucose drink over a minute. Further blood samples were then taken at times 15, 30, 60, 90 and 120 minutes after the glucose load. These samples were immediately stored on ice for further processing (see section **2.3**). The OGTT was performed in OCDEM, but where individuals could not travel it was performed by the research team in people's own homes using portable centrifuges, ice for storage and dry ice for transportation of spun samples back to OCDEM. Home visits were conducted for 32% of subjects (22/68) recruited for OGTTs.

Glucose tolerance was assessed using fasting glucose and glucose at 120 minutes of the OGTT using WHO criteria (http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf). Specific other measures of insulin sensitivity or beta cell function were obtained depending on which parameter was being assessed for the study participant and will be discussed in more detail in the relevant chapters (**Chapters 3 and 5**).

### 2.2.4 Intravenous glucose tolerance test

The intravenous glucose tolerance test (IVGTT) is an established test of beta cell function (Pacini and Mari 2003) and was used for assessment of insulin secretion in *CDKN2A* mutation carriers and control subjects (see **Chapter 5**). Models of insulin secretion enable evaluation of beta-cell function following intravenous injection of a bolus of glucose (Toffolo et al. 1995). The OGTT is a more physiological test than the IVGTT with oral ingestion provoking the 'incretin effect', i.e. the insulinotropic effects of the gut hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Elrick et al. 1964; Kazakos 2011). Insulin action and hepatic insulin extraction can be assessed by models from both OGTT and IVGTT data but models using intravenous protocols are considered simpler and more reliable since the glucose input into the circulation is known, in contrast to OGTT protocols which require assessment of the systemic rate of appearance of the ingested glucose (Cobelli et al. 2007). The IVGTT attains independent measures of beta cell

function and insulin sensitivity, is deemed a particularly good test for assessing beta cell function and is well validated against the hyperglycaemic clamp (DeFronzo et al. 1979; Pacini and Mari 2003). Beta cell function and insulin sensitivity are derived by changing plasma insulin and glucose concentrations analysed by a computer programme, 'The Minimal Model' programme developed by Richard Bergman (Bergman et al. 1979). Further details of the IVGTT results analysis are discussed in **Chapter 5**.

IVGTTs were performed by myself and a research nurse, Beryl Barrow, in OCDEM. Subjects attending for IVGTT were asked to undergo an overnight fast for at least 12 hours and had taken only water on the morning of the test. Subjects were asked to lie supine for at least 15 minutes prior to the test. A 20 gauge cannula was placed in each antecubital vein: one was used for administration of 50% dextrose and the other was used for blood sampling. Baseline fasting blood samples were taken through the sampling cannula, then a dose of 50% dextrose (dose calculated based on weight 0.5mg/kg) was given through the other cannula over 3 minutes and flushed well with 20ml saline. Further blood samples were then taken at time 2, 4, 6, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150 and 180 minutes (after the end of the saline flush) from the sampling cannula. These samples were immediately stored on ice for further processing (see section **2.3**)

### 2.2.5 Hyperinsulinaemic euglycaemic clamp

This is deemed the 'gold standard' test to measure insulin sensitivity (DeFronzo et al. 1979) and was used to assess this parameter in *PTEN* mutation carriers and control subjects (see **Chapter 3**). The clamp studies were performed by myself and two research nurses, Beryl Barrow and Rachel Craven-Todd, in OCDEM. Subjects were asked to undergo an overnight fast for at least 12 hours and had taken only water on the morning of the test. Venous cannulae were inserted into the right antecubital vein for administration of insulin and glucose, and retrogradely into a left dorsal hand

vein for blood sampling. A priming insulin infusion was administered at an increasing rate for the first 10 minutes, then a constant rate (dependent on weight, BMI and estimated body surface area) for the remainder of the 120 minute clamp (DeFronzo et al. 1979): a variable rate infusion of 20% glucose was administered to maintain euglycaemia (5.0mmol/L). Glucose was infused from 4 to 120 minutes and the glucose infusion rate was adjusted manually to achieve a blood glucose level as close as possible to 5mmol/L. At 5 minute intervals, 2ml blood samples were collected from the right dorsal hand vein that had been placed in a heated box to 'arterialize' the venous blood (Liu et al. 1992). The glucose infusion rate was adjusted according to the glucose level in this 2ml sample (analysed immediately via the Haemacue which measures glucose via a glucose dehydrogenase method) aiming for a target of 5mmol/L. Insulin-stimulated glucose uptake (M) was calculated from the mean 20% glucose infusion rate for all subjects and this index of insulin sensitivity was used for the analysis of *PTEN* mutation carriers vs control subjects. For further details see **Chapter 3**.

### 2.2.6 Adipose and muscle tissue biopsies

Adipose and muscle tissue biopsies were taken from a subset of *PTEN* mutation carriers and matched control subjects to analyse components of the insulin signalling pathway in these tissues. Having attended after a 12 hour fast, subjects had biopsies taken from muscle and adipose tissue. Skeletal muscle biopsies were excised from the vastus lateralis muscle under local anaesthesia using a modified Bergstrom needle and suction in the fasting state. Subcutaneous abdominal adipose tissue was taken under local anaesthesia using a 14 gauge sterican needle at time 0 and 120 minutes of an OGTT. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C.

## 2.3 Biochemical assays

### 2.3.1 Sample preparation and storage

Blood samples (plasma in lithium heparinised tubes, serum in SST (serum for storage) tubes and glucose in fluoride oxalate tubes) were immediately kept on ice and spun at 3000rpm for 10 minutes at 4°C within 30 minutes of being taken. The supernatant from each spun sample was then pipetted into smaller 500uL aliquots and immediately stored in -80°C freezers.

### 2.3.2 Glucose, Insulin and Lipid assays

Glucose, insulin and lipid assays were all undertaken via collaboration with Tim James (Biochemistry, John Radcliffe Hospital). Samples were sent for batch analysis.

Glucose was analysed with a standard hexokinase assay using Siemens ADVIA 2400 analyser (Siemens, Frimley, UK). Insulin was analysed by chemiluminescence immunoassay using a Siemens ADVIA Centaur analyser (Siemens). Glucose precision values were 1.9% at 3.8mmol/L and 1.4% at 24.0mmol/L. Insulin precision was 7.3% at 139 pmol/L, 7.4% at 397pmol/L and 7.0% at 1470 pmol/L. Lipid analyses were undertaken using the Siemens ADVIA 2400. Total cholesterol was analysed using a cholesterol oxidase method. Triglyceride was analysed using a method based on glycerol kinase and glycerol phosphate oxidase.

For details of other specific biochemistry assays, please see **Chapter 3**.

## 2.4 RNA extraction

### 2.4.1 Tissue/cell processing and storage for RNA extraction

Human tissues used for this project were either human islets (see **Chapter 4**) made available through collaboration with Paul Johnson and the Oxford Consortium for Islet Transplantation (OXCIT), or human adipose and muscle tissue from CS and control subjects (see **Chapter 3**).

Human islets in media were transferred from tissue culture flasks to 15ml centrifuge tubes. This cell suspension was centrifuged slowly (300rpm for 3 minutes at 4°C) to pellet formation. The supernatant suspension media was removed and the pellet was 'washed' with 1X phosphate

buffered saline (PBS) (Sigma Aldrich, Gillingham, UK). This was re-centrifuged (300rpm for 3 minutes at 4°C) and the PBS 'wash' step was repeated 2 further times before finally resuspending the islets in 5 volumes of RNA*later* (Applied Biosystems, Warrington, UK) to prevent RNA degradation. These samples were kept at 4°C for 24 hours before transfer to freezers.

Muscle and adipose tissue biopsies were washed with saline directly after being taken, weighed and divided into 200mg aliquots. These were wrapped in foil and snap frozen in liquid nitrogen. Samples were transferred to labelled tubes and stored at -80°C.

**2.4.2 RNA extraction using the phenol-chloroform methodology**

RNA extraction was performed by the guanidinium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987).

**Human islets** were thawed on ice and centrifuged at 600rpm for 3 minutes at 4°C to form a pellet. The supernatant RNA*later* was removed before adding 1ml TRIZOL (Applied Biosystems, Warrington, UK). Cells were homogenised and lysed rapidly by passing the solution 5-10 times through a 20 gauge needle and syringe and left to incubate at room temperature for 5 minutes. The lysate was transferred to a 1.5ml eppendorf tube and 200µL chloroform (FisherScientific, Loughborough, UK) was added. The tube was shaken vigorously for 15 seconds for organic and aqueous phase separation to start. Tubes were incubated at room temperature for a further 5 minutes before centrifugation at 12000xg for 10 minutes at 4°C. Following this 3 phases were apparent: an upper aqueous (RNA) layer for transfer; a white (DNA) interphase layer; a pink organic phenol phase (protein) at the bottom of the tube. The clear upper aqueous phase was transferred to a fresh 1.5ml RNase free eppendorf and 500µL isopropranol (FisherScientific, Loughborough, UK) was added to precipitate the RNA. After incubation at room temperature for 5 minutes and inversion to ensure total mixing of the RNA and isopropranol, the tube was left overnight at -20°C. The following day, the sample was centrifuged at 12000xg for 50 minutes at 4°C to pellet the RNA. If

a pellet was not seen the sample was spun for a further 30 minutes at the same speed. The isopropranol was removed carefully to ensure minimal disturbance of the pellet, which was resuspended in 1ml of 75% ethanol (Sigma Aldrich, Gillingham, UK) and centrifuged at 12000xg for 30 minutes at 4°C. The ethanol was removed and this step was repeated with a further 1ml of 75% ethanol. As much as possible of the final ethanol was then removed and the RNA pellet was allowed to air dry for 10 minutes before resuspension in 20µL RNase free water (Ambion, Warrington, UK).

**Adipose tissue and muscle biopsies** were rapidly dissected on dry ice and transferred to 2ml eppendorfs. A ball bearing and 1ml of TRIZOL was added per 100mg tissue. This was homogenised for 5 minutes on the highest frequency of the Retsch free standing homegeniser (Retsch uk, Leeds, UK). The lysate was transferred to a 1.5ml RNase-free eppendorf and centrifuged at 12,000xg for 20 minutes at 4°C to remove insoluble fragments and lipids. The supernatant was removed through the lipid layer and transferred into another RNase-free eppendorf and 200µL chloroform was added. Following this, the RNA extraction protocol for human islets using the guanidinium-thiocyanate-phenol chloroform method was followed as described above.

### 2.4.3 RNA extraction using the RNA spin column methodology

When starting with smaller quantities of tissue for RNA extraction (for example 50-100 human islets in **Chapter 4**) the Purelink™ RNA Mini kit (Ambion, Warrington, UK) was used. RNA precipitation occurs directly onto a Spin Cartridge, therefore not requiring RNA pellet visualisation which can be very challenging when extracting very small quantities of RNA.

Cells were lysed with TRIZOL as described above. After separation into the 3 phases (upper aqueous RNA phase, white DNA interphase and lower pink protein phase) approximately 400µL of the colourless, upper RNA phase was transferred to a fresh RNase-free 1.5ml tube. An equal volume of 70% ethanol was added and mixed well by vortexing. Seven hundred microlitres of this sample was transferred to a Spin Cartridge and collection tube and centrifuged at 12000xg for 15 seconds at

room temperature. The flow-through was discarded and cartridge replaced into the collection tube. The remainder of the starting sample that had been diluted with 70% ethanol was centrifuged similarly until the entire sample had been processed. 700µL of Wash Buffer I was added to the spin cartridge and centrifuged at 12000xg for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was placed into a fresh collection tube. 500µL of Wash Buffer II with ethanol was added to the spin cartridge and centrifuged at 12000xg for another 15 seconds at room temperature. The flow-through was discarded and this wash and spin was repeated once. The spin cartridge and collection tube were centrifuged at 12000xg for 1 minute at room temperature to dry the membrane with attached RNA. The spin cartridge was placed into a recovery tube and 20µL of RNase-free water was added to the centre of the spin cartridge and incubated at room temperature for 1 minute. This was centrifuged at 12000xg for 2 minutes at room temperature. The flow-through was collected and re-eluted through the spin cartridge. The flow-through was transferred to a fresh RNase-free tube and kept on ice for RNA quality determination (see **section 2.5**) or put directly into -80°C freezers.

### 2.5 RNA quality determination

RNA quality was assessed by two methods; spectrophotometric determination of RNA purity and Agilent BioAnalyser determination of RNA integrity.

#### 2.5.1 RNA purity testing using spectrophoto NanoDrop™ technology

A Thermoscientific NanoDrop™ 8000 spectrophotometer (Labtech International Ltd, East Sussex) was used to determine RNA purity. The nucleic acid platform was selected (RNA versus DNA) and the instrument was initialised by placing 1.5µL of water onto the pedestal. The instrument was set to measure RNA using the RNA-40 programme and the water sample was also used to blank the spectrophotometer. The pedestals were wiped clean and 1.5µL of each sample to be analysed was added to each pedestal to determine the concentration of the sample and assess purity through

260/280 and 260/230 ratios. RNA was deemed 'pure' if 260/280 ratio was greater than 2 and 260/230 value was between 2 and 2.2. Sample report and plot are shown in Figure 1a and 1b.

**Figure 1a** Sample report window

Test type: Nucleic Acid 4/6/2009 12:32 PM

apama_nanodrop1_6.4.9 Report Full Mode Ignore

| Plate ID | Well | Sample ID | Date | Time | ng/ul | A260 | A280 | 260/280 | 260/230 | Constant |
|---|---|---|---|---|---|---|---|---|---|---|
| | A1 | B1 | 4/6/2009 | 12:20 PM | 336.13 | 8.403 | 4.066 | 2.07 | 1.68 | 40.00 |
| | B1 | B2 | 4/6/2009 | 12:20 PM | 453.45 | 11.336 | 5.582 | 2.03 | 1.80 | 40.00 |
| | C1 | B3 | 4/6/2009 | 12:20 PM | 413.70 | 10.343 | 5.045 | 2.05 | 1.87 | 40.00 |
| | D1 | X51 | 4/6/2009 | 12:20 PM | 273.21 | 6.830 | 3.318 | 2.06 | 1.75 | 40.00 |
| | E1 | X52 | 4/6/2009 | 12:20 PM | 292.21 | 7.305 | 3.556 | 2.05 | 1.68 | 40.00 |
| | F1 | X53 | 4/6/2009 | 12:20 PM | 240.47 | 6.012 | 2.901 | 2.07 | 1.77 | 40.00 |
| | G1 | 13181 | 4/6/2009 | 12:20 PM | 268.51 | 6.713 | 3.299 | 2.03 | 1.59 | 40.00 |
| | H1 | 13182 | 4/6/2009 | 12:20 PM | 233.49 | 5.837 | 2.821 | 2.07 | 1.74 | 40.00 |
| | A2 | 13183 | 4/6/2009 | 12:27 PM | 419.58 | 10.490 | 5.148 | 2.04 | 1.50 | 40.00 |
| | B2 | 131 | 4/6/2009 | 12:27 PM | 2240.74 | 56.018 | 26.426 | 2.12 | 2.02 | 40.00 |
| | C2 | 132 | 4/6/2009 | 12:27 PM | 1812.63 | 45.316 | 21.588 | 2.10 | 1.93 | 40.00 |
| | D2 | 133 | 4/6/2009 | 12:27 PM | 629.36 | 15.734 | 7.634 | 2.06 | 1.69 | 40.00 |
| | E2 | X751 | 4/6/2009 | 12:27 PM | 1059.61 | 26.490 | 12.588 | 2.10 | 1.77 | 40.00 |
| | F2 | X752 | 4/6/2009 | 12:27 PM | 211.60 | 5.290 | 2.712 | 1.95 | 1.32 | 40.00 |
| | G2 | X753 | 4/6/2009 | 12:27 PM | 278.69 | 6.967 | 3.452 | 2.02 | 1.30 | 40.00 |
| | H2 | X1731 | 4/6/2009 | 12:27 PM | 1239.41 | 30.985 | 14.785 | 2.10 | 1.93 | 40.00 |
| | A3 | X1732 | 4/6/2009 | 12:29 PM | 918.89 | 22.972 | 12.405 | 1.85 | 1.49 | 40.00 |
| | B3 | X1733 | 4/6/2009 | 12:29 PM | 250.09 | 6.252 | 3.046 | 2.05 | 1.54 | 40.00 |

**Figure 1a** Sample readout from Nanodrop 8000 spectrometer with RNA concentration, 260/280 and 260/230 ratios given.

**Figure 1b** Sample plot

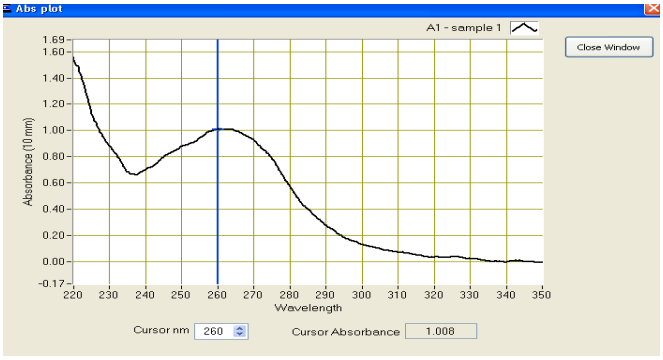



**Figure 1b** Sample RNA nanodrop spectrophotometer wavelength plot showing expected well defined peak absorbance at 260nm. If this peak shifts it is an indication of sample contamination.

### 2.5.2 RNA integrity determination using Agilent Bioanalyser technology

RNA integrity was assessed using a total eukaryotic RNA 6000 nano kit (Agilent, Wokingham, UK). The Agilent 2100 Bioanalyser and chip priming station were set up according to the manufacturer's guidelines. Gel solutions were prepared through filtration in a spin column at 15000xg for 10 minutes. Sixty-five microlitre aliquots of filtered gel were stored in 0.5ml RNase-free tubes at 4°C and were stable for 4 weeks. For the RNA analysis, 1µL of RNA 6000 Nano dye concentrate was added to one of the 65µL aliquots of gel matrix. This was centrifuged at 13000xg for 10minutes and 9µL was added to well 12 of the RNA chip. The chip priming station was locked for 30 seconds to

permit gel dispersal through the chip, then 9µL of gel-dye mix was added to positions 4 and 8 of the chip. Five microlitres of marker was added to all other wells, 1µL of sample to be analysed in wells 1-11 and 1µL of RNA ladder into well 16. The chip was vortexed at 2400rpm for 1 minute before being placed on the Bioanalyser. The Bioanalyser detects RNAs through a laser and emitting fluorescence. RNA integrity was calculated using a computer based model algorithm and was presented as RIN scores: RIN scores > 5.5 were taken as acceptable. A sample trace is shown in Figure 3.

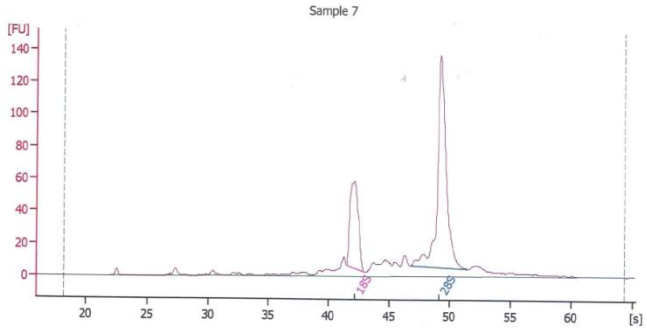



**Figure 3** Sample trace from RNA integrity testing by the Agilent Bioanalyser. Sample shown is from good quality RNA with RIN score 9.10. There are clearly identifiable ribosomal 18S and 28S peaks which are no longer present if there has been significant RNA degradation.

**2.6 Reverse transcription**

This was used to make cDNA from all RNA samples where further analysis by QRT-PCR was required.

**2.6.1 DNase I treatment for removal of genomic contamination**

To remove genomic DNA contamination (and therefore amplification in any subsequent qPCR reaction), RNA samples were treated with DNase I (Amnion, Warrington, UK) prior to cDNA synthesis. One microlitre of DNase I and 10 x DNase I buffer were added to each sample followed by incubation at 37°C for 30 minutes. Two microliters of DNase inactivation buffer was then added and incubated for 2 minutes at room temperature to terminate the reaction. The sample was centrifuged at top speed in a desk-top centrifuge for 1.5 minutes to pellet the DNase inactivation reagent. The DNA free RNA supernatant was transferred to a fresh RNase-free tube and stored at -20°C.

### 2.6.2 cDNA synthesis

RNA was transcribed to single stranded cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK) which contained a recombinant Moloney murine leukaemia virus reverse transcriptase. Reactions were performed both with (RT positive) and without (RT negative) the reverse transcriptase enzyme. The 20µL reaction mix comprised 2µL of 10X RT buffer, 2 µL of 10X RT random primers, 0.8µL of 100mM dNTP mix, 1µL of 50U/µL reverse transcriptase (this was replaced with 1µL RNase free water in the RT negative reaction) and up to 14.2µL of RNA (depending on the concentration of RNA extracted and starting quantity of RNA – if <14.2µL RNA was used the remainder was made up with RNase free water). The 20µL samples were placed on a thermocycler and underwent the following cycle: 25°C for 10 minutes; 37°C for 120 minutes and 85°C for 5 minutes. Samples were immediately used for qRT-PCR (see section 2.8.5) or stored at -20°C until required.

## 2.7 mRNA quantification

Taqman technology (Applied Biosystems, Warrington, UK) was selected for mRNA quantification in these studies due to its high specificity. The method was first reported in 1991 (Holland et al. 1991) and has been adapted for researchers by Applied Biosystems.

### 2.7.1 Taqman methodology and probe selection

Taqman oligonucleotide probes are designed to bind to a DNA region amplified by a specific primer pair. The probe has a fluorophore covalently attached to the 5'end of the oligonucleotide and a quencher at the 3'end which, due to its proximity, quenches any fluorescence signals. As the Taq polymerase extends the primer and synthesises the new strand, the 5' to 3' exonuclease activity hydrolyses the oligonucleotide which consequently releases the fluorophore from its proximity to the quencher, thereby allowing fluorescence. This is detected in the real-time PCR thermal cycler

and as fluorescence is directly proportional to the amount of fluorophore released, one can quantify the amount of DNA present in the PCR.

All primers and probes were selected from the Applied Biosystems inventoried list if available. Probes that crossed exon-exon boundaries (_m1) were selected in preference to those lying within a single exon (_s1) or within genomic DNA (_g1), to prevent non-specific amplification.

### 2.7.2 Analysis method

RT-PCR data may be used to quantify mRNA in two ways: relative quantification is based on housekeeping genes (internal reference genes) to determine fold-differences in expression of the target gene whereas absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards. Relative quantification was the method used in all contained experiments using the comparative $C_T$ method ($\Delta\Delta C_T$). As detailed above, fluorescence was emitted exponentially during successive rounds of the PCR. $C_T$ values were recorded for each sample as the cycle number at which fluorescence crossed a pre-determined threshold value (during the exponential phase of the PCR reaction). Expression levels of genes of interest were determined in two steps; firstly results were calibrated to a control sample ($\Delta C_T$) followed by normalisation to a housekeeping gene(s) ($\Delta\Delta C_T$).

### 2.7.3 Efficiency calculations and standard curves

The $\Delta\Delta C_T$ method requires that the Taqman gene expression assays have similar efficiencies during the PCR reaction. To check this and correct for differences between assay efficiencies during analysis, standard curves of five serial dilutions of known concentration were plated out alongside samples to be tested for each assay. Pools of concentrated cDNA were formed from 1µL of each sample to be tested in the experiment. Five standards were generated through serial dilution with concentrations both above and below the dilution at which the samples would be run: if samples

were to be run at 1:20 dilution, standards were generated at 1:10, 1:20, 1:40, 1:80 and 1:160 using 0.01M TRIS HCL. Linear regression was used to calculate a line of best fit and assay efficiency calculated using the equation: **Efficiency = (10^[-1/slope])$^{-1}$.** Assay efficiency was incorporated into $\Delta\Delta C_T$ calculations using (1 + Efficiency)$^{-\Delta\Delta CT}$ (Pfaffl 2001).

### 2.7.4 Housekeeping genes

Housekeeping genes (HKGs) were selected on the basis that their expression would not change appreciably between tissues or reaction conditions tested within the experiment. For example if the mRNA expression of particular genes was being assessed across a range of tissues to investigate expression in relevant tissues, HKGs were selected that were known to be ubiquitously and reasonably uniformly expressed across these tissues. Similarly, if the experiment was being used to quantify success of gene knockdown by different methods (eg transfection or adenoviral infection) it was important to select HKGs that were minimally affected by the siRNA delivery system. Thus for the human tissue panel experiments (**Chapter 4**), well established HKGs were used: beta-2-microglobulin (B2M), beta glucuronidase (GUSB) and hypoxanthine-guanine phosphoribosylytransferase 1(HPRT1). The gene knockdown experiments were optimised for use in human islets and HKGs were chosen for their stable expression in this tissue and included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HPRT1, PPIA (peptidylprolyl isomerise A) and B2M (Neville et al. ; Yang et al.). At least two HKGs were included per experiment for analysis as using the geometric mean of more than one HKG has been demonstrated as a more accurate method of normalisation (Vandesompele et al. 2002).

### 2.7.5 Quantitative real time PCR (QRT-PCR)

cDNA samples were diluted to reaction concentrations most appropriate for the gene expression assays being used. For example if genes of interest were thought to be lowly expressed and starting quantities of RNA for the cDNA synthesis had been low (<1µg), then samples were run at more concentrated dilutions (1:5, 1:10 or 1:20) depending on quantities of sample cDNA. If starting quantities of RNA were greater (1-2µg), samples were run at dilutions of 1:50 or 1:100. All starting quantities of RNA and sample dilutions were consistent within each individual experiment to permit relative quantification using the comparative $C_T$ method ($\Delta\Delta C_T$).

All samples for each specific assay were run on the same plate as the standard curve for that assay to ensure accuracy of the efficiency calculation. The 10µL sample reaction volume consisted of: 4µL sample cDNA (at the appropriate dilution); 5.5µL gene expression mastermix (Applied Biosystems, Warrington, UK); and 0.5µL gene assay/probe. The standard reaction volume consisted of 4µL of standard with all remaining components the same. All reactions were performed in triplicate and amplified on an ABI7900 HT machine using the amplification programme: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds; and 60°C for 1 minute.

**2.8 RACE PCR**

RACE (rapid amplification of cDNA ends) PCR is used to obtain full length cDNAs for mRNAs for which part of the sequence is known and to identify alternative 5' or 3' ends of fully sequenced genes (Frohman et al. 1988). The method consists of using PCR to amplify, from extractions of tissue mRNA, the regions between the known parts of the sequence and non-specific tags appended to the ends of the cDNA. The poly(A) tail acts as the tag at the 3' end of the mRNA: however an artificial one needs to be generated at the 5' end which can make the RACE more technically challenging at this end (see **Chapter 4** for more detail).

**2.9 Statistical analysis**

All analyses were performed in SPSS version 20.0. Given the small sample sizes and distribution of the data, non-parametric tests were employed for most comparisons. The Mann-Whitney U test (two-tailed) was used to analyse the clinical data; all P values stated are two-sided. Further details on specific statistical analyses are given in the relevant chapters.

# Chapter 3

*PTEN* mutations as a cause of constitutive insulin sensitivity and obesity in humans

# Chapter 3

## 3.1 Introduction

### 3.1.1 PTEN is a component of metabolic and cellular growth pathways

There is considerable epidemiological evidence linking T2D and cancer (Giovannucci et al. 2010; Smith and Gale 2010) with an increased risk of liver, pancreatic, breast and endometrial cancers in particular (Giovannucci et al. 2010). This literature extends to associations between anti-diabetes therapies and cancer risk with increased cancer incidence observed with use of some insulin analogues and a purported reduction of cancer risk with metformin therapy (Smith and Gale 2009; Giovannucci et al. 2010). Following this, genetic data solidify the links between cancer and T2D given the discovery of T2D-susceptibility loci within or proximal to genes involved in cell-cycle regulation (Voight et al. 2010; Morris et al. 2012).

Biological mechanisms that may underlie the connections between T2D and cancer include the hyperactive insulin and IGF-1 axis in T2D (Pollak 2008), the chronic hyperglycaemic (Vander Heiden et al. 2009) and inflammatory (Park et al. 2010) states that can exist in T2D, all of which potentially provide conditions promoting cancer growth. Other plausible explanations for this overlap are the possible impact of inherited pro-proliferative effects upon functioning beta-cell mass and the existence of common signalling pathways to both cell growth and metabolism, as exemplified by metformin (Zhou et al. 2001; Zakikhani et al. 2006).

An example of a component common to both cellular growth and metabolic pathways, is the tumour suppressor phosphatase and tensin homolog (PTEN), a protein and lipid phosphatase that antagonises the phosphatidylinositol 3-kinase (PI3K) pathway and has roles in both cell-cycle and

metabolic pathways (Maehama and Dixon 1998; Goberdhan and Wilson 2003)(Figure 3.1). The PI3K-AKT pathway has roles in cell growth, proliferation, apoptosis, cell migration and cytoskeletal rearrangement (Vivanco and Sawyers 2002), as well as mediating many of the metabolic downstream effects of insulin.

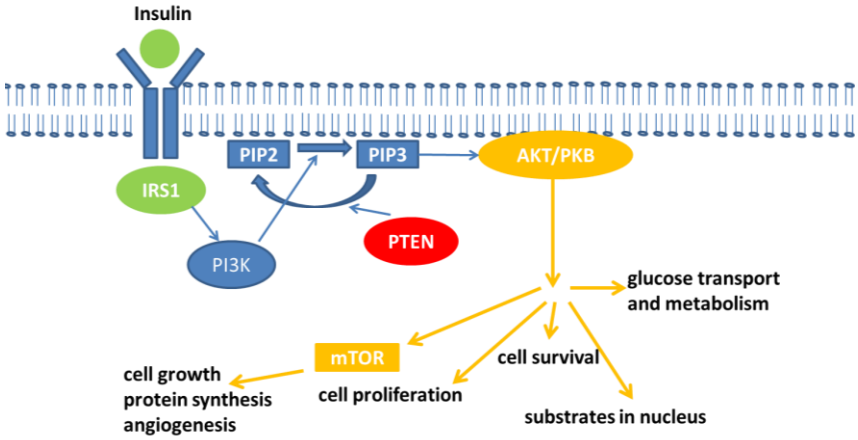



**Figure 3.1** Insulin signalling via the PI3K/AKT pathway. PI3K is activated by IRS1 (amongst other cell receptor and ligands) and phosphorylates phosphatidylinositol 4,5-biphosphate ($PIP_2$) to phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) which subsequently recruits AKT to the plasma membrane where it is in turn phosphorylated and activated. Activated AKT has multiple downstream effects in the cell needed for cellular growth and metabolism. PTEN negatively regulates this pathway by dephosphorylating $PIP_3$ to $PIP_2$.

### 3.1.2 *PTEN* is a tumour suppressor gene and germline mutations cause Cowden syndrome (CS)

By antagonising the PI3K-AKT pathway, PTEN dysregulates many of the processes required for cell cycling and growth. Hence *PTEN*, on chromosome 10q23.3, is a well-recognised tumour suppressor gene (Cantley and Neel 1999; Simpson and Parsons 2001) and is amongst the most commonly somatically mutated genes in a wide range of tumours (Hollander et al. 2011). Furthermore, germline inactivating *PTEN* mutations cause a number of autosomally dominantly inherited cancer predisposition syndromes also known as the PTEN hamartoma tumour syndromes and include CS, Lhermitte-Duclos diseases, Bannayan-Riley-Ruvalcaba syndrome and Proteus syndrome (Liaw et al. 1997; Marsh et al. 1998; Marsh et al. 1998; Blumenthal and Dennis 2008; Hobert and Eng 2009). CS is the most common of these and is associated with increased risk of breast, thyroid and endometrial cancer (Lloyd and Dennis 1963; Weary et al. 1972) and in around 80% cases is due to

germline loss-of-function *PTEN* mutations (Nelen et al. 1996; Marsh et al. 1998). CS is now well recognised as a very variable, autosomal dominant hereditary cancer predisposition syndrome characterised by multiple hamartomas and increased risk of developing malignant transformation (Eng 2000; Farooq et al. 2010).

CS is rare with estimated prevalence 1 in 200, 000 and only around 300 recognised cases in the UK (Nelen et al. 1999; Blumenthal and Dennis 2008; Farooq et al. 2010). This is most likely an underestimation as there is a varied phenotype not readily recognised as a syndrome by many clinicians. Most reported cases are in Caucasians and it is more commonly diagnosed in females (probably reflecting the diagnostic criteria which are biased towards diagnosing females through breast and endometrial cancers) (Starink et al. 1986; Farooq et al. 2010) with age at diagnosis ranging between 13 and 65 years (Salem and Steck 1983; Eng 2000).

Features of CS include benign hamartomas particularly in the skin and gastrointestinal tract, facial trichilemmomas, acral keratoses, papillomatous papules, gastrointestinal polyps: a hallmark feature of the syndrome is macrocephaly (Blumenthal and Dennis 2008). Diagnostic criteria are based on clinical findings and summarised in Table 3.1. Diagnosis is made with presence of any pathognomonic criteria; presence of two major criteria one of which must be macrocephaly; one major criterion and three minor criteria; four minor criteria (Eng 2000; Farooq et al. 2010).

| Pathognomonic criteria |
|---|
| • Adult Lhermitte-Duclos disease (LDD) (cerebellar tumours)<br>• Mucocutaneous lesions:<br>• Facial trichilemmomas<br>• Acral keratoses<br>• Papillomatous papules |
| **Major criteria** |
| • Breast carcinoma<br>• Non-medullary thyroid carcinoma<br>• Macrocephaly (megalocephaly) >97th percentile<br>• Endometrial carcinoma |
| **Minor criteria** |
| • Other thyroid lesions (e.g. adenoma, multinodular goitre)<br>• Mental retardation (IQ <75)<br>• Gastrointestinal hamartomas<br>• Fibrocystic disease of the breast<br>• Lipomas<br>• Fibromas<br>• Genitourinary tumours (especially renal cell carcinoma)<br>• Genitourinary structural manifestations<br>• Uterine fibroids |

**Table 3.1** Diagnostic criteria for CS based upon Nation Comprehensive Cancer Network (NCCN) criteria <http://www.nccn.org>

### 3.1.3 Relevance of PTEN to glucose homeostasis

As mentioned above the PI3K-AKT pathway is also key in the metabolic downstream effects of the insulin signalling pathway (Figure 3.2).

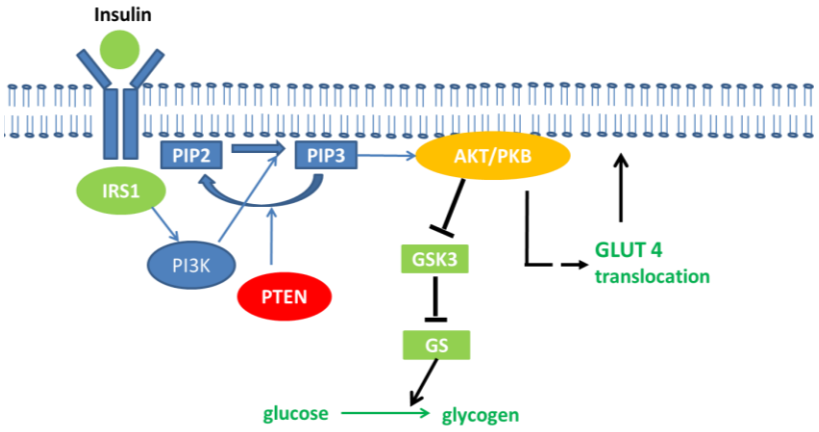



**Figure 3.2** PI3K-AKT pathway and glucose metabolism in an insulin target tissue such as adipose or muscle tissue. Activated AKT/PKB leads to metabolic downstream effects of insulin; inhibition of glycogen synthase kinase-3 (GSK3) and glucose uptake via increased GLUT 4 translocation to the cell surface (Burgering and Coffer 1995; Watson et al. 2004).

As PTEN inhibits the PI3K-AKT pathway, it is a negative regulator of glucose uptake and transport in the cell. Consistent with this, *Pten* haploinsufficient mice, display increased phosphorylation and activation of the PI3K-AKT pathway, resulting in increased insulin sensitivity and improved glucose tolerance when challenged with insulin and glucose (Wong et al. 2007). Tissue-specific knockout of *Pten* in rodents also supports a role for reduced Pten levels in improved glucose tolerance: muscle specific *Pten* deletions prevented development of insulin resistance and diabetes in mice fed a high-fat diet (Wijesekara et al. 2005); liver specific *Pten* deletion led to increased hepatic insulin sensitivity and better systemic glucose tolerance (Stiles et al. 2004); adipose tissue *Pten* knockouts led to enhanced insulin sensitivity and glucose tolerance in mice (Kurlawalla-Martinez et al. 2005); pancreatic beta-cell specific deletion has led to increased islet mass and also lower fasting glucose levels in mice (Nguyen et al. 2006; Stiles et al. 2006). Global *Pten* knockouts are embryonic lethal (Di Cristofano et al. 1998; Podsypanina et al. 1999), however Butler et al investigated the effects of suppressing *Pten* expression with an antisense oligonucleotide inhibitor and showed reduced hyperglycaemia and improved insulin sensitivity in diabetic mice (Butler et al. 2002). This was confirmed by the work of Wong and colleagues who, as aforesaid, demonstrated enhanced insulin sensitivity and glucose tolerance in haploinsufficient *Pten* mice (Wong et al. 2007). Interestingly, in a recent study overexpressing *Pten* in mice, 'Super-PTEN' mutants displayed metabolic effects of reduced glucose uptake, reduced body size and body fat and increased energy expenditure as well as tumour suppression (Garcia-Cao et al. 2012).

CS provides a unique opportunity to study the effect of *PTEN* haploinsufficiency on insulin action and glucose tolerance in humans. Given the dual role of PTEN in both cell growth and metabolism, it was hypothesised that subjects with inactivating *PTEN* mutations would have increased insulin sensitivity and beta-cell function (Figure 3.3). The aims were to recruit individuals with CS due to *PTEN*

mutations and matched (age, gender and BMI) control subjects, and investigate glucose tolerance, insulin sensitivity and beta-cell function.

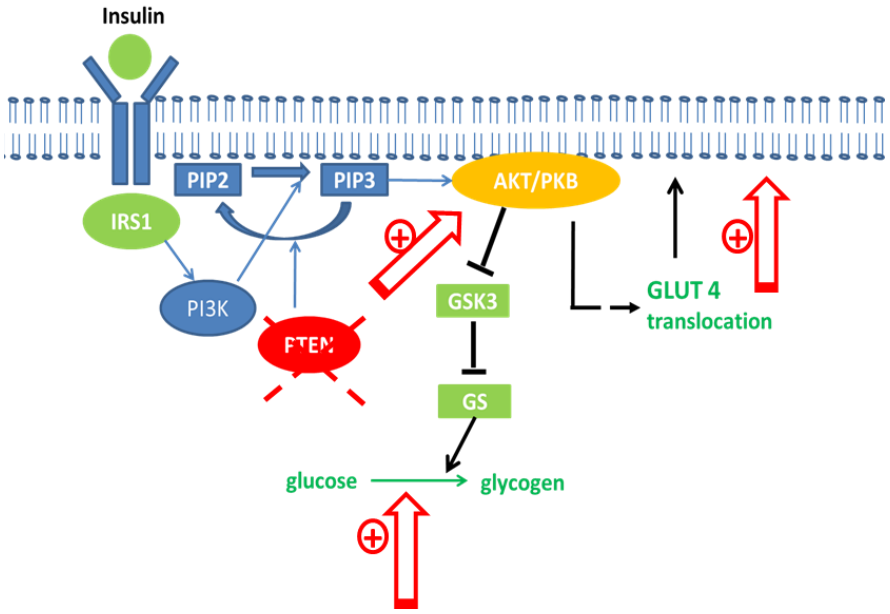



**Figure 3.3** Schematic of hypothesis: individuals with *PTEN* mutations may have amplified PI3K-AKT signalling through less conversion of PIP3 to PIP2, subsequently leading to enhanced glucose uptake via increased GLUT 4 translocation and increased glycogen synthase (GS) activity (highlighted by hollow red arrows).

## 3.2 Methods

### 3.2.1 Subjects studied

Recruitment of subjects with CS due to heterozygous loss-of-function *PTEN* mutations was initiated in Oxford through a collaboration with a Clinical Geneticist, Dr Lisa Walker (Churchill Hospital). To increase numbers, subjects were also recruited from around the UK via the Clinical Genetics Network. In collaboration with Clinical Geneticists at 5 other centres [Dr Louise Izatt (London); Dr Mary Porteus (Edinburgh); Dr Nicola Cooper (Birmingham); Dr Katharine Lachlan (Southampton)], invitation letters and information sheets about the study were posted to eligible potential participants. All interested subjects who fulfilled inclusion criteria were enrolled into the study. Inclusion criteria for the study were:-

- Clinical diagnosis of CS: major criteria being macrocephaly and one of thyroid, breast or endometrial carcinoma; minor criteria are other thyroid lesions, mental retardation, gastrointestinal hamartomas, lipomas, fibrocystic disease of the breast, uterine fibroids, fibromas and genitourinary tumours or malformations. Diagnosis requires the presence of two major criteria, one major and three minor criteria or four minor criteria (Table 3.1) (Eng 2000).
- *PTEN* mutation confirmed by a certified molecular diagnostics laboratory (Table 3.2)
- Subject should have been cancer free for at least 2 years prior to the study.
- Subject should not be taking any medications that may interfere with glucose tolerance e.g corticosteroids.
- Age of subject should be 18 – 80 years.

Recruitment of control subjects was via the Oxford Biobank (collaboration with Professor Fredrik Karpe). The Oxford Biobank (OBB www.oxfordbiobank.org.uk) is a collection of healthy men and women living in Oxfordshire who have undergone detailed assessment, given DNA and consent to be re-approached for research purposes. As the primary investigative measures were regarding glucose

tolerance and metabolic parameters, every effort was made to identify well matched (age, gender, BMI, activity) control subjects. Once CS subjects with *PTEN* mutations were recruited into the study, a search of the OBB was conducted (using age, gender and BMI) to identify potential participants to contact. Appropriate approval was sought and gained from the Oxfordshire Local Research Ethics Committee REC B, and all subjects gave written informed consent.

| Nucleotide Effect | Protein Effect | Exon | Protein Domain | | Functional consequence |
|---|---|---|---|---|---|
| *Deletions* | | | | | |
| c.141delG | - | 2 | Catalytic | Phosphate | Frameshift, truncates |
| c.141delG | - | 2 | Catalytic | Phosphate | Frameshift, truncates |
| *Nonsense* | | | | | |
| c.81T>A | Tyr27X | 2 | Catalytic | Phosphate | Truncates |
| c.176C>G | Ser59X | 3 | Catalytic | Phosphate | Truncates |
| c.388C>T | Arg130X | 5 | Catalytic | Phosphate | Truncates |
| c.654C>A | Cys218X | 7 | Lipid membrane-binding | C2 lipid | Truncates |
| c.697C>T | Arg223X | 7 | Lipid membrane-binding | C2 lipid | Truncates |
| c.697C>T | Arg223X | 7 | Lipid membrane-binding | C2 lipid | Truncates |
| c.954-957delTACT | Thr391X | 8 | Lipid membrane-binding | C2 lipid | Frameshift, truncates |
| *Missense* | | | | | |
| c.144C>G | Asn48Lys | 2 | Catalytic | Phosphate | Phosphatase null |
| c.202T>G | Tyr68Asp | 3 | Catalytic | Phosphate | Phosphatase null |
| c.379G>A | Gly127Arg | 5 | Catalytic | Active Site | Phosphatase null |
| c.379G>A | Gly127Arg | 5 | Catalytic | Active Site | Phosphatase null |
| c.512A>G | Gln171Arg | 6 | Catalytic | Phosphate | Phosphatase null |
| c.518G>A | Arg173His | 6 | Catalytic | Phosphate | Phosphatase null |

**Table 3.2** *PTEN* mutation details for all subjects studied. All sequence information is based on Genbank reference sequence NM_000314.4.

### 3.2.2 Clinical assessment

A full history and examination was conducted taking particular note of any cancers and treatments undergone by the participant. Subjects were asked specifically about a personal history or family history of diabetes. Any features of CS were noted (e.g macrocephaly, macroglossia, lipomas, skin tags, papillomas, other hamartomas). A detailed family history of each subject with CS was taken, noting all other family members with a diagnosis of the syndrome.

Basic anthropometric measures were taken: two systolic and diastolic blood pressures taken at least ten minutes apart; height (metres); weight (kilogrammes); waist circumference (measured midway between the uppermost border of the iliac crest and the lower border of the costal margin); hip circumference (measured around the widest portion of the buttocks). Body mass index (BMI) was calculated according to BMI = weight (kg)/height ($m^2$).

### 3.2.3 Oral glucose tolerance test

All subjects underwent a standard 75g OGTT (described in Chapter 2 Methods **section 2.2.3**). This was used to assess glucose tolerance (using fasting and 2 hour glucose levels) as well as a tool to measure insulin sensitivity and beta-cell function through a range of indices (Table 3.3). Although not a 'gold-standard' test to measure insulin sensitivity or beta-cell function, the OGTT was selected as a simple dynamic test that could be performed reproducibly in all study participants and in people's homes should they be unable to travel to Oxford. As the test does stimulate insulin secretion and glucose uptake, it gives reasonable estimates of beta-cell function and insulin sensitivity: the indices described in Table 3.3 have been well validated against recognised gold standard tests of insulin sensitivity (hyperinsulinaemic euglycaemic clamp) and beta-cell function (frequently sampled intravenous glucose tolerance test and hyperglycaemic clamp studies) (Stumvoll et al. 2000; Pacini and Mari 2003). All measures of insulin sensitivity and beta-cell function derived from the OGTT were calculated according to the formulae described in Table 3.3. HOMA IR and

HOMA B were calculated using the computer model available via the University of Oxford Diabetes Trials Unit website (http://www.dtu.ox.ac.uk/homacalculator/download.php.).

**Table 3.3** Surrogate measures of insulin sensitivity and beta-cell function derived from the oral glucose tolerance test

| Name of measure/index | Physiological parameter measured | OGTT time-point (min) and assay used | Formula or mathematical model used | Reference |
|---|---|---|---|---|
| HOMA B | Beta-cell function | Fasting (time 0) insulin and glucose | Computer model<br>Approximate equation: (20 X insulin)/(glucose -3.5) | (Matthews et al. 1985) |
| HOMA IR | Insulin resistance | Fasting (time 0) insulin and glucose | Computer model<br>Approximate equation: (glucose X insulin)/22.5 | (Matthews et al. 1985) |
| Fasting insulin | Insulin sensitivity | Fasting (time 0) insulin | NA | |
| Insulinogenic index | Beta-cell function | Time 0 and time 30 insulin and glucose | $(insulin_{30} – insulin_0)/(glucose_{30} - glucose_0)$ | (Phillips et al. 1994) |
| Disposition index | Beta-cell function | Time 0 and time 30 insulin and glucose | Insulinogenic index/HOMA IR | (Phillips et al. 1994) |
| Area under curve for insulin ($AUC_{insulin}$) | Insulin sensitivity | Time 0,15,30,60,90 and 120 insulin | Calculate AUC using trapezoidal rule | |
| Stumvoll index | Insulin sensitivity | Time 0 insulin and time 120 glucose and insulin | $0.156-0.0000459 \times insulin_{120} – 0.000321 \times insulin_0 -0.00541 \times glucose_{120}$ | (Stumvoll et al. 2000) |
| Matsuda index | Insulin sensitivity | Time 0,15,30,60,90 and 120 glucose and insulin | $10000/ \sqrt{[( glucose_0 \times insulin_0) \times ( glucose_{mean} \times insulin_{mean} )]}$ | (Matsuda and DeFronzo 1999) |

### 3.2.4 Biochemical assays

All samples were collected and spun immediately, kept on ice and stored in -80˚ freezers as described in Chapter 2 Methods (**section 2.3.1**) by myself. Glucose, insulin and lipid assays are described in Chapter 2 Methods (**section 2.3.2**) and were performed by Dr Tim James (Biochemistry, John Radcliffe Hospital). Apolipoprotein B was analysed as an additional lipid marker using a kit supplied by Randox Laboratories Ltd, (Crumlin, County Antrim) adapted for use on an ILab650 analyser, (Instrumentation Laboratory UK Ltd, Warrington Cheshire) by Sandy Humphries (OCDEM). The immunoturbidimetric assay is based on the reaction between a sample containing human Apo B and a specific antibody forming an insoluble complex which can be measured turbidometrically at 340nm which is read against a standard curve.

In addition to glucose, insulin and lipids, fasting leptin and adiponectin were measured as markers of adipocyte function and also for their association with insulin resistance (Trujillo and Scherer 2006; Groeneveld et al. 2012). Highly sensitive C-reactive protein (HsCRP) was measured for its association with insulin resistance (Visser et al. 1999; Corrado et al. 2010). Leptin and total adiponectin were assayed on a 1235 AutoDELFIA (PerkinElmer Lifesciences, Boston, US) automatic immunoassay system using two-step time-resolved fluorometric assays. HsCRP was analysed using Siemens ADVIA chemistry wide range CRP kit (Siemens, Camberley, UK) adapted for use on an Instrumentation Laboratory ILab 650 Analyser (Instrumentation Laboratory (UK) Ltd., Warrington, UK).

### 3.2.5 Hyperinsulinaemic euglycaemic clamp assessment

This test was performed as described in Chapter 2 Methods (**section 2.2.5!**). Insulin-stimulated glucose uptake (M) was calculated from the mean glucose infusion rate during the final 40 minutes of the clamp in mg/min and adjusted for adiposity; $M_{LBM}$ = M/lean body mass (measured with a bioimpedence meter) in mg/kg/min. As the level of hyperinsulinaemia varied between cases, $M_{LBM}$/I

(insulin sensitivity index) was calculated as M divided by the mean plasma insulin level during the last 40 min of the clamp. This index of insulin sensitivity (M/I) was used for comparison between *PTEN* mutation carriers and control subjects (DeFronzo et al. 1979).

### 3.2.6 Assessment of body composition and body fat distribution

Body fat was measured by bioimpedance using a Bodystat 500 (Bodystat, Douglas, UK). This method measures body resistance by recording a voltage drop in the applied electric current. Muscle and viscera which are water and electrolyte-rich tissues give minimal impediment to the passing current. In contrast, adipose tissue has little intracellular fluid compared to fat content which is a natural insulator and therefore triggers voltage reduction and consequently resistance elevation (Jaffrin 2009).

In a subset of CS subjects (who had consented to attend for DXA) and their matched control subjects more detailed anthropometry, including skin fold thickness measurements, were taken. Suprailiac and triceps measurements were taken in duplicate using skinfold calipers and the mean measurement recorded to the nearest millimetre. A third measurement was recorded if a difference of >1mm was noted between the first two measurements. Suprailiac skinfolds were measured immediately above the crest of the ilium with skin pinched following the natural fold along a line from the suprailiac to the umbilicus at an angle of approximately 30 degrees. Triceps skinfolds were measured midway along the posterior aspect of the arm between the top of the acromial process to the bottom of the olecranon process of the ulna with skin pinched parallel to the vertical axis of the arm.

### 3.2.6.i Dual X-ray Absorptiometry (DXA) Scan

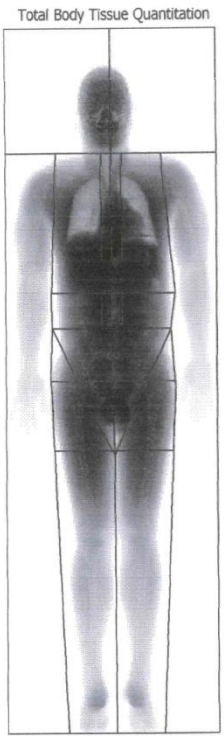
DXA is considered the gold standard for measurement of body composition and body fat measurements (Hind et al. 2011). For this study the DXA was performed using a Lunar iDEXA Bone Densitometer scanner (Lunar Radiation Corporation, Madison, WI) with the patient in the supine position and total body scans taken from the cranium downwards to the feet. One operator (Jane Cheeseman) performed all scanning and analyses and followed the manufacturer's guidelines for patient positioning and for scan acquisition. All scans were analysed using enCORE Software, version 11.0 (GE Lunar) which calculates the grams of fat tissue, fat mass percentage, grams of lean tissue and grams of bone mineral mass (Figure 3.4).

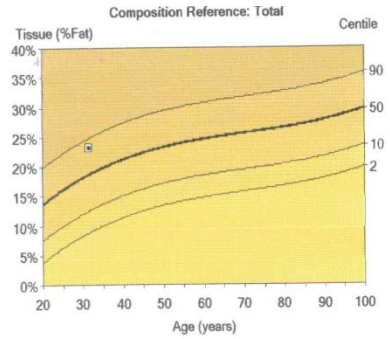
**BODY COMPOSITION**

| Region | Tissue[1] (%Fat) | Region (%Fat) | Tissue[1] (g) | Fat[1] (g) | Lean[1] (g) | BMC (g) | Total Mass (kg) |
|---|---|---|---|---|---|---|---|
| Left Arm | 14.4 | 13.8 | 5,575 | 803 | 4,772 | 234 | 5.8 |
| Left Leg | 20.8 | 20.0 | 14,917 | 3,102 | 11,815 | 612 | 15.5 |
| Left Trunk | 27.3 | 26.7 | 23,060 | 6,296 | 16,763 | 496 | 23.6 |
| Left Total | 23.3 | 22.5 | 45,663 | 10,635 | 35,028 | 1,611 | 47.3 |
| Right Arm | 13.4 | 12.8 | 5,477 | 731 | 4,745 | 231 | 5.7 |
| Right Leg | 20.3 | 19.5 | 14,884 | 3,021 | 11,863 | 612 | 15.5 |
| Right Trunk | 28.3 | 27.7 | 22,394 | 6,342 | 16,053 | 503 | 22.9 |
| Right Total | 23.4 | 22.5 | 46,474 | 10,858 | 35,616 | 1,800 | 48.3 |
| Arms | 13.9 | 13.3 | 11,051 | 1,535 | 9,517 | 465 | 11.5 |
| Legs | 20.5 | 19.7 | 29,801 | 6,123 | 23,678 | 1,223 | 31.0 |
| Trunk | 27.8 | 27.2 | 45,454 | 12,638 | 32,816 | 999 | 46.5 |
| Android | 32.3 | 32.0 | 6,662 | 2,151 | 4,511 | 58 | 6.7 |
| Gynoid | 28.2 | 27.5 | 14,126 | 3,977 | 10,149 | 333 | 14.5 |
| Total | 23.3 | 22.5 | 92,137 | 21,493 | 70,644 | 3,411 | 95.5 |

**FAT MASS RATIOS**

| Trunk/ Total | Legs/ Total | (Arms+Legs)/ Trunk |
|---|---|---|
| 0.59 | 0.28 | 0.61 |




| Region | Tissue[1] (%Fat) | Centile[2,3] | Total Mass (kg) | Fat[1] (g) | Lean[1] (g) | BMC (g) |
|---|---|---|---|---|---|---|
| Legs | 20.5 | - | 31.0 | 6,123 | 23,678 | 1,223 |
| Trunk | 27.8 | - | 46.5 | 12,638 | 32,816 | 999 |
| Total | 23.3 | 84 | 95.5 | 21,493 | 70,644 | 3,411 |

**Figure 3.4** Sample body composition readouts produced from the Lunar iDEXA scanner using encore software.

Adipose and muscle tissue biopsies were taken from a subset of CS subjects (who had consented to re-attending for clamp and biopsy studies) and matched control subjects (5 vs 5) to analyse components of the insulin signalling pathway in these tissues. Having attended after at least a 12 hour fast, subjects had biopsies taken from muscle and adipose tissue. Skeletal muscle biopsies were excised from the vastus lateralis muscle under local anaesthesia using a modified Bergstrom needle and suction in the fasting state. Subcutaneous abdominal adipose tissue was taken under local anaesthesia using a 14 gauge sterican needle at time 0 and 120 minutes of an OGTT to obtain fasted and stimulated samples. The tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C.

**3.2.7 Preparation of biopsies for immunoblot analysis**

Biopsies samples were attained from subjects and stored in liquid nitrogen by myself, Fredrik Karpe and Jane Cheeseman (Oxford). All further sample preparation and immunoblot analysis described below was performed by Simon Rudge and Qifeng Zhang in Michael Wakelam's group at the Babraham Institute in Cambridge.

Frozen adipose and muscle tissue samples were pulverized to a fine powder under liquid nitrogen and subsequently suspended in ice-cold lysis buffer (50 mM Hepes, 150 mM NaCl, 0.1% Igepal-CA650, pH7.5, and Roche complete protease inhibitor tablets) followed by further disruption in a Dounce glass homogenizer on ice. Samples were then allowed to lyse for an additional thirty minutes at $4^0$C with continual end-on-end rotation. Insoluble material was removed by centrifugation (16,000 x g for 30 minutes at $4^0$C), and the resultant supernatant measured for protein content by the Bradford protein assay (Bio-Rad). Sample buffer was added directly to the muscle tissue lysates but due to the low protein content of the adipose tissue lysates, they were first subjected to trichloroacetic acid precipitation, and the resultant acetone-washed protein pellets were subsequently re-suspended in 8 M urea and sample buffer. Muscle (25 µg) and adipose (15 µg)

tissue proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and then blotted for total AKT (cell signalling #9272) and phospho-AKT (p-AKT) (Ser473, cell signalling #9271). Blots were quantified using imageJ software http://rsbweb.nih.gov/ij/.

### 3.2.7.i Analysis of $PIP_2$ and $PIP_3$ in adipose and muscle tissue samples

This was done using a methodology based on phosphate methylation coupled to high-performance liquid chromatography-mass spectrometry (HPLC-MS) as described by our collaborators (Clark et al. 2011). Pulverized muscle samples were extracted of their lipids and following the removal of neutral lipids, $PIP_2$ and $PIP_3$ were assayed (Clark et al. 2011).

### 3.2.8 *PTEN* gene expression levels in muscle and adipose tissue

Adipose and muscle tissue RNA extraction and mRNA quantification using the Taqman methodology (as described in **Chapter 2**) was performed by Amy Barrett (Oxford). Adipose tissue and muscle biopsies were rapidly dissected on dry ice and then transferred to 2ml eppendorfs. A ball bearing and 1ml of TRIZOL was added per 100mg tissue. This was homogenised for 5 minutes on the highest frequency of the Retsch free standing homegeniser (Retsch uk, Leeds, UK). The lysate was then transferred to a 1.5ml RNase-free eppendorf and centrifuged at 12,000xg for 20 minutes at 4˚C to remove insoluble fragments and lipids. The supernatant was removed through the lipid layer and transferred into another RNase-free eppendorf and 200µL chloroform was added. Following this the RNA extraction protocol for human islets using the guanidinium-thiocyanate-phenol chloroform method was followed as described in Chapter 2 Methods (**section 2.5.2**).

cDNA was generated via random primed first stand synthesis from 1µg RNA in accordance with standard protocols (Applied Biosystems, Warrington, UK). Total gene expression was quantified using TaqMan gene expression assays (Applied Biosystems) which were run on an ABI 7900HT

machine (Applied Biosystems). Analysis of mRNA expression was performed by myself, using the ΔΔ Ct method and normalised to the geometric mean of the endogenous controls as described in Chapter 2 Methods.

### 3.2.9 *PTEN* mutation screening of control subjects

All control subjects were screened for *PTEN* mutations by Martijn van de Bunt (Oxford). The *PTEN* gene was sequenced by capillary sequencing using the primers detailed below (Table 3.4).

| Fragment | Region amplified | Primer direction | Primer sequence | Annealing temperature |
|---|---|---|---|---|
| 1 | Exon 1 | Forward | TGTAAAACGACGGCCAGTAGTCGC CTGTCACCATTTC | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCACTACG GAC ATTTTCGCATC | 58˚C |
| 2 | Exon 2 | Forward | TGTAAAACGACGGCCAGTGTTTGA TTGCTGCAT ATTTCA G | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCTCT AAA TGA AAA CAC AAC ATG | 58˚C |
| 3 | Exon 3 | Forward | TGTAAAACGACGGCCAGTATA TTC TCT GAA AAG CTC TGG | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCTTA ATC GGT TTA GGA ATA CAA | 58˚C |
| 4 | Exon 4 | Forward | TGTAAAACGACGGCCAGTCAT TAT AAA GAT TCA GGC AAT G | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCGAC AGT AAG ATA CAG TCT ATC | 58˚C |
| 5 | Exon 5 | Forward | ACC TGT TAA GTT TGT ATG CAA | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCTCC AGG AAG AGG AAA GGA AA | 58˚C |
| 6 | Exon 6 | Forward | TGTAAAACGACGGCCAGTCAT AGC AAT TTA GTG AAA TAA CT | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCGAT ATG GTT AAG AAA ACT GTT C | 58˚C |
| 7 | Exon 7 | Forward | TGTAAAACGACGGCCAGTCAG TTA AAG GCA TTT CCT GTG | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCGGA TAT TTC TCC CAA TGA AAG | 58˚C |
| 8 | Exon 8 | Forward | CTC AGA TTG CCT TAT AAT AGT C | 58˚C |
| | | Reverse | CAGGAAACAGCTATGACCTCA TGT TAC TGC TAC GTA AAC | 58˚C |
| 9 | Exon 9 | Forward | TGTAAAACGACGGCCAGTAGG GCC TCT TAA AAG ATC ATG | 58˚C |
| | | Reverse | ATT TTC ATG GTG TTT TAT CCC TC | 58˚C |

**Table 3.4** *PTEN* was amplified in 9 fragments spanning coding regions and intron-exon boundaries

### 3.2.10 Analysis of common variation in *PTEN*

To establish whether there was association between common genetic variation at the *PTEN* locus and measures of insulin sensitivity/glucose homeostasis in a healthy population (fasting glucose, fasting insulin or 2 hour glucose levels after an OGTT), Martijn van de Bunt (Oxford) examined and analysed the association p-values for SNPs in or within 10 Kb of *PTEN* in the publicly available data

from the Meta-Analysis of Glucose and Insulin related traits Consortium (MAGIC) (Dupuis et al. 2010) downloaded from http://www.magicinvestigators.org/downloads/.

## 3.3 Results

### 3.3.1 Characteristics of subjects studied

Fifteen subjects with CS due to *PTEN* mutation were recruited from around the UK: 7 from Oxford, 4 from Birmingham, 3 from Southampton and 1 from Edinburgh. All *PTEN* mutations had been reported by a certified Molecular Genetics diagnostic laboratory. All missense mutations were located in the exons encoding the catalytic domain of PTEN and all mutations are expected to result in haploinsufficiency (Table 3.2) (Berger et al. 2011). Two CS subjects were first-degree relatives (siblings), no other individuals were known to be related. Removal of either of the two siblings from the analysis did not alter the levels of significance of any parameter measured. All control subjects were negative for *PTEN* mutations and all study subjects were white European.

Oral glucose tolerance tests (OGTTs) were performed in all 15 subjects with CS due to *PTEN* mutation and in 15 gender, age and BMI-matched controls (Table 3.5).

- There was no significant difference in glucose excursion after glucose challenge between the groups (Table 3.5 and Figure 3.4A).
- None of the subjects had diabetes; one participant with CS had impaired fasting glycaemia but normal glucose tolerance (2-h glucose 5.3mmol/L).
- There was no difference in levels of fasting lipids (total cholesterol, HDL cholesterol, LDL cholesterol, apolipoprotein B and triglycerides) between *PTEN* mutation carriers and controls (Table 3.5).
- There was no difference in hsCRP levels measured between the groups (Table 3.5).

**Table 3.5** Baseline characteristics of subjects undergoing OGTT

| | ***PTEN* mutation carriers** | **Controls** | **P value** |
|---|---|---|---|
| **n** | 15 | 15 | NA |
| **% Male** | 47 | 47 | 1.0 |
| **Age (years)** | 44 [29, 62] | 45 [34, 67] | 0.59 |
| **BMI (kg/m$^2$)** | | | |
| Males | 33 [28, 40] | 31 [26, 40] | 0.51 |
| Females | 31 [23, 42] | 30 [23, 37] | 0.40 |
| **Body Fat (%)** | | | |
| Males | 27 [15, 34] | 35 [28, 44] | 0.13 |
| Females | 37 [20, 51] | 35 [22, 47] | 0.57 |
| **Waist circumference (cm)** Males | 115 [85, 136] | 121 [89, 137] | 0.53 |
| Females | 103 [92,126] | 102 [91, 110] | 0.96 |
| **WHR** | | | |
| Males | 1.03 [0.80, 1.15] | 1.04 [0.85, 1.16] | 0.80 |
| Females | 0.89 [0.85, 0.93] | 0.90 [0.85, 0.96] | 0.57 |
| **Systolic BP (mmHg)** | 124 [104, 136] | 124 [107, 140] | 0.93 |
| **Diastolic BP (mmHg)** | 75 [52, 96] | 73 [60, 86] | 0.57 |
| **Fasting glucose (mmol/l)** | 5.0 [4.2, 6.9] | 4.9 [4.5, 5.8] | 0.20 |
| **Fasting Insulin (pmol/L)** | 29.2 [8.7, 99.3] | 74.3 [22.1, 184.6] | 0.001 |
| **Triglycerides (mmol/L)** | 1.34 [0.74, 2.64] | 1.40 [0.58, 2.68] | 0.62 |
| **Total cholesterol (mmol/L)** | 4.94 [3.31, 6.54] | 5.35 [3.62, 6.81] | 0.13 |
| **HDL cholesterol (mmol/L)** | 1.21 [0.91, 1.72] | 1.18 [0.92, 1.77] | 0.54 |
| **LDL cholesterol (mmol/L)** | 3.41 [1.93, 4.86] | 3.87 [2.33, 5.41] | 0.07 |
| **Non-HDL cholesterol (mmol/L)** | 3.69 [2.11, 5.34] | 4.17 [2.53, 5.62] | 0.07 |
| **Apolipoprotein B (g/L)** | 0.95 [0.41, 1.37] | 0.88 [0.50, 1.14] | 0.27 |
| **hsCRP (mg/L)** | 0.88 [0.05, 5.08] | 0.82 [0.07, 6.71] | 0.98 |
| **Adiponectin (µg/ml)** | | | |
| Males | 3.22 [1.0, 4.7] | 3.51 [3.0, 7.6] | 0.03 |
| Females | 3.42 [2.2, 5.4] | 6.47 [3.1, 35.41] | <0.001 |

| | | | |
|---|---|---|---|
| **Leptin (ng/ml)** | | | |
| Males | 12.89 [1.3, 36.6] | 5.93 [2.7, 12.7] | 0.12 |
| Females | 32.51 [6.2, 84.8] | 29.32 [12.5, 68.1] | 0.55 |

Data are geometric means [range]. P value refers to Mann-Whitney testing for each of the traits across groups; a Bonferroni-adjusted p value of 0.0025 was interpreted as being statistically significant to account for increased probability of a type 1 error. NA=not applicable

Two of the fifteen subjects (13%) with CS and two of the fifteen matched control subjects (13

fulfilled International Diabetes federation (IDF) criteria for the diagnosis of metabolic syndrome

(Alberti et al. 2005).

### 3.3.2 Glucose tolerance, insulin sensitivity and beta-cell function measures from the OGTT: *PTEN* mutation carriers are more insulin sensitive

Insulin levels throughout the OGTT were significantly lower in *PTEN* mutation carriers (CS subjects) than controls ($p<0.001$) (Figure 3.5B) despite similar glucose levels ($p=0.35$) (Figure 3.5A), demonstrating their higher insulin sensitivity.

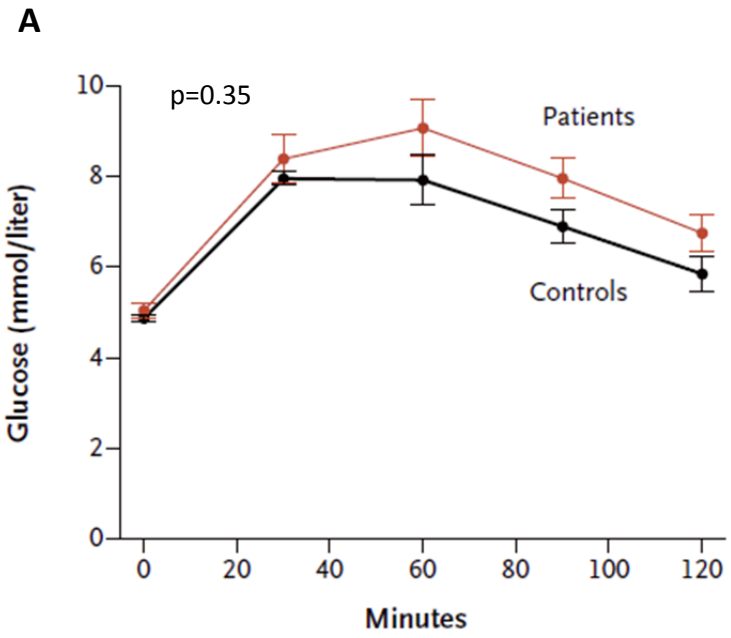
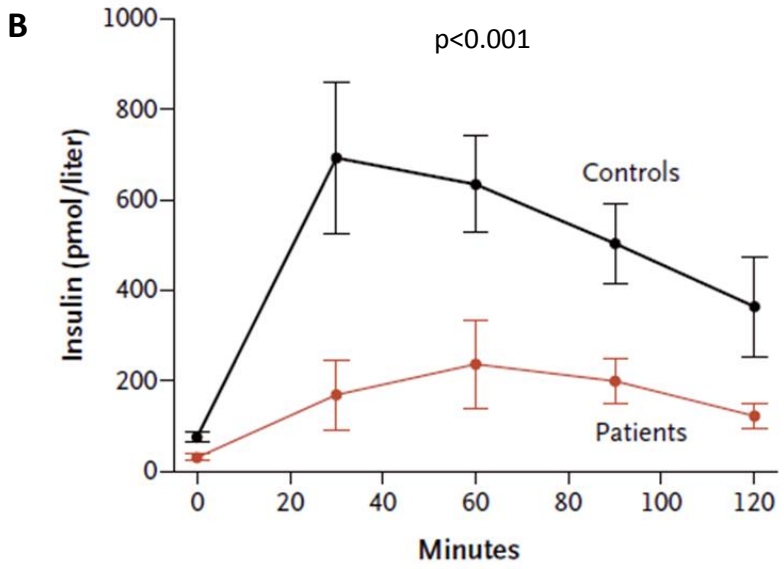



**Figure 3.5 A and B** Mean +/- SEM glucose and insulin levels of CS subjects (*PTEN* mutation carriers) and controls during the OGTT. P values refer to Mann-Whitney testing of area under the curve values, for glucose and insulin during the OGTT, between groups.




All measures of insulin resistance were lower in the *PTEN* mutation carriers compared with control subjects, including fasting insulin (-60%, p=0.001), area under the curve for insulin ($AUC_{insulin}$; -67%, p<0.001), and HOMA IR (-59%, p=0.001) (Table 3.6). Measures of insulin sensitivity were higher in the *PTEN* mutation carriers compared to the control group including the Stumvoll index (+67%, p=0.02) and Matsuda index (+120%, p=0.001) (Table 3.6).

| | ***PTEN* mutation carriers** | **Controls** | **P value** |
|---|---|---|---|
| **n** | 15 | 15 | NA |
| **Fasting Insulin (pmol/L)** | 29.2 [8.7, 99.3] | 74.3 [22.1, 184.6] | 0.001 |
| **AUC Insulin** | 361 [102, 1608] | 1095 [350, 2528] | <0.001 |
| **HOMA IR** | 6.5 [1.9, 29.3] | 16.0 [5.0, 40.2] | 0.001 |
| **HOMA %B** | 67 [45, 107] | 125 [50, 229] | 0.04 |
| **Stumvoll index** | 0.10 [0.07, 0.12] | 0.06 [0.02, 0.12] | 0.02 |
| **Matsuda index** | 19.6 [6.3, 54.1] | 8.9 [4.6, 33.8] | 0.001 |
| **Insulinogenic index** | 47 [8, 510] | 199 [91, 512] | <0.001 |
| **Disposition index** | 187 [22, 1509] | 128 [27, 359] | 0.89 |

**Table 3.6** OGTT measures of insulin sensitivity and beta-cell function. Data are geometric means [range]. P value refers to Mann-Whitney testing across groups. NA=not applicable

Although the insulinogenic index and HOMA %B were lower in *PTEN* mutation carriers than the control group, there was no difference in beta-cell function between groups when insulin resistance was accounted for using the disposition index (Table 3.6).

### 3.3.3 Clamp studies confirm higher insulin sensitivity in *PTEN* mutation carriers

Hyperinsulinaemic euglycaemic clamp studies were performed in a subset of *PTEN* mutation carriers who had consented to re-attend for clamp studies and biopsy studies. Control subjects were recruited from the OBB based upon age, gender, BMI and activity matching as far as possible to *PTEN* mutation carriers (Table 3.7). These studies confirmed higher insulin sensitivity in the *PTEN* mutation carrier group (p=0.009) (Figure 3.6).

| | ***PTEN* mutation carriers** | **Controls** | **P value** |
|---|---|---|---|
| **n** | 5 | 5 | NA |
| **% Male** | 40 | 40 | 1.0 |
| **Age (years)** | 39 [29, 62] | 42 [31, 60] | 0.62 |
| **BMI (kg/m$^2$)** | 31 [29, 33] | 30 [28, 33] | 0.57 |
| **Activity : Low**<br>**Medium**<br>**High** | 2<br>2<br>1 | 2<br>2<br>1 | NA |

**Table 3.7** Baseline characteristics of subjects undergoing hyperglycaemic euglycaemic clamp studies. Data are geometric means [range]. P value refers to Mann-Whitney testing across groups. NA=not applicable

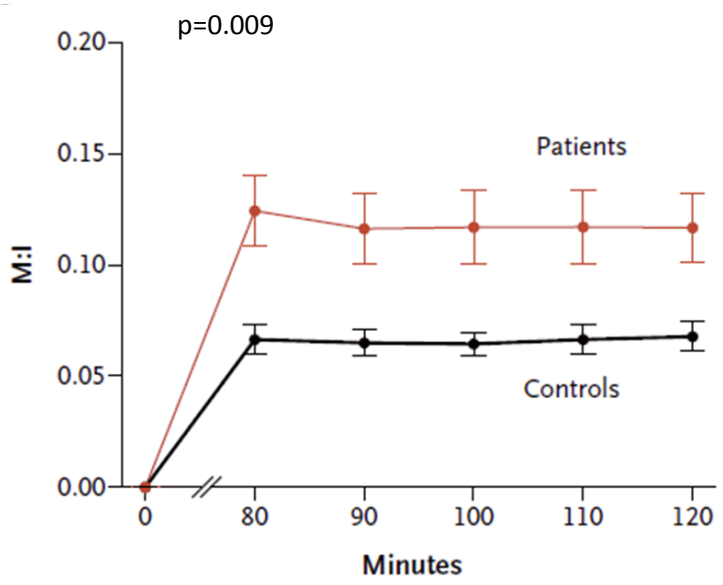



**Figure 3.6** Hyperinsulinaemic euglycaemic clamp studies in *PTEN* mutation carriers and controls. Data points represent the mean +/-SEM M/I component for 5 *PTEN* mutation carriers and 5 matched controls. The M component (mg/Kg/min) was calculated from the mean 20% glucose infusion rate (ml/hr) for all subjects: the M/I component was calculated by dividing M by the ambient insulin concentration (mU/L). The p value refers to Mann-Whitney testing of the mean M/I component for subjects during the last 40 minutes of the clamp, between groups.

### 3.3.4 *PTEN* mutation carriers have elevated BMIs

BMIs were noted to be elevated (>25) for all but one of the recruited subjects with CS (mean 32 range [23, 42] kg/m$^2$). To confirm this observation in relation to the general population, the BMIs of these subjects were compared with a large population-based control cohort (Oxford Biobank, www.oxfordbiobank.org.uk) (Figure 3.7). Those with CS (the *PTEN* mutation carriers) had significantly higher BMIs than the OBB population controls ($p<0.001$; Figure 3.7).

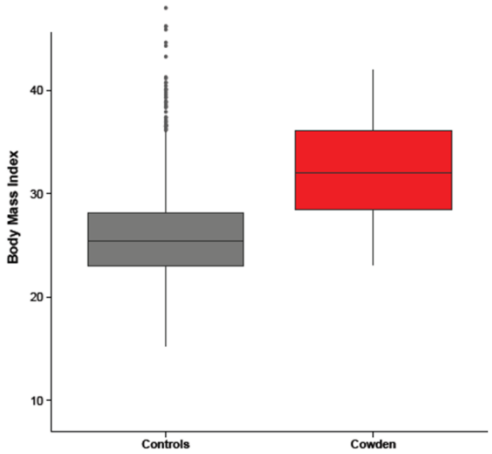



**Figure 3.7** BMIs of 15 *PTEN* mutation carriers [mean BMI 32 kg/m$^2$ range 23-42] and a healthy control population of 2,097 individuals (OBB) [mean BMI 26 kg/m$^2$ range 15-48]: bars represent median, the box represents interquartile range, whiskers represent the range, with outliers shown. The groups were well matched for both gender and age: 47% males in the *PTEN* mutation carriers and 49% males in the control population; mean [range] age of *PTEN* mutation carrier group 44 years [29, 62] and control group 41 years [29, 53].

To investigate if the recruited group of 15 *PTEN* mutation carriers were representative of CS due to *PTEN* mutations, and therefore if elevated BMI was a feature of CS *per se,* six additional *PTEN* mutation carriers were identified via the UK Clinical Genetics Network. It was observed that their BMIs were also elevated (>25) as a group (mean (kg/m$^2$) [range], 32 [24, 43]). Moreover, comparison with unaffected, non-carrier siblings (n=18; mean (kg/m$^2$) [range], 25 [20, 34]) and spouses (n=11, 27 [22, 34]) demonstrated that the BMIs of *PTEN* mutation carriers were greater (vs. unaffected siblings p=0.001; vs spouses p=0.004).

Figure 3.8 shows the largest single pedigree with CS in the study and demonstrates co-segregation of higher BMIs with the *PTEN* mutation in this family.

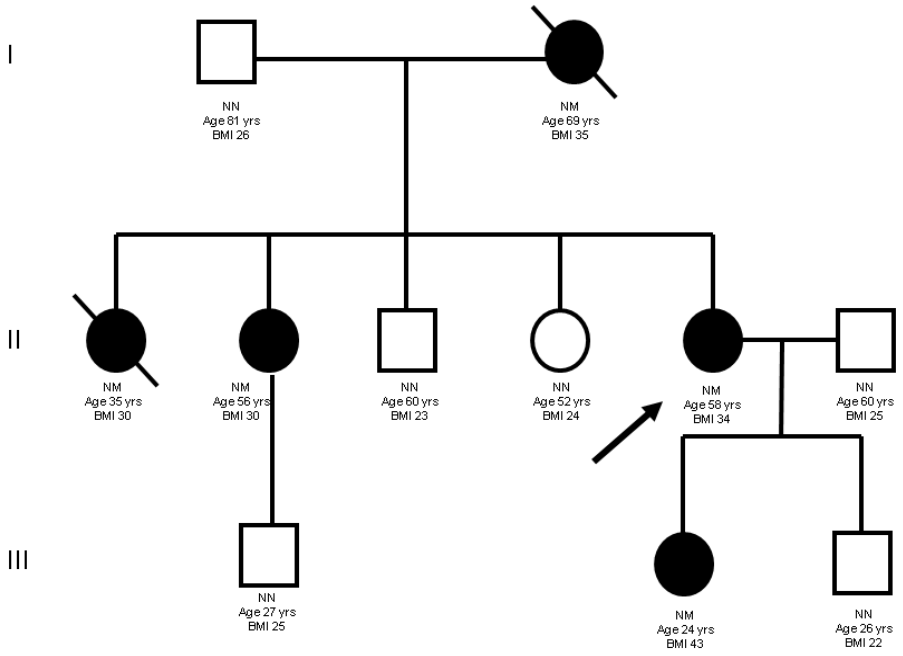



**Figure 3.8** Pedigree of family with the c.141delG *PTEN* mutation. Males are shown as squares, females as circles. Shaded shapes represent affected individuals. Mutation status is shown as NN = no mutation, NM = heterozygous mutation carrier. Age (years) BMI (kg/m$^2$) is shown underneath each subject. The proband is indicated by an arrow.

### 3.3.5 *PTEN* mutation carriers are insulin sensitive despite their relatively high BMI

To assess the relationship between insulin sensitivity and BMI, fasting insulin levels against BMI were plotted for *PTEN* mutation carriers and 2,097 individuals from a control population (Figure 3.9). The *PTEN* mutation carriers and controls were well matched for gender (with 47% and 49% men, respectively) and age (mean, 44 years [range, 29 to 62] and 41 years [range, 29 to 53], respectively). Clustering of the *PTEN* mutation carriers around the 5% percentile of the control population was observed, i.e. the lower end of the normal range of fasting insulin given their BMI.

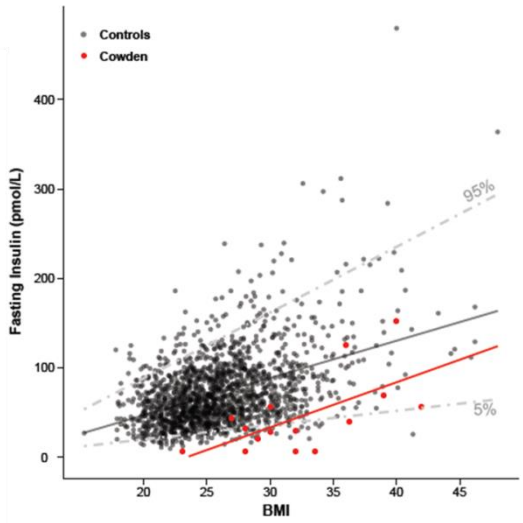



**Figure 3.9** Assessment of insulin sensitivity and BMI in individuals with loss of function *PTEN* mutations relative to a population control cohort demonstrating the relationship between fasting insulin levels and BMI (corrected for age and gender) of the population control cohort (grey) and *PTEN* mutation carriers (red). A darkening of the data points represents superimposed points for the control population. Lines for the 5% and 95% percentiles of the control population are plotted as grey dotted lines whilst the regression slopes between fasting insulin and BMI are shown as solid lines.

The regression slopes between fasting insulin and BMI were parallel between CS subjects and the general population (Figure 3.9). Therefore the absolute increase in insulin per unit of BMI was similar across the range of BMI in the groups, yet at a lower level in the *PTEN* mutation carriers, and fasting normoglycaemia could be maintained in the *PTEN* mutation carriers with a significantly lower output of insulin, consistent with the higher insulin sensitivity of this group.

### 3.3.6 *PTEN* mutation carriers have increased body mass due to enhanced adiposity but no regional differences in fat distribution

The composition of the observed greater body mass in the *PTEN* mutation carriers was investigated through anthropometric measurements and DXA in a subset of subjects (who had consented to re-

attending for further investigations) (Table 3.8). By comparing DXA scans in 6 CS subjects and at least eight matched controls per case, it was found that there was no difference in lean body mass (p=0.49), bone mineral content (p=0.94) or total fat (p=1.0) between the groups (Table 3.8).

The DXA scans and skinfold thickness measurements (a specific measure of subcutaneous adipose tissue) were used to assess any regional differences in fat distribution, particularly with respect to areas that are associated with insulin sensitivity. There were no specific trends in fat distribution between the *PTEN* mutation carriers and controls (Table 3.8). There was no increase in subcutaneous adipose tissue in *PTEN* mutation carriers compared to matched controls, no compartmentalisation towards subcutaneous from visceral adipose tissue and no difference in the android or gynoid adipose tissue distribution, or the android/gynoid ratio (analogous to the waist/hip ratio) between groups. In addition, the DXA scans were reanalysed by Professor Fredrik Karpe (Oxford) for android visceral fat mass (Kaul et al. 2012), and showed no difference between the six *PTEN* mutation carriers and their controls (1.57±0.38kg vs 1.24±0.42kg, p=0.18). The higher insulin sensitivity of the *PTEN* mutation carriers cannot therefore be attributed to an increase in the amount of metabolically protective gluteofemoral fat or any other trends in fat distribution.

To investigate if the observed increased body mass in CS is present in childhood, data was obtained on one individual with CS. This female steadily gained weight along the 98$^{th}$ centile, throughout the first year with birth weight (4.4kg; 98$^{th}$ centile), birth length (56cm; 99$^{th}$ centile) and head circumference (39cm; >99$^{th}$ centile). Between the ages of twelve and sixteen her BMI was above the 85$^{th}$ centile. Currently the subject is overweight (BMI = 26.1) at the age of 21 years. This case is consistent with a report of markedly increased weight gain relative to height, leading to obesity in children and adolescents with *PTEN* mutations (Cole and Hughes 1991).

**Table 3.8** Body Composition for CS subjects and matched controls

| Subject | Gender | BMI ($kg/m^2$) | Age (yrs) | Bone mineral content (kg) | Lean Body Mass (kg) | Total Fat (%) | Android Fat (%) | Gynoid Fat (%) | Android/ Gynoid ratio | Suprailiac Skin Fold Thickness (mm) | Triceps Skin Fold Thickness (mm) | Insulin (mU/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 30.3 | 30 | 3.4 | 70.6 | 23.3 | 32.3 | 28.2 | 1.1 | 10 | 12 | 2.2 |
| 2 | F | 29.6 | 62 | 2.3 | 46.8 | 42.7 | 46.2 | 53.9 | 0.9 | 35 | 21 | 3.2 |
| 3 | F | 33.6 | 58 | 2.2 | 45.8 | 43.9 | 53.7 | 48.0 | 1.1 | 53 | 52 | 33.8 |
| 4 | M | 34 | 35 | 3.8 | 60.4 | 34.1 | 47.6 | 35.4 | 1.3 | 23 | 14 | 3.7 |
| 5 | F | 31.6 | 35 | 2.7 | 47.5 | 43.9 | 58.5 | 48.3 | 1.2 | 39 | 29 | 5.9 |
| 6 | F | 42.9 | 24 | 3.3 | 61.2 | 47.2 | 56.1 | 57.3 | 1.0 | 38 | 53 | 10.7 |
| mean | | 33.4 | 38.3 | 2.9 | 54.6 | 38.2 | 48.2 | 43.9 | 1.1 | 29.3 | 25.6 | 6.2 |
| **BMI, age and sex matched control group** | | | **Age (yrs) [95% CI]** | **Bone mineral content kg [95% CI]** | **Lean Body Mass kg [95% CI]** | **Total Fat % [95% CI]** | **Android Fat % [95% CI]** | **Gynoid Fat % [95% CI]** | **Android/ Gynoid ratio** | **Suprailiac Skin Fold Thickness mm [95% CI]** | **Triceps Skin Fold Thickness mm [95% CI]** | **Insulin mU/L [95% CI]** |
| 1 (n=10) | M | 30.4 [0.8] | 31.3 [0.9] | 3.6 [0.2] | 66.5 [4.6] | 30.7 [2.2] | 43.0 [3.5] | 34.6 [3.2] | 1.2 | 27.6 [7.2] | 19.0 [6.0] | 22.4 [5.9] |
| 2 (n=8) | F | 28.9 [0.9] | 59.3 [1.5] | 2.5 [0.3] | 43.4 [4.1] | 41.4 [1.4] | 51.2 [1.8] | 49.5 [1.9] | 1.0 | 27.3 [4.0] | 26.0 [4.6] | 13.6 [1.6] |
| 3* (n=8) | F | 28.9 [0.9] | 59.3 [1.5] | 2.5 [0.3] | 43.4 [4.1] | 41.4 [1.4] | 51.2 [1.8] | 49.5 [1.9] | 1.0 | 27.3 [4.0] | 26.0 [4.6] | 13.6 [1.6] |
| 4 (n=10) | M | 34.2 [3.0] | 34.3 [1.0] | 3.5 [0.2] | 67.9 [6.9] | 36.5 [4.2] | 51.1 [6.4] | 41.5 [4.3] | 1.2 | 35.9 [6.7] | 22.7 [3.9] | 22.9 [6.0] |
| 5 (n=10) | F | 31.6 [0.9] | 32.8 [0.9] | 2.6 [0.2] | 45.6 [3.1] | 46.0 [1.8] | 57.1 [2.4] | 54.7 [1.6] | 1.0 | 34.7 [6.3] | 31.5 [5.3] | 18.5 [5.0] |
| 6 (n=10) | F | 41.0 [2.2] | 36.0 [2.1] | 2.7 [0.2] | 50.9 [1.5] | 50.7 [2.1] | 61.6 [2.1] | 57.3 [2.8] | 1.1 | 42.0 [6.9] | 30.6 [5.5] | 23.6 [6.7] |
| mean | | 32.2 | 40.6 | 2.9 | 52.0 | 40.6 | 52.2 | 47.2 | 1.1 | 32.0 | 25.6 | 18.6 |

Differences between cases and control groups were evaluated by the Mann Whitney test. Each CS subject was matched with at least 8 BMI, age and gender matched OBB control subjects. All p values were >0.5 except fasting insulin p=0.06. *Due to difficulties in identifying post-menopausal female controls for subject 3 (age 58 years, BMI 33.6 $kg/m^2$) the mean BMI in the matched group is 28.9 $kg/m^2$.

### 3.3.7 *PTEN* mutation carriers have lower adiponectin levels

To investigate the role of PTEN in adipose tissue further, and for their association with insulin resistance (Trujillo and Scherer 2006; Groeneveld et al. 2012), two adipokines (fasting adiponectin and leptin) were assayed (Table 3.5). The total adiponectin was significantly lower in the *PTEN* mutation carriers ($p<0.001$) and there was no difference in plasma leptin levels or lipid profiles, between the groups (Table 3.5). To assess if there was a relationship between adiponectin levels and BMI, adiponectin levels were plotted against BMI for *PTEN* mutation carriers and the matched control group (Figure 3.10).

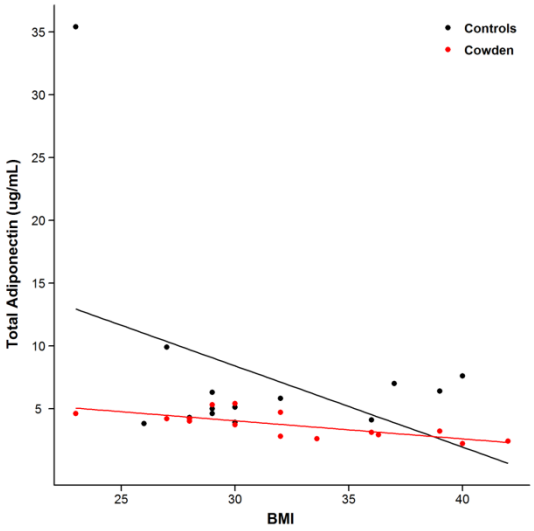



**Figure 3.10** Relationship between total plasma adiponectin levels and BMI in subjects with Cowden syndrome and matched control subjects. Total adiponectin levels (ug/mL) were plotted against BMI for Cowden subjects (red) and control subjects (black). Lines of regression are solid lines.

On examining the correlation with BMI, the expected relationship was observed for the control group but adiponectin levels were consistently lower for any given BMI in the CS group.

### 3.3.8 Evidence of increased signalling through AKT-PI3 Kinase pathway as mechanism for heightened insulin sensitivity in *PTEN* mutation carriers

Fasting muscle and adipose tissue biopsies, and stimulated adipose tissue biopsies (taken at time 120min of an OGTT) were taken from a subset of 5 *PTEN* mutation carriers (who had consented to re-attend for biopsies and DXA scan investigation) and 5 age, gender, BMI and activity matched controls. In collaboration with the Babraham Institute in Cambridge, phosphatidylinositol (3,4,5)-

trisphosphate ($PIP_3$) levels in muscle, and AKT phosphorylation in muscle and adipose tissue were assayed by Simon Rudge and Qifeng Zhang of Michael Wakelam's group (Babraham Institute, Cambridge) to investigate if the increased insulin sensitivity observed in *PTEN* mutation carriers was associated with amplified signalling through the PI3K-AKT pathway.

Despite significantly lower circulating insulin levels in *PTEN* mutation carriers in the fasting state, muscle $PIP_3$ levels were similar to control samples. This was consistent with results observed in total lipid mass and for the $PIP_3$: phosphatidylinositol 4,5-bisphosphate ($PIP_2$) ratio (which corrects for variation in the cellular membranes between samples) (Table 3.9).

| | Pair | 38:4 $PIP_3/PIP_2$ | 38:3 $PIP_3/PIP_2$ | 36:2 $PIP_3/PIP_2$ | 36:1 $PIP_3/PIP_2$ | Total $PIP_3/PIP_2$ |
|---|---|---|---|---|---|---|
| **Case** | **A** | 0.0060 | 0.0012 | 0.0010 | 0.0009 | 0.0091 |
| **Control** | **A** | 0.0056 | 0.0012 | 0.0008 | 0.0013 | 0.0089 |
| **Case** | **B** | 0.0042 | 0.0010 | 0.0009 | 0.0009 | 0.0070 |
| **Control** | **B** | 0.0044 | 0.0009 | 0.0007 | 0.0004 | 0.0064 |
| **Case** | **C** | 0.0061 | 0.0019 | 0.0016 | 0.0011 | 0.0108 |
| **Control** | **C** | 0.0060 | 0.0024 | 0.0023 | 0.0033 | 0.0141 |
| **Case** | **D** | 0.0043 | 0.0016 | 0.0015 | 0.0022 | 0.0096 |
| **Control** | **D** | 0.0070 | 0.0024 | 0.0022 | 0.0014 | 0.0131 |
| **Case** | **E** | 0.0063 | 0.0013 | 0.0009 | 0.0013 | 0.0098 |
| **Control** | **E** | 0.0075 | 0.0012 | 0.0013 | 0.0013 | 0.0113 |
| **Case** | Mean | 0.0053 | 0.0014 | 0.0011 | 0.0012 | 0.0092 |
| **Control** | Mean | 0.0060 | 0.0015 | 0.0013 | 0.0013 | 0.0104 |
| **P value** | | 0.34 | 0.46 | 0.22 | 1 | 0.22 |

**Table 3.9** $PIP_3$ levels expressed as a ratio of $PIP_2$ levels in muscle from *PTEN* mutation carriers and their matched controls. Analysis of Phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and Phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$)in muscle tissue samples**.** P value refers to nonparametric Wilcoxon Signed-Rank test (two-tailed).

AKT levels were similar between *PTEN* mutation carriers and controls in the same muscle samples. A trend for lower (although not significantly lower) phosphorylated AKT levels (pAKT) was observed in four of the five samples (Figure 3.11A, 3.11B).

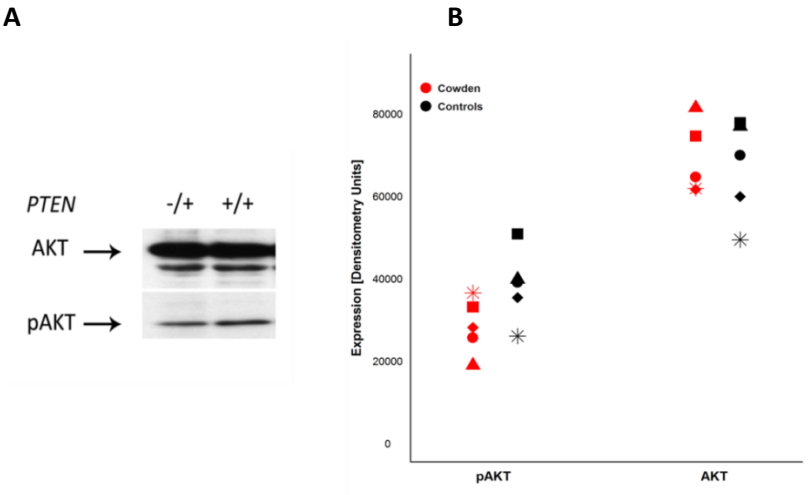



**Figure 3.11** AKT phosphorylation status in muscle tissue from *PTEN* mutation carriers and matched controls. **Fig 3.11A** A representative blot for AKT and phosphorylated-AKT (pAKT) levels in muscle samples from 5 fasted *PTEN* mutation carriers and 5 age, gender, BMI and activity matched controls. *PTEN* +/- indicates a Cowden syndrome subject, *PTEN* +/+ indicates a control subject. **Fig 3.11B** The individual values for protein expression for AKT and pAKT from the quantification of 5 muscle tissue age, gender, BMI and activity matched pairs. Wilcoxon Signed-Rank test (two-tailed) between the groups gave a p value of p=0.14 and p=0.69 for AKT and pAKT respectively.

In contrast, when AKT phosphorylation was examined in adipose tissue from three of the same individuals, a trend for higher pAKT levels (although not signficantly higher) was observed in the *PTEN* mutation carriers compared to their matched controls (Figure 3.12A, 3.12B). A trend for increased AKT phosphorylation following glucose stimulation was also observed in both groups (Figure 3.12A, 3.12B), although this was in a small sample size and hence difficult to analyse statistically.

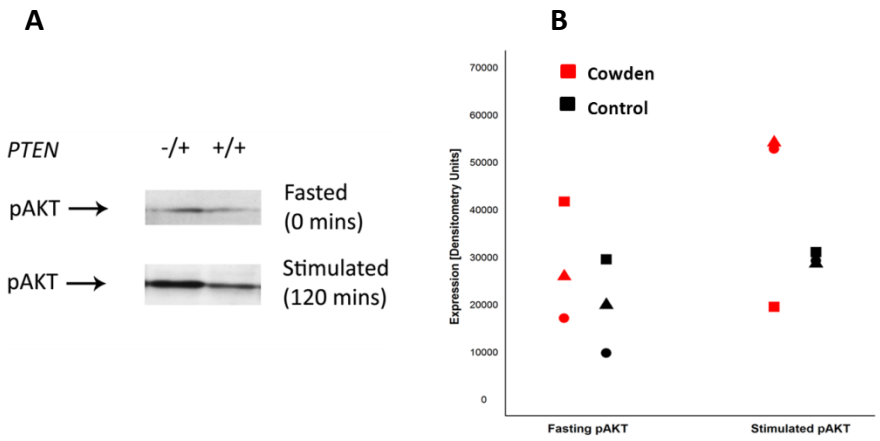



**Figure 3.12** AKT phosphorylation status in fasted and stimulated adipose tissue from *PTEN* mutation carriers and matched controls. **Fig 3.12A** A representative blot for pAKT levels in adipose tissue in the fasted (0 mins) state and glucose stimulated (120 mins) state. *PTEN* +/- indicates a Cowden syndrome subject, *PTEN* +/+ indicates a control subject. **Figure 3.12B** Individual values for protein expression for pAKT from the quantification of 3 adipose tissue age, gender, BMI and activity matched pairs before (fasted) and after stimulation with glucose (stimulated). For two samples technical difficulties resulted in insufficient data for analysis. A non-parametric Wilcoxon Signed-Rank test (two tailed) between the groups gave a p value of p=0.11 for fasted samples and p=0.28 for stimulated samples.

### 3.3.9 *PTEN* mRNA levels are lower in adipose and muscle tissue from *PTEN* mutation carriers

A tendency for lower *PTEN* mRNA levels was observed in fasted adipose tissue and muscle tissue from *PTEN* mutation carriers compared to matched controls, although only achieving statistical significance in the fasted adipose tissue (Figure 3.13)

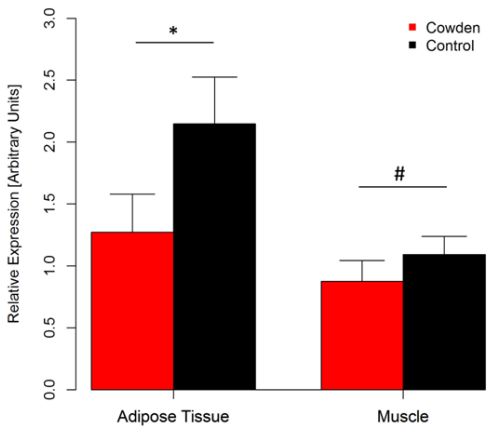



**Figure 3.13** Messenger RNA (mRNA) expression profiles for *PTEN* in fasted muscle and adipose tissue from Cowden syndrome subjects and age, gender and BMI matched controls as determined by QRT-PCR. Figures on the y axes refer to the test transcript levels relative to three separate endogenous control genes (*PPIA*, *B2M* and ubiquitin C (*UBC*) for muscle and *PPIA*, *B2M* and phosphoglycerate kinase 1 (*PGK1*) for adipose tissue). * p<0.05, # non significant.

### 3.3.10 Common variation in *PTEN* and fasting glucose and insulin traits in general population

Given that studies in individuals with rare penetrant *PTEN* mutations demonstrated significantly higher insulin sensitivity as described above, an association between common genetic variation at the *PTEN* locus and measures of fasting glucose or insulin in healthy non-diabetic individuals was tested for. SNPs in or within 10Kb of *PTEN* in the publically available data from the Meta-Analysis of Glucose and Insulin related traits Consortium (MAGIC)(Dupuis et al. 2010) were examined and analysed by Martijn van de Bunt. No variant reached genome wide significance by stringent genome wide significance criteria ($p=5 \times 10^{-8}$), but there were suggestive associations for both fasting glucose (rs11202613; $p=7.13 \times 10^{-7}$) (Figure 3.14A) and fasting insulin levels (rs2142136; $p=1.17 \times 10^{-5}$) in the same region of the *PTEN* locus (Figure 3.14B).

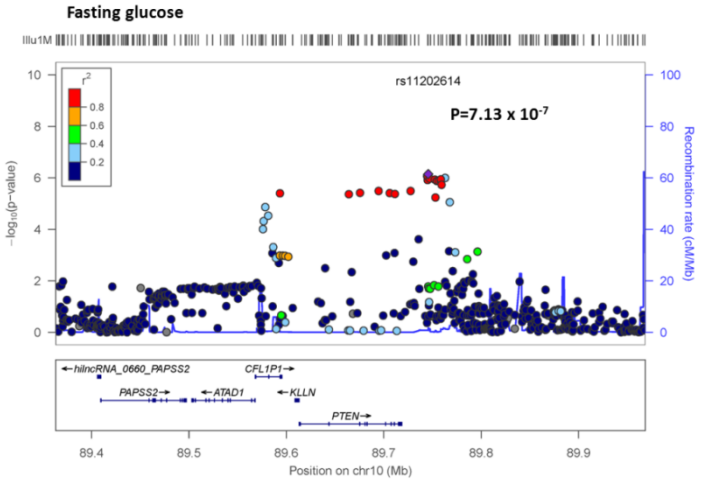



**Figure 3.14A** Plot of all SNPs within 1Kb of *PTEN* associated with fasting glucose. Strength of association with fasting glucose is shown on the y axis and position along chromosome 10 is shown on the x axis. SNPs most strongly associated with fasting glucose are shown by purple diamond.

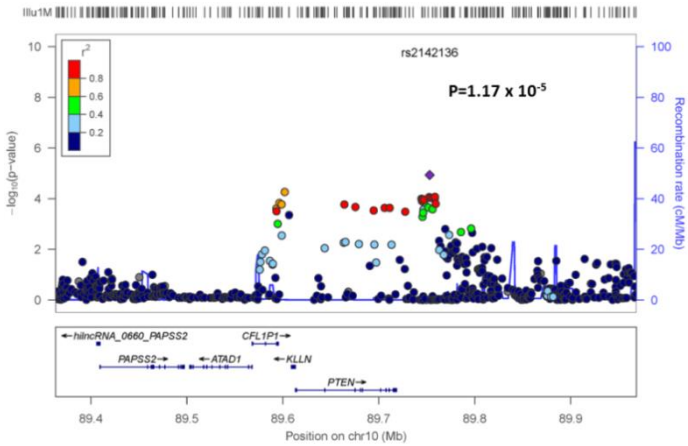



**Figure 3.14B** Plot of all SNPs within 1Kb of *PTEN* associated with fasting insulin. Strength of association with fasting insulin is shown on the y axis and position along chromosome 10 is shown on the x axis. SNPs most strongly associated with fasting insulin are shown by purple diamond.

## 3.4 Discussion

In this study a human model of *PTEN* haploinsufficiency has been utilised to investigate the role of a common signalling pathway in cell growth and metabolism. The results presented demonstrate that individuals with a cancer predisposition syndrome due to inactivating mutations in the tumour suppressor gene *PTEN* are sensitised to insulin. This is in line with the original hypothesis and is consistent with an earlier case report (Iida et al. 2000). By recruiting 15 individuals (~ 5% UK population with CS) with CS due to *PTEN* mutations and 15 well-matched control subjects it has been shown that all surrogate measures of insulin sensitivity derived from the OGTT were significantly higher in the *PTEN* mutation carriers. This finding was confirmed using the gold standard test of insulin sensitivity, the euglycaemic hyperinsulinaemic clamp (DeFronzo et al. 1979), in a subset of *PTEN* mutation carriers and control subjects (5 vs 5), which confirmed that *PTEN* haploinsufficient subjects are more insulin sensitive.

As the liver is the principal insulin-responsive tissue, fasting insulin levels reflect insulin sensitivity of the liver predominantly: fasting insulin levels were significantly lower in *PTEN* mutation carriers ($p=0.001$). This is consistent with liver-specific *Pten*$^{-/-}$ knockouts where fasting insulin levels were also significantly lower than in wild type mice (Stiles et al. 2004). The euglycaemic clamp reflects the impact of muscle in glucose homeostasis as the induced hyperinsulinaemia inhibits insulin action in the liver and, despite relatively small numbers, a significant effect of *PTEN* haploinsufficiency on insulin sensitivity was shown ($p=0.009$). This is also consistent with effects observed in muscle-specific *Pten*$^{-/-}$ knockout mice which were protected from the insulin resistance and diabetes that developed in the wild type mice when fed on a high fat diet (Wijesekara et al. 2005).

These studies show that PTEN deficiency enhances insulin signalling in both muscle and liver in humans, and in order to demonstrate the molecular mechanism underlying the enhanced insulin sensitivity of the CS subjects components of the PI3K-AKT pathway were studied (Figure 3.15).

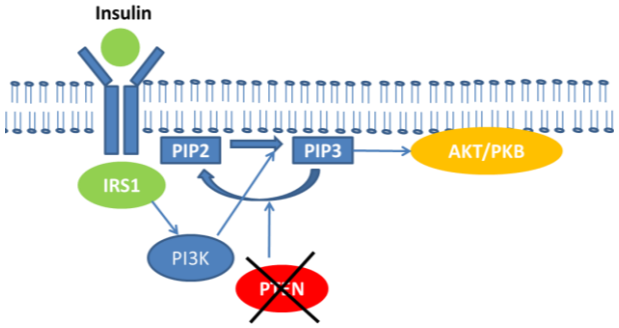



**Figure 3.15** PI3K-AKT pathway in CS subjects with *PTEN* mutations. In *PTEN* mutation carriers one would expect to find higher levels of $PIP_3$ and phosphorylated AKT (pAKT) than in control subjects where there is more inhibition of the PI3K-AKT pathway from higher levels of PTEN.

In collaboration with colleagues at the Babraham Institute in Cambridge, $PIP_3$ levels were determined in muscle, and AKT phosphorylation in muscle and adipose tissue biopsies from subjects with CS and matched (for age, gender, BMI and activity) controls. In the fasting state similar levels of $PIP_3$ in muscle were observed despite significantly lower circulating stimulatory insulin levels in those with CS, demonstrating amplified PI3K signalling in *PTEN* haploinsufficiency. This was observed both in the total lipid mass and when examining the $PIP_3$: $PIP_2$ ratio which corrects for variation in the cellular membranes between samples (Table 3.9). In the same muscle samples AKT phosphorylation levels were similar between cases and controls, again, despite significant differences in circulating insulin levels (Figure 3.11 A and B). In contrast, when AKT phosphorylation in adipose tissue taken from these same fasted individuals was examined, increased pAKT levels in *PTEN* haploinsufficient individuals compared to matched controls was observed (although this did not reach statistical significance p=0.11) (Figure 3.12 A and B). After glucose stimulation there was a clear increase in AKT phosphorylation in adipose tissue which was consistent between the groups (Figure 3.12 A and B). Taken together, these data support enhanced signalling through the PI3K-AKT pathway in subjects with *PTEN* haploinsufficiency.

The observation that *PTEN* mutation carriers have a tendency to higher levels of AKT phosphorylation in adipose but not muscle tissue indicates that adipose tissue may govern insulin sensitivity in the fasted state, consistent with the view that adipose tissue is exquisitely sensitive (Karpe and Tan 2005). The data presented are supported by rodent studies which have shown

tissue-specific roles for *Pten* in liver, muscle and adipose tissue and increased phosphorylation and activation of components of the PI3K-AKT pathway in *Pten* haploinsufficient mice (Stiles et al. 2004; Kurlawalla-Martinez et al. 2005; Wijesekara et al. 2005; Wong et al. 2007). Consistent with the human data presented here, these rodent studies have also demonstrated dominant roles for both muscle and adipose tissue *Pten* in insulin sensitive phenotypes (Kurlawalla-Martinez et al. 2005; Wijesekara et al. 2005). Data from *Pten* knockout rodent studies regarding beta-cell function is inconsistent: mice with beta-cell specific *Pten* deletions (Nguyen et al. 2006; Stiles et al. 2006) demonstrated islet hyperplasia and increased insulin secretion in contrast to *Pten* haploinsufficient mice (Wong et al. 2007) which showed no differences in islet mass or beta-cell function. The data from humans, presented here, do not support increased beta-cell function or insulin secretion suggestive of increased functional beta-cell mass in *PTEN* mutation carriers. The disposition index was derived from the OGTT data and used as a measure of beta-cell function, as it accounts for prevailing insulin resistance, and found no difference between *PTEN* mutation carriers and control subjects.

One of the most striking observations of this study was that subjects with *PTEN* mutations are apparently significantly overweight and yet display markedly higher insulin sensitivity compared to matched controls. This observation was strengthened by firstly recruitment of 6 additional *PTEN* mutation carriers who also displayed elevated BMI, and secondly unaffected sibling and spouse analyses that confirmed *PTEN* mutation carrier subjects to be the more overweight group. The comparison of DXA scans and anthropometric measures between CS subjects and healthy age, gender and BMI matched controls demonstrated that the increased body mass observed in *PTEN* mutation carriers is not attributable to increased bone or lean mass but rather, an increase in adiposity. In addition, there were no demonstrable regional differences in fat distribution that could account for the increased insulin sensitivity observed in *PTEN* mutation carriers, namely no observed

increases in subcutaneous adipose tissue or the metabolically protective gluteofemoral fat in *PTEN* mutation carriers, and no difference in the android/gynoid ratio (analogous to the waist/hip ratio) between groups compared to matched controls.

Although systematic measurements of height and weight throughout childhood and adolescence were not available for the majority of the subjects studied (as most individuals had presented well into adulthood), those data we were able to obtain for one subject, together with those previously reported (Cole and Hughes 1991), suggest there is a steady increase in weight throughout childhood and adolescence. No particular appetite or dietary differences in recruited CS subjects was observed, although this study was not specifically designed to investigate this. Hence appetite, diet and energy expenditure were not formally assessed. Interestingly, in *Pten* adipose-specific knockout mice there were no differences in body fat content/adipose tissue mass, adiponectin or leptin levels, however the effects were sufficient to give rise to improved systemic glucose tolerance and lower fasting insulin levels (Kurlawalla-Martinez et al. 2005).

The complexity of the dual roles of PTEN in growth and metabolism are illustrated by the effects of systemic PTEN elevation in mice (Garcia-Cao et al. 2012): the "Super-PTEN" mutant mice have reduced body fat accumulation, a smaller body size and increased energy expenditure. Cells derived from the mice display reduced glucose uptake and, increased mitochondrial oxidative phosphorylation and are resistant to oncogenic transformation via PI3K-dependent and PI3K-independent effects. These data are consistent with another study overexpressing *Pten* in mice, which in addition to demonstrating increased energy expenditure (and tumour suppression), showed this may be due to hyperactivity of brown adipose tissue (BAT) in the mutated mice (Ortega-Molina et al. 2012). Macroscopically the BAT of mice over expressing *Pten* displayed histological

features of hyperactivity, and 18F-deoxyglucose (FDG) uptake by positron emission tomography (PET) confirmed increased glucose uptake of BAT in vivo (Ortega-Molina et al. 2012). BAT is increasingly recognised for its relevance in energy homeostasis in humans (Cypess et al. 2009; Lidell and Enerback 2010) and has been demonstrated to be functionally relevant in adult humans using FDG-PET and CT guided biopsy techniques (Virtanen et al. 2009; Zingaretti et al. 2009).

These data demonstrating increased energy expenditure and reduced cancer risk, from Pten elevation at the level of the organism, are in line with our own observations in *PTEN* haploinsufficient humans being of higher BMI, having increased adiposity and being at higher risk of cancer. Glucose uptake and energy expenditure in cells derived from recruited CS subjects was not investigated, but this would be an interesting further study to undertake. In addition, given the BAT data in mice over expressing *Pten* it would be very interesting to investigate the possible relevance of this tissue in *PTEN* mutation carriers. Finally, these mice with elevated *Pten* levels were also hyperphagic (and yet small) (Garcia-Cao et al. 2012; Ortega-Molina et al. 2012) and although no particular appetite disturbances were noted in CS subjects recruited for studies presented here, it would be desirable to undertake formal calorimetric and appetite studies to give more insight into energy homeostasis and the hypothalamic axis in *PTEN* mutation carriers.

Adiponectin and leptin are hormones secreted by adipocytes involved in regulation of metabolism. Leptin has a key role in regulating energy intake and energy expenditure (Brennan and Mantzoros 2006) and we did not find levels to be significantly different between *PTEN* mutation carriers and matched control subjects (Table 3.5). Adiponectin is associated with insulin sensitivity and insulin-sensitive obese individuals have been shown to have raised circulating adiponectin levels compared to insulin-resistant obese individuals (Kloting et al. 2010). In this context it is interesting that despite

marked insulin sensitivity in *PTEN* mutation carriers, circulating adiponectin levels were paradoxically lower. On assessment of the correlation of adiponectin with BMI (Figure 3.10), the expected relationship was observed for the control group but adiponectin levels were consistently lower for any given BMI in the *PTEN* mutation carriers.

The relationship between adiponectin and insulin sensitivity is complex and others have found unexpected results when measuring this hormone in relation to insulin resistance syndromes; patients with severe insulin resistance due to genetically defective insulin receptors have hyperadiponectinaemia (Semple et al. 2006). In CS, the relationship between adiponectin and insulin sensitivity is further complicated due to recognised relationships between adiponectin levels and the risk of cancer (Wei et al. 2005). A number of studies have shown that circulating adiponectin levels are inversely associated with risk of malignancies that are associated with obesity and insulin resistance, such as cancers of the endometrium, breast and colon (Barb et al. 2006). Lower adiponectin levels in patients with *PTEN* mutations, in whom breast and endometrium cancers are common, would be in line with these studies. Therefore definitive conclusions on the causal relationship between adiponectin and insulin sensitivity cannot be drawn from our observation that insulin sensitive patients with *PTEN* mutations have lower adiponectin levels than matched controls.

The clear impact of rare, penetrant *PTEN* mutations upon insulin sensitivity demonstrated by the data presented here, prompted analysis of the publically available MAGIC data (Dupuis et al. 2010) for an association between common genetic variation at the *PTEN* locus and fasting glucose and insulin levels in healthy non-diabetic individuals. The suggestive associations described here have since been replicated and reanalysed with BMI adjusted data (important given the inverse

relationship with BMI observed in Cowden syndrome) by Scott et al, and revealed an association between fasting insulin and common variation at the *PTEN* locus (Scott et al. 2012).

Finally, the data presented in this chapter, support the hypothesis that common signalling pathways may underlie links between glucose homeostasis/T2D and cancer, with PTEN and the PI3K-AKT signalling pathway a prime example of this mechanism. The PI3K-AKT pathway has been found to harbour other mutations of metabolic and cellular growth effects: loss-of-function *AKT2* mutations have been described as a cause of severe insulin resistance (George et al. 2004) and activating *AKT2* mutations as a cause of hypoglycaemia (Hussain et al. 2011). The latter phenotype displayed asymmetrical overgrowth anomalies, also described with *PIK3CA* mutations that lead to increased PI3K activity (*PIK3CA* encodes the p110α catalytic subunit of PI3K) which have been shown to cause a distinct overgrowth syndrome (Lindhurst et al. 2012). Of note, hypoglycaemia was reportedly not a feature of the syndromes described due to *PIK3CA* mutations (Lindhurst et al. 2012). However, interestingly, *PIK3CA* mutations (as well as *AKT1* mutations) have been recently described as a cause of CS (Orloff et al. 2013) demonstrating the merit in investigating other components of the PI3K-AKT pathway in individuals with CS not due to *PTEN* mutations.

In conclusion, these data demonstrate a monogenic cause of profound insulin sensitisation in humans which paradoxically occurs in the face of adiposity. This also illustrates a divergent effect on diabetes and cancer risk as *PTEN* haploinsufficiency leads to increased risk of obesity and cancer whilst presumably decreasing T2D risk via enhanced insulin sensitivity and reduced insulin demand. Indeed the heightened insulin sensitivity could become a characteristic of CS itself, and included as an additional clinical feature of this condition.

These studies provide a clear example of how intimately pathways in cell growth and metabolism may be linked, and is evidence for the theory that the epidemiological and genetic links between cancer and T2D may rest on common signalling pathways that link tumour suppressor genes to metabolic pathways impacting on glucose homeostasis.

# Chapter 4

Molecular characterisation of the *CDKN2A*, *CDKN2B* and *ANRIL* locus in human pancreatic islets

# Chapter 4

## 4.1 Introduction

### 4.1.1 GWAS identify novel loci giving insight into T2D pathogenesis

The tally for susceptibility loci implicated in T2D is now >70, and continues to grow steadily as larger numbers and populations are included in increasingly collaborative and better powered meta-analyses. This has provided several clues to aspects of diabetes pathogenesis such as the connection between T2D, insulin secretion, obesity and insulin resistance (Dupuis et al. 2010; Speliotes et al. 2010; Voight et al. 2010; Morris et al. 2012; Scott et al. 2012). However, pointers to specific underlying mechanisms have been relatively limited for two main reasons; firstly most of the GWAS signals map to noncoding regions of the genome for which it has been challenging to determine the functional link to a 'causal' transcript and therefore begin appropriate experimental follow-up and secondly, most of the identified susceptibility loci are of modest effect size (OR ~1.05 -1.35) (Frayling 2007; Voight et al. 2010; Morris et al. 2012) making definite biological deductions difficult.

Insights gained into T2D pathogenesis from GWAS include new perspectives on our understanding of the relative contributions of defects in insulin secretion and defects in insulin action (i.e. insulin resistance) to T2D pathogenesis. The majority of the common variant T2D signals are driven predominantly by defects in insulin secretion (Voight et al. 2010), thus highlighting the importance of islet dysfunction in T2D development. Investigating the underlying mechanisms by which these variants affect islet function is therefore of prime importance in understanding which aspects of islet function are vulnerable in T2D pathogenesis. The biology agnostic approach of GWAS also offers the potential to uncover previously unknown participants in glucose homeostasis, such as the key role of

zinc transport in insulin processing and secretion in beta-cells, established via the causal coding variant in *SLC30A8* which encodes the zinc transporter ZnT8 (Sladek et al. 2007; Nicolson et al. 2009).

### 4.1.2 T2D-susceptibility loci are enriched for cell-cycle genes including those at the Chr9p21 locus

One of the most unexpected observations to result from GWAS, has been the enrichment for genes involved in cell cycle regulation within T2D-susceptibility loci (Voight et al. 2010). Many of these genes are known to be expressed in islets, raising the possibility that the T2D-predisposing effects of these variants are related to variation in beta cell mass (Dupuis et al. 2010; Voight et al. 2010). The evidence for this 'cell-cycle' link is strongest at the Chr9p21 association signal (Figure 4.1) where the T2D-association signals map in the vicinity of the cyclin dependent kinase inhibitors *CDKN2A* and *CDKN2B* (Saxena et al. 2007; Scott et al. 2007; Zeggini et al. 2007; Morris et al. 2012). These are well recognised tumour suppressor genes: germline *CDKN2A* mutations are a cause of familial melanoma syndromes and somatic *CDKN2A* or *CDKN2B* mutations are amongst the most commonly affected genes in cancer (Kim and Sharpless 2006) and their role is further discussed in **Chapter 5**.

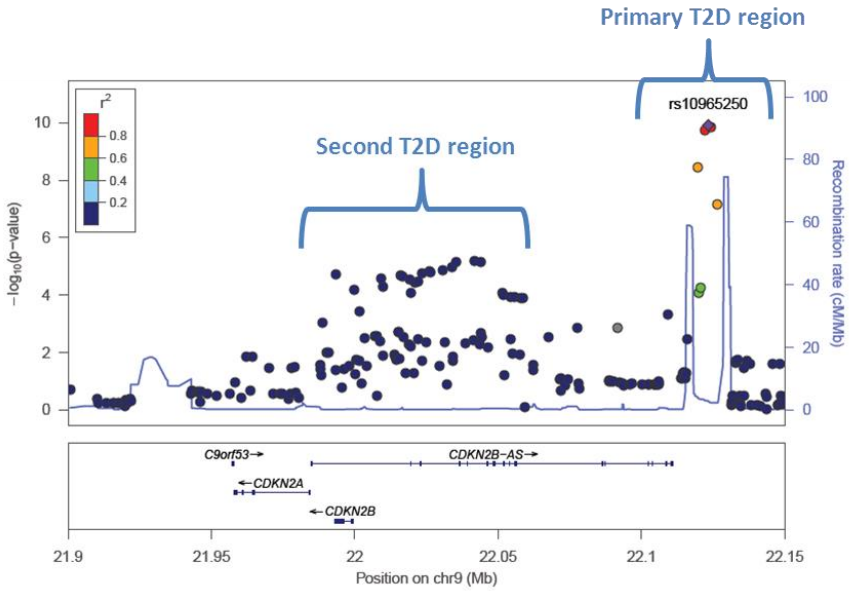



**Figure 4.1** Adapted from Pal and McCarthy (Pal and McCarthy 2013). Regional plot showing position of SNPs in Mb on chromosome 9 with location of the most proximal known genes in the region (x axis): strength of association of the SNPs with T2D shown on y axis.

As well as the association with T2D, the region at Chr9p21 is also independently associated (i.e. through different SNPs) with coronary artery disease (CAD) (Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; Broadbent et al. 2008), abdominal aortic aneurysm (Helgadottir et al. 2008), intracranial aneurysm (Helgadottir et al. 2008; Yasuno et al. 2010), carotid atherosclerosis (Ye et al. 2008), ischaemic stroke (Anderson et al. 2010), peripheral vascular disease (Cluett et al. 2009), endometriosis (Uno et al. 2010) and most recently with glaucoma (Burdon et al. 2012; Nakano et al. 2012; Wiggs et al. 2012). Given the preponderance of vascular associated diseases there is, surprisingly, no association between this locus and other typical CAD-related traits (excluding the independently associated T2D) such as hypertension and hyperlipidaemia (Broadbent et al. 2008; Angelakopoulou et al. 2012). Given the proximity of the tumour suppressor genes *CDKN2A* and *CDKN2B*, it is not surprising that there is also a growing list of cancers associated with Chr9p21 from GWAS: breast cancer (Turnbull et al. 2010), basal cell carcinoma (Stacey et al. 2009), glioma (Shete et al. 2009; Wrensch et al. 2009) and nasopharygeal carcinoma (Bei et al. 2010).

### 4.1.3 *CDKN2A* and *CDKN2B* encode cell-cycle inhibitors

The mechanism by which variation at the Chr9p21 locus affects risk of the non-oncological diseases remains unclear but the nearest protein coding genes are *CDKN2A* and *CDKN2B* (Figure 4.1) which encode the cyclin dependent kinase inhibitors p16 and p15 respectively (Figure 4.2).

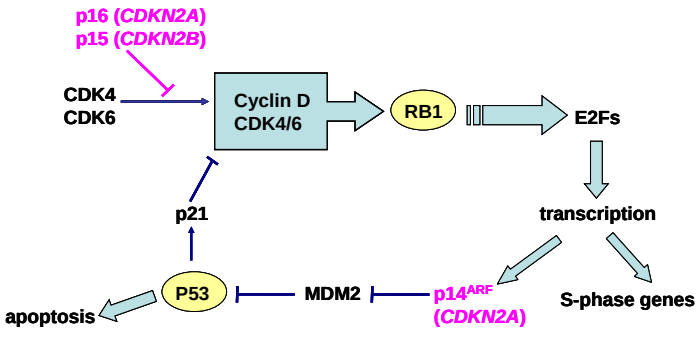



**Figure 4.2** Schematic showing how products of *CDKN2A* and *CDKN2B*, p16 and p15 respectively, suppress cell cycling by inhibiting actions of cyclin dependent kinase 4 and 6 (CDK4 and CDK6) which would otherwise go on to activate the retinoblastoma (RB1) pathway. RB1 would normally activate the E2F transcription factors leading to transcription of the S-phase genes in addition to CDKN2A in its alternate reading frame, giving rise to p14ARF which inhibits MDM2, an important negative regulator of the tumour suppressor p53.

The *CDKN2A*, *CDKN2A*$^{ARF}$ and *CDKN2B* locus is relatively unusual in its transcriptional regulation and location of three important tumour suppressor genes in a relatively small (35kb) region (Ruas and Peters 1998; Kim and Sharpless 2006). The complex transcriptional network that exists in this chromosome 9 region or the so-called 'INK4 locus' (Figure 4.3) may contribute to the identification of so many independently associated SNPs for CAD, T2D and other disease in this region.

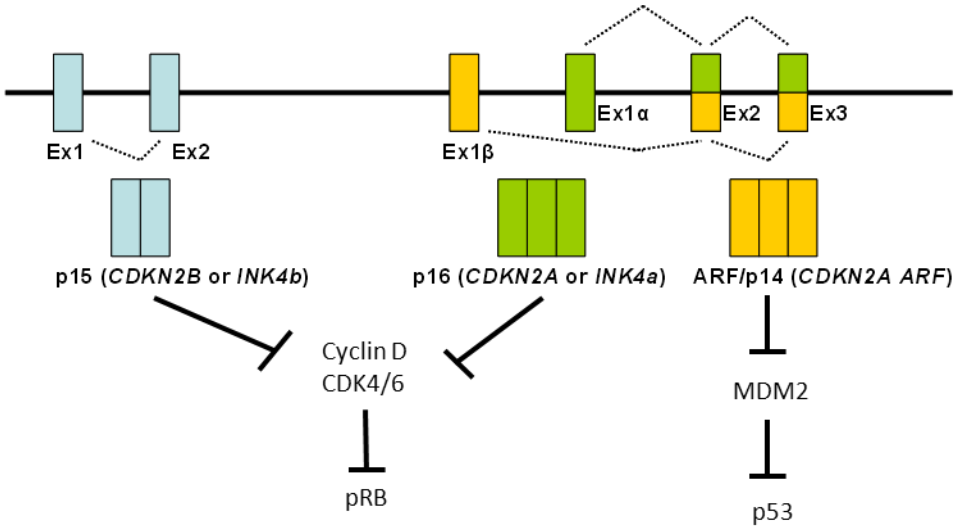



**Figure 4.3** Adapted from Kim and Sharpless (Kim and Sharpless 2006) showing the small 35kb locus (also known as the INK 4 locus) which encodes three genes *CDKN2A* (also known as *INK4a*), *CDKN2A*$^{ARF}$ and *CDKN2B* (also known as *INK4b*). *CDKN2B* has its own open reading frame which is separate from that of *CDKN2A* and *CDKN2A*$^{ARF}$. *CDKN2A* and *CDKN2A*$^{ARF}$ have different first exons which are spliced to a common second and third exon. In addition the proteins, p16 and p14ARF, are encoded in alternate reading frames which results in two entirely different proteins that do not share any amino acid homology.

### 4.1.4 Evidence for role of *CDKN2A* and *CDKN2B* in diabetes pathogenesis

Given the recognised role of vascular smooth muscle proliferation in atherosclerotic plaque biology *CDKN2A*, *CDKN2B* and *MTAP* [a third proximal gene to the Chr9p21 locus – approximately 200kb upstream of *CDKN2A* - which encodes a phosphorylase involved in purine metabolism (Carrera et al. 1984)] are excellent candidates for pathogenetic roles in atherosclerotic disease. The mechanism for association with T2D is less obvious. Rodent data support these genes as plausible candidates for roles in T2D pathogenesis: the main biochemical target of p15 and p16, Cdk4 (Figure 4.2), is a potent

regulator of beta-cell replication and islet mass, and *Cdk4* knockout mice have normal islet mass at birth but as adults develop reduced proliferation, islet mass and eventually diabetes (Rane et al. 1999; Kushner et al. 2005); *Cdk4R24C* knockin mice ($Cdk4^{R24C}$ being a mutated form of Cdk4 that prevents binding to the cell cycle inhibitors p15 and p16) have increased islet mass and beta-cell area (Wolfel et al. 1995; Marzo et al. 2004). More direct evidence comes from murine studies manipulating expression of *Cdkn2a* and *Cdkn2b*. Krishnamurthy et al generated transgenic mice which over expressed *Cdkn2a* (leading to increased Cdk4 inhibition) and showed they developed islet hypoplasia and diabetes (Krishnamurthy et al. 2006); Moritani et al generated transgenic mice which express a constitutively active form of TGF-β1 and displayed increased *Cdkn2b* expression, islet hypoplasia and diabetes (Moritani et al. 2005). However the only evidence to date for *CDKN2A* and *CDKN2B* being implicated in T2D in humans is the relative proximity of these genes to the T2D association signal at chromosome 9p21 (Figure 4.1), as no coding variant associations have been demonstrated.

### 4.1.5 A long non-coding RNA, ANRIL, is transcribed from the Chr9p21 region

Although the ~100kb genomic interval at the Chr9p21 T2D-susceptibility loci do not contain any protein coding genes, the region does include a gene for a long non-coding RNA (ncRNA) known as ANRIL (antisense non-coding RNA in the INK4 locus), also known as *CDKN2BAS* (*CDKN2B* antisense RNA) which was first characterized from a germ-line deletion inherited within a French familial melanoma pedigree (Pasmant et al. 2007). There is a growing understanding of the function of long non-coding transcripts and the suggestion that rather than being redundant elements of the transcriptome, these ncRNAs represent another tier of regulation within the human genome and actually contribute to control of transcription from neighbouring (or distant) coding genes (Mattick 2005; Guttman et al. 2009; Harries 2012). In particular antisense non-coding transcripts may play a

role in the regulation of expression of the coding gene on the opposite strand (Katayama et al. 2005; Yu et al. 2008).

*ANRIL* is made up of 19 exons spanning 126.3Kb and is transcribed in the antisense direction to the *p15/CDKN2B-p16/CDKN2A-p14/ARF* gene cluster (Pasmant et al. 2007). Intron 1 of *ANRIL* overlaps the two exons of *CDKN2B* and the 5' end of the first exon of *ANRIL* is ~ 300bp upstream of the *p14/ARF* gene transcription start site. Exactly how variants at the Chr9p21 locus affect disease risk is not clear but multiple studies investigating the expression of *ANRIL* with the CAD Chr9p21 genotype have demonstrated strong associations between *ANRIL* expression and the Chr9p21 risk allele, although the direction of effects are variable (Jarinova et al. 2009; Liu et al. 2009; Burd et al. 2010; Cunnington et al. 2010; Holdt et al. 2010). These expression studies (mainly in peripheral blood cells) also indicate co-regulation of transcription of *ANRIL*, *CDKN2A* and *CDKN2B* as they show a relationship between CAD-associated SNPs and *CDKN2A* and *CDKN2B* expression, as well as expression of *ANRIL*. This association has also been confirmed in vascular smooth muscle cells (VSMCs): Motterle and colleagues showed that the Chr9p21 CAD-risk genotype was associated with reduced *CDKN2A*, *CDKN2B* and *ANRIL* expression and with increased VSMC proliferation (Motterle et al. 2012) which would be the most plausible mechanism underlying accelerated atheroma formation.

Evidence for the noncoding CAD risk interval at Chr9p21 regulating *CDKN2A* and *CDKN2B* expression also comes from Visel and colleagues who generated a knockout mouse with a 70Kb region deleted on chromosome 4, apparently orthologous to the 58Kb CAD risk interval on chromosome 9 (Visel et al. 2010). Interestingly a dramatic (10-fold) reduction in cardiac expression of *Cdkn2a* and *Cdkn2b* was observed in the mice with null alleles compared to wild type, and an increase in VSMC

proliferation was found (Visel et al. 2010). However the suitability of this model for studying the Chr9p21 locus is not proven, as although the entire region was deleted in this study, it is not certain that this region contains the specific regulatory elements relating to *ANRIL* in humans, as the chromosome 4 region in mice shares a modest 50% DNA homology with the risk interval in humans and is not known to actually encode an equivalent ncRNA.

Thus the majority of evidence to date linking the Chr9p21 region to regulation of genes at the Chr9p21 locus comes from studies investigating possible mechanisms for the CAD variants. There have been no equivalent studies in tissues relevant to T2D and glucose homeostasis. Understanding the relationship between *CDKN2A*, *CDKN2B* and *ANRIL* is pivotal to understanding the pathophysiology of this locus and how the Chr9p21 T2D variants are affecting disease risk.

My aims were to assess the expression of these genes in tissues relevant to glucose homeostasis and T2D; to characterise the *ANRIL* transcript(s) present in human islets; to down regulate *ANRIL* in human islets and assess firstly the effects on *CDKN2A* and *CDKN2B* expression and secondly the effect upon islet function.

## 4.2 Methods

### 4.2.1 Tissue samples

A commercially available tissue panel containing total RNA from 20 human tissues (Clontech) was used to determine *ANRIL*, *CDKN2A* and *CDKN2B* mRNA expression across a range of tissues. Each sample was pooled from at least 3 individuals.

Human islets were made available through the Oxford Consortium for Islet Transplantation (OXCIT). Pancreases from human donors (not known to have diabetes) were procured by regional organ retrieval teams following appropriate consent for islet research and local ethics approval (ref 09/H0605/2). Human islet isolation was performed in the Human Islet Isolation Facility in OCDEM using principles of the semi-automated method (Ricordi et al. 1988). The islets were then cultured in CMRL medium (PAA Laboratories, Austria) (5.5 mM (99 mg/dl) glucose) overnight prior to experiments. As far as possible I conducted my experiments on human islets within 24h of their isolation to ensure maximum viability of the islets for the duration of my experiments.

FACS enriched beta-cells were made available via Martijn van de Bunt (Oxford) who, for his own experiments, performed FACS of human islet preparations at the Flowcytometry Core Facility of the Jenner institute, University of Oxford, using an in-house protocol, modified from Parmoud et al (Parnaud et al. 2008). RNA was extracted from these tissues, and reverse transcription performed as previously described in **Chapter 2**.

### 4.2.2 *ANRIL* siRNA testing in HUVEC

Prior to work in human islets, siRNAs targeting *ANRIL* were tested in HUVEC (human umbilical vein epithelial cells) in collaboration with Theo Kyriakou (Oxford). This cell system was selected for its relevance in CAD biology and significance of *ANRIL* given the association signals at Chr9p21 for CAD as well as T2D. Exons 5 and 6 were selected for knockdown as these have been found to be

expressed in all tissues extracted (Folkersen et al. 2009). Exons 13 and 18 are present in longer transcripts and were also selected to attempt to differentially down regulate these longer transcripts (Folkersen et al. 2009). siRNAs targeting exons 5,6, 13 or 18 of *ANRIL* were custom designed by Applied Biosystems (Table 4.1).

| **siRNA target** | **Sequence (5'→3')** |
|---|---|
| *ANRIL* exon 5 | GGUUCAAGCAUCACUGUUAtt |
| *ANRIL* exon 6 | GCUUCAUUCUAUACCAGGAtt |
| *ANRIL* exon 13 | AAAGCACAUUGGACAAAAAtt |
| *ANRIL* exon 18 | GAGUCAAGAUUUAUGCUUUtt |

**Table 4.1** Sequence of *Silencer*®Select Custom Designed siRNA (Applied Biosystems) used to target specific *ANRIL* exons.

Theo Kyriakou (Oxford) performed the siRNA transfections in HUVEC and maintained cells in culture. HUVECs were grown in 6 well plates. For each well 100µL of siRNA (2µM) was initially incubated with 100µL of serum free media (SFM) for 5 min. Concurrently, 2µL of Dharmacon Dharmafect 1 (Thermo Scientific), a lipid-based transfection reagent, was incubated with 198µL of SFM. After 5 min the transfection reagent was mixed with the siRNA and incubated at room temperature for 20min. It was then added to 1600µL of complete media and added to the cells. Each individual siRNA was tested. Appropriate controls with transfected reagent only or scrambled siRNA were also prepared. In addition a positive control GAPDH siRNA was also used. After they had been incubated for 48h, I harvested the cells, extracted RNA, made cDNA and performed the QRT-PCR and analysis to assess the degree of gene knockdown using methods described in **Chapter 2**.

### 4.2.3 Adenoviral siRNA vector construction, initial testing and use in human islets

An adenoviral construct [type 5 (dE1/E3)] containing shRNA (a sequence of RNA that forms a short hairpin turn that can be used to knockdown a target gene) targeting *ANRIL* exon 5 was custom made

by Vector Biolabs (Philadelphia, US). Exon 5 of *ANRIL* was selected as the target due to gene knockdown results in comparison to other exons targeted by siRNAs used in HUVEC (see results **section 4.3.3**). An adenoviral construct containing scramble shRNA was also made [Vector Biolabs (Philadelphia, US)]. The virus was purified by caesium chloride column purification and the virus titre determined using the plaque formation assay method (all performed by Vector Biolabs prior to use). The viruses co-expressed green fluorescent protein (GFP) to facilitate estimates of infection efficiency in the target cells: expression of shRNA was under a U6 promoter, and GFP co-expression was under a separate CMV promoter.

The adenoviral constructs were trialled in human embryonic kidney (HEK) 293 cells prior to use in human islets. HEK 293 cells were grown in 24 well plates and infected with a range of adenovirus (2, 4, 10, 20, 50, 100 X $10^7$ PFU) in 1ml media (EMEM + 2mM glutamine +1% non essential amino acids +10% fetal bovine serum). These were incubated for 24, 36 and 48h with all preparations grown in duplicate. Widespread cell death occurred with inoculated adenovirus ≥ 10 X $10^7$ PFU and where incubation time >48h. Gene expression and knockdown was assessed as described above. The protocol achieving highest *ANRIL* knockdown (compared to scramble and untransfected control conditions) was taken forward for optimisation in human islets.

Pure human islets of medium or small size (to increase penetration of adenovirus to central core of the islet) were handpicked from isolated islets. Numbers varied depending on the purity and quality of the islet prep received. A minimum of 300 islets were picked into 3 X $10cm^2$ petri dishes containing 2ml of CMRL media each (100 islets per dish). Multiple replicates were prepared if larger quantities of islets were available. The islets were then incubated overnight (if islet preparation was available in the evening) or left for 2h (if islet preparation became available in the morning) before

being infected with the adenoviral vector. Human islets were transferred into 300µL media and after infection with either 2, 4, 6, 7 or 10 X $10^7$ PFU of adenovirus, were incubated for 4 hours. Media was then increased to 2ml and islets were incubated for 24, 36 or 48h. Infection efficiency was then assessed using confocal microscopy. Infection of human islets was optimised over a range of viral concentrations and a range of incubation times. Maximum infection efficiency was achieved with 7 X $10^7$ PFU of adenovirus and 48h incubation time and therefore this was the protocol used for all experiments reported in islets. The main barrier to maximal infection of all cells contained within the islet was penetration of the adenovirus to the central core of the islet which is notoriously difficult (Leibowitz et al. 1999; Mahato et al. 2003). After required incubation time and estimation of infection efficiency, RNA was extracted from the islets using spin column based RNA Minikits (Invitrogen) according to manufacturer's protocols, and RNA quantity assessed using the nanodrop as described in **Chapter 2**. Gene expression and knockdown was assessed using QRT-PCR as described in **Chapter 2** and below.

To demonstrate that lack of penetration of adenovirus to islet core was not due to cell death or absence of cells, human islets were stained with propidium iodide (for dead cells) and also DAPI (4',6-diamidino-2-phenylindole) staining (a nuclear stain) in one islet preparation. For propidium iodide staining, 1µL of stock solution (1ml/ml) was added to 500µL binding buffer, 2ml of this solution was added to the islets which were then incubated for 5 min at room temperature in the dark. Staining was then assessed with confocal microscopy (emission wavelength 620). For DAPI staining the dye was diluted to 300nM in PBS (phosphate-buffered saline) and 15µL of this solution was added to each well of a terasaki plate containing 2 islets per well. This was then incubated for 5 min in the dark and rinsed with PBS. Staining was next assessed with confocal microscopy (emission wavelength 460).

### 4.2.4 Insulin secretion assays

Insulin secretion was measured from batches of 10 similar sized islets (small to medium) in quadruplicate, which were handpicked using a 10µl pipette from islets that had undergone adenoviral siRNA infection (or controls). The islets were picked into 0.5 ml eppendorf tubes and washed in RPMI-1640 (Sigma, UK) supplemented with 100 U/ml penicillin (Lonza, Belgium), 10 µg/ml streptomycin (Lonza, Belgium) and 10% Fetal Calf Serum (Sigma, UK) to remove cell debris. The islets were pre-incubated for 1 hr in a wet chamber at 37°C (5 % $CO_2$ / 95 % air) in 300 µl of Krebs-Ringer buffer (KRB) which contained the following (in mM) 140 NaCl, 3.6 KCl, 2.6 $CaCl_2$, 0.5 $MgSO_4.7H_2O$, 0.5 $NaH_2PO_4$, 2 $NaHCO_3$, 5 HEPES and 2 mg/ml Bovine serum albumin (BSA) (Sigma, UK) (pH adjusted to 7.4 with NaOH). The KRB was supplemented with a 1mM (18 mg/dl) glucose concentration. The pre-incubation buffer was discarded. Islets were incubated for 1 hr in a wet chamber at 37°C (5 % $CO_2$ / 95 % air) in 300 µl of KRB supplemented with the test conditions as indicated. The test buffer contained 1 mM (18 mg/dl) or 20 mM (360 mg/dl) glucose. Samples of the supernatant (290µl) were collected at the end of each test period and immediately frozen at -20°C. The remaining supernatant was discarded. The islets were then lysed in 100µl ice-cold acid-ethanol solution (containing 90% ethanol, $H_2O$ and 12M HCl in a ratio of 52:17:1) to release their hormone content. The lysed islet pellets were immediately frozen at -20°C.

The supernatants and pellets from the human hormone secretion experiments were assayed using human insulin (Millipore, UK) radioimmunoassay (RIA) kits (Millipore, UK) according to the manufacturer's instructions: this was performed by David Wiggins (Oxford). Prior to RIA, the supernatants and pellets were diluted in Dilution Buffer which contained HBSS (Hank's buffered salt solution) supplemented with 0.25 % RIA grade BSA and 0.008 % sodium azide. The dilutions used for the insulin assays were 1 in 4 for supernatant and 1 in 1600 for the pellet.

### 4.2.5 Alternate methods of siRNA delivery - human islet dispersal and transfection

Given the challenges of delivering the adenoviral vector to the central core of cells in the whole human islets, the experiments were also attempted on dispersed islets. Islets were handpicked into 3.5mm dishes and swirled around in 2ml PBS. Using a 20µL pipette, these were picked into a 15 mL falcon tube containing 1 mL of PBS and placed into a 37˚C water bath for 9 min. The islets were next pipetted up and down 10-20 times using a 1mL pipette so that the islet structures dissolved into a cloudy solution. These were diluted with 9 ml of RPMI media and spun for 5 minutes at 250G. The supernatant was then removed and the cells were resuspended in 3ml RPMI. Next this was decanted into three 35mm cell culture dishes containing 1ml RPMI which were incubated for 1-2 hours. Following this, 2ml of PBS was added to each and dishes were gently swirled to remove dead cells. The PBS was then removed and 1.5-2ml RPMI was added and cells were incubated overnight, prior to infection with adenovirus the following morning.

As one of the aims was to assess function of islets post *ANRIL* knockdown, experiments in whole/intact human islets were prioritised rather than single cells from dispersed islets. As the adenoviral vector method for siRNA delivery to the islets seemed variably toxic to the islets, an alternative method of siRNA delivery was attempted: lipid based transfection. Prior to transfection, the siRNA was diluted to 100nM in 250µL of Optimem (Life Tech). The lipid based transfection reagent RNAiMAX (Life Tech), was diluted in Optimem to a ratio of 1:20 (50uL RNAiMAX into 950uL Opti = 1000uL total). The diluted siRNAs were mixed with 250uL of the RNAiMAX to a total volume of 500uL. The siRNA and transfection reagent mixes were incubated at RT for 20min and then added to the islets in a drop wise fashion. Fifty islets (per well) were picked as there was limited availability of islets, and 100nM of *ANRIL* ex5 siRNA or scramble siRNA was transfected. After 48hr transfection islets were harvested, RNA extracted and gene expression determined as described above.

### 4.2.6 QRT-PCR

QRT-PCR was performed on cDNA made from RNA extracted from human islets after incubation with adenovirus or control human islets using methods and data analysis described in **Chapter 2**. Each sample was amplified in triplicate to ensure accuracy of quantification. cDNA was generated from 200ng to 1µg RNA (depending on availability of islets for each experiment) but the same starting quantity was used for all samples in each individual experiment to enable comparisons between siRNA adenovirus, scramble and control conditions. Where individual experiments were repeated, data was amalgamated and number of replicates reported in figure legends. Each 'biological prep' refers to an individual islet isolation. QRT-PCR interpretation of target gene knockdown was reliant on relatively constant housekeeping gene expression between experimental conditions. All QRT-PCR data is represented graphically as relative expression of the gene of interest (GOI) to the housekeeping genes (HKGs). Where housekeeping genes (HKGs) had been effected by experimental conditions (for example being much less expressed in adenoviral versus control conditions), data were not used as the HKG results would skew interpretation of the

expression of genes of interest.

A range of HKGs were used (*B2M, GUSB, HPRT1, RPLP, UBC, HMBS, LRP10*) to determine those most unaffected by adenoviral infection. All behaved comparably; therefore *B2M*, *GUSB* and *HPRT1* were used for the majority of the experiments.

| **Gene ID** | **Gene Name** | **Assay ID** |
|---|---|---|
| *B2M* | Beta-2-microglobulin | Hs99999907_m1 |
| *GUSB* | Glucuronidase beta | Hs99999908_m1 |
| *HPRT1* | Hypoxanthine Phosphoribosyltransferase 1 | Hs99999909_m1 |
| *RPLP* | Ribosomal protein L16p | Hs99999902_m1 |
| *UBC* | Ubiquitin C | Hs00824723_m1 |
| *HMBS* | Hydroxymethylbilane synthase | Hs00609297_m1 |
| *LRP10* | Low-density lipoprotein receptor-related protein 10 | Hs00204094_m1 |
| *CDKN2A* | Cyclin-dependent kinase inhibitor 2A | Hs00233365_m1 |
| **Custom probes** | | **Sequence** |

| | | |
|---|---|---|
| *CDKN2B* | Cyclin-dependent kinase inhibitor 2B | Forward primer CGGATCCCAACGGAGTCA<br>Reverse primer TGGAGCAGCAGCAGCT<br>Probe ATCATGACCTGGATCGCG |
| *ANRIL ex5* | ANRIL or CDKN2BAS (CDKN2B'antisense') | Forward primer TGTCCCTTTTGATGAGAAGAATAAGC<br>Reverse primer GTGGCCAGAAAACAGAAGTCTTT<br>Probe TCTCTGCTGTTGAATCAG |

**Table 4.2** Gene names and gene IDs of probes used to amplify the chromosome 9 genes and all housekeeping genes used. Sequences of primers and probes given for custom probes. All probes were purchased from Applied Biosystems and selected on the basis that they covered all RefSeq genes and specifically amplified mRNA (_m1) where possible.

### 4.2.7 Statistical analysis

Where data are represented graphically the mean +/- SEM is plotted unless otherwise stated. Where sufficient independent experiments were repeated, statistical analysis of expression between paired samples (transfected cells and controls) was performed using a Wilcoxon Signed Ranks Test, the non-parametric equivalent of the paired t-test. This option was selected on the basis that with limited numbers the data are unlikely to be normally distributed making parametric tests unsuitable. All statistical analysis was performed using SPSS statistics programme version 20.0.

## 4.3 Results

### 4.3.1 *CDKN2A*, *CDKN2B* and *ANRIL* mRNA expression in human tissues

mRNA expression of the most proximal genes to the T2D association signals at Chr9p21 was assessed in a range of tissues including those most relevant to glucose homeostasis: human islets and FACS beta-cells. QRT-PCR was used to determine the expression profile of *CDKN2A*, *CDKN2B* and *ANRIL* across a tissue panel containing total RNA from 20 human tissues (Clontech) and in house samples of human islets and FACS beta-cells (Fig 4.4).

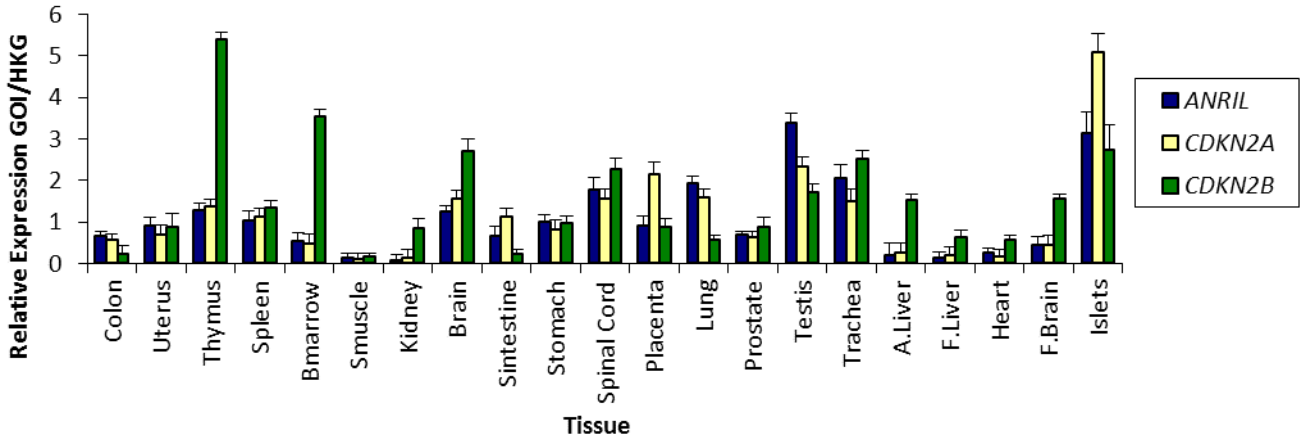



**Figure 4.4** *CDKN2A* (and *CDKN2A*$^{ARF}$), *CDKN2B* and *ANRIL* mRNA expression across a human total RNA tissue panel and human islets. Expression is relative to 3 housekeeping genes (*GUSB*, *B2M* and *HPRT1*). Data shown are from one experiment with QRT-PCR samples amplified in triplicate.

*CDKN2A*, *CDKN2B* and *ANRIL* are widely expressed across human tissues with highest levels observed in human islets and thymus tissue. High expression was also seen in lung and testis. Expression in human islets was validated in 3 further isolations of human islets (i.e. from 3 separate individuals) (Fig 4.5).

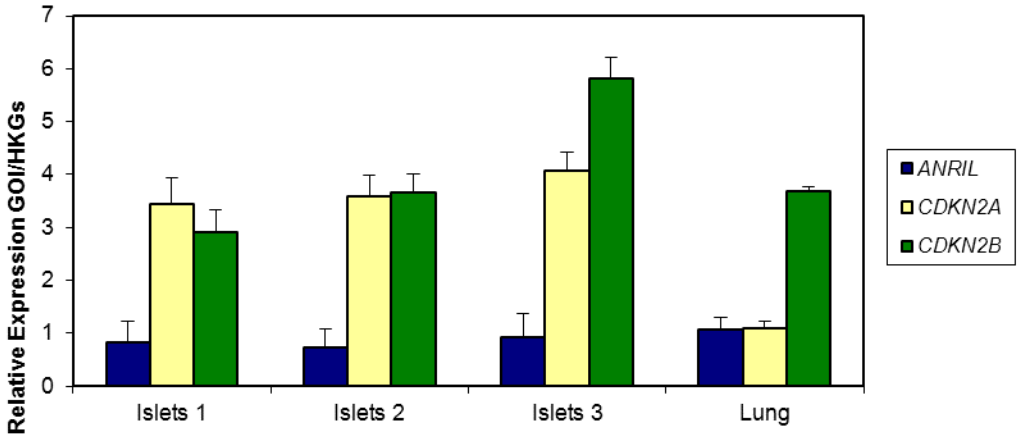



**Figure 4.5** Consistent expression of *CDKN2A* (and *CDKN2A*$^{ARF}$), *CDKN2B* and *ANRIL* in 3 further human islet isolations, alongside lung tissue which is known to express these genes. Data shown are from one experiment with QRT-PCR samples amplified in triplicate.

To investigate *CDKN2A*, *CDKN2B* and *ANRIL* expression in the insulin secreting beta-cell component of human islets, QRT-PCR was performed on cDNA synthesised from RNA extracted from FACS beta-cells (Figure 4.6). All 3 genes of interest were expressed with particularly high relative expression of *CDKN2A* (compared to *CDKN2B* and *ANRIL*).

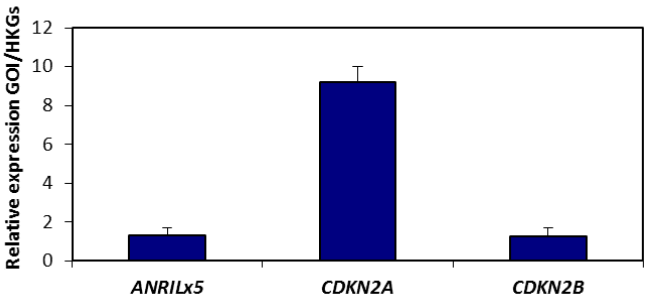



**Figure 4.6** Expression of *CDKN2A* (and *CDKN2A*$^{ARF}$), *CDKN2B* and *ANRIL* mRNA in FACS beta-cells. Data shown are from one experiment with QRT-PCR samples amplified in triplicate.

### 4.3.2 *ANRIL* transcript in human islets sequenced

To design siRNAs to target *ANRIL* for gene knockdown in human islets, transcripts of *ANRIL* were amplified and sequenced from RNA extracted from human islets. PCR and capillary sequencing performed by myself and Theo Kyriakou (Oxford), demonstrated a ~1000bp transcript in human islets with exons 1-5-6-15-17-18-19 present. Several alternatively spliced transcripts in different

human tissues have been demonstrated (Folkersen et al. 2009); exons 5 and 6 were found to be consistently expressed in all tissues tested. RACE-PCR was used to define the 3'end of the *ANRIL* transcript present in human islets (Fig 4.7). I was unable to get the RACE-PCR to work for the 5'end of the transcript, despite repeated attempts using higher concentrations of starting RNA where possible, ensuring RNA purity and adjusting the manufacturer's protocol (prolonged spin times, repeated elutions, prolonged incubation times and repeated rounds of PCR). As RNA quantities extracted from human islets were limited, the 5'RACE-PCR was not pursued.

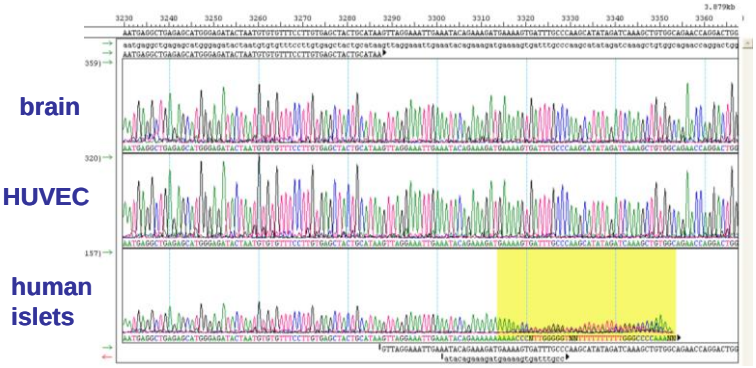



**Figure 4.7** Electropherogram showing *ANRIL* transcripts present in human islets alongside HUVEC and brain. This demonstrates the comparatively shorter transcript present in human islets and the 3'end RACE-PCR product.

### 4.3.3 Down regulation of *ANRIL* in HUVEC and effects on *CDKN2A* and *CDKN2B* expression

siRNAs targeting exons 5, 6, 13, or 18 were custom designed by Applied Biosystems and were initially tested in HUVEC, in collaboration with Theo Kyriakou (Fig 4.8). The siRNA against exon 5 *ANRIL* resulted in the highest level of gene knockdown of 80% and was therefore selected for further experiments in HUVEC to assess the subsequent effects on *CDKN2A* and *CDKN2B* expression (Fig 4.9).

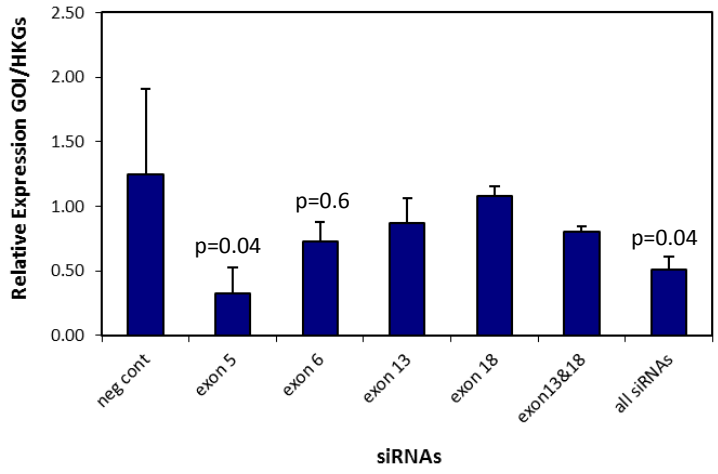



**Figure 4.8** *ANRIL* knockdown with individual siRNAs to exon 5,6,13,18 and 13/18 in combination in HUVEC. Best gene knockdown (80%) was achieved with siRNA against exon 5. Observed transfection efficiency was 90%. n=6 (6 individual transfections and QRT-PCR in triplicate). P values refer to Wilcoxon paired analysis of *ANRIL* expression in siRNA treated samples vs negative control.

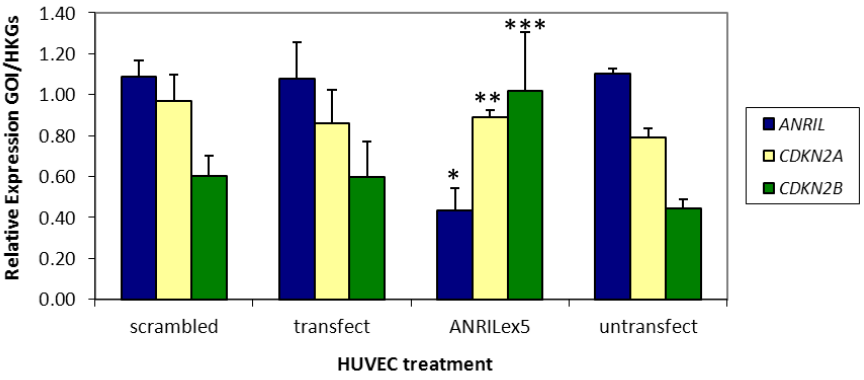



**Figure 4.9** *CDKN2A*, *CDKN2B* and *ANRIL* expression determined in HUVEC transfected with siRNA against exon 5 *ANRIL*. Expression of these genes was also assessed in the controls; cells transfected with scrambled siRNA, cells treated with transfection reagent only & untransfected cells. Observed transfection efficiency was 90%. n=9 (9 independent transfections, QRT-PCR in triplicate). *p=0.068, **p=0.21, ***p=0.068 in Wilcoxon paired analyses of relative expression of the labelled gene in ANRILex5 siRNA treated cells vs transfected controls.

A mean of 60% [SD range 48.7, 71.3] *ANRIL* gene knockdown was achieved in HUVEC treated with siRNA against *ANRIL* exon 5 (Fig 4.9) which did result in an effect on *CDKN2B* expression. In HUVEC with *ANRIL* down regulation there was a 1.7 fold *increase* [SD range 1.3, 2.2] in *CDKN2B*. There was no detectable effect on *CDKN2A* expression with this extent (60%) of *ANRIL* down regulation. There was no effect on *ANRIL*, *CDKN2A* or *CDKN2B* expression in any of the control conditions as expected.

Adenovirus harbouring siRNA against exon 5 were then validated in 2 cell systems (HUVEC and HEK293 cells) prior to experiments in human islets (Fig 4.10).

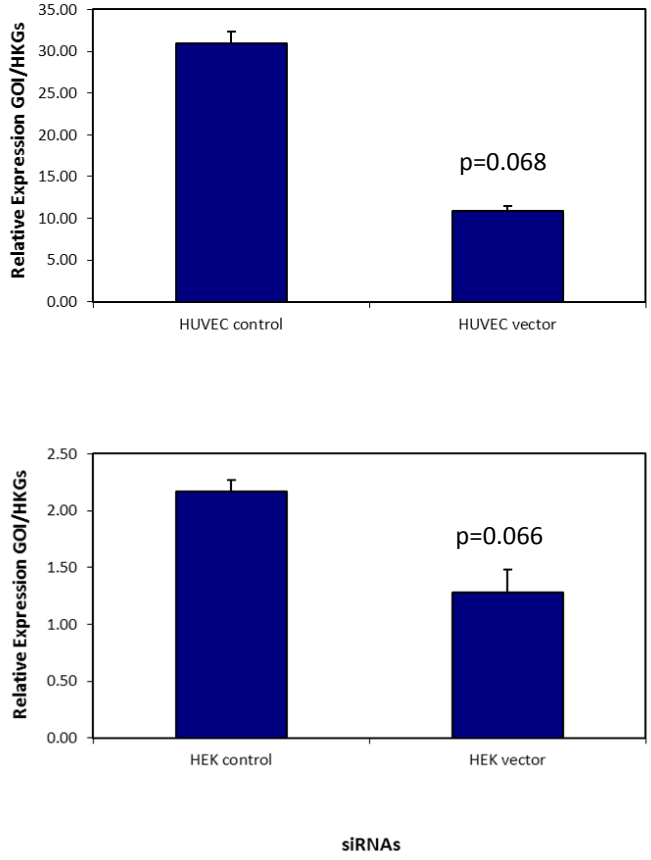



**Figure 4.10** *ANRIL* expression from QRT-PCR results in HUVEC and HEK293 cells demonstrating reduced *ANRIL* expression in HUVEC and HEK293 cells infected with the adenoviral siRNA vector compared to uninfected cells. n=4 (4 individual infections and QRT-PCR in triplicate). Observed infection efficiency was 60% (HUVEC) and 50% (HEK). P values refer to Wilcoxon paired analysis of *ANRIL* expression in infected cells vs controls.

A 68% *ANRIL* knockdown was observed in HUVEC after incubation with the adenoviral siRNA vector. In an alternative cell system, HEK293 cells, a more modest knockdown of 43% was observed. However as the adenoviral siRNA vector system was working, albeit with a modest target gene knockdown, it was assumed that knockdown could be improved with optimisation in the target cell system and therefore this adenoviral vector was taken forward for use in human islets.

### 4.3.4 Down regulation of *ANRIL* expression in human islets – infection efficiency

After infection and incubation of intact human islets with GFP-tagged adenoviral vectors, infection efficiency was assessed using confocal microscopy with Matthias Braun (Oxford) (Fig 4.11). The main barrier to maximal infection of all cells contained within each individual islet was penetration of the adenovirus to the central core of the islet which is notoriously challenging (Leibowitz et al. 1999; Mahato et al. 2003).

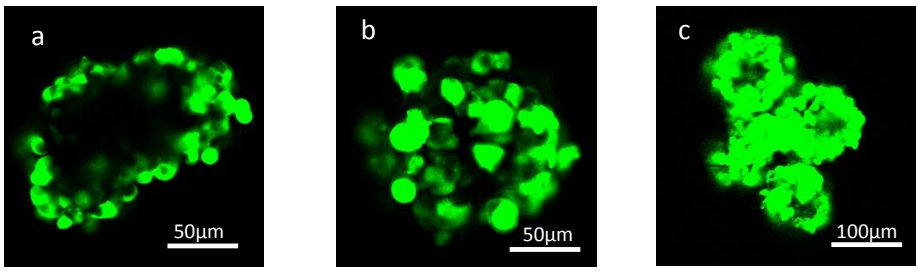



**Figure 4.11** Confocal microscopy images of human islets infected with GFP-tagged adenoviral vectors. Fig 4.8a shows approximately 15% infection efficiency; fig 4.8b approximately 40% infection efficiency; fig 4.8c approximately 60% infection efficiency of a cluster of 4 islets at optimum conditions.

It was evident from the confocal microscopy that the adenovirus was not penetrating the islet core i.e. due to the black appearance at the centre of each individual intact islet (Fig 4.11). To ensure this was not due to either absence of cells or cell death, islets were stained with propidium iodide and DAPI (Fig 4.12). Staining of both was present at the islet core (Fig 4.12a&b), demonstrating that cells were present in the region where GFP absent (Fig 4.12c&d) and that some were viable and some were dead (although the dead cells were less evident).

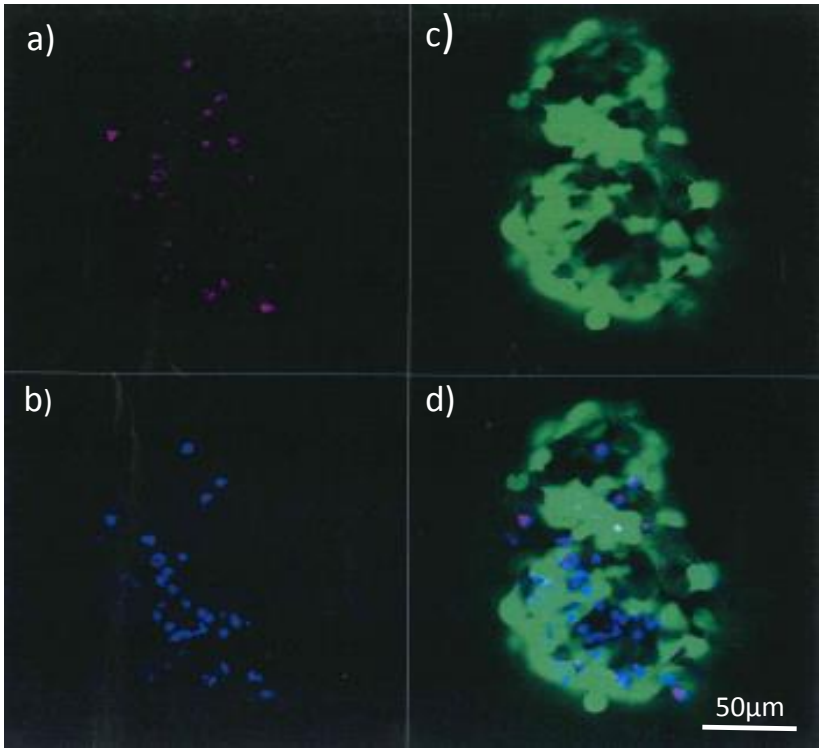



**Figure 4.12** Confocal microscopy images of human islets stained with propidium iodide (a), DAPI (b), GFP (c) and an overlay of all images in (d). DAPI staining is evident in the central areas not penetrated by GFP demonstrating the presence of viable cells here. Propidium iodide staining (for dead cells) was less evident demonstrating that most cells at islet core were still viable.

### 4.3.5 Down regulation of *ANRIL* expression in human islets – effects on *CDKN2A* and *CDKN2B* expression

Data from all experiments where human islets remained intact and viable after incubation with adenovirus were combined for analysis (Fig 4.13). A mean 40% *ANRIL* knockdown was achieved [range 16-66%] (Fig 4.13). In islets where there was *ANRIL* down-regulation, a 42% [range 0-75%] reduction in *CDKN2A* mRNA expression and 26% [range 0-41%] reduction in *CDKN2B* expression was observed (Fig 4.13). There was no detectable effect on *ANRIL*/*CDKN2A*/*CDKN2B* expression in human islets infected with the control adenovirus containing scrambled siRNA compared to the uninfected human islets.

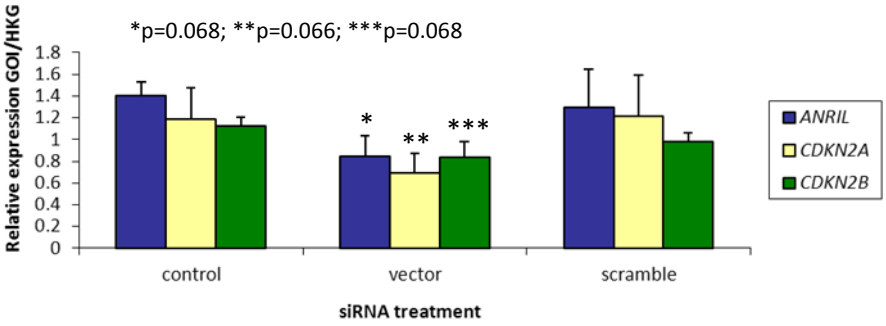



**Figure 4.13** *CDKN2A*, *CDKN2B* and *ANRIL* expression in human islets infected with siRNA against exon 5 *ANRIL*. Expression of these genes was also assessed in the controls: islets infected with adenovirus containing scrambled siRNA; uninfected islets treated with identical media changes and incubation conditions; n=4 (4 independent infections in 4 individual islet preps, QRT-PCR in triplicate). Observed infection efficiency was 60%. P values denote Wilcoxon paired analyses of mRNA expression in vector treated islets vs scramble controls.

### 4.3.6 Down regulation of *ANRIL* expression in human islets – effects on insulin secretion

Insulin secretion data was available on one preparation of human islets where *ANRIL* was down-regulated with 65% knockdown and consequent 75% reduction in *CDKN2A* expression and 40% reduction in *CDKN2B* expression (Fig 4.14A&B). The expected increase in insulin secretion at a higher concentration of glucose (20 mmol/L) was observed for all samples of islets (Figure 4.14B) but there was no difference in insulin secretion in relation to adenoviral treatment. Secretion studies were also performed on 2 other biological preparations of islets but no appreciable *ANRIL* knockdown was achieved in those experiments.

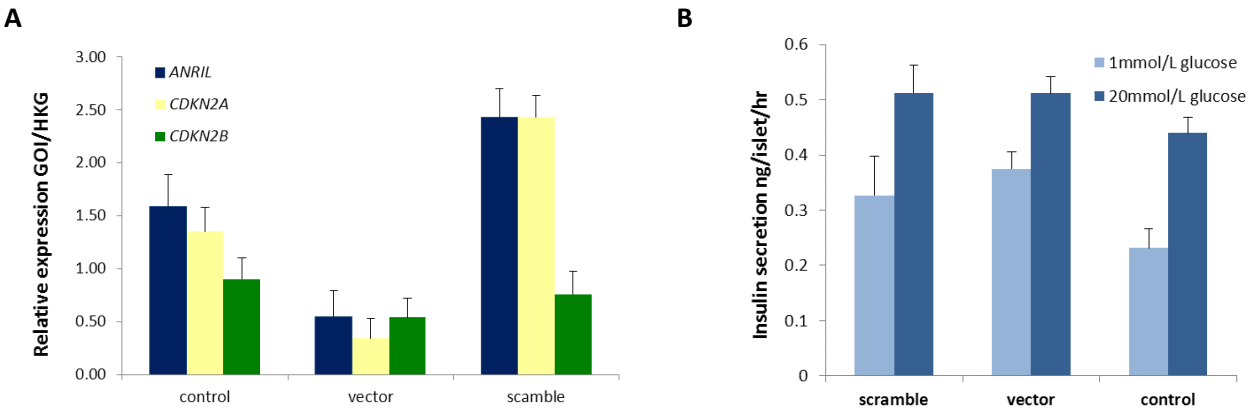



**Figure 4.14A** *CDKN2A*, *CDKN2B* and *ANRIL* expression in human islets infected with siRNA against exon 5 *ANRIL*; n=1 (1 biological prep, QRT-PCR in triplicate). Observed infection efficiency was 60%. **Figure 4.14B** Insulin secretion studies in these human islets after 48h incubation with scramble/vector adenovirus or control prior to RNA extraction for QRT-PCR. Light blue bars represent insulin secretion (ng/islet/h) at 1mmol/L glucose and dark blue bars represent insulin secretion (ng/islet/h) at 20mmol/L.

### 4.3.7 Adenoviral *ANRIL* knockdown in dispersed human islets

It was difficult to achieve greater target gene knockdown and penetration of adenovirus to the central core of the human islet without islet toxicity (using higher concentrations of adenovirus or prolonging incubation times resulted in non-viable islets upon which further experiments could not be performed). Therefore *ANRIL* down regulation was attempted in dispersed human islets twice. In the first experiment cells died within 24 hours of incubation with the adenovirus and therefore could not be further assessed; in the second experiment no knockdown of the target gene *ANRIL* was achieved (Fig 4.15). As this work was focussed upon intact human islets in order to ultimately assess if *ANRIL* knockdown had any effect upon physiological islet function, further experiments in dispersed human islets were not attempted as dispersed cells are noted to behave differently to intact human islets (Rorsman et al. 2011) and the islet dissociation process itself can lead to increased cell loss and apoptosis (Todorov et al. 2010).

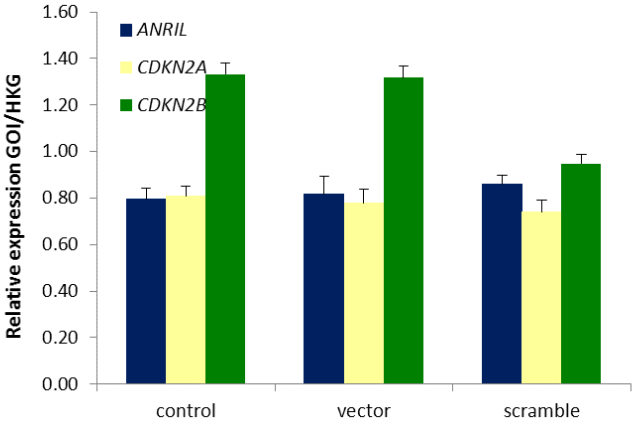



**Figure 4.15** *CDKN2A*, *CDKN2B* and *ANRIL* expression in dispersed human islets infected with siRNA against exon 5 *ANRIL*. Expression of these genes was also assessed in the controls: dispersed islets infected with adenovirus containing scrambled siRNA; uninfected dispersed islets treated with identical media changes and incubation conditions; n=1 (1 biological prep, QRT-PCR in triplicate). Observed infection efficiency was 50%.

### 4.3.8 Down regulation of *ANRIL* expression in human islets using transfection

*ANRIL* knockdown was also attempted using lipid based transfection of siRNA against *ANRIL* exon 5 to see if this method could be utilised whilst causing less toxicity to the islets than adenoviral infection.

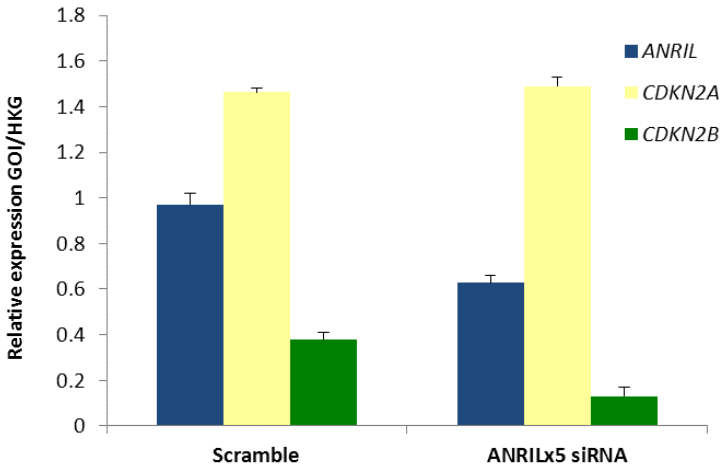



**Figure 4.16** *CDKN2A*, *CDKN2B* and *ANRIL* expression in intact human islets transfected with siRNA against exon 5 *ANRIL* or scramble siRNA; n=1 (1 biological prep, QRT-PCR in triplicate). Observed transfection efficiency was 70%.

This experiment was performed once (Fig 4.16) and resulted in 35% *ANRIL* knockdown with no change in *CDKN2A* mRNA expression but 65% reduction in *CDKN2B* mRNA expression. The reduction in *CDKN2B* expression was consistent with my results from adenoviral *ANRIL* knockdown in islets.

## 4.4 Discussion

### 4.4.1 *CDKN2A, CDKN2B* and *ANRIL* are expressed in tissues relevant to glucose homeostasis

To investigate the role of the tumour suppressor genes, *CDKN2A* and *CDKN2B*, and the neighbouring ncRNA, *ANRIL*, in the mechanism underlying the T2D variants at the Chr9p21 locus, I first assessed their tissue expression. Across a panel of human tissues I have shown that these genes are widely expressed, consistent with data previously published (Zeggini et al. 2007). In this previous study *CDKN2A* and *CDKN2B* expression was assessed in a range of fetal tissues, brain tissue, human islets, adipose and muscle tissue: high expression was observed in human islets and pituitary (Zeggini et al. 2007). My expression analyses have also demonstrated wide expression of *CDKN2A* and *CDKN2B*, in addition to expression of *ANRIL*, in a larger range of 22 human tissues (overlap with the previous study being in liver, kidney, muscle and brain). In particular, high levels of expression of these genes have been demonstrated in human islets which support a role for these tumour suppressor genes in a tissue highly relevant to glucose homeostasis. In addition I have shown novel data demonstrating expression of these genes in FAC sorted human beta-cells, the predominant and most relevant cell type to glucose homeostasis in the human islet. These molecular data, supporting a role for the Chr9p21 genes in insulin-secreting human islets, are consistent with the findings from association studies which have shown the Chr9p21 T2D effect is mediated via a primary effect on insulin secretion and beta-cell function (Grarup et al. 2007).

### 4.4.2 *ANRIL* has a regulatory role upon *CDKN2A* and *CDKN2B* expression

To investigate the potential regulatory role of *ANRIL* upon *CDKN2A* and *CDKN2B* expression it was important to define the *ANRIL* transcript/transcripts present in human islets. Theo Kyriakou and others have shown that *ANRIL* transcription is complex with ≥8 alternatively spliced transcripts and tissue-specific expression (Folkersen et al. 2009). Exons 5 and 6 were found to be consistently

expressed in all tissues tested, including human islets, and therefore the gene knockdown experiments were based around manipulating expression of *ANRIL* exon 5 (which had the best performing siRNA and gene expression assays). It is important to acknowledge the significant methodological challenges in these studies which do limit interpretation of the ANRIL knockdown data in particular. This will be discussed in more detail in section 4.4.3.

Taking limitations in methodology into account, I have shown some evidence supporting the theory that the ncRNA *ANRIL* regulates *CDKN2A* (in human islets) and *CDKN2B* expression (in HUVEC and human islets). In HUVECs a 60% *ANRIL* knockdown led to a 1.7 fold increase in *CDKN2B* expression in HUVECs: there was no detectable effect on *CDKN2A* expression at this level of *ANRIL* knockdown in HUVECs. In human islets a 40% *ANRIL* knockdown led to a 42% reduction in *CDKN2A* expression and 26% reduction in *CDKN2B* expression.

The selective up-regulation of *CDKN2B* in HUVECs makes sense given that *ANRIL* (also known as *CDKN2BAS*) is antisense to *CDKN2B*. There is evidence that *ANRIL* is involved in transcriptional regulation of *CDKN2A* and *CDKN2B* via recruitment of chromatin modifying multiprotein complexes, such as Polycomb repressive complexes (PRCs), to the Chr9p21 locus (Yap et al. 2010; Kotake et al. 2011). Two main PRCs, PRC1 and PRC2 have been described: PRC1 recruitment leads to ubiquitinylation of histone H2A on lysine 119, which is thought to be required for maintaining repression of transcription (Margueron and Reinberg 2011) and PRC2 is thought to be involved in initiating transcriptional repression via trimethylation of the lysine 27 residue on histone 3 (H3K27me3) (Margueron and Reinberg 2011). Yap et al demonstrated that *ANRIL* associates with the Polycomb-group protein CBX7 (which is part of the PRC1 complex) and proceeds to repress expression of *CDKN2A* (Yap et al. 2010). Down-regulation of *ANRIL* RNA expression was associated with increased *CDKN2A* expression, decreased H3K27me3 methylation, and CBX7 binding to the

*CDKN2A* locus, however *CDKN2B* expression was unaffected (Yap et al. 2010). Kotake et al showed that *ANRIL* binds to a polycomb-group protein of the PRC2 complex, SUZ12, and that *ANRIL* knockdown led to increased *CDKN2B* expression but *CDKN2A* expression remained unaffected (Kotake et al. 2011), consistent with my results in HUVECs. These two studies suggest that *ANRIL* has a cis regulatory effect on *CDKN2A* and *CDKN2B* but the *ANRIL* knockdown results are conflicting as different genes are regulated. However both these studies did demonstrate reduced cell proliferation in *ANRIL* knockdown cells, which is the mechanism assumed to be involved in the pathophysiology of atherosclerosis (Yap et al. 2010; Kotake et al. 2011).

Thus my results in HUVECs, showing up-regulation of *CDKN2B* in *ANRIL* knockdown cells, are consistent with evidence demonstrating *ANRIL* as a transcriptional repressor. They are also consistent with studies that have shown CAD risk alleles at the Chr9p21 locus having an inverse effect on *ANRIL* and *CDKN2B* expression in peripheral blood cells (Cunnington et al. 2010).

My observation of unaffected *CDKN2A* expression in the *ANRIL* knockdown HUVEC may firstly be due to the moderate level of knockdown (60%) and/or secondly due to the complex transcriptional network that exists at the locus (Fig 4.3). Certainly, co-clustering of *ANRIL* and *p14/ARF* expression has been observed in a range of human tissues and also tumours, suggesting their coordinated transcription (Pasmant et al. 2007); in addition transcription factor binding sites have been mapped to the CpG island overlapping the *ANRIL-p14/ARF* promoters also supporting coordinated expression of these genes (Rodriguez et al. 2010). However it may be that I did not observe any effects of *ANRIL* knockdown upon *CDKN2A* expression in HUVECs due to a tissue specific effect (in that an effect on *CDKN2A* expression was evident in human islets).

My observation of down-regulation of *CDKN2A* and *CDKN2B* subsequent to *ANRIL* knockdown in human islets was unexpected in light of the prior HUVECs results and also did not fit with my initial hypothesis that T2D causal variants on chromosome 9 reduce *ANRIL* expression which therefore leads to reduced islet mass via an increase in *CDKN2A* and/or *CDKN2B* expression. This may well be due to the technical limitations to the methodologies I employed for studying ANRIL and its potential regulatory role in human islets (discussed below). However, the results are consistent with the observations of Visel et al who demonstrated reduced expression of *Cdkn2a* and *Cdkn2b* in cardiac tissue from the knockout mouse which had a deleted region on chromosome 4, orthologous to the 58Kb CAD risk interval on chromosome 9 (Visel et al. 2010). One could speculate that the T2D causal variants lead to an *increase* in *ANRIL* expression in human islets and therefore also an increase *CDKN2A* and/or *CDKN2B* expression leading to reduced islet mass as the T2D risk alleles may not necessarily lead to a reduction in *ANRIL* expression. Alternatively *CDKN2A* and/or *CDKN2B* may have an as yet undefined role in metabolic pathways and the T2D link may not actually be via a proliferative effect.

It is difficult to unify my observations in different cell types with one overarching mechanism and mode of regulation of *ANRIL* and its neighbouring genes. As discussed earlier, there are also conflicts in the literature with regard to *ANRIL* expression and the effects upon *CDKN2A* and *CDKN2B.* A plausible explanation for this and the differences in gene regulation subsequent to *ANRIL* knockdown both in terms of which genes are regulated (*CDKN2B* in HUVEC; *CDKN2A* and *CDKN2B* in islets) and also direction of effect (*CDKN2B* up-regulation in HUVEC; *CDKN2A* and *CDKN2B* down-regulation in islets) could be the fact that several different *ANRIL* transcripts have been reported with tissue specific expression (Folkersen et al. 2009; Jarinova et al. 2009; Burd et al. 2010; Holdt et al. 2010). The different *ANRIL* isoforms may have very distinct roles: a 'short' and 'long' transcript are described with studies showing that expression of the long transcript of *ANRIL* are

decreased and the short variants increased, in individuals homozygous for the 9p21 CAD high-risk allele compared with those homozygous for the low-risk allele (Jarinova et al. 2009; Liu et al. 2009; Motterle et al. 2012). A correlation has also been observed between expression of the long transcript of *ANRIL* and *CDKN2A* and *CDKN2B* expression (Folkersen et al. 2009; Jarinova et al. 2009; Cunnington et al. 2010; Holdt et al. 2010). The hypothesis proposed for this is that the Chr9p21 CAD risk allele reduces expression of the long transcript but increases expression of the short transcripts and that the short transcripts down-regulate *CDKN2A* and *CDKN2B* expression (Jarinova et al. 2009). Circular isoforms of *ANRIL* have also been described and their significance with regard to gene regulation have yet to be elucidated (Burd et al. 2010).

In summary, I firstly acknowledge the difficulties in reaching clear conclusions from the studies presented here, as there were significant limitations to the methodology which will be expanded upon in the following section. However the results are consistent with the existing literature in this field and show some support for a regulatory role of *ANRIL* on *CDKN2A* and *CDKN2B* expression. This regulation may well be tissue- and transcript-specific: my data apply to transcripts that include exon 5: however there may be alternatively spliced transcripts not including exon 5 that have a different regulatory role in human islets. Tissue specific regulation at the Chr9p21 region may underlie the fact that the T2D association signals are independent of the CAD association signals. Whilst CAD-risk SNPs in the region may have an effect in for example endothelial cells and impact upon CAD risk, they may have no regulatory effect in pancreatic beta-cells. Similarly the T2D Chr9p21 risk SNPs could be affecting the transcriptional regulation of genes at this locus in the pancreatic beta-cells, whilst having no (or a different) effect in tissues relevant to CAD.

### 4.4.3 Methodological challenges limit interpretation of these studies

There are several aspects to the methodology of this work that may have contributed to inconsistencies in results or challenges in reaching clear conclusions regarding regulation at the Chr9p21 locus and the role of *ANRIL* in human islets.

Firstly, the methodology employed for gene knockdown, namely adenoviral siRNA mediated gene knockdown in whole/intact human islets resulted in only a modest (40%) gene knockdown. Although, at this level, changes in *CDKN2A* and *CDKN2B* expression were observed, ideally a 60-80% knockdown would have been achieved. It is notoriously difficult to attain reliably good gene knockdown in intact human islets (without impairing islet function) given the difficulties in penetrating the cells at the islet core (Leibowitz et al. 1999; Mahato et al. 2003). At the time of planning these experiments arguably the best method for delivering siRNAs into intact human islets was via viral vectors (Rehman et al. 2005) of which I attempted the adenoviral method. Unfortunately this proved to be toxic to the cells at higher concentrations and longer time periods that may otherwise have resulted in higher gene knockdown. A major limitation of this toxicity was the effect it had on the expression of housekeeping genes, as their relatively constant expression (between experimental conditions) was a requirement for accurate interpretation of QRT-PCR results. It was technically challenging to maintain reasonably uniform expression of HKGs between siRNA treatments i.e. HKGs unaffected by scramble or target siRNA containing adenovirus. Where the HKG mRNA expression was significantly disrupted, data were not used, as this skewed relative expression of the target genes (*ANRIL, CDKN2A, CDKN2B*).

Alternative methods of delivery of siRNAs to whole human islets have since been used by other groups with some success, including transfection using accutase distention and microporation which achieved around a 70% gene knockdown with apparently little effect on islet function (Lefebvre et al. 2010). Traditional lipid mediated transfection methods have had limited success previously (40-50%

transfection efficiency), but encountered the same difficulties as viral methods in penetrating the islet core (Lakey et al. 2001; Mahato et al. 2003). However, more recently lipid-based transfection was used with some success (around 50% gene knockdown) and with interpretable effects of gene knockdown upon islet function (Rosengren et al. 2010). Hence, although remaining laboratory time was limited, I attempted lipid based transfection and achieved one set of results with modest *ANRIL* knockdown (35% Fig 4.16). If time had not been limiting this alternative strategy for *ANRIL* knockdown in whole human islets would have been pursued.

As attaining gene knockdown without toxicity, using adenovirus, was challenging in whole human islets, this methodology was also attempted in single cells i.e. dispersed human islets (Fig 4.15) with the rationale that some data might be gained on gene regulation. However the adenovirus was toxic to the cells at concentrations required to achieve any gene knockdown. This strategy was not pursued given one of the major experimental aims was to assess insulin secretion of the intact human islets, post incubation with the adenovirus, to investigate what effect *ANRIL* knockdown had upon glucose stimulated insulin secretion.

A final alternative strategy considered, for investigating gene regulation by *ANRIL* in cells relevant to glucose homeostasis was investigating the effects of gene knockdown in a relevant cell line. HUVEC was a very relevant cell line to use in the context of cardiovascular disease, but at the time of planning these experiments there was no such readily available human beta-cell line. As there is no convincing evidence to date of the existence of a homologue of the *ANRIL* ncRNA in rodents it was not relevant to test the siRNAs in either the MIN6 cell line [an insulinoma cell line derived from a transgenic mouse (Ishihara et al. 1993)] or INS1 cells [(a rat insulinoma derived cell line (Asfari et al. 1992)] which are often used in metabolic experiments.

Subsequent to my experiments a human beta-cell line has become available (Ravassard et al. 2011) and it will be interesting to assess the effects of *ANRIL* knockdown in such a cell line which theoretically would have fewer of the technical challenges faced when performing similar work in human islets. A cell line provides potential for higher experimental replicates, speedier data collection and greater consistency of results given that cell lines are less vulnerable to the variability experienced with experiments in islets which are isolated with differing donor characteristics impacting upon islet function. The experiments in human islets are also significantly limited by donor availability and quantity, purity and viability of islets isolated. However, despite all these challenges, the primary aim of this project had been to investigate the effects of *ANRIL* knockdown in intact human islets in order to use a model approximating the in vivo physiology and cell architecture as far as possible.

The complexities of transcription at the Chr9p21 locus make it interesting in terms of potential variability in regulation in different tissues but consequently it does provide challenges in terms of defining the transcripts of the genes studied at this locus in different cell types. For instance although exon 5 *ANRIL* was selected as the siRNA target due to its expression in many defined transcripts to date, there are multiple splice variants and it is possible that some *ANRIL* transcripts would not be targeted by the siRNA used. Given the limitation on islet numbers available this did restrict the quantity of RNA extracted after adenoviral incubation as well as limit replicates (a minimum of 50 islets was required in order to reliably extract the minimum amount of RNA required for the QRT-PCR experiments). Had I been able to extract more RNA in experiments where the HKG expression and islet viability had been unaffected by adenovirus, it would have been desirable to perform microarrays on RNA extracted from these knockdown cells to assess the impact of *ANRIL* knockdown on genes involved in other pathways (for example those involved in proliferation) in human islets.

Finally although QRT-PCR was the method used here to assess *ANRIL* expression given its status as a non-coding RNA, it would have been desirable to assess its impact upon the protein expression of *CDKN2A* and *CDKN2B*, i.e. to have used western blotting methods to assess p15 and p16 expression in *ANRIL* knockdown cells, to see if this confirmed reduced expression in the *ANRIL* knockdown cells. There are no data on the half-life of these targets in human islets and one could argue that to draw true conclusions regarding the impact of *ANRIL* knockdown in human islets one should assess not only the mRNA but also the protein expression of the genes of interest.

### 4.4.4 Conclusions

In summary with regard to T2D risk and the *CDKN2A, CDKN2B* and *ANRIL* locus, it is known firstly, that the Chr9p21 T2D risk SNPs are near to these genes and that the risk alleles act via a loss of beta-cell function (Grarup et al. 2007; Saxena et al. 2007; Scott et al. 2007; Zeggini et al. 2007). Secondly, rodent data demonstrate the importance of *Cdkn2a* and *Cdkn2b* in islet mass and diabetes pathogenesis (Moritani et al. 2005; Krishnamurthy et al. 2006) and also that deleting the entire region with 50% homology to the Chr9p21 region results in down-regulation of *Cdkn2a* and *Cdkn2b* (Visel et al. 2010). My cellular studies implicate *CDKN2A* and perhaps *CDKN2B* more so in the mechanism of association between the Chr9p21 region and T2D given that a relationship between *ANRIL* (transcribed from the region of maximal T2D association) expression and *CDKN2B* expression existed in HUVEC, and both *CDKN2A* and *CDKN2B* expression with *ANRIL* in human islets. These data are consistent with the theory of tissue-specific and species-specific differences in the regulation of this region, with one, both or neither of *CDKN2A* and *CDKN2B* implicated depending on the tissue, disease or species examined.

# Chapter 5

Studies of glucose homeostasis in individuals with inactivating *CDKN2A* mutations

# Chapter 5

## 5.1 Introduction

### 5.1.1 GWAS reveal enrichment for cell-cycle genes amongst susceptibility loci for T2D

An unforeseen link to cell proliferation has been observed amongst novel T2D susceptibility loci (Voight et al. 2010; Morris et al. 2012), as discussed in previous chapters (see **Chapter 1 Section 1.4** and **Chapter 4 Section 4.2**). Pathway and protein-protein interaction analyses performed in an effort to assign susceptibility loci to specific biological processes, have highlighted enrichment for genes involved in cell cycle regulation (Voight et al. 2010; Morris et al. 2012). Many of these genes encode cyclin-dependent kinase (CDK) inhibitors (e.g. *CDKN2A* and *CDKN2B*, *CDKN1C*, *CDKN2C*) and also cyclins that activate CDKs (e.g. *CCNE2*, *CCND2* and *CCNA2*) (Morris et al. 2012).

The proposed mechanism for these cell cycle genes influencing diabetes pathogenesis is via their proliferative effect upon islet mass. Many of the CDK inhibitors implicated through T2D-associated loci regulate CDK4 or CDK6, which are known to have a role in pancreatic beta-cell proliferation (Rane et al. 1999). Rane et al demonstrated that *Cdk4* knockout mice developed diabetes due to lower circulating insulin levels and showed that this was due to a reduction in the number of pancreatic beta-cells in these mice (Rane et al. 1999): histological analysis of the pancreatic tissue showed islet hypoplasia and when component cell-types were analysed it was beta-cells that were specifically affected. More recently Fiaschi-Taesch and colleagues demonstrated the importance of both CDK4, CDK6 and cyclin D3 in beta-cell replication both in vitro and vivo and that whilst combinations of these molecules were able to drive human beta-cell replication, CDK6 alone was enough to induce human beta-cell replication, and enhance beta-cell transplantation in vivo: this

conferred superior glucose tolerance and protected against streptozotocin-induced diabetes in transplanted mice (Fiaschi-Taesch et al. 2010).

### 5.1.2 T2D-associated loci have shown appreciable links to glucose physiology, specifically beta-cell function

The fact that many of the implicated cell cycle genes in diabetes pathogenesis are expressed in pancreatic islets (as well as other tissues) (Zeggini et al. 2007) supports a role for them in mediating an islet-beta-cell mass/insulin secretory effect. In addition the observation that the T2D-association effects are mediated primarily via beta-cell dysfunction (rather than insulin resistance) (Florez 2008; Voight et al. 2010) also highlights the potential contribution of beta-cell mass regulation to maintenance of normal glucose homeostasis in the long-term.

As alluded to in Chapters 1 and 4, one of the major challenges in deciphering the underlying mechanisms by which T2D-associated loci exert their effect, is that the majority occur in non-coding regions, sometimes >1000kb from coding genes. One approach to understanding what mechanism underlies a T2D-associated loci, is to perform molecular functional studies of the most plausible biological candidates in the region (e.g. molecular studies performed in Chapter 4). Another approach is to demonstrate the physiological effects of T2D-associated loci in individuals harbouring T2D risk alleles. If one can demonstrate an effect on 'whole body' glucose physiology, this is a powerful indicator of how causal variants influence T2D disease risk.

Despite modest odds ratios, researchers have managed to demonstrate appreciable effects of T2D risk alleles on glucose homeostasis. Steinthorsdottir et al demonstrated defects in insulin secretion in risk allele carriers at *CDKAL1*: insulin secretory response at time 30 minutes of a 75g OGTT was measured and it was found that homozygous risk allele carriers had a 20% lower insulin secretory response than heterozygotes or non-carriers (Steinthorsdottir et al. 2007). Pascoe et al also

demonstrated that *CDKAL1* variants associated with reduced beta-cell function as measured by the 30 min insulin response in OGTTs (Pascoe et al. 2007). Finally, Palmer and colleagues showed that the diabetes risk allele at *CDKAL1* was associated with reduced acute insulin response during an IVGTT (Palmer et al. 2008). Collectively, these studies linked the T2D-associated *variants* at the *CDKAL1* locus (not the gene itself) to altered secretion: rodent studies have shown similar effects to be mediated by *Cdkal1* specifically, as *Cdkal1* knockout mice showed glucose intolerance and decreased insulin secretion (Wei et al. 2011). Other T2D-associated loci have also been shown to influence insulin secretion/beta-cell function including those at *TCF7L2*, *SLC30A8*, *HHEX* and *IGF2BP2* (Pascoe et al. 2007; Staiger et al. 2007; Steinthorsdottir et al. 2007; Palmer et al. 2008).

Thus it has been possible to show effects of T2D risk alleles on glucose homeostasis using basic physiological tests, despite small effect sizes and the presumed subtle differences that may exist between carriers and non-carriers. However, it would be informative to demonstrate a physiological effect of mutations in the genes associated with these loci to confirm which genes mediate the effects displayed by the T2D-risk variants.

### 5.1.3 T2D association loci at Chr9p21 map close to tumour suppressor genes *CDKN2A* and *CDKN2B*

The evidence for a cell-cycle link is strongest at Chr9p21 (as discussed in **Chapter 4 Section 4.2**) where T2D associated loci map in the proximity of the tumour suppressor genes, *CDKN2A* and *CDKN2B* (see **Chapter 4 Figure 4.1**) (Saxena et al. 2007; Scott et al. 2007; Zeggini et al. 2007; Morris et al. 2012). *CDKN2A* and *CDKN2B* encode the cyclin dependent kinase inhibitors p16 (and p14ARF) and p15 respectively. These suppress cell cycling by inhibiting the actions of cyclin dependent kinase 4 and 6 (CDK4 and CDK6) which would normally go on to activate the retinoblastoma (RB1) pathway. RB1 activates the E2F transcription factors leading to S-phase gene transcription and also *CDKN2A* in its alternate reading frame, p14ARF. The *CDKN2A* gene locus encodes two separate but related

proteins involved in cell cycle regulation: p16 is encoded by exons 1α, 2 and 3 whilst p14ARF is encoded by exons 1β, 2 and 3 (Figure 5.1, also shown in **Chapter 4**). Both have distinct but complementary roles as tumour suppressors (Figure 5.1) and the relatively unusual arrangement of three tumour suppressor genes in such a small region is well conserved between mice (orthologous region on chromosome 4) and humans (Zhang et al. 1998).

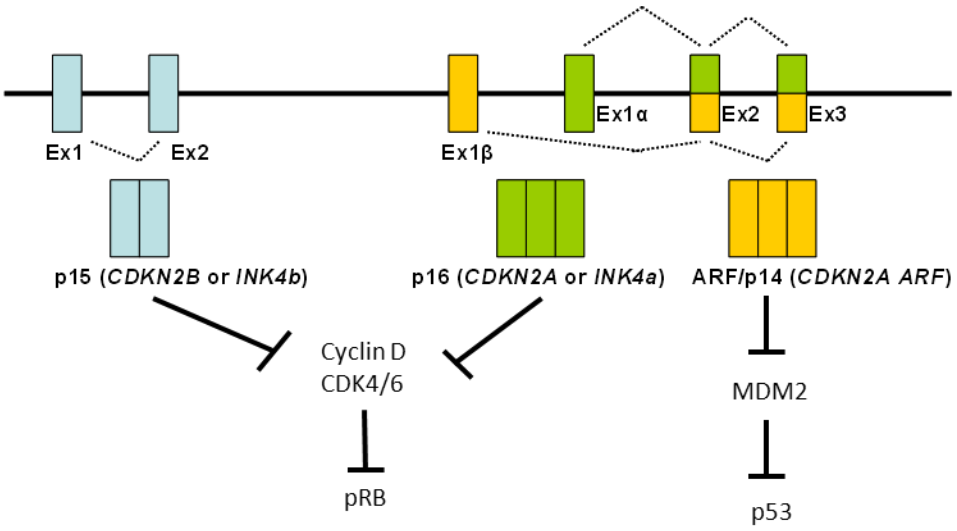



**Figure 5.1** Adapted from Kim and Sharpless (Kim and Sharpless 2006) showing the small 35kb locus (also known as the INK 4 locus) which encodes three genes *CDKN2A* (also known as *INK4a*), *CDKN2A*$^{ARF}$ and *CDKN2B* (also known as *INK4b*). *CDKN2B* has its own open reading frame which is separate from that of *CDKN2A* and *CDKN2A*$^{ARF}$. *CDKN2A* and *CDKN2A*$^{ARF}$ have different first exons which are spliced to a common second and third exon. In addition the proteins, p16 and p14ARF, are encoded in alternate reading frames which results in two entirely different proteins that do not share any amino acid homology.

Of the three tumour suppressors encoded at Chr9p21 (p16, p14ARF and p15), somatic point mutations or small deletions affecting p16 alone are the most reported in human cancers (Forbes et al. 2006). Similarly many distinct germline *CDKN2A* mutations affecting p16 but sparing p14ARF and p15 have been described (Greenblatt et al. 2003). By contrast, selective somatic or germline inactivation of p14ARF alone has only been reported in a small number of human cancers (Esteller et al. 2001; Hewitt et al. 2002; Kim and Sharpless 2006). Likewise, specific genetic lesions affecting p15 which do not also inactivate p16 and 14ARF are not well described (Kim and Sharpless 2006): specific epigenetic silencing of *CDKN2B* has only been demonstrated in rare glial tumours and a minority of

haematological neoplasms including leukaemia and myelodysplasia (Esteller et al. 2001). However due to their overlapping biological function, co-deletion affecting both p15 and p16, may be more oncogenic and a few malignancies such as T cell acute lymphoblastic leukaemia show much higher frequency of both tumour suppressors affected than p16 alone (Kim and Sharpless 2006). In addition rodent studies comparing *Cdkn2a* knockout mice to *Cdkn2a* and *Cdkn2b* knockout mice demonstrate that p15 is a crucial tumour suppressor in the absence of p16 (Krimpenfort et al. 2007).

### 5.1.4 Rodent data link *CDKN2A/CDKN2B* tumour suppressor genes to glucose homeostasis

Although much is known about *CDKN2A* and *CDKN2B* as oncogenes, their potential role in glucose homeostasis has largely come from rodent data, but also been inferred by their association with the Chr9p21 T2D susceptibility locus. Grarup et al demonstrated reduced insulin secretion on oral glucose and intravenous tolerance testing in individuals homozygous for the T2D risk allele at the Chr9p21 locus (Grarup et al. 2007) but it is not known if the T2D-risk variants at this locus are acting via *CDKN2A* and/or *CDKN2B*. *Cdk4* knockout mice have normal islet mass at birth but as adults develop reduced proliferation, islet mass and eventually diabetes: Cdk4 is the main biochemical target of p15 and p16 (encoded by *Cdkn2a* and *Cdkn2b* respectively) (Rane et al. 1999; Kushner et al. 2005). *Cdk4R24C* mice ($Cdk4^{R24C}$ being a mutated form of Cdk4 that prevents binding to the cell cycle inhibitors p15 and p16) have increased islet mass and beta-cell area (Wolfel et al. 1995; Marzo et al. 2004). Murine studies manipulating expression of *Cdkn2a* and *Cdkn2b* add further evidence for this role in glucose metabolism: Krishnamurthy et al generated transgenic mice which over expressed *Cdkn2a* and showed they developed islet hypoplasia and diabetes (Krishnamurthy et al. 2006); Moritani et al generated transgenic mice which express a constitutively active form of TGF-β1 which showed increased *Cdkn2b* expression, islet hypoplasia and diabetes (Moritani et al. 2005).

There have been no equivalent metabolic studies in humans bearing loss-of-function *CDKN2A* and/or *CDKN2B* mutations as previously these genes have been mainly recognised for their role in cancer.

### 5.1.5 Germline mutations in *CDKN2A* are a cause of familial melanoma

Germline mutations in *CDKN2A* are a cause of familial melanoma, also known as familial atypical multiple mole melanoma syndrome (FAMMM) (Cannon-Albright et al. 1992; Goldstein et al. 1995; Ranade et al. 1995). Mutations are autosomally dominantly inherited and show incomplete inheritance (Begg et al. 2005; Berwick et al. 2006; Goldstein et al. 2007). The familial melanoma syndrome is characterised by multiple melanocytic naevi (usually >50) and a family history of melanoma (Bergman et al. 1990; Lynch et al. 2002). Some *CDKN2A* melanoma-prone families also have an increased risk of pancreatic cancer (Goldstein et al. 1995). Diagnostic criteria are described in Table 5.1 with all 3 criteria required for diagnosis of FAMMM.

| |
|---|
| **1. Malignant melanoma in one or more first- or second-degree relatives** |
| **2. High total body naevi count (often >50) including some of which are clinically atypical** (asymmetric, raised, colour variegation present, of variable sizes) |
| **3. Naevi with certain histologic features on microscopy*** |
| * architectural disorder with asymmetry, subepidermal fibroplasia, and lentiginous melanocytic hyperplasia with spindle or epithelioid melanocytes gathering in nests of variable size and fusing with adjacent rete ridges to form bridges; variable dermal lymphocyte infiltration and the “shouldering" phenomenon wherein intraepidermal melanocytes extend alone or in groups beyond the main dermal component may also be present |

**Table 5.1** Diagnostic criteria for Familial Atypical Multiple Mole Melanoma syndrome. National Cancer Institute 2008; NIH Consensus 1992

The exact prevalence of familial melanoma or FAMMM is unknown given its variable phenotype and incomplete penetrance, but it is thought that around 5-12% of melanoma is hereditary (Parker et al. 2003) and approximately 10-40% of familial cases are due to *CDKN2A* mutations (Goldstein et al.

2006). The likelihood of detecting a *CDKN2A* mutation increases with number of melanomas in the family, multiple primary melanomas in one individual and early age of onset; patients with a family history of melanoma in 3 or more relatives may have as high as a 50% risk of carrying a *CDKN2A* mutation (FitzGerald et al. 1996; Bishop et al. 2007).

*CDKN2A* mutation carriers have a high risk of developing melanoma: by age 80 mutation carriers living in Europe have approximately 58% risk of developing melanoma, in the USA approximately 76% and in Australia approximately 91% (Bishop et al. 2002). It has been suggested that selection criteria for genetic testing should include 3 patients with melanoma in a family, age <45 at diagnosis in one member and one member with multiple primary melanoma and/or pancreatic cancer (Bishop et al. 2007; Newton Bishop and Gruis 2007; Leachman et al. 2009).

### 5.1.6 Patients with *CDKN2A* haploinsufficiency provide opportunity to study glucose homeostasis in human 'knockout' model

Given the working hypothesis for the T2D-associated loci at Chr9p21 is via *CDKN2A* and/or *CDKN2B* function, it would be very informative to demonstrate their potential role in glucose homeostasis in humans. Subjects with *CDKN2A* mutations provide a unique opportunity to study the role of p16 and p14ARF in insulin secretion and beta-cell function, the hypothesis being that loss-of-function *CDKN2A* mutations would lead to increased beta-cell proliferation and higher functional beta-cell mass.

My aims were to recruit individuals with familial melanoma due to *CDKN2A* mutations and study their glucose homeostasis by performing a series of investigations and analyses focussed upon defining their beta-cell function and comparing these to non-mutation carrier control subjects.

## 5.2 Methods

### 5.2.1 Subjects studied

Subjects with familial melanoma due to *CDKN2A* mutations and first degree familial controls were recruited from around the UK, through a collaboration with Professor Tim Bishop and Professor Julia Newton-Bishop (Leeds Institute of Molecular Medicine) who have access to a large number of pedigrees of familial melanoma patients, and are specialists in the field of melanoma genetics. All interested subjects who fulfilled inclusion criteria were enrolled into the study. Inclusion criteria for the study were:-

- Strong family history of melanoma – 3 or more cases of melanoma within a pedigree with at least one case presenting at age <45yrs – these criteria (agreed with Professors Tim Bishop and Julia Newton-Bishop) increased chance of recruiting familial melanoma cases due to *CDKN2A* mutations as researchers were blind to mutation status until after biochemical investigations were complete (see explanation below).
- Subject should have been cancer free for at least 2 years prior to the study
- Subject should not be taking any medications that may interfere with glucose tolerance e.g. corticosteroids
- Age of subject should be 18-80 years

Current Ethics in place for the Familial melanoma cohort in Leeds precludes disclosure of mutation status to subjects (due to incomplete penetrance of *CDKN2A* mutations). Therefore I was not able to recruit by genotype to this study but agreed the above criteria with Professors Tim Bishop and Julia-Newton Bishop to ensure a high chance of achieving recruitment of desired numbers of *CDKN2A* mutation carriers. One of the advantages of this method of recruitment was that subjects

subsequently found to be non-mutation carriers could be utilised as familial control subjects for the study as discussed below.

As mutation status was disclosed to myself after subjects were recruited for biochemical investigations, mutation-negative subjects were used as familial controls. As far as possible control subjects were matched (post hoc) for age, gender and BMI to the mutation carriers studied. Additional control subjects were recruited as required from the Oxford Biobank.

Appropriate approval was sought and gained from the Oxfordshire Local Research Ethics Committee REC B, and all subjects gave informed consent.

Although there was no available estimate of the likely effect size, initial power calculations were performed based on preliminary data available on similar studies performed in *PTEN* mutation carriers (completed studies reported in **Chapter 3**). I aimed to recruit 25 cases and 25 controls in the first instance. This would provide 80% power (for an alpha of 0.05) to detect a between group difference of 0.8 SD in any of the traits of interest.

### 5.2.2 Clinical assessment

A full history and examination was taken, noting in particular the timing and treatment of any melanoma or other cancers in the past medical history of the participant. Subjects were asked specifically about a personal or family history of diabetes. A detailed family history of each subject was taken, noting all other family members with a diagnosis of the syndrome.

Basic anthropometric measures were taken: two systolic and diastolic blood pressures taken at least ten minutes apart; height (metres); weight (kilogrammes); waist circumference and hip circumference. BMI was calculated according to BMI = weight (kg)/height ($m^2$).

### 5.2.3 Oral glucose tolerance test and measures of beta-cell function/insulin secretion

All subjects underwent a standard 75g OGTT (described in **Chapter 2 section 2.2**). This was used to assess glucose tolerance (using fasting and 2 hour glucose levels) and further indices were derived to measure beta-cell function and insulin sensitivity. Indices used focussed upon assessment of beta-cell function as it was the insulin secretory parameter that was specifically being investigated in these subjects (Table 5.2). Two insulin sensitivity measures were included (HOMA IR and BIGTT-$S_I$) to assess any significant differences between the groups as insulin sensitivity will affect an individual's beta-cell function (Table 5.2) (Kahn et al. 1993).

As discussed in Chapter 3 section 3.2.3, the OGTT is not a 'gold-standard' test for measuring beta-cell function/insulin secretion, but was selected as a simple dynamic test that could be performed reproducibly in all study participants and in people's homes should they be unable to travel to Oxford. As the test stimulates insulin secretion and glucose uptake, it does give reasonably well-validated estimates of beta-cell function and insulin sensitivity (Stumvoll et al. 2000; Pacini and Mari 2003). Additional measures of beta-cell function and insulin sensitivity, the BIGTT-$AIR_{0\text{-}30\text{-}120}$ and BIGTT-$S_{I\_0\text{-}30\text{-}120}$ respectively, were also used to maximise the OGTT data, as these measures have been well validated against the IVGTT, the gold standard test of beta-cell function (Table 5.2) (Hansen et al. 2007).

Measures of insulin sensitivity and beta-cell function derived from the OGTT were calculated according to the formulae described in Table 5.2. HOMA IR and HOMA B were calculated using the computer model available via the University of Oxford Diabetes and Trials Unit website (http://www.dtu.ox.ac.uk/homacalculator/download.php.).

**Table 5.2** Measures of beta-cell function and insulin sensitivity derived from the OGTT for familial melanoma subjects and controls

| Name of measure/index | Physiological parameter measured | OGTT time-point (min) and assay used | Formula or mathematical model used | Ref |
|---|---|---|---|---|
| HOMA B | Beta-cell function | Fasting (time 0) insulin and glucose | Computer model<br>Approximate equation: (20 X insulin)/(glucose -3.5) | (Matthews et al. 1985) |
| $AUC_{insulin}$ | Beta-cell function | Time 0,15,30,60,90 and 120 insulin | Calculate AUC using trapezoidal rule | (Pacini and Mari 2003) |
| Insulinogenic index | Beta-cell function | Time 0 and time 30 insulin and glucose | $(insulin_{30} – insulin_0)/(glucose_{30} - glucose_0)$ | (Phillips et al. 1994) |
| Disposition index | Beta-cell function | Time 0 and time 30 insulin and glucose | Insulinogenic index/HOMA IR | (Phillips et al. 1994) |
| $BIGTT\text{-}AIR_{0\text{-}30\text{-}120}$ | Beta-cell function | Time 0,30 and 120 insulin and glucose | *exp[8.20 + (0.00178 X $insulin_0$) + (0.00168 X $insulin_{30}$) - (0.000383 X $insulin_{120}$) - (0.314 X $glucose_0$) - (0.109 X $glucose_{30}$) + (0.0781 X $glucose_{120}$) + (0.180 X gender (where male=0 and female=1)) - (0.032 X BMI)] | (Hansen et al. 2007) |
| $BIGTT\text{-}S_{I\_0\text{-}30\text{-}120}$ | Insulin sensitivity | Time 0,30 and 120 insulin and glucose | exp[4.90 - (0.00402 X $insulin_0$) - (0.000556 X $insulin_{30}$) - (0.00127 X $insulin_{120}$) - (0.152 X $glucose_0$) - (0.00871 X $glucose_{30}$) - (0.0373 X $glucose_{120}$) - (0.145 X gender (where male=0 and female=1)) - (0.0376 X BMI)] | (Hansen et al. 2007) |
| HOMA IR | Insulin sensitivity | Fasting (time 0) insulin and glucose | Computer model<br>Approximate equation: (glucose X insulin)/22.5 | (Matthews et al. 1985) |

*exp denotes exponential function

### 5.2.4 Intravenous glucose tolerance test (IVGTT)

An accurate and physiological test was required to assess beta-cell function and although the hyperglycaemic clamp is recognised as the 'gold standard' test for assessment of beta-cell function (DeFronzo et al. 1979), it only measures insulin-stimulated glucose uptake at insulin levels in the upper physiological range and not in the basal condition which is physiologically important. In addition it would not be possible to perform this test outside of the hospital/specialised research unit setting, and this study necessitated a test that could be reliably performed in another centre if required (for example in order to facilitate recruitment if subjects could not travel to Oxford). Therefore the intravenous glucose tolerance test was selected as a test of beta-cell function that has been well-validated against clamp studies (Bergman et al. 1979; Bergman 1989; Pacini et al. 1998). In this test, glucose and insulin are measured at various time points after a glucose bolus injection is given intravenously, following which blood samples are collected at regular intervals over 3 hours and analysed for glucose, insulin and/or C-peptide.

An advantage of the IVGTT (over the OGTT alone) is that the frequent sampling over the first 10 minutes and the prolonged sampling (3 hours in total) give more detailed data on the 'first phase' and 'second phase' of insulin secretion (**Figure 5.2**).

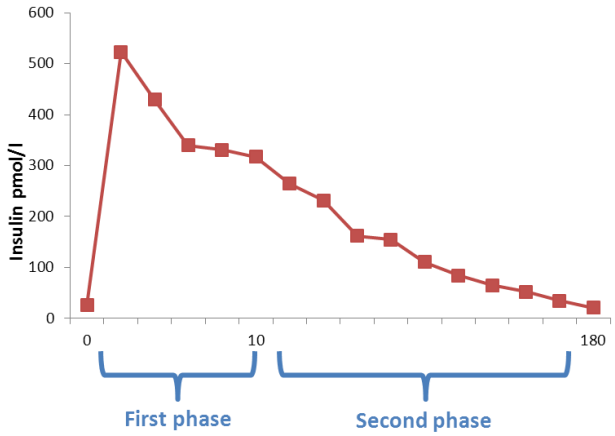



**Figure 5.2** Phases of insulin secretion in response to IV glucose bolus at time 0

It has been established for some time that secretion of insulin is biphasic (Curry et al. 1968). In response to a food stimulus/glucose load, there is a rapid and sizable release of preformed insulin from storage granules within the beta cell. This is the first phase of insulin secretion and stimulates peripheral uptake of the nutrient load, suppresses hepatic gluconeogenesis, and limits postprandial glucose excursion. First-phase insulin secretion is initiated within 2 minutes of food ingestion and continues for 10 to 15 minutes. The second phase of prandial insulin secretion follows, and is sustained until normoglycemia is restored. Although the OGTT and oral mixed meal tests are more physiological tests as they also stimulate the gastrointestinal system, the IVGTT is a more standardized beta-cell stimulus that has by-passed the 'incretin effect'(Elrick et al. 1964; Kazakos 2011), resulting in an insulin secretory response which can be compared between subjects. The IVGTT allows a direct stimulation of the beta-cell without the confounding effects of the gastrointestinal factors of the oral test, provides high dynamics of both glucose and insulin concentration, and the dose of glucose given is known, as well as the appearance in the systemic circulation (Pacini et al. 1998). These factors allow for accurate and precise extrapolation of the metabolic parameters derived from the test. As both OGTT and IVGTT data were to be collected, these approaches were deemed complementary and optimal for assessing insulin secretion between groups.

Subjects who had undergone an OGTT in the study were invited to have the IVGTT. A subset of subjects (8) who had attended for OGTT consented to have the IVGTT. Control subjects, matched for age, gender, BMI and activity were recruited from the Oxford Biobank. Subjects attending for IVGTT were asked to undergo an overnight fast for at least 12 hours and had taken only water on the morning of the test. Subjects were asked to lie supine for at least 15 minutes prior to the test. A 20 gauge cannula was placed in each antecubital vein: one was used for administration of 50% dextrose and the other was used for blood sampling. Baseline fasting blood samples were taken through the

sampling cannula, then a dose of 50% dextrose (dose calculated based on weight 0.5mg/kg) was given through the other cannula over 3 minutes and flushed well with 20ml saline. Further blood samples were then taken at time 2, 4, 6, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150 and 180 minutes (after the end of the saline flush) from the sampling cannula. These samples were immediately stored on ice and subsequently kept at -80˚C before batch analysis for insulin, glucose and C-peptide.

### 5.2.5 Biochemical assays

Samples were prepared, stored and glucose and insulin assays were performed as described in **Chapter 2 Methods section 2.3.** C-peptide was measured by Tim James (John Radcliffe Hospital, Oxford) using a standard chemiluminescence immunoassay with a Siemens ADVIA Centaur analyser (Siemens).

### 5.2.6 Minimal Model analyses and Statistical analyses

The IVGTT data were analysed through a collaboration with Dr Ian Godsland (Imperial College, London) who performed all minimal modelling analyses of the IVGTT data. He used Bayesian hierarchical analysis to identify the minimal model for all the IVGTTs in the study (Agbaje et al. 2003; Godsland et al. 2006). The basic principles of models he utilized are described below and definitions of the data output from the models used are given in **Table 5.3**. I performed all the IVGTTs and collated the biochemical data which was then sent to Ian Godsland for minimal modelling analyses. I performed all statistical analyses of the indices derived from the minimal model analysis.

**5.2.6.i Pancreatic insulin secretion measures from the IVGTT**

C-peptide data gives additional information (to insulin data alone) as a measure of pre-hepatic insulin secretion. Plasma insulin concentration is an index of pancreatic insulin secretion, but may be confounded by hepatic extraction of recently secreted insulin and peripheral insulin removal. C-peptide is secreted from the pancreas simultaneously and in equimolar quantities to insulin but is not extracted by the liver and has simpler characteristics of peripheral distribution (Horwitz et al. 1975). This has permitted assumptions about C-peptide behaviour that enable model-based estimates of true pancreatic insulin secretion to be derived: in the combined model of pancreatic insulin secretion it is assumed that newly secreted insulin and C-peptide enter single compartments of distribution after passing through the liver and that hepatic extraction of newly secreted insulin during the IVGTT is constant (Volund et al. 1987). This analysis gives an index of fractional hepatic insulin throughput (f), the plasma insulin elimination constant ($k_i$) and the plasma C-peptide elimination constant ($k_c$) (Watanabe et al. 1989) (see **Table 5.3**).

The net increment in IVGTT insulin area under the curve was calculated by myself and confirmed by Ian Godsland using the trapezium rule: net increments from 0 to 10 minutes and 10 to 180 minutes were taken as measures of first-phase and second-phase insulin secretion respectively. Similar calculations were made for IVGTT C-peptide area under the curve. In addition net pancreatic insulin secretion during the IVGTT was calculated by Ian Godsland (Imperial College, London) from the model-derived estimates of pancreatic insulin secretion rates at each IVGTT sampling point (see **Table 5.3**).

### 5.2.6.ii Insulin sensitivity measures from the IVGTT

As insulin sensitivity cannot be derived directly from the data accurately, a glucose-insulin model, the *minimal model*, is used to analyse the data (Bergman et al. 1979; Bergman et al. 1981; Swan et al. 1994) and the protocol requires a 'frequently sampled intravenous glucose tolerance test' that entails frequent sampling over the first hour to give the most precise modelling data (Bergman 1989; Pacini et al. 1998). The relatively high glucose dose used in this study (0.5g/Kg as opposed to 0.3g/Kg) gives a high rate of model identification and good correlation with insulin sensitivity derived from the 'gold standard' euglycaemic clamp technique ($r$=0.92) (Walton et al. 1992; Swan et al. 1994).

| IVGTT index | Definition of parameter described |
|---|---|
| **di** | The disposition index, equal to insulin sensitivity multiplied by the acute insulin response to glucose (i.e. the area under the IVGTT insulin profile from 0-10 minutes) - reference measure of the ability of the pancreas to maintain normal glucose levels |
| **cpepdi** | A variant of the disposition index calculated using insulin sensitivity multiplied by the area under the IVGTT C-peptide profile from 0-10 minutes |
| **secdi** | A variant of the disposition index calculated using insulin sensitivity multiplied by the area under the modelling analysis-derived IVGTT insulin secretion rate profile from 0-10 minutes |
| **iarea1** | Area under the IVGTT insulin concentrations profile from 0-10 minutes |
| **iarea2** | Area under the IVGTT insulin concentrations profile from 10-180 minutes |
| **carea1** | Area under the IVGTT C-peptide concentrations profile from 0-10 minutes |
| **carea2** | Area under the IVGTT C-peptide concentrations profile from 10-180 minutes |
| **netsec1** | Area under the IVGTT insulin secretion modelling analysis-derived IVGTT insulin secretion rate profile from 0-10 minutes |
| **netsec2** | Area under the IVGTT insulin secretion modelling analysis-derived IVGTT insulin secretion rate profile from 10-180 minutes |
| **si** | Minimal model-derived insulin sensitivity |
| **f** | IVGTT insulin secretion modelling analysis-derived fractional hepatic insulin throughput |
| **ki** | IVGTT insulin secretion modelling analysis-derived plasma insulin elimination rate |

**Table 5.3** IVGTT minimal model indices and definitions

## 5.3 Results

### 5.3.1 Subjects studied

Thirty-four subjects who fulfilled all inclusion criteria (see **section 5.2.1**) were recruited into the study. All study subjects were white European. On completion of OGTTs I was unblinded to mutation status of all subjects and found of the thirty-two subjects remaining in the study, 14 were *CDKN2A* mutation carriers (Table 5.4) and 18 were wild-type, and hence familial controls. The largest single pedigree taking part in the study is shown (Figure 5.3).

| ***CDKN2A* Mutation** | **Intron/Exon** | **Protein change** | **Protein affected: $p14^{ARF/}$, p16 or both** |
|---|---|---|---|
| IVS2-89C>T | Intronic | - | Both |
| IVS2-89C>T | Intronic | - | Both |
| 88delG | Exon 1 | - | p16 |
| 88delG | Exon 1 | - | p16 |
| 88delG | Exon 1 | - | p16 |
| IVS2-105A>G | Intronic | - | Both |
| IVS2-105A>G | Intronic | - | Both |
| IVS2-105A>G | Intronic | - | Both |
| IVS2-105A>G | Intronic | - | Both |
| c.52_57dup | Exon 1 | Thr18_Ala19dup | p16 |
| c.52_57dup | Exon 1 | Thr18_Ala19dup | p16 |
| c.159G>C | Exon 2 | Met53Ile | Both |
| c.159G>C | Exon 2 | Met53Ile | Both |
| c.159G>C | Exon 2 | Met53Ile | Both |

**Table 5.4** Mutation details of recruited *CDKN2A* mutation carriers

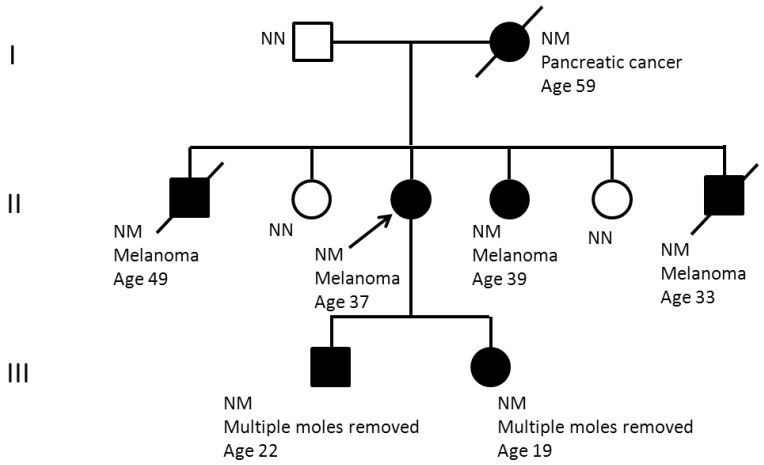



**Figure 5.3** Pedigree of family with the IVS2-105A>G *CDKN2A* mutation. The mutation creates a false GT splice donor site 105 bases 5' of exon 3 and has been demonstrated to result in aberrant splicing of the mRNA (Harland et al. 2001). Males are shown as squares, females as circles. Shaded shapes represent affected individual. Mutation status is shown as NN = no mutation, NM = heterozygous mutation carrier. Age at diagnosis of FAMMM related illness is shown by each affected subject. The proband is indicated by an arrow.

### 5.3.2 Baseline characteristics of subjects studied

OGTTs were performed in all 32 subjects. Of the 18 non-mutation carriers, 14 were selected as best 'matched' controls for the 14 mutation carriers, using age, gender, BMI and activity (see Table 5.5). Groups were reasonably well matched for age [age (yrs) 39 vs 48; p=0.19] and gender [% males 43 vs 33; p=0.58] (Table 5.5). Importantly, groups were well matched for BMI (26.6 vs 27.5Kg/$m^2$) given metabolic parameters were being assessed.

- There was no significant difference in glucose excursion after glucose challenge between the groups (Table 5.5 and Figure 5.4A).
- Two participants were subsequently excluded after their OGTT which diagnosed diabetes or impaired glucose tolerance (2h glucose 12.1 and 10.1mmol/l). Both were non-mutation carriers.
- There was no difference in fasting total cholesterol levels or triglyceride levels between *CDKN2A* mutation carriers and non-mutation carriers (Table 5.5).
- None of the mutation carriers and one of the control subjects fulfilled International Diabetes Federation (IDF) criteria for the diagnosis of metabolic syndrome (Alberti et al. 2005).

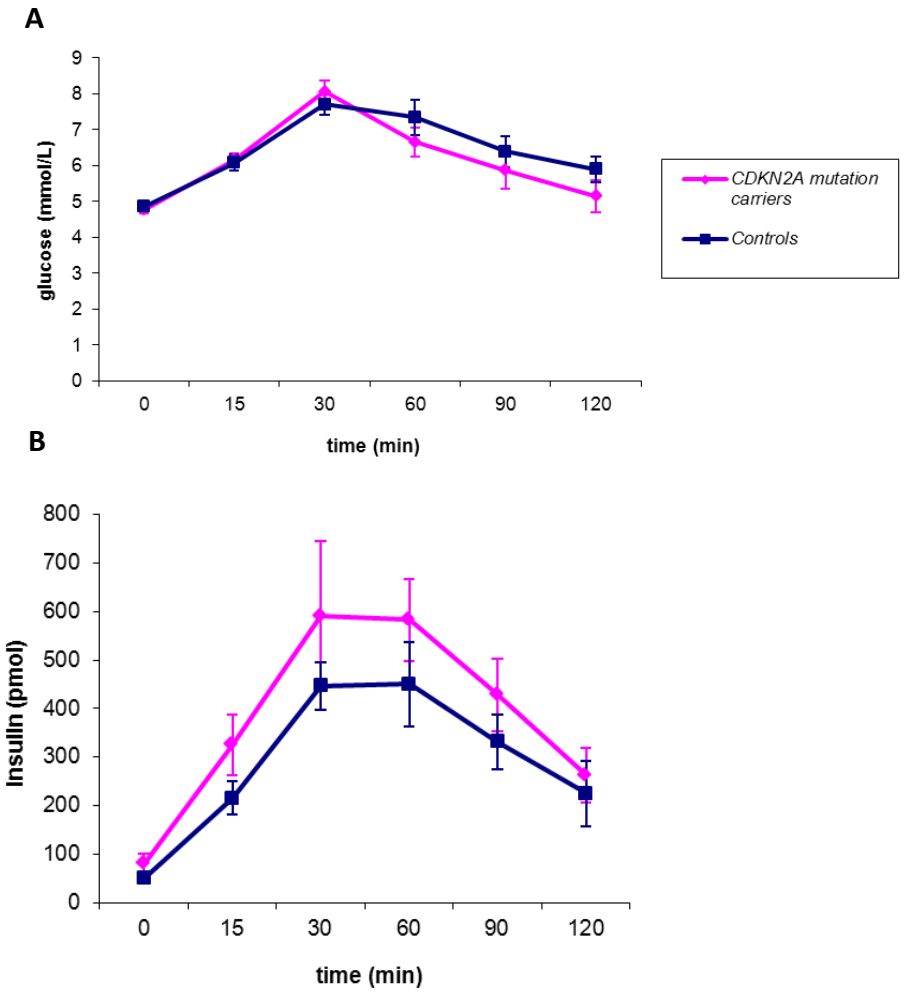
| | *CDKN2A* mutation carrier | Control | P value |
|---|---|---|---|
| **n** | 14 | 14 | NA |
| **% Male** | 43 | 33 | 0.58 |
| **Age (years)** | 39 [21, 64] | 48 [25, 79] | 0.19 |
| **BMI (Kg/m$^2$)** | 26.6 [23, 37] | 27.5 [19, 37] | 0.27 |
| **Waist circumference (cm)** | 88 [75, 110] | 89 [76, 106] | 0.56 |
| **WHR** | 0.86 [0.74, 1.01] | 0.86 [0.77, 1.01] | 0.90 |
| **BP (mmHg)**<br>Systolic<br><br>Diastolic | <br>121 [93, 138]<br><br>71 [61, 83] | <br>125 [105, 169]<br><br>75 [55, 86] | <br>0.95<br><br>0.14 |
| **Fat bioimpedance (%)** | 26.5 [15, 47] | 31.3 [10, 47] | 0.29 |
| **Fasting glucose (mmol/l)** | 4.75 [4.3, 5.3] | 4.76 [3.9, 5.8] | 0.59 |
| **Glucose at 120 min of OGTT (mmol/l)** | 5.15 [3.1, 8.9] | 5.42 [4.0, 8.5] | 0.20 |
| **Total cholesterol (mmol/l)** | 5.26 [4.7, 6.8] | 5.03 [3.6, 6.8] | 0.53 |
| **Triglycerides (mmol/l)** | 1.12 [0.63, 2.19] | 0.94 [0.54, 2.68] | 0.16 |

**Table 5.5** Baseline characteristics of subjects undergoing OGTT. Data are geometric means [range]. P value refers to Mann-Whitney testing across groups. NA=not applicable.

### 5.3.3 OGTT results give some evidence for tendency to higher beta-cell function in *CDKN2A* mutation carriers

Glucose levels were similar between *CDKN2A* mutation carriers and control subjects (Figure 5.4A). By contrast there was a tendency for higher insulin levels during the OGTT in the *CDKN2A* mutation carriers, although this was not statistically significant (Figure 5.4B). Calculated $AUC_{insulin}$ during the OGTT had a tendency to be higher in *CDKN2A* mutation carriers (Table 5.6; 1074 vs 801 p=0.29).

**Figure 5.4** Glucose and insulin levels during the OGTT (data shown as mean+/-SEM)




| | *CDKN2A* mutation carrier | Control | P value |
|---|---|---|---|
| **n** | 14 | 14 | NA |
| **HOMA B** | 157 [78, 441] | 101 [70, 177] | 0.03 |
| **Insulinogenic index (InsI)** | 221 [39, 561] | 160 [67, 251] | 0.88 |
| **Disposition Index (DI)** | 161 [15, 467] | 192 [70, 297] | 0.43 |
| **$AUC_{insulin}$** | 1074 [398, 1886] | 801 [181, 1762] | 0.29 |
| **HOMA IR** | 1.8 [0.5, 5.6] | 1.0 [0.4, 2.0] | 0.08 |
| **BIGTT_AIR** | 5006 [949, 25819] | 3136 [1398, 6562] | 0.48 |
| **BIGTT_si** | 4.79 [1.45, 12.78] | 6.49 [1.55, 17.75] | 0.27 |

**Table 5.6** OGTT measures of beta-cell function and insulin sensitivity. Data are geometric means [range]. P value refers to Mann-Whitney testing across groups. NA=not applicable.

Few of the derived values of beta-cell function, other than HOMA B, were significant at $p<0.05$, but did reveal a consistent tendency to higher levels of insulin secretion in *CDKN2A* mutation carriers (Table 5.6).

- HOMA B, a surrogate marker of beta-cell function derived from fasting insulin and glucose levels, was significantly higher in the *CDKN2A* mutation carriers [157 vs 101; p=0.03 Table 5.6)].
- Insulinogenic index (InsI), disposition index (DI) and $AUC_{insulin}$ were also taken as measures of beta-cell function. InsI and $AUC_{insulin}$ showed a tendency to be higher in *CDKN2A* mutation carriers but did not reach statistical significance [InsI 221 vs 160; p=0.88; $AUC_{insulin}$ 1074 vs 801 pmol/L; p=0.29 (Table 5.6)].
- HOMA IR tended to be higher in the mutation carriers (although not statistically so; p=0.08) and contributes to the tendency for lower DI in the mutation group as DI = InsI X 1/HOMA IR.
- BIGTT_AIR, also a measure of beta-cell function, was not significantly different between *CDKN2A* mutation carriers and control subjects. BIGTT_si, a measure of insulin sensitivity, was not significantly different between groups. However both these measures (which take BMI and gender into account in the formulae (see Table 5.2) did give the same picture as other measures i.e. BIGTT_AIR had a tendency to be higher in mutation carriers [5006 vs 3136; p=0.48 (Table 5.6)] as with other beta-cell function measures; BIGTT_si tended to be higher in control subjects [6.49 vs 4.79; p=0.27 (Table 5.6)].
- Subanalysis of OGTT results in *CDKN2A* mutation carriers, comparing groups where different isoforms affected (i.e. p16 alone vs p16 and $p14^{ARF}$) (Table 5.4) did not show any significant differences in glucose tolerance, HOMA B, InsI, DI, $AUC_{insulin}$, HOMA IR or the BIGTT indices.

### 5.3.4 Subjects studied for IVGTT

Eight *CDKN2A* mutation carriers (who had also undergone OGTT) and 8 healthy, well-matched controls (for age, gender, BMI and activity) from the Oxford Biobank were recruited for IVGTT (Table 5.7). Activity level was assessed and graded as 'low', 'medium' or 'high' depending on formal weekly exercise levels and defined according to groups (described in **section 2.2.1** of **Chapter 2**).

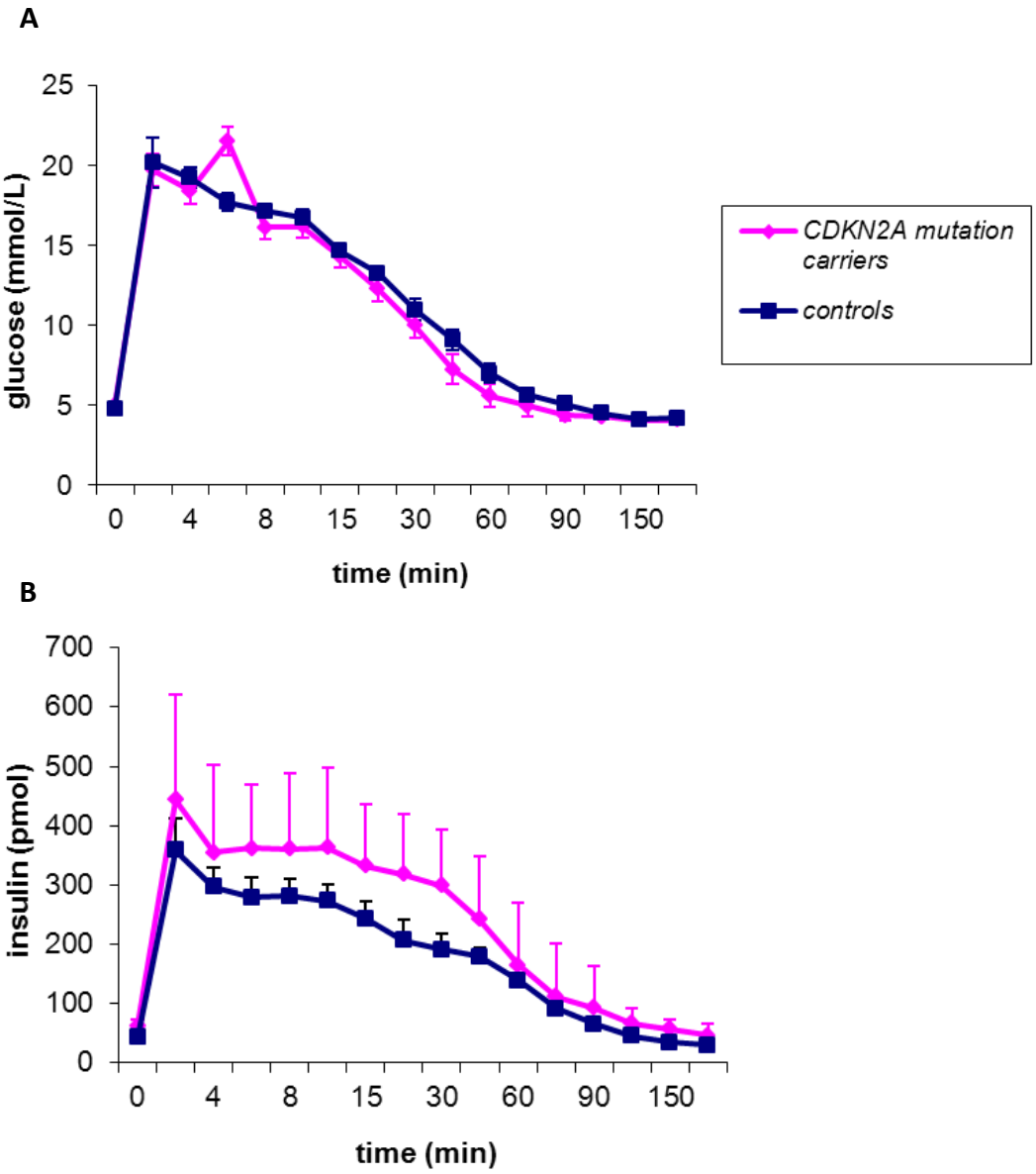
| | ***CDKN2A* mutation carrier** | **Control** | **P value** |
|---|---|---|---|
| **n** | 8 | 8 | NA |
| **% Male** | 50 | 50 | 0.50 |
| **Age (years)** | 43 [22, 64] | 43 [25, 59] | 0.96 |
| **BMI (Kg/m$^2$)** | 27.1 [23, 37] | 27.5 [23, 36] | 0.72 |
| **Activity**<br>Low<br>Medium<br>High | <br>2<br>5<br>1 | <br>2<br>5<br>1 | <br><br>NA |

**Table 5.7** Baseline characteristics of subjects studied for IVGTT. Data are geometric means [range]. P value refers to Mann-Whitney testing across groups. NA=not applicable.

### 5.3.5 IVGTT results give some evidence for tendency to higher beta-cell function in *CDKN2A* mutation carriers

Glucose excursion during the IVGTT was similar between *CDKN2A* mutation carriers and control subjects (Figure 5.5A). By contrast, although not statistically significant, there was a tendency for higher insulin levels during the IVGTT in the *CDKN2A* mutation carriers (Figure 5.5B). This was evident in the area under the curve measurements for insulin (Table 5.8), particularly iarea2, although this did not reach statistical significance [27559 vs 16687 pmol/L; p=0.14 (Table 5.8)].

**Figure 5.5** Glucose and insulin levels during the IVGTT (data points represent means +/-SEM)




There was no significant difference in minimal model-derived insulin sensitivity (Si) between *CDKN2A* mutation carriers and control subjects studied with IVGTTs. Although there were no significant differences between beta-cell function/insulin secretion measures derived from the IVGTT data, there was a consistent tendency for all beta-cell function measures to be higher in the mutation

carriers, with the most appreciable difference in iarea2 (measuring the second phase of insulin secretion during the IVGTT from 10-180 minutes).

| | *CDKN2A* mutation carrier | Control | P value |
|---|---|---|---|
| **n** | 8 | 8 | NA |
| **di**<br>disposition index | 2232 [1138, 2870] | 1760 [767, 3401] | 0.46 |
| **cpepdi**<br>disposition index using C-peptide profile | 8.0 [3.6, 12.6] | 7.0 [4.0, 13.7] | 0.75 |
| **secdi**<br>disposition index using modelling analysis-derived insulin secretion rate | 0.3 [0.1, 0.7] | 0.3 [0.2, 0.9] | 0.67 |
| **iarea1**<br>$AUC_{insulin}$ 0-10 min | 3857 [1730, 13177] | 2785 [1841, 4276] | 0.46 |
| **iarea2**<br>$AUC_{insulin}$ 10-180 min | 27559 [13717, 94854] | 16687 [12512, 22232] | 0.14 |
| **carea1**<br>$AUC_{C\text{-}peptide}$ 0-10 min | 13.8 [9.0, 31.9] | 11.3 [9.9, 14.5] | 0.92 |
| **carea2**<br>$AUC_{C\text{-}peptide}$ 10-180 min | 179 [103, 364] | 150 [101, 186] | 1.00 |
| **netsec1**<br>Minimal model derived insulin secretion rate 0-10 min | 0.5 [0.3, 0.8] | 0.4 [0.1, 1.0] | 0.60 |
| **netsec2**<br>Minimal model derived insulin secretion rate 10-180 min | 3.2 [1.2, 11.8] | 2.6 [1.1, 5.0] | 0.60 |
| **Si**<br>Minimal model-derived insulin sensitivity | 0.6 [0.2, 1.3] | 0.6 [0.3, 0.9] | 0.67 |
| **f**<br>fractional hepatic insulin throughput | 0.5 [0.3, 1.1] | 0.4 [0.3, 0.7] | 0.21 |
| **ki**<br>plasma insulin elimination rate | 0.1 [0.1, 0.2] | 0.1 [0.1, 0.2] | 0.67 |

**Table 5.8** IVGTT Minimal model indices. Data are geometric means [range]. P value refers to Mann-Whitney testing across groups.

The OGTT and IVGTT data from *CDKN2A* mutation carriers and control subjects require replication in larger numbers in order to conclusively reject or accept the null hypothesis. Recruitment fell short of the desired target: initially it was aimed to recruit 25 cases and 25 controls (**section 5.2.1**). Given the rarity of *CDKN2A* mutations and hence difficulties recruiting sufficient numbers of cases, there was a need to work with international colleagues of Professors Tim Bishop and Julia Newton-Bishop

(Leeds). Further power calculations have been performed based upon the presented IVGTT data. The present aim is to recruit 40 cases and 40 controls in total to provide 80% power (for an alpha of 0.05) to detect a between group difference of ~0.57 SD. These studies are underway through a collaboration with Professor Hans Vasen (Leiden, Netherlends).

## 5.4 Discussion

### 5.4.1 *CDKN2A may* contribute to glucose homeostasis

Using the OGTTs it has been demonstrated that whilst there is no difference in glucose tolerance between groups (as assessed by fasting and 2 hour glucose levels), there is a significantly higher HOMA B value (56% higher) in mutation carriers (p=0.03). This measure of beta-cell function being higher in *CDKN2A* mutation carriers is in line with my original hypothesis that *CDKN2A* haploinsufficient individuals will secrete more insulin than a control group of non-mutation carriers. However, none of the other beta-cell function measures reached statistical significance although there was a consistent trend for other indices of beta-cell function (insulinogenic index, $AUC_{insulin}$, corrected insulin response and BIGTT_AIR) to tend to be higher in the *CDKN2A* mutation carriers.

Importantly, the numbers recruited to the studies (14 vs 14) fell short of the 25 cases vs 25 controls initially aimed for, thus making it difficult to reject or accept the null hypothesis with this scale of study. Given that the control subjects were assigned control status retrospectively (i.e. I was unblinded to *CDKN2A* mutation status after recruitment for OGTT), these were heterogeneous groups of relatively small numbers. As the potential effect size being investigated may have been subtle, this heterogeneity and sample size could have contributed to the difficulties reaching clear conclusions with these data.

The IVGTT results demonstrate consistent findings to the OGTT: there was no statistically significant differences between groups but there was an interesting tendency for all measures of beta-cell function derived from the IVGTT data to be higher in the *CDKN2A* mutation carriers compared to well-matched control subjects (Table 5.8). This is most apparent in the 'second-phase' of insulin secretion as the iarea2 and carea2 measures, reflecting insulin secretion and C-peptide secretion

over 10-180 minutes of the IVGTT respectively, seem to show a more obvious trend (although not significant) than the first phase measures (iarea1, carea1). This is interesting as it is thought that loss of first phase insulin secretion is one of the earliest detectable defects in the natural history of T2D development (Cerasi and Luft 1967; Gerich 1997; Gerich 2002). However others have shown that a reduction in both first and second phase insulin secretion contribute to the hyperglycaemia evident in those with impaired glucose tolerance (Gerich 2002).

### 5.4.2 Insulin resistance may have contributed to tendency to higher beta-cell function measures in *CDKN2A* mutation carriers

Although not statistically significant, the *CDKN2A* mutation carrier group in the subjects analysed for OGTTs did have a tendency towards higher insulin resistance (Table 5.6; HOMA IR 1.8 vs 1.0 p=0.08). Therefore the tendency towards higher insulin secretion in the mutation carriers may be a reflection of the tendency towards higher insulin resistance in this group (or vice versa) given the hyperbolic relationship between insulin sensitivity and beta-cell function (Kahn et al. 1993). I analysed other features of insulin resistance i.e. those included in the metabolic syndrome (higher BP, higher lipid levels, higher WHR) and found no tendency or significantly higher results in the mutation carrier group. For the IVGTT data, the insulin sensitivity results derived from minimal modelling showed no difference between *CDKN2A* mutation carriers and controls subjects [Si 0.6 (0.2, 1.3) vs 0.6 (0.3, 0.9); p=0.67 (Table 5.8)]. This is notable as the observation for a tendency to higher IVGTT beta-cell function measures (although not statistically significant) in *CDKN2A* mutation carriers is in the context of very similar prevailing insulin sensitivity, and therefore reflects the 'true' ambient insulin secretory capacity of the beta-cells rather than any difference between groups reflecting the beta-cells adapting to insulin resistance.

Whilst it was advantageous to this study to use familial controls for mutation carriers, this did not allow for precise matching of metabolic characteristics (e.g. age, gender, BMI): controls were allocated from the 18 subjects who were revealed as non-mutation carriers and selected as the best available matches for the 14 *CDKN2A* mutation carriers. This permitted moderately well matched groups (BMI 26.6 vs 27.5; 43% males vs 33% males) but had I not used familial controls I would have aimed for more precise matching of groups for age, gender and BMI (as achieved with the IVGTT control subjects who were recruited from the Oxford Biobank). However I was able to utilise measures that correct for age and BMI [BIGTT_AIR and BIGTT_si (Table 5.2)] to allow for differences between the groups (although this can also be adjusted for in the statistical analysis).

In summary, null hypothesis cannot be rejected or accepted with the presented sample size. I have shown that one beta-cell function measure (HOMA B) is significantly higher in the *CDKN2A* mutation carrier group studied by OGTT and the trend observed for the remaining beta-cell function measures presented is interesting. Further studies are currently underway through a collaboration formed with Professor Hans Vasen (Netherlands Foundation for the Detection of Hereditary tumours), who has access to a large cohort of individuals and pedigrees of *CDKN2A* mutation carriers, with the aim of obtaining OGTT data on enough subjects to conclusively state if *CDKN2A* has a role in determining beta-cell function.

### 5.4.3 Does any difference in beta-cell function measures observed in *CDKN2A* haploinsufficient individuals reflect a difference in beta-cell mass compared to controls?

The premise of these studies is based upon the theory that any differences in insulin secretion detected between *CDKN2A* mutation carriers and control subjects will be due to an appreciable difference in the mass of beta-cells contained in the pancreases of mutation carriers, the hypothesis

being that mutation carriers will have higher beta-cell proliferation and subsequent mass and therefore higher insulin secretory capacity. This is probably an over-simplification in the complex field of beta-cell mass and its impact (or lack of impact) in the pathophysiology of T2D (discussed in detail in **Chapter 1 section 1.9.5.i**). Of note the study in *Cdkn2a* knockout mice conducted by Krishnamurthy et al, emphasised the importance of senescence, as the observed effects on enhanced islet proliferation and resistance to streptozotocin treatment were only demonstrated in older mice (Krishnamurthy et al. 2006). It is difficult to make definitive conclusions regarding the role of beta-cell mass in T2D pathogenesis, particularly when so much of the evidence is based upon rodent or autopsy data, and a technique to accurately quantify functioning beta-cell mass in humans remains elusive.

Although the scale of this study currently precludes rejection or acceptance of the null hypothesis, other theoretical reasons could be proposed for no obvious impact of *CDKN2A* mutation upon beta-cell function. Firstly, one could argue that the *CDKN2A*/*CDKN2B* T2D associated loci are acting via an alternative mechanism to one affecting beta-cell mass. Secondly, it may be that glucose stimulatory tests are not accurate tests of beta-cell mass and thirdly it may be that *CDKN2A* and/or *CDKN2B* have dual roles as tumour suppressor genes but also are as yet undefined components of metabolic pathways, as demonstrated for the tumour suppressor gene *PTEN* (Pal et al. 2012). I present no evidence for this latter explanation but consider it as another possible reason for not detecting greater differences in beta-cell function between groups tested.

### 5.4.4 The physiological tests used for this study may not have been sensitive enough to detect subtle differences in beta-cell function between groups

One of the limitations of this study could be the methods selected to assess beta-cell function in *CDKN2A* mutation carriers and control subjects. Although a range of dynamic tests were chosen, I am aiming to identify and characterise *improved* beta-cell function, which is challenging given that most of the biochemical methods to date are designed to define deficiencies of beta-cell function in the hyperglycaemic prodrome to diabetes. The hyperglycaemic clamp which involves stepwise increments in glucose concentration that stimulates first and second phase insulin secretion, has been referred to as a 'gold-standard' beta-cell function test (DeFronzo et al. 1979). Whilst it is true that the indices derived from this test are physiological and almost unaffected by confounding factors, it involves a relatively complex experimental procedure that would have been impractical to perform outside of the Clinical Research Unit used for most of my physiological studies. Also, a two hour clamp is often not long enough to achieve a stable steady state.

Thus the IVGTT was selected as the detailed test to assess beta-cell function as this test is extremely well validated against the hyperglycaemic clamp (Bergman et al. 1981) whilst still being a practical choice for reproducibility and completion outside of the Research Unit. It also has the added advantage of giving an accurate assessment of insulin sensitivity using the minimal model.

Although the OGTT is possibly used to more advantage in larger epidemiological studies (where effect size may be smaller) and takes the 'incretin effect' into account (Kazakos 2011), the hypothesis anticipated a potentially large effect of *CDKN2A* haploinsufficiency on islet mass and therefore insulin secretion. This assumption was made given the strength of rodent data that exists on mice under expressing *Cdkn2a* (Krishnamurthy et al. 2006) and also that *CDKN2A* mutations are rare and highly penetrant mutations (and therefore may display large effects). Thus, whilst an OGTT is not the 'best' test of beta-cell function, I was able to derive a number of indices giving me valuable

data on beta-cell function, from a test that I was able to conduct sometimes on multiple family members simultaneously, often in their own homes. This was advantageous as a challenge of this study was to recruit enough individuals with these relatively rare mutations.

In conclusion I present data that lends some support to the hypothesis that *CDKN2A* haploinsufficiency may potentiate beta-cell function. Whilst I acknowledge the data presented are in a sample size too small to assess existence of an effect between groups, further studies are currently underway that will hopefully allow more certain conclusions regarding this hypothesis.

# Chapter 6

Discussion

# Chapter 6

The GWAS era of T2D genetics has brought with it an abundance of proposed susceptibility loci, most previously unconnected to T2D pathogenesis. The subsequent challenge faced by scientists is the functional follow-up of these loci that will hopefully give novel biological insights into T2D pathophysiology. Some novel insights have been gained already, for example, by variants in *SLC30A8*, *GCKR* and *KLF14* (Beer et al. 2009; Hamming et al. 2009; Wijesekara et al. 2010; Small et al. 2011). The aims of the work presented in this thesis were to add insight into T2D pathogenesis through the perspective of a purported aetiological overlap with cancer. The studies presented here aimed to firstly, examine specific examples of potential mechanisms underlying the cancer/T2D overlap and secondly, to add to the functional follow-up of T2D susceptibility loci with arguably the strongest links to cell cycle regulators, those at the Chr9p21 tumour suppressor gene locus.

The intriguing GWAS discovery of several T2D susceptibility loci that map close to/within genes involved in cell cycle regulation (Voight et al. 2010; Morris et al. 2012) is supported by an abundance of epidemiological data connecting T2D and cancer (Giovannucci et al. 2010; Smith and Gale 2010). Various mechanisms have been proposed: the cancer promoting growth properties of high insulin, IGF-1 and glucose levels that occur in T2D; existence of common signalling pathways; and potential effects of T2D risk alleles upon functioning beta-cell mass via a proliferative effect. The latter two mechanisms were tested by hypotheses proposed in this thesis: the *PTEN* studies addressed the existence of common signalling pathways to cell growth and metabolism and the *CDKN2A/CDKN2B* studies addressed if the T2D associated loci at Chr9p21 may be acting via these cell-cycle regulators and subsequent effects on functioning beta-cell mass.

Common signalling pathways to growth and metabolism are one of the most likely mechanisms to underlie links between T2D and cancer. Insulin itself has dual roles in both processes via the multiple downstream effects of the PI3K-AKT pathway (Taniguchi et al. 2006), metformin is increasingly recognised as able to modify both cell-cycling as well as insulin sensitisation via MAPK dependent (and independent) pathways (Zakikhani et al. 2006; Foretz et al. 2010; Schultze et al. 2012) and the role of microRNAs have been investigated for their role in modulating multiple points of the PI3K-AKT pathway in order to achieve either cell-cycling or metabolic effects depending on their temporal expression through embryogenesis or later in adulthood (Zhu et al. 2011).

The critical role of the PI3K-AKT pathway in transduction of insulin signalling is well recognised (Taniguchi et al. 2006) and as an inhibitor of this pathway, *PTEN* was a good candidate to pursue for a potential role in glucose homeostasis, given that increased activation of the PI3K-AKT pathway should lead to amplified effects in tissues of insulin action. Taken alongside an earlier case report describing reactive hypoglycaemia after administration of intravenous glucose in an individual with a *PTEN* mutation (Iida et al. 2000), the data from tissue-specific *Pten* knockout (Stiles et al. 2004; Kurlawalla-Martinez et al. 2005; Wijesekara et al. 2005; Stiles et al. 2006; Wijesekara et al. 2010) and haploinsufficient mice (Wong et al. 2007) led to the hypothesis that individuals with CS due to loss-of-function *PTEN* mutations would be insulin sensitive and possibly have altered beta-cell function. The physiological data presented in Chapter 3 demonstrated *PTEN* haploinsufficiency as a cause of constitutive insulin sensitivity in humans, and in collaboration with colleagues at the Babraham institute in Cambridge, amplified signalling through the PI3K-AKT pathway was shown as the underlying mechanism for this heightened insulin sensitivity.

In addition to *PTEN* mutations, the PI3K-AKT pathway itself has been found to harbour other rare mutations leading to metabolic effects: loss-of-function *AKT2* mutations have been described as a cause of severe insulin resistance and diabetes (George et al. 2004) and activating *AKT2* mutations as a cause of severe recurrent hypoglycaemia (Hussain et al. 2011). By contrast, *AKT1* mutations are more frequently associated with cancer (Altomare and Testa 2005), and somatic mosaic activating *AKT1* mutations have recently been reported as a cause of the segmental overgrowth disorder of Proteus syndrome (Lindhurst et al. 2011). In fact, activating mutations in all 3 isoforms of AKT (also known as protein kinase B or PKB) have been described as causes of somatic overgrowth disorders: somatic *AKT1* as a cause of Proteus syndrome (Lindhurst et al. 2011), the activating *AKT2* mutations causing hypoglycaemia were also associated with asymmetrical overgrowth (Hussain et al. 2011) and somatic *AKT3* mutations have been described as a cause of hemimegalencephaly (enlargement of one cerebral hemisphere) (Lee et al. 2012; Poduri et al. 2012). Interestingly, only the reported *AKT2* mutations were associated with a metabolic phenotype, emphasising the different roles of AKT1 and AKT2 isoforms, also evident in rodent data (Gonzalez and McGraw 2009). A final component of the PI3K-AKT pathway found to harbour mutations relevant to CS and also segmental overgrowth disorders, is the p110α catalytic subunit of PI3K, encoded by the *PIK3CA* gene. Somatic activating mutations in *PIK3CA* cause a distinct overgrowth syndrome, particularly affecting adipose and fibrous tissues: interestingly no obvious metabolic phenotype was observed, although it is not clear if insulin secretion, sensitivity or glucose tolerance were formally assessed (Lindhurst et al. 2012). These recently described mutations are also of relevance to CS itself as the spectrum of CS, CS-like syndromes and segmental overgrowth syndromes are recognised as overlapping clinical presentations and germline *PIK3CA* and *AKT1* mutations have now been described in CS (Orloff et al. 2013).

The aforesaid *AKT2* mutations (Hussain et al. 2011), along with the PTEN studies presented here, demonstrate how specific perturbation of a pathway (better known for its alternative roles in cellular growth and cell cycle regulation) translates into appreciable effects on physiological parameters relevant to diabetes. The approach taken in this thesis was that subjects were recruited with a cancer-predispositon syndrome due to a tumour suppressor gene mutation, in order to study metabolic parameters. Many similar studies have an obvious metabolic parameter at the start (e.g. severe insulin resistance or severe hypoglycaemia) and investigations are aimed at defining the causal gene.

The role of PTEN as an inhibitor of the PI3K-AKT pathway and therefore *PTEN* mutations having a stimulatory effect on cancer growth whilst concurrently *reducing* diabetes risk is also an example of a divergence of risk. This phenomenon also applies to one of the clearest examples of a genetic link between T2D and cancer, the *HNF1B* variant that influences both T2D and cancer risk (Gudmundsson et al. 2007). This study initially identified a variant in intron 2 of *HNF1B* that was associated with an *increased* risk of prostate cancer (OR 1.22) and investigators sought an association with T2D risk given the knowledge that rare *HNF1B* mutations are a cause of MODY (Horikawa et al. 1997). Gudmundsson and colleagues showed that the risk allele for the same variant for prostate cancer was associated with *protection* from T2D (OR 0.91) (Gudmundsson et al. 2007). Epidemiological studies, although not always consistent in results and prone to numerous biases and confounding factors, have generally shown an inverse relationship between T2D and prostate cancer risk (Gong et al. 2006; Kasper and Giovannucci 2006; Calton et al. 2007). This is contrary to what is observed, epidemiologically, for many other cancers which show a general *increase* in prevalence with T2D (as discussed in **Chapter 1 section 1.6**). The mechanism for the association with prostate cancer is not clear but hypotheses proposed include changes in the local hormonal environment of the prostate in T2D, including levels of insulin, IGF1, testosterone and leptin (Giovannucci and Michaud 2007). The

fact that the reduction in prostate cancer risk is strongest for longer duration of diabetes (Giovannucci et al. 1998; Rodriguez et al. 2005) may simply point to the protective effects of metformin therapy (Evans et al. 2005), however this effect is not observed for the other cancers positively associated with T2D pointing to a distinct mechanism underlying the T2D/prostate cancer connection.

More generally, a possible explanation for the divergence of cancer and T2D risk and the genetic links between the conditions, could be that genes involved in cell-cycle proliferation, when suppressed may lead to a decrease in functioning beta-cell mass and thereby increased T2D risk but with a simultaneous reduction in cancer risk (the converse being true for variants that may *activate* these genes). Applying this principle to the inverse relationship observed with T2D and prostate cancer risk would imply that this divergence of risk should also be seen with other cancers and T2D: if cell cycle inhibitors are overexpressed in pancreatic beta-cells (suppressing beta-cell mass) and prostate tissue (reducing prostate cancer risk), why is this phenomenon of reduced cancer risk not observed in other tissues? This could be explained by tissue specific differences in cell-cycle regulation: for instance tissues may have differential systems to compensate when there is over or under expression of the relevant cell cycle regulators. This hypothesis was tested with the studies presented on the Chr9p21 T2D susceptibility loci (Chapters 4 and 5), from which the noncoding RNA, ANRIL, is transcribed and most proximal coding genes are the cyclin dependent kinase inhibitors, *CDKN2A* and *CDKN2B* (Pasmant et al. 2007; Saxena et al. 2007; Scott et al. 2007; Zeggini et al. 2007). The hypothesis proposed for the T2D association at this tumour suppressor locus is that the T2D variants attenuate expression of cell-cycle inhibitors, therefore altering beta-cell mass and T2D risk.

The region also contains independently associated CAD susceptibility loci (McPherson et al. 2007; Samani et al. 2007; Broadbent et al. 2008; Helgadottir et al. 2008) and the proposed mechanism for the Chr9p21 T2D and CAD susceptibility loci is that ANRIL regulates expression of *CDKN2B* and/or *CDKN2A*. The risk alleles are proposed to determine specific *ANRIL* transcript expression leading to an effect upon proliferation in tissues related to that disease, namely the VSMCs in CAD and the pancreatic islets in T2D. The Chr9p21 T2D-associated loci appear to be acting via a 'beta-cell' effect given that individuals homozygous for the risk allele have reduced insulin secretion (Grarup et al. 2007) and rodent data supports a role for *Cdkn2a* and *Cdkn2b* in islet mass and beta-cell function (Moritani et al. 2005; Krishnamurthy et al. 2006): the most plausible biological mechanism underlying the T2D-association would be that ANRIL regulates *CDKN2A* and/or *CDKN2B* expression in pancreatic islets leading to an effect on beta-cell mass and consequently insulin secretion. Expression studies give indirect evidence of co-regulation of *ANRIL*, *CDKN2A* and *CDKN2B* transcription with CAD-associated SNPs appearing to show a relationship with expression of these genes (Motterle et al. 2012). In addition a rodent model with a deleted region modestly homologous (50%) to *ANRIL*, provides provisional evidence of a regulatory effect upon *Cdkn2a* and *Cdkn2b* expression (Visel et al. 2010). Before the work presented in this thesis, there had been no *direct* evidence of ANRIL regulating *CDKN2A* and/or *CDKN2B* expression and no molecular characterisation of this locus in tissues relevant to T2D. Thus the aims of the molecular and physiology studies of the Chr9p21 locus were to provide evidence for the mechanism underlying the associations at this locus and indirectly, to investigate if this example could be attributed to an effect on beta-cell proliferation/functioning beta-cell mass as a biological mechanism for linking T2D and cancer.

Expression studies presented (**Chapter 4**) demonstrate mRNA presence of *ANRIL*, *CDKN2A* and *CDKN2B* in tissues relevant to glucose homeostasis and despite significant methodological

challenges, mainly in achieving desired levels of gene knockdown without adenoviral toxicity to human islets, I have been able to provide some evidence to show that *ANRIL* knockdown in human islets leads to reduced expression of *CDKN2A* and *CDKN2B.* These effects were noted to be tissue-specific, in that in contrast to the effect in human islets, *ANRIL* knockdown in HUVECS led to *up-regulation* of *CDKN2B* (perhaps more expected given *ANRIL* is antisense to *CDKN2B*) with no effects upon *CDKN2A* expression. In terms of how these results sit with the hypothesised mechanism of T2D-association at this locus, one could speculate that the T2D causal variants lead to *increased ANRIL* expression and therefore up-regulation of *CDKN2A* and *CDKN2B* in islets, thereby reducing functional beta-cell mass and insulin secretion, thus leading to increased T2D risk. It will be interesting to see the results of *ANRIL* knockdown experiments in a human beta-cell line now available (Ravassard et al. 2011). However, one could argue that the experiments described in this thesis are more relevant to T2D as the ultimate goal was to assess roles of the Chr9p21 genes in *whole* human islets and on physiological insulin secretion, rather than a transformed cell line model where extrapolation particularly of pathway effects on proliferation are possibly less reliable.

To further test the assumption that the Chr9p21 T2D risk variants are acting via *CDKN2A* and/or *CDKN2B*, physiological studies of glucose homeostasis were conducted in human *CDKN2A* 'knockout' model subjects with the aim of defining any effects particularly on beta-cell function. Although accumulating evidence for the CAD-association at the Chr9p21 locus suggests a relationship between *ANRIL*, *CDKN2A* and *CDKN2B* expression and CAD-risk genotypes (Holdt et al. 2010; Motterle et al. 2012), there are no similar studies for the T2D association. Pathway and protein-protein interaction meta-analyses certainly incriminate cyclin dependent kinase inhibitors such as *CDKN2A* and *CDKN2B* in the mechanism underlying T2D susceptibility loci (Voight et al. 2010; Morris et al. 2012) and physiological studies in humans homozygous for the T2D-risk alleles are also consistent with the hypothesis of increased insulin secretion being the mechanism mediating the Chr9p21 association

(Grarup et al. 2007). Rodent data supports the plausibility of a hypothesised mechanism being mediated by T2D-risk variants affecting *CDKN2A* and/or *CDKN2B* expression leading to affects upon islet mass and therefore insulin secretion (Moritani et al. 2005; Krishnamurthy et al. 2006) but interestingly the effects seen in *Cdkn2a* knockout mice were only evident in older mice, suggesting a possible role for the islet proliferation effect in senescence or development. However there is no direct evidence for a role of *CDKN2A* or *CDKN2B* in glucose homeostasis in humans.

As with the studies in *PTEN* mutation carriers, the ambiguity discussed above was approached by recruiting individuals who would be haploinsufficient for the gene of interest, in this case *CDKN2A* mutation carriers, aiming to quantify and characterise any impact of loss-of-function of this gene on beta-cell function and insulin secretion in particular. It was hypothesised that individuals with rare penetrant mutations in the tumour suppressor, *CDKN2A,* would be likely to manifest a metabolic phenotype. Of the measures of beta-cell function used to assess *CDKN2A* mutation carriers versus control subjects, only HOMA B was significantly higher, in line with the hypothesis of *CDKN2A* having a role in determining insulin secretory capacity. All other measures of insulin secretion derived from the OGTT and IVGTT data were not significantly different between groups but did reveal a consistent trend for higher insulin secretion. Therefore these data are currently inconclusive with regard to confirming or refuting a definitive role for *CDKN2A* in glucose homeostasis. However, we have formed a collaboration with Professor Han Vasen (Netherlands Foundation for the Detection of Hereditary tumours), who has access to a large cohort of individuals and pedigrees of *CDKN2A* mutation carriers with the aim of obtaining OGTT data on enough subjects to conclusively state if *CDKN2A* has a role in determining beta-cell function.

The Chr9p21 locus also exemplifies the challenges of functional follow-up of T2D susceptibility loci: disease associated loci map to noncoding regions, causal transcripts are yet to be defined, risk conferred by the risk alleles is modest and the genes ascribed to mediating the T2D association have been proposed for proximity and biologically plausible hypotheses rather than any direct evidence of a role in pathogenic mechanisms giving rise to T2D in humans. Another challenge in the face of functional interpretation of implicated T2D genetic variants, is the ability to study these loci in tissue models of relevance. For instance, one the most notable tissues in glucose homeostasis, the pancreatic beta-cell, is relatively inaccessible, lacks a reliably representative cell system for in vitro study and has significant differences to the related tissue in rodent models. This latter reason is particularly relevant for models of cell-cycle regulation in human pancreatic islets as fundamental differences to rodent models exist, not only in biology, but in several critical components of the cell-cycle pathway (Kushner et al. 2005; Malumbres and Barbacid 2005; Fiaschi-Taesch et al. 2009). Therefore, for studies investigating impact of cell-cycle components in metabolic pathways, it is most relevant to perform such experiments in human models of disease. The molecular studies of the Chr9p21 locus presented in this thesis add to the weight of evidence linking regulatory effects of *ANRIL* upon *CDKN2A* and *CDKN2B* expression as the mechanism underlying T2D-association, and do so in human islets, a cell system directly relevant to T2D.

Apart from results pertaining to hypotheses proposed in this thesis, there were some unexpected findings, particular in relation to the PTEN studies (Chapter 3). An unexpected and apparently paradoxical observation was the higher BMI noted in *PTEN* mutation carriers, which was evident despite their significantly higher insulin sensitivity. It was confirmed that the elevated BMI was due to increased adiposity (as opposed to increased lean or bone mass) and that there were no patterns of adiposity to account for their increased insulin sensitivity (i.e. increased gluteofemoral or subcutaneous fat). Limited data from one case along with data previously reported suggests steady

weight gain in *PTEN* mutation carriers throughout childhood and adolescence (Cole and Hughes 1991). Further work is needed to elucidate why this obesity and energy imbalance occurs, for example through calorimetric studies. Of note, in rodent models overexpressing *Pten*, mutant mice were smaller, had reduced body fat, increased energy expenditure (Garcia-Cao et al. 2012) and interestingly have also been observed to be hyperphagic and have hyperactive brown adipose tissue (Ortega-Molina et al. 2012) (a tissue recognised for its role in energy consumption (Nedergaard et al. 2011)). There was no evidence of hyperphagy or appetite disturbance in the subjects who took part in these studies but further work on appetite regulation and the hypothalamic axis, whole body calorimetry studies and the possible role of white versus brown adipose tissue is required in individuals with *PTEN* mutations.

The finding of lower adiponectin levels in the *PTEN* mutation carrier group was also paradoxical in that this hormone is normally associated with insulin sensitivity (Kloting et al. 2010). However others have observed 'paradoxical' results in relation to monogenic insulin resistance syndromes and postulated that the level of adiponectin is *subsequent* to the severe insulin resistance and not a clear-cut 'causal' factor in determining prevailing insulin sensitivity (Semple et al. 2006; Groeneveld et al. 2012). In this cancer predisposition syndrome, with newly ascribed constitutive insulin sensitivity, the role of adiponectin is further complicated by its association with cancer: an inverse relationship has been observed in relation to malignancies associated with obesity and insulin resistance (Wei et al. 2005; Barb et al. 2007). It would be interesting to measure adiponectin levels in larger numbers of *PTEN* mutation carriers (and analyse these results in the context of what cancers participants had previously been diagnosed with), and confirm the findings reported here.

Finally, the work presented in this thesis has some wider implications. The demonstration of constitutive insulin sensitivity in *PTEN* mutation carriers highlights the importance of this phosphatase and the PI3K-AKT pathway in insulin signalling. Observations prompting further investigation have been highlighted but the knowledge that patients with CS due to *PTEN* mutations will display heightened insulin sensitivity is of interest to Clinical Geneticists. Increased insulin sensitivity (which can be most simply assessed with fasting insulin and glucose levels) may be considered a feature of CS, and if an insulin sensitive CS patient was found not to have a *PTEN* mutation, might motivate examination of other components of the PI3K-AKT pathway.

The wider applicability of this work is also demonstrated by the preliminary data on common variation at the *PTEN* locus giving suggestive associations for fasting glucose and insulin levels in the general population reported in Chapter 3. This has since been replicated and the *PTEN* locus is now reported as one of 17 additional loci influencing fasting insulin concentrations by Scott and colleagues who performed genome wide association meta-analyses on BMI-adjusted results to account for the variance in fasting insulin due to BMI (Scott et al. 2012).

As discussed previously, the PTEN studies also illustrate how intimately cell cycle and metabolic pathways can be linked and are of interest to those seeking to manipulate PTEN, amongst other lipid phosphatases as therapeutic agents in T2D (Sasaoka et al. 2006; Beguinot 2007; Mak et al. 2010). These studies also confirm the cautions around *systemic* targeting of PTEN given the adverse oncogenic side effects (Di Cristofano et al. 1998; Podsypanina et al. 1999). Tissue-specific knockout rodent models in insulin target organs (Stiles et al. 2004; Kurlawalla-Martinez et al. 2005; Wijesekara et al. 2005) are more promising and demonstrate the potential for novel insulin sensitisers in T2D (Sasaoka et al. 2006; Beguinot 2007). Targeted deletion of further downstream negative regulators of the PI3K-AKT pathway, such as TRIB3, may also confer beneficial metabolic effects without oncogenic side-effects (Koo et al. 2004; Okamoto et al. 2007).

In conclusion, the data presented in this thesis illustrate some mechanisms underlying the aetiological overlap between T2D and cancer by focussing on two loci known for their tumour suppressor gene function. The *PTEN* studies demonstrate how signalling pathways can be common to both metabolism and cell growth, and link two apparently disparate conditions in a divergent manner, i.e. with loss-of-function *PTEN* mutations *increasing* cancer risk but presumably *decreasing* T2D risk via heightened insulin sensitivity and the subsequent reduced demand on pancreatic beta-cells. The molecular studies of the T2D-associated chr9p21 loci demonstrate how a ncRNA may be regulating expression of the proximal tumour suppressors *CDKN2A* and *CDKN2B* in human islets, potentially leading to altered glucose physiology via an altered functioning beta-cell mass effect.

Collectively this work is an exemplar of the power of human models to demonstrate the function and relevance of genes not previously ascribed to specific pathways or physiological parameters and provides an interesting perspective on the pathogenic mechanisms underlying T2D via its commonality with cancer.

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