UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA
Departamento de Ciencias Médicas y Quirúrgicas

Anexo I

D. JUAN RAMÓN HERNÁNDEZ HERNÁNDEZ, SECRETARIO DEL DEPARTAMENTO DE CIENCIAS MÉDICAS Y QUIRÚRGICAS DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA

Que el Consejo Ordinario de Departamento de Doctores en su sesión de fecha 7 de octubre de 2015, tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "STUDY OF THE PATHOGENESIS AND TREATMENT OF DIABETES MELLITUS THROUGH ANIMAL MODELS", presentada por el/la doctorando/a, Don Yeray Brito Casillas y dirigida por los doctores Doña Ana M. Wägner, Don Carlos Melián Limiñana, Don Julia C. Wiebe.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del Reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a siete de octubre de dos mil quince.

Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento/Instituto/Facultad: Ciencias Médicas y Quirúrgicas
Programa de doctorado: Avances en Medicina Interna

Título de la Tesis

'Study of the pathogenesis and treatment of diabetes mellitus through animal models'

Tesis Doctoral presentada por D. Yeray Brito Casillas

Dirigida por la Dra. Dª. Ana M. Wägner

Codirigida por los codirectores Dr. D. Carlos Melián Limiñana y Dra. Dª Julia C. Wiebe

El/la Director/a, **El/la Codirector/a** **El/la Doctorando/a,**

Las Palmas de Gran Canaria, a 1 de Octubre de 2015

Dª. ANA M. WÄGNER, PROFESORA TITULAR DEL DEPARTAMENTO DE CIENCIAS MÉDICAS Y QUIRÚRGICAS DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA (ULPGC) Y SECRETARIA DEL INSTITUTO UNIVERSITARIO DE INVESTIGACIONES BIOMÉDICAS Y SANITARIAS DE LA ULPGC.

CERTIFICA,

Que D. Yeray Brito Casillas, Licenciado en Veterinaria, ha realizado bajo su dirección y asesoramiento el trabajo de investigación titulado:

'Study of the pathogenesis and treatment of diabetes mellitus through animal models'

Una vez revisada la presente memoria, la encuentra apta para su defensa ante el tribunal para la obtención del título de Doctor, mención Doctor Internacional, por la Universidad de Las Palmas de Gran Canaria.

Y para que así conste y surta los efectos oportunos, extiende la presente certificación en Las Palmas de Gran Canaria a 1 de Octubre de 2015.

Fdo. Dra. Ana M. Wägner

D. CARLOS MELIÁN LIMIÑANA, GERENTE DEL HOSPITAL CLÍNICO VETERINARIO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA (ULPGC) E INVESTIGADOR DEL INSTITUTO UNIVERSITARIO DE INVESTIGACIONES BIOMÉDICAS Y SANITARIAS DE LA ULPGC.

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Fdo. Dr. Carlos Melián Limiñana

Dª. JULIA WIEBE, INVESTIGADORA POSTDOCTORAL DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA (ULPGC) E INVESTIGADORA DEL INSTITUTO UNIVERSITARIO DE INVESTIGACIONES BIOMÉDICAS Y SANITARIAS DE LA ULPGC.

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Fdo. Dra. Julia C. Wiebe

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

Study of the pathogenesis and treatment of diabetes mellitus through animal models

Yeray Brito Casillas

Las Palmas de Gran Canaria, 2015

A mi familia.

A mi padre. Sé que la hubieras disfrutado como el mejor de los regalos.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A1c	Glycated Haemoglobin
ADA	American Diabetes Association
Asn	Asparagine
Asp	Aspartic Acid
AIDS	Acquired Immunodeficiency Syndrome
AUC	Area under the curve
AV	Aloe vera
BCA	Bicinchoninic acid
BB-DP	Bio Breeding Diabetes-Prone Rat
BB/OK	BioBreeding/Ottawa Kalsburg Rat
BW	Body Weight
CEBA	Comité Ético de Bienestar Animal
CEIC	Comité Ético de Investigación Clínica
CEN	European Committee for Standardisation
db/db	See *Lepr*^{db}
DM	Diabetes Mellitus
C57Bl/6J	Mouse strain
cDM	Canine Diabetes Mellitus
CTLA4	Cytotoxic T-Lymphocyte-Associated Protein 4
DCCT	Diabetes Control and Complications Trial
DD	Diestrus Diabetes
DIAMOND	Diabetes Mondiale, World Health Organization Multinational Project
DLA	Dog Leukocyte Antigen
DM	Diabetes Mellitus
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immunosorbent assay
EURODIAB	European Diabetes Studies
fa/fa	See *Lepr*$^{fa/fa}$
FPG	Fasting plasma glucose
FTO	Fat Mass And Obesity Associated
GAD or GAD65	Glutamate Decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCK	Glucokinase (Hexokinase 4)
GDH	Glucose Dehydrogenase
GDM	Gestational Diabetes Mellitus
GFR	Glomerular filtration rate
GIR	Glucose Infusion Rate
GO	Glucose Oxydase
GTT	Glucose Tolerance Test
HbA1c	Glycated Haemoglobin
HFDID	High-Fat Diet-Induced Diabetic
HIV	Human Immunodeficiency Virus
HNF1A	HNF1 Homeobox A
HPLC	High-performance Liquid Chromatography
HLA	Human Leukocyte Antigen
IA2	Islet Antigen 2
ICA	Islet Cell Antibodies
IDD	Insulin Deficiency Diabetes
IDF	International Diabetes Federation

IGF1	Insulin-Like Growth Factor 1 (Somatomedin C)
ILR2	Interleukin Receptor 2
INS	Insulin gene
IPGTT	Intraperitoneal GTT
IPITT	Intraperitoneal ITT
IR	Insulin Resistance
IRS1, IRS2	Insulin Receptor Substrate 1/2
IS	Insulin Sensitivity
ISO	International Organization for Standardization
ITT	Insulin Tolerance Test
Jci-ICR	Mouse Strain
KCNJ11	Potassium Channel, Inwardly Rectifying Subfamily J, Member 11 gene
KO, Ko	Knock out
LADA	Latent Autoimmune Diabetes of the Adult
Lepr^{db}	Mutation in Leptin receptor
Lepr^{fa}	Mutation in Leptin receptor
Lep^{ob}	Mutation in Leptin gene
LETL	Long Evans Tokushima lean (rat)
MDRD4	Modification of Diet in Renal Disease-4
MHC	Major Histocompatibility Complex
MODY	Maturity onset diabetes of the young
NGSP	National glycohemoglobin standardisation program
NOD	Non Obese Diabetic (mouse)
ob/ob	See *Lep^{ob}*
OGTT	Oral GTT
PBGM	Portable Blood Glucose Meter
Pcmt, PCMT1	Protein-L-Isoaspartate (D-Aspartate) O-Methyltransferase
PG	Plasma glucose
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha gene
PIMT	PCMT1 protein
POC	Point Of Care
PPARG	Peroxisome Proliferator-Activated Receptor Gamma
PROX1	Prospero Homeobox 1
PTM	Post-translational modification
PTPN22	Protein Tyrosine Phosphatase, Non-Receptor Type 22 (Lymphoid)
RT-PCR	Real-Time Polymerase Chain Reaction
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitacion
SAH	S-adenosylhomocysteine
SAM	S-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
STZ	Streptozotocin
TCF7L2	Transcription Factor 7-Like 2 (T-Cell Specific, HMG-Box)
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
UA	*Uromastyx Achantinura*
WB	Whole Blood
WHO	World Health Organisation
ZFR	Zucker Fatty Rat
ZDF	Zucker Diabetic Fatty Rat
ZnT8	Zinc Transporter Eight

INTRODUCTION

I. INTRODUCTION

1. Diabetes Mellitus: definition, classification and epidemiology

Diabetes mellitus (DM) comprises a set of chronic metabolic disorders, characterised by hyperglycaemia, that are originated by deficient insulin secretion, insulin action or both [1, 2].

The diagnosis of DM can be made by the coexistence of symptoms (polyuria, polydipsia) and random blood glucose above 200 mg/dL or by a glycated haemoglobin (HbA1c or A1c) ≥ 6.5%, among other criteria (see table 1) [2]. Its early diagnosis is crucial to reduce the risk of complications. Indeed, 22-50% of patients diagnosed with type 2 diabetes (T2D) have chronic complications of the disease at the time of diagnosis [3, 4].

Diagnostic Criteria of Diabetes Mellitus	
A1c ≥ 6.5%	The test should be performed in a laboratory using a method that is NGSP certified and standardised to the DCCT assay*
Fasting plasma glucose (FPG) ≥ 126 mg/dL	Fasting is defined as no caloric intake for at least 8 h*
2h plasma glucose (PG) ≥ 200 mg/dL during an oral glucose tolerance test (OGTT)	The test should be performed as described by the WHO using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water *
Classic symptoms of hyperglycaemia or hyperglycemic crisis and a random PG ≥ 200 mg/dL	
*In the absence of unequivocal hyperglycaemia, results should be confirmed by repeat testing	

Table 1: Criteria for the diagnosis of diabetes mellitus. Adapted from *'Classification and diagnosis of diabetes'*, ADA [1]. NGSP: National Glycohemoblogin Standardisation Program. DCCT: Diabetes Control and Complications Trial. WHO: World Health Organisation

Even though DM has been known for millennia and great advances have been achieved in its diagnosis and management, there is currently no cure for the disease and its public health consequences are only growing [5]. Implications for the patients include need for lifestyle changes and lifelong treatments with pills and/or injections, as well as increased risk for complications, such as blindness, end-stage renal disease, lower-limb amputations, cardiovascular disease and increased mortality [1, 6-10]. Indeed, every seven seconds one person dies as a consequence of this disease [11] and, in 2014, 4.9 million people died worldwide, including 24,428 in Spain [11]. From an

economical point of view, costs of DM and its complications for the European health systems added up to 2291€ per patient in that same year [11].

The Canary Islands are not an exception: in fact the region has the highest incidence of end-stage diabetic kidney disease [12] and a high diabetes-related mortality compared to the rest of Spain [13, 14].

Diagnostic and classification criteria of DM have been revised several times in the last few years[1, 2, 15-22]. The current, most accepted classification, proposed by the American Diabetes Association (ADA), considers four main types of diabetes: type 1 (T1D), T2D, other specific types of diabetes (which includes genetic defects of insulin secretion and action, endocrine disorders such as Cushing's syndrome and pancreatic diseases, among others) and gestational diabetes mellitus (GDM) **(see table 2)** [1], although there are recent proposals to reconsider this classification again [15]. T1D is characterised by β-cell destruction that usually leads to absolute insulin deficiency, whereas patients with T2D have a combination of insulin resistance (IR) and relative insulin deficiency and GDM is defined as diabetes diagnosed during the second or third trimester of pregnancy [1, 23].

Classification of Diabetes Mellitus	
Type 1 diabetes (T1D)	Due to β-cell destruction, usually leading to absolute insulin deficiency
Type 2 diabetes (T2D)	Due to a progressive insulin secretory defect on the background of insulin resistance
Gestational diabetes mellitus (GDM)	Diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes
Specific types of diabetes	Due to other causes e.g., monogenic diabetes syndromes (i.e. neonatal diabetes and maturity-onset diabetes of the young [MODY]), diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced diabetes (i.e. in the treatment of HIV/AIDS or after organ transplantation)

Table 2: Diabetes classification. Adapted from *'Classification and diagnosis of diabetes'*, ADA [1].

T1D represents 5-10% of all cases of DM and is one of the most common chronic diseases in childhood [24]. Age-adjusted incidence varies among populations, with Finland in one extreme (>60 cases per 100,000 people/year), followed by Sardinia and Sweden, and Venezuela, China or

India (0.1 cases per 100,000 people/year) in the other extreme [25]. National and international registries show that the incidence of T1D is rising worldwide at a rate of 3-5% per year [26-31] and a doubling in the number of cases has been estimated for the next ten years[26]. Reports from the Canary Islands show a T1D incidence ranging between 23 and 32 cases/100,000 people/year, which would place it behind Finland, Sardinia and Sweden's [32-34].

The predominant form of diabetes is T2D, representing 90% of all cases of diabetes worldwide [35, 36]. Directly related to the degree of obesity, its incidence rises when countries become richer and adopt a western lifestyle: high caloric intake combined with poor physical activity [37]. In fact, 80% of the present cases of DM live in developing countries and are still rising [5, 38]. In the Canary Islands, the prevalence of T2D, of 13.2%, is similar to that of other Spanish regions [39, 40].

2. Pathogenic mechanisms in Type 1 (T1D) and Type 2 Diabetes (T2D)

2.1 Pathogenesis of T1D

T1D results from T cell-mediated autoimmune destruction of insulin-producing, pancreatic β-cells, elicited by complex gene-environment interactions [41, 42]. Most of the genes associated with T1D either affect the regulation of immune tolerance, the character of the immune response and β-cell defence mechanisms, or insulin and cytokine/monokine production [43]. The genes encoding the major histocompatibility complex (MHC) class II molecules, the HLA region [44, 45], account for about 50% of the genetic risk associated with T1D [45]. Other genes, with a less prominent effect on T1D risk, include the insulin gene (*INS*) [46], the cytotoxic T lymphocyte antigen 4 gene (*CTLA-4*) [47, 48], protein-tyrosine phosphatase non-receptor type 22 (*PTPN22)* and interleukin-2 receptor (*IL2R*), although more than 50 susceptibility loci have now been identified [43, 49, 50].

Autoantibodies are present in 85-90% of individuals with recently diagnosed T1D [2, 51]. Four major, specific islet autoantibodies have been identified that are targeted against insulin, glutamic acid decarboxylase 65 (GAD65), protein-tyrosine phosphatase-2 (IA-2) and zinc transporter eight (ZnT8) [52-55]. These antibodies can be measured years, even decades, before the onset of the disease, though there is no evidence of their pathogenic role [56].

The reason for the first event initiating the destructive process in the ß-cell, the loss of tolerance and the activation of T-cells, is still not known. The Copenhagen model, first proposed by Nerup in 1994 [57] and recently updated by Størling et al [58], suggests that a combination of environmental factors interact with genetic susceptibility and attack the ß-cell, which thereafter releases some of its components, such as insulin or GAD, possibly in forms not previously "seen"

by the immune system [59]. These proteins are believed to have suffered posttranslational modifications, eliciting new antigenic epitopes that are taken up and presented by dendritic cells in the islets. They are then transported to regional pancreatic lymph nodes, where the antigens are processed and presented to T-cells and the immune response is amplified and perpetuated. Other autoimmune diseases in which modified self-antigens are known to play a role in autoimmune response include multiple sclerosis, rheumathoid arthritis and systemic lupus erythematosus [60].

Typical histopathological findings in human pancreas at the onset of T1D, known as insulitis, include immune cell infiltration in the pancreatic islets and reduced β-cell area [61-63]. The following criteria for the definition of insulitis have been proposed: ≥15 $CD45^+$ (common leukocyte antigen) cells in a minimum of three islets of approximately 150 μm in diameter [62]. The majority of infiltrating cells are of lymphocytic nature, with predominant $CD3^+CD8^+$ T cells in early stages of the disease and an increment of B lymphocytes ($CD20^+$) later on [64, 65].

The list of environmental factors which have shown an association with the development of T1D is long and includes enterovirus infections [66, 67], air pollution and environmental toxins (i.e. nitrosamines) [68, 69], early exposure to cow's milk proteins, cereals or gluten [70-74] and vitamin D deficiency [75, 76]. Furthermore, environmental factors traditionally related to the pathogenesis of T2D, such as increased body mass index, increased body size and IR, have also been considered as predictors of T1D [77-79].

2.2 Pathogenesis of T2D

T2D is caused by a combination of ß-cell dysfunction and IR. In the presence of IR, ß-cells are driven to increase their insulin production and this situation, if maintained, can lead to ß-cell failure, especially in genetically predisposed individuals. The mechanisms behind this process, although extensively studied, are still poorly understood [80]. The proposed mechanisms leading to ß-cell dysfunction include glucotoxicity, lipotoxicity and amyloid deposition [81].

More than 80 genetic loci have now been associated with T2D and with inter-individual variation traits such as levels of glucose, insulin, proinsulin and hemoglobin A1c [82]. Most of the genes involved are responsible only for a very modest increase in the risk of the disease and their roles reflect the pathogenesis of DM2. Indeed, although most of the associated variants described are related to β-cell function (*TCF7L2*, *HNF1A, GCK, PROX1,...*), others are associated with impaired insulin sensitivity (*PPARG, IRS1, IGF1*) or obesity (*FTO*) [81, 83-86]. In fact, beside the mentioned genes, advancing age, obesity and physical inactivity are the most important risk factors for T2D [5]. Central adiposity and waist circumference, closely associated with IR, are even better predictors than BMI *per se* [87, 88]. Given the rapid increase in the prevalence of obesity (33% in

2005; estimated 57.8% of adults in 2030) [89], the parallel predictions in DM prevalence by the IDF are not unexpected [11].

3. Animal models of diabetes

The consequences of DM on people's lives have motivated the search for better understanding of the mechanisms of the disease, as well as better treatments [90]. Even though *in vitro* and *in silico* studies have improved in the last decades, they cannot completely replace the information inferred from animal models, given the complex aetiology and multi-systemic interactions present in diabetes [91, 92]. Most of the research in diabetes is performed in animals [93, 94] and animal replacement or reduction [95] is still a chimera. The combined approach of human, *in vitro* and animal studies is probably the best strategy to improve our understanding of underlying mechanisms [94].

The modern, more standardised use of animal models of DM started during the 17th century by JC Brunner [96], though even earlier uses of animal research have been reported [97]. Claude Bernard, father of 'vivisection', identified the existence of differentiated endocrine and exocrine pancreas using animal models [97]. Some of the most important discoveries of biomedical research and DM were made with pancreatectomised dogs: the discoveries of pancreatic function by Von Mering and Minkowski (1890s) [98] and the insulin hormone by Banting and Best (1920s) [99]. Presently, a variety of animal models of DM are present and many additional advances have been possible: leptin's discovery, new insights into pathogenesis and complications and the development of new treatments, among others [100-102].

These models are mainly classified based on which type of diabetes they mimic and whether they are spontaneous or induced [92, 103-108]. Furthermore, progress in genetics has allowed to generate specific transgenic models, almost *à la carte*, extending the range of spontaneous or more susceptible/resistant models [109]. Sometimes, when they develop DM, the disease can be inconsistently classified as spontaneous or induced depending on the author [92, 107], as they are genetically induced, but clinical signs and symptoms of DM appear spontaneously.

3.1 Spontaneous diabetes models

Diabetes occurs spontaneously in many animal species, including the horse, dolphin, and even hippopotamus, among many others (*reviewed by E. Gale*) [93]. Some of these species have provided important results as veterinary patients or as animal models, enabling better understanding of underlying mechanisms of DM. The principal advantages when compared with induced models

are that they are presumed to share mechanisms of disease with the human condition, especially in polygenic models [93, 104, 105].

The past decade has seen remarkable advances in the understanding of genetics and pathophysiology of spontaneous models of immune mediated diabetes and the creation of new models. The most commonly used spontaneous models of T1D are the non-obese diabetic (NOD) mouse and the BioBreeding Diabetes-Prone (BB-DP) rat [103]. Other spontaneous T1D models include Long Evans Tokushima lean (LETL) rat and the New Zealand white rabbit. These models provide useful tools for the study of the autoimmune process and prevention of T1D. For T2D, the most frequently used spontaneous models are the Zucker fatty (ZFR) and Zucker diabetic fatty rats (ZDF) and the *ob/ob* and db/db mice.

Nevertheless, although their contribution is considerable, successful animal outcomes have failed to be translated to humans [94, 110, 111]. Consequently, the choice of appropriate, single or combined animal models should be made to fit specific purposes, according to their validation to the aim [92, 94]. The most relevant, spontaneous models are described below.

a) The Non-Obese-Diabetic (NOD) mouse and the BioBreeding Diabetes-Prone (BB-DP) rat

The NOD mouse and the BB-PD rat are the most prominent and frequently used spontaneous models of T1D since they emerged 40 years ago [103, 104].

The first NOD mouse was established by intercrossing females derived from the JcI-ICR strain, a model for cataracts [112]. NOD mice develop insulitis already at the age of 3-4 weeks, preceding subclinical β-cell destruction and insulinopenia. Final establishment of DM occurs between 12 to 30 weeks of age and animals can survive without insulin injections for weeks. Evident sexual dimorphism is present: 90% of females develop DM, whereas only 60% of males do [113, 114].

The BB-DP rat derived from Wistar strain intercrosses in the Bio-Breeding Laboratories [115]. This rat shows classic clinical signs of insulinopaenia at 12 weeks or earlier. In contrast to NOD, insulin treatment is mandatory to assure survival of the BB rat, as ketoacidosis is severe and fatal [116-118].

The pathogenic mechanisms leading to DM in both models are close to those described for human T1D [103]. In the endocrine pancreas, autoimmune processes mediated by T cells, B cells, macrophages and natural killer cells lead to insulitis and islet loss [117-121].

Autoantibodies have also been identified in both models, but, as in humans, their pathogenic role has not been confirmed [122, 123]. Genetic studies have confirmed the role of the MHC region, both in NOD and BB-PD [124-127]. The role of virus infections, dietary factors, vitamin D or

immunosupressors like cyclosporine-A in the pathogenesis of DM, have been widely studied in both BB and NOD, also for the development of new therapies [128, 129].

b) Zucker fatty rat (ZFR) and Zucker diabetic fatty rat (ZDF)

The ZFR was first identified by Zucker in 1961, in the rat stock of Sherman and Merck, USA [105]. Also named as (*fa/fa*) fatty or obese rat ($Lepr^{fa}$), its phenotype is derived from an autosomal recessive mutation (*fa)* in a gene on chromosome 5, which leads to dysfunction in leptin receptor signalling in the hypothalamus [105]. Following this mutation, hyperphagia and obesity develop already at four weeks of age. This phenotype leads to mild hyperglycaemia and IR, glucose intolerance, hyperlipidaemia, hyperinsulinaemia and hypertension. The principal causes of the mild glucose intolerance are hepatic metabolic defects [105, 130]. ZFR have mostly been used to study insulin sensitisers and antiobesity agents [131, 132].

Derived from the ZFR, the ZDF was selectively inbred for hyperglycaemia. Both ZFR and ZDF are models of the metabolic syndrome and T2D. ZDF is less obese and more insulin resistant and develops mild diabetes [133]. There is marked sexual dimorphism and males are more susceptible to develop diabetes, normally from 7-10 weeks of age. On the contrary, females show a phenotype, which is closer to the ZFR. They are normally used as non-diabetic controls for their male littermates [105].

Unlike the ZFR, they do not show compensatory insulin over-secretion in response to peripheral IR and β cells are ultimately damaged, following apoptosis, due to the higher secretion demand, even though proliferative mechanisms are not affected [134]. Marked lipotoxicity and down-regulation of GLUT-2 and GLUT-4 glucose transporters have also been demonstrated [105]. ZDF have been used for the study of mechanisms of IR and β-cell dysfunction [135, 136].

c) The *ob/ob*, *db/db*, Lep^{ob}, $Lepr^{db}$ mouse

The *ob/ob* or Lep^{ob} mouse (obese mouse), with C57BL/6J strain background, has a monogenic, autosomal, recessive mutation (obese) on chromosome 6. The mutation in *ob/ob* mice has been pinpointed to the leptin gene. Leptin, which is absent in this homozygously 'obese' mouse, is mainly synthesised in adipose tissue and causes appetite suppression and energy expenditure, and modulates IR [137]. The lack of leptin leads to hyperphagia, decreased energy expenditure and obesity, high levels of neuropeptide Y (an orexigenic peptide) and hypercorticosteronism, which also contributes to IR [105]. Mice gain weight early and rapidly and end up weighing three times wildtype controls. The diabetes-like syndrome of the *ob/ob* mouse is characterised by hyperglycaemia, mildly impaired glucose tolerance, severe hyperinsulinaemia, sub fertility and

impaired wound healing [138]. Histological examination of the pancreas shows hypertrophy and hyperplasia of pancreatic islets [139]. IR is caused by reduced insulin binding to its receptors, impaired insulin receptor autophosphorylation, and reduced signal transduction [105]. This model has been used to test body weight loss and antiobesity treatments, insulin sensitisers and antihyperglycaemic agents [140-142]. The *db/db* or *Lepr*^{db} mouse shares the same characteristics of *ob/ob*, but its mutation is sited in the leptin receptor gene [105].

d) The Diabetic Cat

Diabetes is one of the most common spontaneous endocrine diseases in cats, and it is estimated that approximately 80% of naturally occurring diabetes is similar to human T2D, with important IR as the principal cause [143], although the reasons for this IR are not fully understood [143].

Genetics play a role in the pathogenesis of T2D in cats, as reflected by the increased risk associated with certain breeds (e.g Burmese and Russian blue) [144-147], but environmental factors are also important [143]. Indeed, the major risk factor for the development of DM in this model is obesity, together with physical inactivity [145, 148-150]. In fact, obese cats are approximately 4 times more likely to develop diabetes compared with those with optimal body weight [151]. Moreover, weight gain in cats is also associated with IR, which is worse in males and older animals [145, 148, 149, 152, 153]. However, there are no available reports on the effect of sex steroids on weight and IR [143]. In early stages of feline diabetes, IR can be reversed by weight loss [154] and diabetes remission has been reported (*reviewed by Gostelow et al.*) [155].

Some similarities to human obesity and T2D are observed, comparing findings from obese and lean cats. Indeed, the expression of insulin signalling genes (*IRS-1, IRS-2, PI3-K*...) in liver and skeletal muscles is lower in obese cats [156]. Adipose tissue-derived hormones' mRNA expression, secretion and action are affected, as is the case of adiponectin (anti-inflammatory and insulin sensitising hormone), which decreases with obesity and DM [152, 157]. Cats with diabetes show higher blood leptin concentrations [157, 158], although no differences have been found in mRNA expression when compared to lean cats [157].

Obesity is also considered a chronic inflammatory process, where adipose tissue secretes pro-inflammatory cytokines, such as TNFa, which is increased in the fat of obese cats [159].

However, not all obese cats become diabetic. In this sense, the most suitable explanation is probably the existence of secretory dysfunction of the β-cell, but available data are insuficient to draw conclusions, for the time being [143]. Amyloid deposition, glucotoxicity and lipotoxicity have all been proposed as possible mechanisms [143, 160]. Glucotoxicity has been shown to damage β-cell function and survival in cats [161]. In a recent study, after ten days of prolonged, induced

hyperglycaemia in healthy cats, 50% of their β-cell mass was lost. Apoptosis and systemic inflammatory responses were detected [162]. Similar mechanisms have been described in humans [163, 164]. The role of lipotoxicity in feline diabetes, as in humans, seems to enhance the effects of glucotoxicity [143, 162].

The most accepted hypothesis to explain the loss of β-cell function in cats is their destruction by amyloid deposition. Obese cats with IR [165], as humans and other primates [166, 167] show amyloid deposits, which can, however, also be found in healthy cats [168]. On the other hand, these deposits are absent in other species that like dogs and rodents [166, 169].

e) The Diabetic Dog

In 1921, Marjorie, an induced diabetic crossbreed dog, received insulin therapy for the first time, paving the way for treatment of human patients [99].

Although the first report of spontaneously diabetic dogs was published in 1951 [170], canine DM (cDM) was only very recently proposed as a spontaneous animal model of human autoimmune diabetes [171]. This is supported by clinical presentation, the existence of both purebred and outbred animals, shared environment and common pathogenesis, with the presence of auto-antibodies and genetic risk [171, 172]. Our shared environment represents an important advantage, when compared to other animal models of DM and special interest in comparative research can be undertaken to investigate the interaction between genetic and environmental factors. Despite the importance of rodent models, dogs are genetically closer to humans.

(1) Clinics, aetiology and epidemiology of canine diabetes

Diabetic dogs show classical symptoms of T1D: polydipsia, polyuria and weight loss, associated with hyperglycaemia and glucosuria [143]. Diagnosis is mostly based on these and other clinical signs, like cataracts (10-20% cases), coexisting with hyperglycaemia (>200 mg/dL) or increased fructosamine (>350 micromol/L) and glucosuria **(see figure 1)**. Clinical history, physical examination and diagnostic tests are crucial to help differentiate the type of cDM, needed to provide the appropriate treatment and to determine prognosis. Treatment includes insulin injections in most of the cases, administered once or twice daily, but also exercise, dietary modifications and sterilisation of intact females [143].

Types of cDM are established based on aetiology [143, 171, 172], following the most accepted classification, adapted from human classification [2]. Although certain differences among populations exist [173, 174], the most common form is insulin-deficient diabetes (IDD), which is similar to human T1D. Other common types of cDM are dioestrus and secondary DM (to pancreatitis or hyperadrenocorticism).

Figure 1. Clinical manifestations of canine diabetes. A: Cataracts in a neutered male griffon with autoimmune diabetes. B: Female with diabetes secondary to hyperadrenocorticism, incipient cataracts and evident abdominal comedones. C: Cachexia in an entire male with juvenile diabetes. D: Duodenum and marked atrophic pancreas, findings that are also common in diabetes. Pictures: Dr. Melián & Dr. Quesada.

The role of immunity in cDM is supported by the presence of islet cell- (ICA), GAD65-, IA2- and proinsulin antibodies [171, 175-179], although a recent report questions this role [180]. An association between cDM and the MHC class II genes, the *Dog Leukocyte Antigen* (*DLA*), has also been demonstrated and protective and risk haplotypes have been identified [181]. *DLA* haplotype distribution in different dog breeds has been compared to that found for HLA in different human ethnic groups [172, 181]. Age of onset ranges from 5 to 12 years in most studies [173], although rare, juvenile forms of cDM also exist [178]. Autoimmune processes, clinical presentation and middle-age onset, suggest that cDM could be a model for human latent autoimmune diabetes of the adult (LADA) [15, 172].

Prevalence of cDM ranges between 0.0005-1.5%, depending on both geographical regions and breeds [171, 173, 174, 182-186], suggesting gene-environment interactions. However, most epidemiological studies have been performed in northern European and North American populations [173, 174, 182, 184, 185], with specific cultural and demographic characteristics.

Attending to these reports, the most frequently affected breeds are Australian terrier, Keeshond, Schnauzer, Miniature Poodle, Samoyed and Cairn Terrier [173, 182, 183, 186]. Other breeds, like Boxer and German shepherd, seem to be protected against the development of cDM [181, 186].

One of the purposes of the present thesis was to evaluate the epidemiology and clinical features of a diabetic dog population in Gran Canaria.

The methods and results of this characterisation are presented in section V:

'Characterisation of canine diabetes in a population from the Canary Islands'

(2) Glucose monitoring and disease management in the diabetic dog

Management of cDM requires a broad clinical approach, including symptom assessment and laboratory and point-of-care (POC) tests. Total blood or plasma glucose concentrations (>200 mg/dL) and glucosuria confirm the diagnosis in a dog with the classical clinical symptoms of cDM [143].

The treatment of cDM is mainly based on porcine insulin, administered subcutaneously, with some other strategies depending on the type of cDM, such as oophorectomy and histerectomy for entire female dogs. Once clinical signs are evident and permanent, insulin is needed [187].

Initial insulin regime usually starts with 0.3-0.5 UI/Kg and is adjusted thereafter, according to individual response, assessed by serial glucose measurements. The most frequently used methods, both for owners and practitioners, are urine test strips [188] and blood glucose determinations performed by specialised laboratories or by domestic, or POC, portable blood glucose meters (PBGM). Continuous blood glucose monitoring has also been tested [189, 190], but its clinical use is still exceptional.

For long-term evaluation of glucose control, glycated protein assays are also available [191, 192]. Fructosamine, the most frequently used measurement, reflects glucose control for the previous 2-3 weeks and may allow distiction of DM from stress hyperglycaemia. It is considered a standard diagnostic and monitoring tool for cDM.

Self-monitoring with PBGMs in humans is pivotal in the management of insulin-treated diabetes, where frequent glucose measurements and insulin dose adjustment have proved to reduce disease complications [193]. In veterinary medicine, adequate glycaemic control increases survival in dogs with cDM. In this context, PBGM allow owners and practitioners to obtain glucose determinations easily and make immediate therapeutic decisions.

Specific PBGMs have been developed for small animals [194], which offer reliable glucose measurements and might be the best option for portable glucose meters in dogs, but are not widely available. Thus, those initially developed for humans are commonly used in cDM and other

animals, too, although there has been some concern regarding their reliability [194-201]. Furthermore, more recent, presently available PBGMs had not been systematically assessed before and a need was detected during the clinical management of the dogs included in the study mentioned above.

Therefore, we tried to answer the question of which widely available PBGM could be recommended for blood glucose monitoring in dogs.

The methods and results of this evaluation are presented in section V:

'*ISO-Based Assessment of Accuracy and Precision of Glucose Meters in Dogs'*

3.2 Induced diabetes models

Induced animal models are probably the most frequently used type of DM models, since they are easier to generate than the spontaneous models. They fit adequately to many research purposes, which are less focussed on the autoimmune process and more on hyperglycaemia itself, obesity or the metabolic syndrome.

Two major ways of developing diabetes are described for induced models. Surgical models consist of partial or complete ablation of the pancreas, whereas non-surgical models can be generated by the administration of toxic substances with β-cell trophism (i.e. alloxan or streptozotocin (STZ), hyper-caloric diets (e.g high-fat, or high-sucrose), immunosuppresors or even by viral infections (e.g Coxsackie B virus, Kilham rat virus) [202-208]. Furthermore, both surgical and non-surgical methods can be combined to mimic special or more complex types of DM (e.g combining high-fat diet with STZ, or under specific physiological conditions, like in pregnancy) [209].

a) Surgically induced models

The most common way of inducing diabetes surgically is pancreatectomy, although other methods are available [210]. Total pancreatectomy, which led to the famous discovery of insulin in dogs [98] and was later translated to rodents for the generation of T1D or T2D-like diseases, is less frequently used nowadays, except in specific areas, such as islet transplantation or β-cell regeneration [211-214]. Partial pancreatectomy has been performed in many species (dog, pig, rabbit, rat, mouse) [205, 209, 215], including large animals [216]. After the removal of 50-95% of their pancreatic mass [209, 214], animals develop a mild diabetic state, with hyperglycaemia and IR, which can be combined with many other physiological conditions (obesity, pregnancy, etc). In fact, it has been used to study intrauterine developmental disorders in gestational diabetes [217, 218]. The development of mild, insulin-independent hyperglycaemia (from 150 to 200 mg/dl) is the

main advantage of this technique [209]. However, it is invasive and requires a high degree of expertise, since up to 20% post-surgical mortality has been described, and severe hypoglycaemia and pancreatic exocrine insufficiency are also common [209, 219].

b) Chemically, toxic or drug induced models

Several drugs and toxics with affinity for the β-cell [2, 103, 220, 221] have been used to develop models of DM. The most commonly chosen are STZ and alloxan, although others, like vacor or dithizone, have also been used [103, 222, 223].

Administered at different doses, intervals or routes (i.e. intraperitonial vs intravenous), both STZ and alloxan may lead to disorders mimicking T1D, T2D, pre-DM or other conditions [209, 224]. If high doses are applied, the model mimics human T1D by irreversible loss of β-cells. If lower doses are given, mild impairment of insulin secretion, a condition resembling T2D, is obtained [207].

A single, large dose (i.e. 200 mg/Kg for mice) of STZ leads to fulminant diabetes by direct toxic effects, but of different severity depending on the route of administration and animal susceptibility [217, 226, 227]. On the other hand, the same drug, administered in multiple, small doses (i.e. 40 mg/Kg for mice) for several days (1-5 daily applications), causes a process that resembles human T1D, with immune destruction and insulinopenia [225, 226]. This application has shed light on the immunological pathways of insulitis and β cell death [227, 228]. However, unlike spontaneous DM, the disease develops even without the intervention of T and B lymphocytes [226].

Alloxan is a synthetic pyrimidine derivative first synthesised in the XIX century [229], that causes necrosis, *via* a selective, toxic effect on β-cells, like STZ [233, 234][230, 231]. Its use was first reported in rabbits (1943) [105], where STZ is inefficacious. On the other hand, Guinea pigs seem to be resistant to alloxan *(reviewed by Srinivasan et al.)* [105].

c) Diet induced models

The ongoing epidemics of obesity, metabolic syndrome and T2D are mainly attributed to over-nutrition and reduced physical activity. In animals, dietary manipulation is commonly employed to study this human situation. Many species are prone to develop diet-induced IR and glucose-impairment: rodents, cats, squirrels, pig, apes..., but rodents are the most extended for research purposes [232]. Since the first experiments with high-fat diets (70% content) were performed in rats in the 1940s [233], diet-induced models have become one of the most widespread models for the study of T2D [234].

Rodent diets are formulated attending to their daily caloric needs (10-15 kcal/day) and are provided ad libitum. Standard "chow diet" usually consists of 65-70% carbohydrate, 20-25%

vegetable proteins and 5-12% fat, for an approximate caloric intake of 2900 kcal/Kg. Increasing the proportion of fat (up to 85% of total caloric content) or simple carbohydrates (30-60% caloric intake of i.e. sucrose), or adding high salt or cholesterol to the diet, provides a variety of animal models to study obesity, IR, hypertriglyceridaemia, hypertension, atherosclerosis and other related disorders.

The most established rodent model in this context is the C57BL/6J mouse, given a hypercaloric, high-fat (4730kcal/Kg, 40% fat) or very high fat (5240kcal/Kg, 60% fat) diet. Variable, strain-dependent response to dietary intervention has been reported [202, 235].

Additionally, dietary intervention on genetically manipulated animals gives a wide range of models with known genetic roles, or can be combined with chemical induction of DM [234].

(1) The High-Fat Diet Induced Diabetic C57Bl/6J mouse (HFDID)

Marked obesity **(see figure 2)**, IR, hyperinsulinaemia, hyperglycaemia and glucose intolerance are the main features of HFDID C57Bl/6J mice [202, 236]. They also manifest peripheral leptin resistance [108], and a prolonged hyperglycaemic response to stress [202].

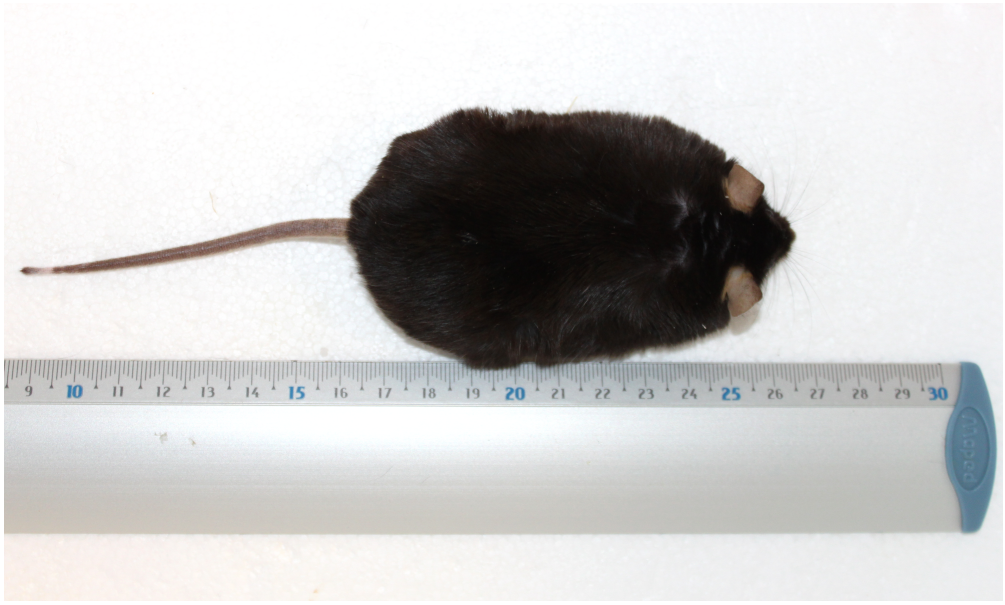

Figure 2. C57Bl/6J HFDID female with evident obesity, after being fed with a 60% fat content diet during six months.

Following the most common protocol with young animals (4-6 weeks of age), the development of hyperglycaemia occurs over long periods of time, since β-cells maintain high insulinaemia to compensate for IR [237]. At least 10-12 weeks are necessary to obtain an evidently

obese and diabetic phenotype, and although the strain is considered to be obesity-prone, individual variability exists [232, 238].

The main advantage of this animal model is that it reflects the interaction between complex genetic and environmental risk factors, compared to the Lep$^{ob/ob}$ mouse (C57Bl/6J background), which is extremely genetically determined [105]. In this sense, the HFDID C57Bl/6J mouse has been used in the study of impaired glucose tolerance [239], T2D and its complications [240-242] as well as for preclinical drug testing [241, 243].

(2) Evaluation of glucose metabolism in the C57Bl/6J mouse

Characterisation of glucose metabolism in mice is mainly performed using FPG, glucose tolerance tests (GTT) and the insulin tolerance test (ITT), but model assessments and clamps can be applied, too. Although all the procedures are extensively applied in rodent phenotyping, there is a lack of standardisation among protocols, glucose/insulin doses, analytical devices employed, etc. [234, 244, 245]. No single test is suitable under all circumstances and each one should be applied following uniform criteria [245].

Initial screening is based on single blood glucose measurements, usually performed with a "human" PBGM. FPG concentrations represent an estimation of IR, especially if simultaneous insulinaemia is measured. Both parameters are used to calculate the homeostatic model assessment (HOMA) indices, that estimate IR (HOMA-IR) or β-cell function (HOMA-%B) [246-248]. In humans, this equation shows strong linear correlation with the euglycaemic, hyperinsulinaemic clamp, the gold-standard in the assessment of glucose metabolism [248], but in rodents it has not been validated, although it is used for group comparison.

Glucose tolerance tests (GTT) consist of the administration of a standardised glucose load and the measurement of glycaemic response thereafter. Performed orally (OGTT), intravenously (IVGTT) or intraperitoneally (IPGTT), the route of administration can be chosen to appraise certain aspects, such as gut absorption or incretin activity.

The reviewed literature [234], demonstrates a lack of consistency among the different protocols for GTT, and errors that affect rodents' normal physiology and interfere with the results are common [234]. One of the most frequent is an excessively long fasting period (18h, overnight), which leads to weight loss and hepatic glycogen depletion [249]. The fact that mice are nocturnal eaters intensifies this catabolic state [250]. In this sense, the most carefully standardised protocol for GTT in mice was described in 2008 by Andrikopoulos et al. [244], establishing that in conscious or anaesthetised animals, after a fasting period of 6 h (from 8:00-14:00), the most homogeneous results between groups are obtained following an oral glucose load of 2 g/Kg [244]. The area under

the curve (AUC), peak, and baseline glucose values are the most established glucose variables in the GTT.

The insulin tolerance test (ITT), also known as insulin sensitivity test, consists of the evaluation of glucose response to a known insulin load, intraperitoneally (IPITT) or intravenously (IVITT), after a fasting period. Baseline glucose, glucose decline, AUC and nadir (lowest glucose value) are the most frequently used variables in this case, to obtain an estimation of IR [245].

The hyperinsulinaemic-euglycaemic clamp is the gold standard for the evaluation of IR. Its name derived from the "clamping" on glucose at a desired fixed concentration (e.g. 100 mg/dL). Glucose infusion rate (GIR), the glucose amount necessary to maintain euglycaemia in the presence of a constant insulin infusion, is the main outcome of the test, and is inversely related to IR.

In addition to these procedures, long-term evaluation of glucose metabolism is also performed in mice by means of HbA1c [251-253], which, in humans, also serves as a diagnostic test for DM **(see table 1)**. Different methods have been used to measure HbA1c in mice: from ion-exchange high-performance liquid chromatography (HPLC) to antibody based approaches [251, 254]. However, a lack of consistency among studies is present and there is no cut-off point defined for DM diagnosis. From the point of view of animal welfare [95], standardisation of a test such as HbA1c would be very relevant: POC analysers could provide reliable results with small, single blood samples (1 μL) [251].

In conclusion, for correct glucose monitoring in mice, standardisation of all of these procedures and analyses is desirable, since their variability makes comparisons between studies difficult. This fact complicates the *per se* demanding inter-species extrapolation [234].

During the performance of the present thesis, HbA1c determinations from different studies in HFDID C57Bl/6J mice were correlated with other variables related to glucose metabolism. The methods and results of this study are presented in section V:

'*Can we use HbA1c as a diagnostic tool in mouse models of diabetes?'*

Main advantages of induced or spontaneous models	**Induced Diabetes Models**			**Spontaneous Diabetes Models**				
	Some methods can be combined to develop DM with different severity in specific situations. Larger animals can be used, as more adequate for surgical or other procedures. Rodents are cheaper to maintain and have a short life-cycle.			Presumed to share mechanisms of disease with the human condition, especially in polygenic models. Rodents: Minimum variability resulting from inbreeding and control of environmental factors. This rends to a minimum sample size. They also have a short life-cycle. Pet models: share human environment. The role of the owner can be an advantage or a disadvantage.				
Specific models	**Surgical Partial Pancreatectomy**	**Chemical STZ/Alloxan**	**Diet C57Bl/6J HFDID**	***Lep*$^{ob/ob}$, *Lep*$^{db/db}$ mice**	**NOD mouse BB-DP rat**	**ZFR, ZDF rats**	**The diabetic Cat**	**The diabetic Dog**
Type of DM modelled	T1D/T2D	T1D/T2D	Obesity/T2D	Obesity/T2D	T1D	Obesity/T2D	T2D	T1D/GDM
Advantages by specific model	Multiple species. Versatility to adjust to type and severity of DM Mild insulin-independent hyperglycaemia can be reached: good model of human T2D due to reduced β-cell mass Rapidly generated	Multiple species Versatility to T1D or T2D Insulin not needed (dose-dependent). Reduced costs Easy to perform Selective loss of β-cell (STZ/alloxan), rest of islet cells remain intact Fast generation.	Multiple species Complex genetic and environment interaction Similar to humans, resulting from overnutrition and insulin resistance	Phenotype of metabolic syndrome, similar to the human condition Pancreas also shows similar patterns	Mechanisms close to human T1D: autoimmunity, insulitis, islet loss MHC role demonstrated. Subclinical insulinopaenia due to β-cell destruction. NOD: No insulin required (easy to maintain)	Phenotype derived by a single mutation. Phenotype of metabolic syndrome, similar to the human condition	Similar mechanisms of human T2D, with environmental factors and polygenic interactions Inflammatory response β-cell loss with gluco- and lipotoxicity roles Common genes to human T2D	Gene-environment interaction Clinical presentation similar to T1D Similar autoantibodies Genetic risk demonstrated (MHC role) Similar pattern of human β-cell loss Other subtypes of DM are also modelled, like GDM
Disadvantages by specific model	Highly invasive High degree of expertise required Loss of other islet cells (affected counter regulatory response) High mortality Pancreatic exocrine insufficiency	In T2D models hyperglycaemia is reached by β-cell destruction Reversible effects Toxicity on other organs and handling Variability on the development of hyperglycaemia, also by species Absent of autoimmune processes	Heterogeneous response Only mild hyperglycaemia in non-genetically modified animals Time-consuming	Highly inbred and genetically determined Phenotypes from a monogenic mutation in leptin or its receptor Loss of human heterogeneity Expensive, Limited Insulin required	BB-DP: insulin required, high mortality due to fatal ketoacidosis High mortality due to ketosis Require sophisticated maintenance NOD: marked sexual dimorphism (only 60% males develop DM)	ZFD: marked sexual dimorphism Males more diabetic High mortality due to ketosis Females closer to the ZFR, mostly used as controls for male littermates (potential source of bias)	Mechanisms of IR still not fully understood Not maintained in repositories Difficult to obtain	Mechanisms of β-cell loss still not fully understood Not maintained in repositories Difficult to obtain

Table 3. Advantages by group, and advantages and disadvantages of each described animal model.

4. Applications of the animal models of diabetes

4.1 Pathogenesis of Type 1 diabetes: Post-translational protein modifications

The causes leading to the autoinmune destruction of ß-cells in T1D still remain unclear and part of this thesis focuses on this aspect.

Posttranslational modifications (PTM) of proteins are involved in the development of new antigenic epitopes. They can trigger autoantibody production and T-lymphocyte activation and could initiate autoimmune diseases where new tissue-specific epitopes are targeted by the immune system [57, 58, 255, 256]. In T1D, several modified autoantigens have been shown to trigger a more intense immune response than the native protein. Indeed, antibodies from patients with T1D bind to modified GAD65 with higher affinity than to native GAD65 [257, 258], and T-lymphocytes show increased recognition of the modified A-chain of human insulin [259]. Furthermore, insulin is prone to in vitro isomerisation, i.e, spontaneous conversion of asparagine (Asn) or aspartic acid (Asp) residues into isoaspartyl (IsoAsp) at susceptible protein sites [260]. Some autoimmune diseases, such as systemic lupus erythematosus [261] and celiac disease [256, 262], show immune responses directed toward post-translationally modified self-antigens. A recent report showed that post-translationally modified (oxidised) insulin bound to serum autoantibodies present in T1D patients more frequently than native insulin [263].

The repair enzyme Protein-L-isoaspartate (D-aspartate) O-methyltransferase EC 2.1.1.77 (PIMT) [264], encoded by *PCMT1* [265], catalyses the conversion of IsoAsp back to normal aspartyl residues and prevents the accumulation of damaged proteins **(see figure 3)**.

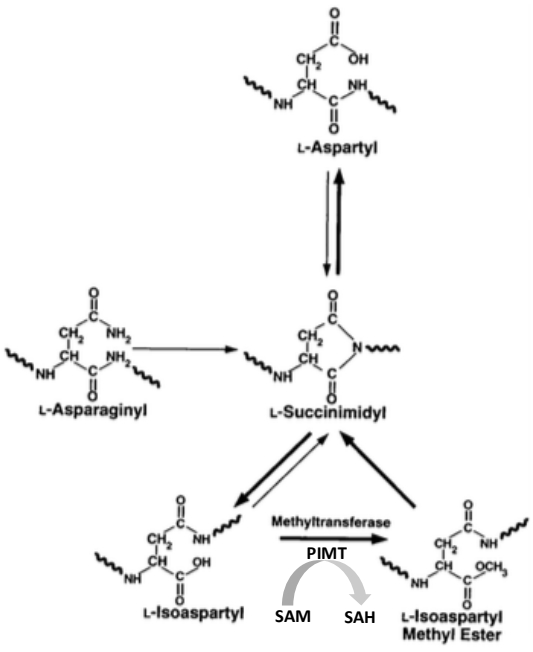

Figure 3. Isoaspartate (IsoAsp) formation and repair by PIMT. Spontaneous deamidation and isomerization aspargine and aspartic acid residues in proteins (i.e. insulin) generate L-isoaspartyl linkages. PIMT repairs this Isoasp residues by methylation donored by S-adenosyl-L-methionine (SAM), resulting in the intermediate S-adenosylhomocysteine (SAH).

(Adapted from: Kim *et al., 'Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice.' PNAS* 1997)

In the human pancreas, PIMT is selectively and highly expressed in ß-cells [266] and, in families with T1D, a SNP in *PCMT1* (rs4816, allele A), associated with increased PIMT enzyme activity, tended to be less frequently transmitted to the affected than to the unaffected offspring [267]. *Pcmt* knock-out mice exhibit hyper-responsive T cells, and transfer of their bone marrow to irradiated wild-type mice leads to autoimmunity [268]. Furthermore, in diabetes-prone BB/OK rats, the administration of the PIMT-inducer CGP3466B delayed the onset and reduced the severity of diabetes and was associated with preserved ß-cell mass [266].

The deletion of PIMT in mice, results in the development of fatal epileptic seizures associated with the accumulation of damaged proteins in the brain **(see fig. 4)** [269]. Rescue experiments show that transgenic expression of PIMT effectively cures the PIMT-deficient mice [270]. Also, $Pcmt^{-/-}$ mice show a higher peak level of blood glucose during a GTT, suggesting mild impairment in glucose tolerance [271].

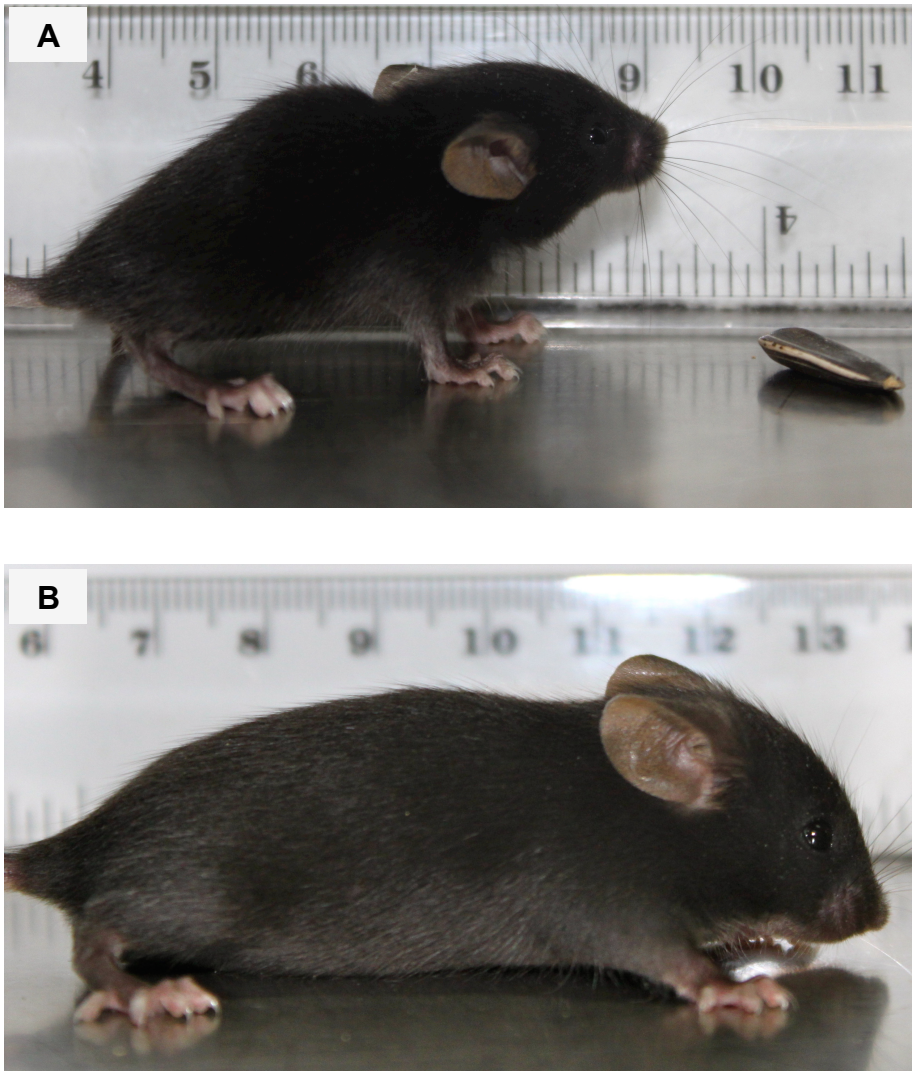

Figure 4. PIMT KO (A) and its littermate wildtype (B) (males, 43 days of age). KO show growth-delay, kyphosis, cachexia, macrocephaly, and abnormal behaviours such as anhedonia, although heterogeneity is seen.

In the present Thesis, the role of the repair protein PIMT was evaluated in spontaneous dogs with diabetes, together with results from human patients with T1D and T2D. In addition, the *Pcmt*$^{-/-}$ mouse were characterised for glucose metabolism.

The methods and results derived from this evaluation are presented in section V:

'Role of the repair enzyme Protein Isoaspartyl Methyl-Transferase in Type 1 Diabetes'

4.2 Natural treatments as a source of new bioactive compounds for diabetes

Traditional medicine and ethnomedical approaches can serve as foundation for drug discovery [91]. They are mainly based on the use of natural products that also are, or have been, widely employed for many diseases, either as complementary or alternative remedies to conventional medicine [272]. These products are a potential source of treatments also for DM and related disorders. Indeed, the number of traditional remedies claimed to have glucose-lowering properties is high [272-279], but most have not been evaluated for efficacy or safety. Moreover, the used of complementary and alternative medicine therapies for diabetes is common all over the world [280, 281]. Thus, research on and standardisation of natural treatments is needed [277, 282].

In fact, some of the currently used glucose-lowering agents (e.g metformin and exenatide) were originally discovered from natural sources [283, 284]. Once a bioactive product is identified through ethnomedical or ethnopharmacological approaches (i.e. oral testimonies and interviews, population based questionnaires...) [285], *in vitro* and/or *in vivo* screening is recommended. In the case of DM, due to the complexity of the disease, *in vivo* evaluations are required [93].

In the present thesis, we evaluated several natural products and extracts *in vivo*, using an animal model of DM, the HFDID C57Bl/6J mouse [91]. These studies are described in section V.

"*Uromastyx acanthinura as a natural treatment in a mouse model of type 2 diabetes*"

"Acute and sub-chronic effects of Aloe vera barbadensis in a mouse model of type 2 diabetes"

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HYPOTHESIS

III HYPOTHESIS

By using spontaneous and induced animal models of diabetes, different hypothesis were tested:

1. Since most epidemiological and clinical studies of canine diabetes have been performed in northern populations, the study of a canine population from a southern region may add valuable information to the understanding of the pathogenesis of the disease.

2. As previously suggested by Catchpole and colleagues, dogs with spontaneous diabetes can serve as a model for human type 1 diabetes.

3. Protein Isoaspartyl Methyl-Transferase (PIMT), a repair enzyme of posttranslational protein modifications, may play a role in the pathogenesis of type 1 diabetes.

4. The C57Bl/6J mouse with diabetes induced by a high fat diet can be used to test natural treatments for diabetes.

5. A cross-over experimental design could be used to assess the short-term effects of potential glucose-lowering substances and allow for a reduction in the number of animals required.

6. Both botanical and zoological products, traditionally used to treat diabetes, could have glucose-lowering effects.

7. The choice of portable blood glucose meter is relevant in the management of canine diabetes.

8. Specific ISO criteria, defined for the evaluation of portable blood glucose meters in humans, can be adapted and applied to veterinary medicine.

9. HbA1c measurement with a Point-Of-Care analyser can be a useful tool in the characterisation of diabetes in mice.

AIMS

IV AIMS OF THE STUDY

1 To characterise the local canine diabetic population

a. Identifying and sampling dogs with spontaneous diabetes
b. Clinically analysing, DLA-typing, measuring autoantibody reactivity and performing histopathology studies in samples from the studied population

2 To evaluate the potential role of post-translational protein modifications and the repair enzyme PIMT in the pathogenesis of T1D

a. Assessing *PCMT1* genotypes and expression and PIMT protein concentrations and activity in patients with T1D
b. Assessing *PCMT1* expression in blood and PIMT immunoreactivity in pancreas of dogs with spontaneous diabetes
c. Phenotyping a *Pcmt1*$^{-/-}$ model for glucose metabolism

3 To evaluate new treatments for type 2 diabetes using natural products from our region

a. Evaluating the acute effects on glucose metabolism of both zoological and botanical products in the HFDID C57Bl/6J mouse
b. Evaluating the long-term effects on glucose metabolism of both zoological and botanical products in the HFDID C57Bl/6J

4 To improve the assessment of glucose metabolism in the animal models studied

a. Evaluating nine portable blood glucose meters in spontaneously diabetic and non diabetic dogs, based on the ISO 15197:2013
b. Evaluating the diagnostic role of HbA1c in the high fat diet induced diabetic C57Bl/6J mouse

RESULTS AND DISCUSSION

V RESULTS AND DISCUSSION

1. Paper 1

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'Characterisation of canine diabetes in a population from the Canary Islands'

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Keywords
Spontaneous diabetes, canine diabetes, autoimmune diabetes, dioestrus diabetes

Abbreviations
Canine Diabetes Mellitus (cDM), Type 1 Diabetes (T1D), Dioestrus Diabetes (DD), GAD65, IA2,

Abstract

Background

Canine diabetes mellitus (cDM) has been proposed as a spontaneous animal model of human autoimmune diabetes (Catchpole et al., 2005). Previous epidemiological and histological studies on cDM have mostly been performed in northern populations, with specific cultural and demographic characteristics. The study of a canine population from a southern country may add valuable information to the understanding of the pathogenesis of canine diabetes mellitus.

Objectives

Our purpose was to evaluate the epidemiological, clinical and histological features of a diabetic dog population from the Canary Islands, with special focus on immune-mediated disease.

Animals

Diabetic dogs attending the Veterinary Teaching Hospital of the University of Las Palmas de Gran Canaria (ULPGC) from January 2009 to January 2012 were included in the study. Previously diagnosed and new cases were considered.

Methods

Prevalence was calculated as number of cDM/total number of dogs attending the hospital during that period and incidence as newly diagnosed cases divided by the total number of dogs seen per year. Clinical features were analysed and recorded. Blood samples were drawn from most dogs and serum and genomic DNA was obtained. All diabetic cases were clinically classified for the different types of cDM. Anti-insulin antibodies were assessed in all insulin treated dogs. In those with suspected immune-mediated cDM, Dog leukocyte antigen (DLA) sequences were obtained and evaluated, and canine anti-glutamate decarboxylase 65 (GAD65) and canine anti-tyrosine phosphatase (IA-2) were assessed. Pancreata from nine diabetic dogs were examined and compared with **6** controls.

Results

Twenty-nine diabetic dogs were identified in a population of 5213 dogs (mean population: 1738 dogs/year; mean prevalence of diabetic dogs/year: 0.53%; mean incidence/year: 0.33%). Most diabetic cases were female (79%) and intact (87% of females, 83% of males). Clinically, diestrus (58.6%) and immune-mediated (17.2%) cDM were the most frequent types. Anti-GAD65 or anti-IA2 antibodies were positive in two out of five cases. cDM was distributed among eleven breeds and the Andalusian wine-cellar rat-hunting was first described as at high risk of diabetes. Asevere reduction in the number of pancreatic islets and β-cell mass was seen, with vacuolation of islet cells and ductal epithelium, among other lesions.

Conclusions and clinical importance

These results, obtained in a small population, support the heterogeneous pathogenesis of cDM, where autoimmunity has a role in a small proportion of dogs. In our population, where neutering is less common than in other populations, dioestrus cDM was the most frequent type. Further population-based studies are needed to assess this heterogeneous nature, and a proper clinical characterisation of cDM patients would offer a better understanding for each studied process.

Introduction

Canine diabetes mellitus (cDM) has been proposed as a spontaneous animal model of human autoimmune diabetes (Catchpole et al., 2005). If this were proved, the dog could be considered the best animal model of human type 1 diabetes (T1D) (Gale, 2005). In addition, given our shared habitat, comparative research on the interaction between genetic and environmental factors would be of special interest. Previous epidemiological studies on cDM have mostly been performed in northern European and North American populations (Ahlgren et al., 2014; Davison et al., 2005; Fall et al., 2007; Fracassi et al., 2004; Guptill et al., 2003; Mattin et al., 2014; Shields et al., 2015), with varying incidences and breed, age and sex distributions. Discrepancies on the involvement of autoimmunity in the pathogenesis of the disease have also been described (Ahlgren et al., 2014).

Although the role of the pancreas in the pathogenesis of cDM is still poorly understood, β-cell loss seems to be the main underlying mechanism of disease (Catchpole et al., 2005; Shields et al., 2015). Descriptions of pancreatic lesions are heterogeneous, including degenerative changes in pancreatic islets with vacuolisation, insulitis with lymphocytic infiltration and generalised pancreatic inflammation (Ahlgren et al., 2014; Alejandro et al., 1988; Atkins et al., 1979; Atkins et al., 1988; Gepts, 1965; Gepts and Toussaint, 1967; Jouvion et al., 2006; Shields et al., 2015).

The purpose of the present study was to evaluate the epidemiological, clinical and histological features of a diabetic dog population from the Canary Islands, with special focus on immune-mediated disease, for further application to human T1D studies.

Methods

Diabetic dogs attending the Veterinary Teaching Hospital of the University of Las Palmas de Gran Canaria (ULPGC) from January 2009 to January 2012 were included in the study. The study was approved by the Animal Welfare Ethics Committee (*Comité Ético de Bienestar Animal, ULPGC*). Previously diagnosed and new cases were considered. Prevalence was calculated as number of cDM cases/total number of dogs attending the hospital during the given period and

incidence as number of newly diagnosed cases divided by the total number of dogs seen per year. Clinical features including age, breed, sex, neutering state, time of diagnosis, clinical signs and survival time after diagnosis were recorded.

Since sex and neutering state were not available for the whole population, from the 23,710 hospital's clinical records, 14,513 were selected based on the information available indicating they were dogs. A random (computer-based) list was generated and the dogs' owners who were available were consecutively contacted and interviewed until 100 dogs were reached. Each owner was attempted to be reached **5** times before the next available record was chosen. This procedure was repeated until 151 owners were interviewed (not all of them owned dogs, after all), obtaining the neutering state data of 108 dogs.

Blood samples were drawn from 26 diabetic dogs and serum and genomic DNA were obtained (GenElute Blood Genomic DNA Kit, Sigma-Aldrich; St. Louis, US). Anti-insulin antibodies were assessed in 19 insulin-treated dogs (Davison et al., 2003), by ELISA. Diabetic dogs without a history of pancreatitis, hyperadrenocorticism, recent dioestrus or any other known cause of insulin resistance were considered to have idiopathic or immune-mediated diabetes (Catchpole et al., 2005). In this group, genomic DNA was isolated and dog leukocyte antigen (*DLA*) was sequence- based genotyped, as previously reported by *Soutter et al.* 2015 (Soutter et al., 2015). Gene alignment and allele assignment were performed using SBT Engine Software version 2.17 (GenDex, The Netherlands). Serological screening for reactivity to canine anti-glutamate decarboxylase 65 (GAD65) and canine anti-tyrosine phosphatase (IA-2) were assessed by a radio-immuno-precipitation assay (RIA), as previously described (Davison et al., 2008). Antibody positive and negative human sera were included as controls.

Pancreata were available from diabetic and non-diabetic dogs of different breeds, naturally deceased or humanely sacrificed after hospitalisation. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin, or directly immersed in tissue embedding compound (Tissue-Tek, O.C.T. Compund, Sakura Finetek) and stored at -80ºC. Five µm sections were

attached to glass slides (Superfrost Plus, Thermo Scientific, Braunschweig), stained with hematoxylin-eosin and assessed by a pathologist (OQC, blinded to diabetes status). Insulin content was evaluated by immunohistochemistry, after permeabilisation with Triton X-100 0.05% in PBS, with primary rabbit anti-insulin antibody (9168, Santa Cruz Biotechnology) and secondary biotinilated goat-antirabbit antibody (B2770, Thermofisher Scientific). Images were acquired on an Olympus microscope (BX51, Olympus, Tokio, Japan).

Descriptive statistical analysis was performed. Continuous variables are described as mean (SD) or median (range), according to their distribution (Gaussian or non-Gaussian) and qualitative variables, as percentages. The distribution of the number of cases diagnosed by month and season were recorded and analysed for seasonality at diagnosis. Odds-ratios (OR) were calculated and chi-squared analysis was performed to compare breed frequencies between the diabetic and the background population (Microsoft Excel 2011, 14.2.2, Microsoft Corporation, Redmond; IBM SPSS Statistics Version 20, SPSS Inc., Chicago).

Results

A total of 29 dogs with cDM were identified from a mean total population of 1,737 (1,747-1,879) dogs per year (mean prevalence 0.56% and mean incidence per year 0.36%) **(see table 1)**. Median age at diagnosis was 9.5 years (1–14 years) and most diabetic dogs were female (79.3%) and not neutered (87% of the females; 83% of the males). The cDM cases were distributed among eleven breeds, of the 106 breeds included in the whole population **(see table 2)**. Miniature poodle was the most frequent breed among cDM, though fox terrier, dachshund, English cocker spaniel, West Highland white terrier and Andalusian wine-cellar rat-hunting dog were also at high risk for diabetes. On the other hand, some breeds seemed to be less prone to develop diabetes, such as the local breeds presa canario, podenco canario and bardino majorero **(see table 2)**. Mean duration of clinical signs until consultation was 23 [3-150] days.

After 180 personal interviews, we obtained the neutering state data of 110 (non-diabetic) dogs (60% female): 42% of females and 49% of males were not neutered.

Regarding seasonality of the diagnosis for new cDM cases, no significant differences were observed among the different months [2.5 (1-4) cases per month, $p = 0.457$)] and seasons [6 (6-7) cases per season, $p = 0.392$].

Diabetes was clinically classified as dioestrus diabetes (DD) (58.6%), idiopathic/immune-mediated (17.2%), iatrogenic (6.9%) and secondary to pancreatitis (13.8%) or other endocrine disorders (3.4%). The most commonly associated disorders were dermatologic diseases (31%), pancreatitis (14%) and pyometra (10%), although other conditions including renal disease (6.9%) and hyperadrenocorticism (3.4%) were also seen. In two dogs with recurrent atopic dermatitis, cyclosporin A and glucocorticoid treatments were suspected to be the cause of diabetes, and exogenous progesterone, in another case.

Out of the 25 dogs with insulin treatment at sampling [porcine insulin (n=23), NPH insulin (n=1) and detemir (n=1); treatment duration 35 (1-1155) days], anti-insulin antibodies were measured in 19 of the porcine insulin treated dogs, without positive response in any case. From the clinically suspected immune-mediated cases (n=5), autoantibody reactivity was shown in two cases (anti-GAD65, n=1; anti-IA2, n=1) but no previously described diabetes-risk DLA-types were identified **(table 3)**.

Pancreata from nine diabetic and nine non-diabetic dogs were collected between 2009 and 2015 and were available for examination. Macroscopically, four of the nine diabetic dogs showed pancreatic atrophy. Histopathologically, severe reduction in islet number and size was seen in diabetic pancreata. They were sparse and scattered, at times even difficult to detect. Vacuolation of islets cells and ductal epithelium was evident throughout all the tissue sections examined **(fig. 1)**.

In addition, in two cases the interlobular connective tissue was moderate and multifocally infiltrated densely by neutrophils and sparsely by macrophages, lymphocytes and plasma cells, with fibrin, edema and necrotic debris. Occasionally, this inflammatory infiltrate extended into the adjacent exocrine parenchyma, with few and discrete areas of parenchymal lytic necrosis.

Multifocal, mild to moderate perivascular and periductal lymphocytes and plasma cell infiltration was observed in one case. The interlobular septa, pancreatic parenchyma and peripancreatic adipose tissue were also affected, multifocally, by a small to moderate number of lymphocytes and plasma cells and scattered neutrophils and macrophages. In a non-diabetic control dog, the same pattern was observed**.**

Another case showed mild to moderate multifocal necrosis and saponification of the peripancreatic adipose tissue, along with moderate neutrophil and macrophage infiltration, rare multinucleated giant cells, and fewer lymphocytes and plasma cells.

Differences with control dogs were remarkable, as pancreatic islets were much more abundant, evident and easily recognized in the latter, which showed no lesions **(figure 1).**

Controls showed strong diffuse cytoplasmic insulin-immunolabelling in the pancreatic islets. In diabetic cases, insulin positive-cells were scant, with weak, or even absent immunolabelling. An insulin-staining grade was identified among all diabetic dogs. The two cDM cases secondary to hyperadrenocorticism showed the most intense immunolabelling among the affected dogs **(fig. 1)**, with mild to moderate granular cytoplasmic immunorreaction.

Discussion

A total of 29 dogs were diagnosed with cDM, with a mean prevalence of 0.56% and an incidence of 0.34% in the three year-period assessed. Most were non-neutered females with a clinical history supporting dioestrus diabetes, although a percentage of idiopathic/immune-mediated cases were identified. Of the 5 dogs selected by their clinical presentation, two showed autoantibody reactivity, and new DLA genotypes were identified.

cDM is a common endocrine disease also in our region, even though only data from the teaching hospital are considered. Age at diagnosis, prevalence and incidence did not differ much from previous studies (Davison et al., 2005; Fall et al., 2007; Fracassi et al., 2004; Mattin et al., 2014). However, a high frequency of DD was seen, which could be explained by the high

proportion of entire females found in our canine population, compared with others (Davison et al., 2005; Mattin et al., 2014). Indeed, these results agrees with previous studies in regions where neutering is less frequent (Fall et al., 2007), and differ form studies that evaluated canine populations with higher percentages of neutered dogs, where DD is considerably less common (Davison et al., 2005; Mattin et al., 2014).

When compared to previous studies, the breed distribution and susceptibility to diabetes remains consistent for some breeds (Poodle, Fox terrier, Yorkshire terrier) but differs for others (German shepherd, Griffon or Dachshund). We should bear in mind however, that breed spectrum varies substantially among regions and that genetic predisposition can be population-specific (Davison et al., 2005; Fall et al., 2007; Fracassi et al., 2004; Guptill et al., 2003; Marmor et al., 1982; Mattin et al., 2014). On the other hand, for some high-risk breeds, the present study shows even higher risks than previously described (Fracassi et al., 2004; Guptill et al., 2003; Marmor et al., 1982; Mattin et al., 2014). The main singularity within the present population is the identification of the Andalusian wine-cellar rat-hunting dog identified as a high-risk breed. In addition, four local, previously unstudied breeds did not show any case of the disease: presa canario, podenco canario, bardino majorero and Spanish water-dog. Nonetheless, our results are inferred from a small population, belonging to a reference centre, and should thus be regarded with caution. Also, lack of accuracy for breed characterisation during clinical recording can be a factor interfering with the assessment of breed distribution.

No seasonal patterns were found for the diagnosis in the present study, as has been demonstrated for cDM and human T1D in some (Ardicli et al., 2014; Atkins and MacDonald, 1987; Davison et al., 2005; Hanberger et al., 2014; Mattin et al., 2014; Moltchanova et al., 2009; Patterson et al., 2009; Samuelsson et al., 2013), but not all previous reports (Guptill et al., 2003; Jensen et al., 2015), including human data from the Canary Islands (Carrillo-Domínguez, 2000). Population specific characteristics and small samples with low statistical power are possible explanations and time elapsed between initial clinical manifestations and diagnosis could add to the imprecision in

the date of onset. When entire females are present, spring peaks in diagnosed cases are hypothesised to be related to increased oestral activity, in the context of onset of DD (Fall et al., 2007). In the present case, the high proportion of entire females does not seem to affect this pattern.

Autoimmunity seems to be infrequent in the described population, and clinical characterisation probably increased the diagnostic yield in this case. Different reasons can account for the lack of reactivity for a-GAD and a-IA2, such as the prolonged period between diagnosis and blood sampling, although the a-GAD65 positive dog was sampled 2.4 years after diagnosis. Furthermore other antibodies, such as anti-pro-insulin or anti-zinc transporter 8, were not assessed in the present study and have been positive in other studies (Ahlgren et al., 2014; Davison et al., 2011; Merger et al., 2013). Indeed, perhaps additional autoimmune epitopes involved in cDM aetiology are still to be discovered. The absence of anti-insulin reactivity is in agreement with previous authors and is explained by the homology between canine and porcine insulin (Davison et al., 2003).

The DLA genotypes observed were unknown and consequently not previously associated with cDM. They might be population-specific, but since no other non-diabetic or diabetic dogs were genotyped, no further conclusions can be drawn at this point.

Cyclosporine A and glucocorticoids can cause insulin resistance and hyperglycaemia (Kovalik et al., 2011; Murray et al., 2009). In the same way, progesterone therapy can induce iatrogenic DD (Selman et al., 1994). In the cases described in this report, maybe the repeated administrations of these therapies in a pre-diabetic situation and/or in high-risk breeds, could trigger the development of diabetes.

A reduction in the number of β-cells, lymphocytic infiltration, insulitis, pancreatic inflammation and β-cell vacuolisation are the most important, previous pathological findings in diabetic dogs, although heterogeneity and discrepancies are found among studies (Ahlgren et al., 2014; Alejandro et al., 1988; Atkins et al., 1979; Atkins et al., 1988; Gepts, 1965; Gepts and

Toussaint, 1967; Jouvion et al., 2006; Shields et al., 2015). The present diabetic pancreata show severe reduction in the number of islets and β-cell mass, represented by sparse, isolated, insulin-positive cells in most cases. On the other hand, control pancreata show numerous islets, mostly constituted by β-cells. Time since diagnosis and cause of cDM seemed to determine the level of β-cell loss. For the majority of the cases, cDM was related to a drastic β-cell loss, reinforcing previous findings and the common proposed pathogenesis with human T1D (Catchpole et al., 2005; Shields et al., 2015).

Finally, these singular findings obtained in such a small population, suggest that more population-based studies in different regions are still necessary to assess the heterogeneous nature of cDM.

Table 1

Number of dogs seen at the hospital and distribution of diabetes between 2009 and 2011

Year	Total number of dogs	Number of diabetic dogs	Newly diagnosed diabetic dogs	Prevalence (%)	Incidence (%)
2009	1747	11	4	0.63	0.23
2010	1587	5	5	0.32	0.32
2011	1879	13	10	0.69	0.53
Total/Annual Mean (SD)	5213/1738 (146.2)	29/9.7 (4.2)	19/6.3 (3.2)	0.56 (0.20)	0.40/0.36 (0.16)

Table 2

Breed distribution among diabetic and non-diabetic dogs.

Breed	Diabetic dogs per breed N (%)	Non-diabetic dogs per breed N (%)	OR (Cross-Breed as reference)	95% CI	P value (Pearson´s Chi squared)	OR (whole population as reference)	95% CI	P value (Pearson´s Chi squared)
Poodle	6 (20.7%)	191 (3.7%)	11.419	3.194 - 40.828	<0.001*	6.819	2.745 - 16.942	<0.001*
Cocker Spaniel	4 (13.8%)	153 (3%)	9.503	2.353 - 38.379	<0.001*	5.261	1.809 - 15.302	0.001*
Cross-Breed	4 (13.8%)	1454 (28%)	1	reference	reference	0.410	0.143 - 1.181	0.088
German Shepherd	3 (10.3%)	202 (3.9%)	5.399	1.200 - 24.295	0.014*	2.846	0.854 - 9.480	0.075
Fox Terrier	2 (6.9%)	29 (0.6%)	25.069	4.415 - 142.359	<0.001*	13.167	2.991 - 57.957	<0.001*
Andalusian Wine-cellar Rat-Hunting	2 (6.9%)	91 (1.8%)	7.989	1.444 - 44.195	0.005*	4.146	0.971 - 17.695	0.037*
Dachshund	2 (6.9%)	34 (0.7%)	21.382	3.787 - 120.744	<0.001*	11.220	2.566 - 49.062	<0.001*
West Highland White Terrier	2 (6.9%)	59 (1.1%)	12.322	2.213 - 68.622	<0.001*	6.434	1.496 - 27.680	0.004*
Yorkshire Terrier	2 (6.9%)	498 (9.6%)	1.460	0.267 - 7.994	0.661	0.697	0.165 - 2.940	0.621
Griffon	1 (3.4%)	37 (0.7%)	9.824	1.072 - 90.047	0.013*	4.968	0.659 - 37.478	0.084
Siberian Husky	1 (3.4%)	49 (0.9%)	7.418	0.814 - 67.605	0.037*	3.743	0.499 - 28.056	0.168
Total	29	5184	* (P<0.05). OR= Odds Ratio					

Table 3

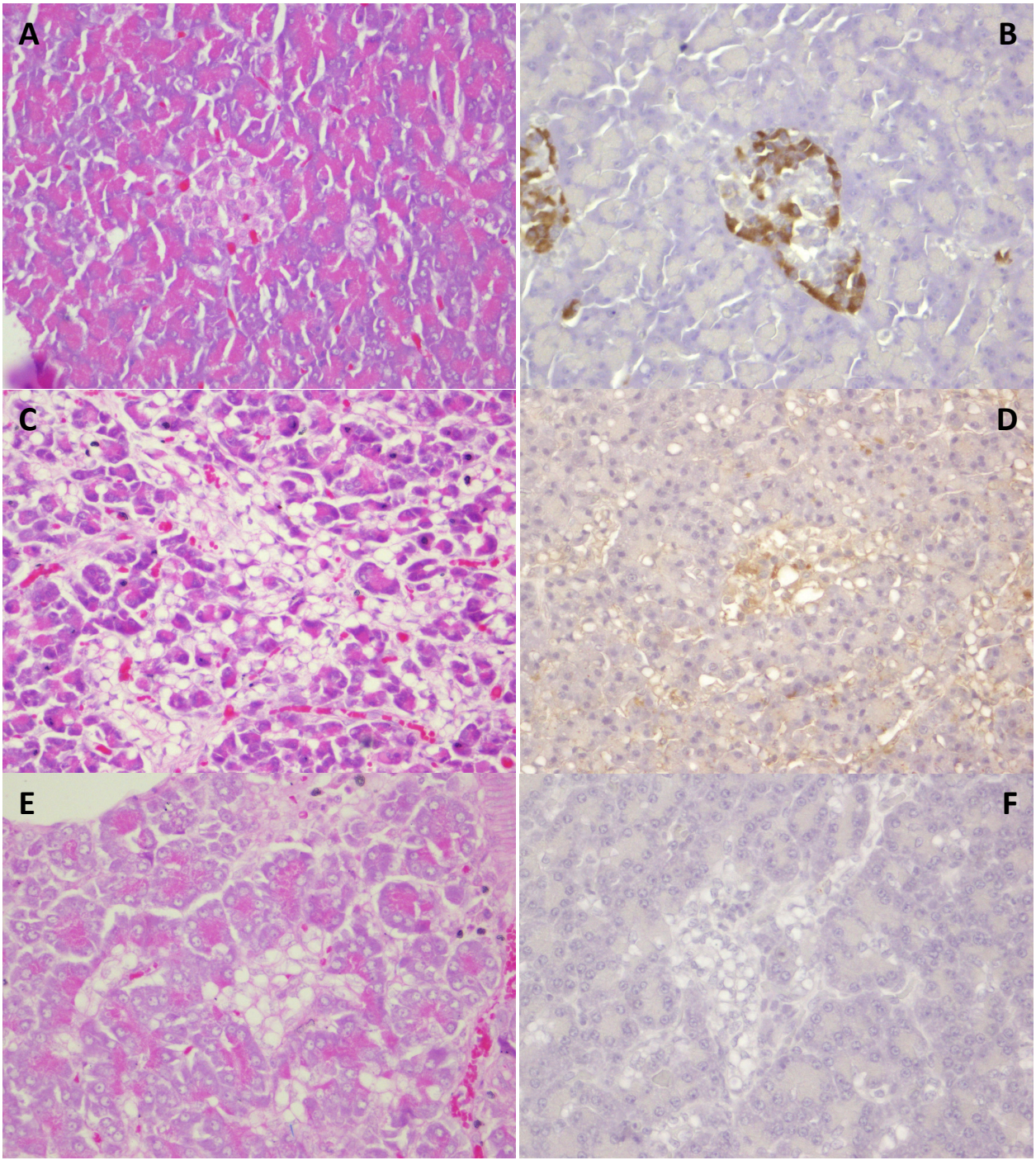
Characterisation of the 5 cases suspected to have auto-immune diabetes.

Breed	Sex	Age at diagnosis (years)	Age at sampling (years)	DLA alleles			Auto-antibody reactivity	
				DRB1	DQA1	DQB1	GAD65	IA2
Griffon	MN	8.6	8.8	Hom. 015	Hom. 006	Hom. 049	-	-
West Highland White Terrier	M	3.17	5.5	Hom. 001	Hom. 009	00101/03001	+	-
Miniature Poodle	FN	10.4	10.9	01501/01302 or 01503/01302	00101/02201	02601/00201	-	-
Poodle	M	12.1	12.1	01501/01301 or 01501/01302	Hom. 009	Hom. 001	-	+
Cross-breed	M	7.8	8	Hom. 001	Hom. 006	Hom. 049	-	-

M= male; MN= male, neutered; FN= female, neutered; Hom.= homozygous

Figure 1

Histopathological evaluation. Hematoxilin-Eosin and Insulin staining of non-diabetic (A-B) and diabetic pancreases (C-F). No lesions are identified in the non-diabetic tissue **(A)**. Langheran's islets, easy recognised by the insulin-positive cells (brown) are evident **(B)** and generalised in the non-diabetic pancreas. Most of the pancreatic islet is formed by β-cells. All diabetic pancreases showed severe reduction in islet number and size, and when detected, vacuolation of islets cells and ductal epithelium was evident **(C-F)**. Insulin positive-cells were scant **(D)**, with weak, or even absent immune-labeling **(F)**. An insulin-staining grade was identified among all diabetic dogs, as is the case of **D**, a recently diagnosed (one week) dog with cDM secondary to hyperadrenocorticism, and **F**, a pancreas from a dog with sustained hyperadrenocorticism.



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2. Paper 2

ISO-Based Assessment of Accuracy and Precision of Glucose Meters in Dogs
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ISO-Based Assessment of Accuracy and Precision of Glucose Meters in Dogs

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Background: Portable blood glucose meters (PBGMs) allow easy glucose measurements. As animal-specific PBGMs are not available everywhere, those for humans are widely used.

Objectives: To assess the accuracy and precision of 9 PBGMs in canine whole blood (WB) and plasma, based on the ISO 15197:2013.

Animals: Fifty-nine client-owned dogs attending the Veterinary Teaching Hospital.

Methods: Analytical evaluation of 100 blood samples was performed for accuracy and 23 for precision (glucose 29–579 mg/dL) following ISO recommendations. A PBGM was considered accurate if 95% of the measurements were within ±15 mg/dL from the reference when glucose was <100 mg/dL and within ±15% when it was ≥100 mg/dL, and if 99% of them were within zones A and B in error grid analysis (EG). A hexokinase-based analyzer was used as reference. Ninety samples were assessed for hematocrit interferences.

Results: Accuracy requirements were not fulfilled by any PBGM in WB (74% of measurements within the limits for the most accurate) and by 1 only in plasma. However, the EG analysis in WB was passed by 6 PBGM and by all in plasma. The most accurate were also the most precise, with coefficients of variation <5% in WB and <3% in plasma. Hematocrit correlated with bias against the reference method in 4 PBGM ($r = -0.243 - [-0.371]$; $P < .021$).

Conclusions and Clinical Importance: This disparity among PBGM suggests that meters approved for humans need to be evaluated before use in other species.

Key words: Diabetes; Dog; ISO 15197:2003; ISO 15197:2013.

In humans, self-monitoring of blood glucose is crucial for the management of insulin-treated diabetes and has allowed more accurate treatment dosing and has contributed to a reduction in disease complications.[1] In veterinary medicine, portable blood glucose meters (PBGM) allow owners and practitioners to obtain glucose measurements easily and make immediate therapeutic decisions. Although specific PBGM have been developed for small animals, they are not available everywhere and those developed for humans are widely used.

Abbreviations:

CEN	European Committee for Standardization
GDH	glucose dehydrogenase
GO	glucose oxidase
PBGM	portable blood glucose meter
WB	whole blood

Many studies have been published where these "human" devices have been tested in dogs and other animals,[2–9] and at the time this study was performed, the previous study in dogs had been published in 2009[9] and new PBGM had been developed and become available since then. In published studies, not all PBGM were accurate enough to be used in dogs and even those specifically developed for animals led to inappropriate clinical interpretation.[3]

Our aim was to assess the analytical accuracy and precision of 9 available PBGM in canine whole blood (WB) and plasma samples, based on a standardized systematic evaluation approved by the European Committee for Standardization (CEN), the recommendations of the ISO 15197:2003.[10] However, during the review of this manuscript the ISO 15197:2013 was released and, therefore, results were reanalyzed based on the current standard.[11]

Both ISO 15197:2003 and 2013 are international standards utilized to evaluate accuracy and precision of these devices for human blood.[10–13] They establish minimum performance criteria for blood glucose monitoring systems, based on analytical precision and accuracy and specify the procedures to demonstrate the systems' conformity to these standards.[10,11]

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All work was performed at the Hospital Clínico Veterinario, ULPGC, Trasmontaña s/n, Arucas, Gran Canaria, Spain.

Two posters containing preliminary results were presented at the 22nd Congress of the European College of Veterinary Internal Medicine, Maastricht, 6–8th September 2012, and the 12th Congress of the Federation for European Laboratory Animal Science Associations, Barcelona, 10–13th June 2013.

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Materials and Methods

Setting and Design

A total of 9 PBGM were assessed for accuracy and precision in canine blood samples based on ISO 15197:2003 guidelines. The samples were obtained in the Veterinary Teaching Hospital and all measurements were performed in its clinical laboratory.

Glucose Monitoring Systems and Reference Method

The following PBGM were assessed by using a single meter of each brand: AccuChek Aviva Nano[a] (Aviva), FreeStyle Freedom Lite[b] (Freestyle), Glucocard G+ meter (GT 1820)[c] (Glucocard), Hemocue Glucose 201^{+}[d] (Hemocue), OneTouch UltraEasy[e] (Ultra), OneTouch VerioPro[e] (Verio) and OneTouch Vita[e] (Vita), Optium Xceed[b] (Optium), and StatStrip Xpress Glucose Hospital Meter[f] (StatStrip). Their main features are summarized in Table 1. The different devices and strips or cuvettes were donated by their manufacturers. All are currently widely available and used in human medicine and were the most recent models at the time this study was performed (April 2011–April 2012).

As a reference method, the hospital's automated laboratory analyzer (Catalyst[g]), based on a multilayered, dry-slide technology, hexokinase method, was employed on plasma samples, as in routine clinical practice.[h] Quality controls for the reference method and each device were performed following manufacturer's instructions. The reference method typically shows coefficients of variation below 5% for most measurements and less than 1% for glucose.[h]

Samples and Protocol

Whole blood samples were collected from 59 client-owned dogs with diabetes, insulinoma and other unrelated diseases, attending the Veterinary Teaching Hospital between May 2011 and April 2012. Most samples were obtained for clinical purposes and opportunistically included in this assessment. The protocol was approved by the Animal Welfare Ethics Committee (Comité Ético de Bienestar Animal, ULPGC; Reference number 007/2011). They were extracted from the jugular, cephalic, or saphenous vein, with a syringe (22G-needle). Immediately after sampling, 100 μL of WB was separated to be used on all of the PBGM and the rest was poured into lithium-heparin tubes[10,11] and centrifuged. After plasma separation, 300 μL were used in the automated laboratory analyzer, to measure glucose and other routine analytes, and the rest, to measure plasma glucose with all the PBGM. To avoid glucose consumption in the sample, all measurements were performed consecutively, with a maximum delay of 20 minutes between sampling and testing. To avoid systematic bias because of sample processing, PBGM were randomly and blindly extracted from an opaque container and measurements were performed in the initial order and then shifted several times during the assay. All devices were operated and calibrated according to the manufacturers' instructions.

Samples were classified according to their glucose concentration, measured by the reference method, into normoglycemic (73–143 mg/dL), hypoglycemic, and hyperglycemic. In addition, for each ISO-established interval samples were collected as recommended: 5 <50 mg/dL, 15 between 51 and 80 mg/dL, 20 between 81 and 120 mg/dL, 30 between 121 and 200 mg/dL, 15 between 201 and 300 mg/dL, 10 between 301 and 400 mg/dL, and 5 >400 mg/dL. Following ISO recommendations, when patient samples were not available for a specific range, euglycemic WB samples were either incubated at room temperature to allow for erythrocyte glucose consumption[8,10,11] or Glucose G7528[i] was added.[10,11] After direct addition of glucose, one of the meters (Glucocard) was used as a preliminary estimation that the sample was within the aimed glucose range. In that case, if this range was confirmed by the reference method, the rest of the procedure was completed.

When analytical errors were warned by a device, the measurement was repeated attempting to correct the error, until it was obtained. If, after a third attempt, the error persisted, the value was defined as missing. When values outside the measurable range were obtained (LO or HI), the value immediately below or above the limit, respectively, was entered.

A total of 100 (WB and plasma) samples were used to assess accuracy: glucose was measured in each sample with the 9 PBGM and the reference method. According to ISO 15197:2003 requirements for human use, a PBGM is considered accurate if 95% of the measurements are within ±15 mg/dL from the reference when glucose is <75 mg/dL, and within ±20% when glucose is ≥75 mg/dL.[10] The results were also analyzed following the ISO 15197:2013 requirements[11] that establish narrower accuracy limits (±15 mg/dL from the reference when glucose is <100 mg/dL, and within ±15% when glucose is ≥100 mg/dL).

Error grid analysis assessment for type 1 diabetes[11] was performed to asses clinical risk for each measurement (see Fig 2).[14] To define a PBGM as accurate, the ISO 15197:2013 requires 99% of the values to be within zones A and B.[11]

Precision was assessed on 23 samples, 8 in the hypoglycemic and hyperglycemic ranges, and 7 in the normoglycemic range. On each sample, 3 measurements were performed with each PBGM.

Hematocrit was assessed in 90 samples, either by a microhematocrit centrifuge (StatSpin VT[g]), or by the hospital's automated analyzer (Lasercyte Dx[g]), and was classified as low

Table 1. Main features of the evaluated devices according to their manufacturers.

PBGM	Sample (μL)	Measurement Range (mg/dL)	Measurement Time (seconds)	Measurement Method
AccuChek Aviva Nano	0.6	10–600	5	GDH
Freestyle Freedom Lite	0.3	20–500	5	GDH
Glucocard G+ meter (GT 1820)	0.6	10–600	5.5	GDH
Hemocue Glucose 201^{+}	5	0–400	40–240	GDH
OneTouch Ultra	1	20–600	5	GO
OneTouch VerioPro	0.4	20–600	5	GDH
OneTouch Vita	0.4	20–600	5	GO
Optium Xceed	1.5	20–500	5	GDH
Statstrip Xpress Glucose H. M.	0.6	10–600	6	GO

PBGM, portable blood glucose meters; GDH, glucose dehydrogenase; GO, glucose oxidase.

(<37%), normal (37–55%), or high (>55%), following the reference of the automated analyzer.

Statistical Analysis

To assess accuracy, PBGM values and the reference method were compared using paired Student's *t* or Wilcoxon's test. The differences between the PBGM and the reference method were plotted against the reference values in Bland-Altman plots. Passing-Bablok linear regression analysis was performed to detect constant and proportional bias. If the 95% CI for the slope did not include 1, this was considered evidence of proportional bias. If the 95% CI for the *y* intercept did not include 0, this was considered evidence of constant bias.[15] To assess precision, mean, standard deviation, and coefficient of variation were calculated for each device. Interference by hematocrit was assessed comparing the differences between PBGM and the reference method in low, normal, and high hematocrit samples (Kruskall-Wallis test) and evaluating their correlation with hematocrit values (Spearman tests).

Statistical analyses were performed by a commercial statistical software package.[j] Differences were considered significant when two-tailed *P* was below .05.

Results

One hundred samples from 57 dogs with glucose concentrations ranging from 29 to 579 mg/dL were included in the study and analyzed for accuracy and 23 of them (same range) were also analyzed for precision. A total of 43 samples were treated to complete the required number for hypo- and hyperglycemic ranges: 6 were incubated at room temperature and to 27, glucose was added, respectively. The other 10 were included in the normoglycemic range, as they did not fall within the expected limits. Analytical errors, warned by Aviva, Ultra, Verio, Optium, and StatStrip, included insufficient volume in the strip chamber, incorrect application of the sample, and defective strips. Verio and Hemocue failed to measure 1 hyper- and 1 hypoglycemic sample, respectively.

Accuracy

Mean differences in glucose concentrations (mean and SD) obtained with the reference method and the 9 PBGM assessed (both for WB and plasma) are displayed in Table 2. WB glucose concentration was lower for all PBGMs compared with the reference method (175.30 [SD 115.74] mg/dL), though the Aviva PBGM was the most accurate (155.98 [SD 105.79] mg/dL) ($P < .005$). Regarding plasma samples, the most exact were Freestyle (174.41 [SD 111.70] mg/dL) ($P = .665$) and StatStrip (179.22 [SD 129.16] mg/dL) ($P = .148$).

When evaluating the different glycemic intervals in WB (Table 2), in the hypoglycemic range, only 2 devices showed similar values to the hexokinase method (51.38 [SD 14.98] mg/dL), Aviva ($P = .678$) and Hemocue ($P = .605$), whereas Verio reached the

Table 2. Deviation from "trueness": Reference mean values and devices' mean differences from reference [mg/dL (SD)] for WB and P, for the whole range and per glycemic interval.

		Mean difference with reference (SD)			
Device	Blood Source	Whole Range (N = 100)	Hypoglycemia (N = 15)	Normoglycemia (N = 38)	Hyperglycemia (N = 47)
Reference value Mean (SD)		175.30 (115.74)	51.38 (14.98)	106.15 (22.30)	265.04 (106.39)
Aviva	WB	19.32 (28.19)	1.38 (12.61)[d]	8.87 (13.05)	32.67 (33.67)[d]
	P	−10.90 (15.20)	−6.54 (3.48)	−5.69 (6.40)	−16.31 (19.82)
FreeStyle	WB	74.15 (55.24)	18.69 (13.60)	40.38 (21.09)	116.60 (48.83)
	P	0.89 (20.48)[b]	−2.00 (4.95)[d]	1.49 (9.85)[d]	1.19 (28.21)[d]
Glucocard	WB	48.18 (30.84)	25.54 (10.62)	40.64 (11.60)	60.44 (39.02)
	P	28.10 (20.21)	15.46 (5.97)	24.97 (6.62)	34.06 (26.97)
Hemocue	WB	47.83 (49.15)[a]	2.75 (20.93)[d]	22.08 (15.92)	80.02 (51.00)
	P	−10.79 (32.66)	−19.15 (9.61)	−18.49 (7.85)	−2.27 (45.07)[d]
Ultra	WB	38.7 (30.99)	16.92 (13.16)	27.15 (13.53)	54.23 (36.56)
	P	−64.71 (43.32)	−6.62 (11.41)[d]	−40.87 (17.16)	−99.81 (31.91)
Verio	WB	29.96 (32.45)[a]	6.77 (11.19)[d]	14.44 (10.09)	49.25 (37.28)
	P	−13.19 (14.42)[a]	−5.42 (5.47)	−8.38 (7.16)	−19.17 (17.81)
Vita	WB	38.82 (30.95)	16.46 (12.69)	25.15 (11.56)	54.58 (36.25)
	P	−59.61 (40.45)	−3.61 (9.61)[d]	−35.80 (13.71)	−94.13 (26.76)
Optium	WB	38.57 (34.76)	10.85 (14.96)	19.03 (15.32)	61.96 (34.78)
	P	−48.56 (31.95)	−13.46 (9.67)	−39.00 (11.99)	−65.83 (35.90)
StatStrip	WB	33.28 (30.28)	12.62 (11.09)	20.44 (15.21)	49.26 (34.57)
	P	−3.92 (26.87)[c]	4.85 (4.78)	5.62 (7.32)	−14.04 (35.64)

WB, whole blood; P, plasma; PBGMs, portable blood glucose meters.

[a]One hundred determinations were obtained for all devices, except Verio (99 WB and 98 P samples) and Hemocue (99 WB samples). Normoglycemia is defined between 73 and 143 mg/dL.

[b,c]All PBGMs showed significant differences with the reference in both whole blood and plasma, for the whole glucose range, with the exception of [b]Freestyle ($P = .665$) and [c]StatStrip ($P = .148$) in plasma samples, and Vita and Ultra for the same samples in the hypoglycemic range ($P = .200$ and $P = .059$, respectively) and Hemocue ($P = .729$), in hyperglycemia.

[d]$P > .05$ (nonsignificant) compared with reference by glycemic ranges.

limit of statistical significance ($P = .05$). For the normoglycemic and hyperglycemic intervals, all devices showed significantly lower values than the reference ($P < .005$).

For the corresponding plasma samples (Table 2), Freestyle and StatStrip were similar to the reference (175.30 [SD 115.74] mg/dL) for all glycemic intervals ($P = .686$ and $P = .148$, respectively), Vita and Ultra, in the hypoglycemic range ($P = .200$ and $P = .059$, respectively) and Hemocue ($P = .729$), in the hyperglycemic range.

None of the devices fulfilled ISO 15197:2013 accuracy requirements for WB (95% of values within the global glycemic range) (Fig 1A). The most accurate PBGM was Aviva with 74% of total measurements within the limits. For plasma, standards were only achieved by Aviva, and approached by FreeStyle and Verio with 97%, 92%, and 91.92% of values within limits, respectively (Fig 1B).

Even the previous, somewhat laxer, ISO 15197:2003 accuracy requirements, were not fulfilled by any device. The 2 most accurate, Aviva and Verio, showed 82% and 64% of total measurements within the limits, respectively. For plasma, the requirements were achieved by Aviva, FreeStyle, and Verio with 99%, 95%, and 99% of values within limits, respectively, and were approached by StatStrip, with 92% of the values within the limits.

Regarding the EG analysis for WB (Fig 2), most satisfied the requirements, with all (Aviva, Verio, and StatStrip) or 99% of the values (Optium, Ultra, and Vita) falling within zones A and B. The rest of the devices approached the EG requirements (Fig 2). In plasma, all of the PBGM passed the analysis with 99% of values in zones A–B for Optium and Hemocue and 100% for the rest (data not shown).

According to Passing-Bablok linear regression analysis, 6 devices showed both proportional and constant errors (Glucocard, Hemocue, Ultra, Verio, Vita, and StatStrip) in WB and in plasma (Aviva, Glucocard, Hemocue, Vita, Optium, and StatStrip). Constant errors alone were shown in WB for Aviva, Freestyle,

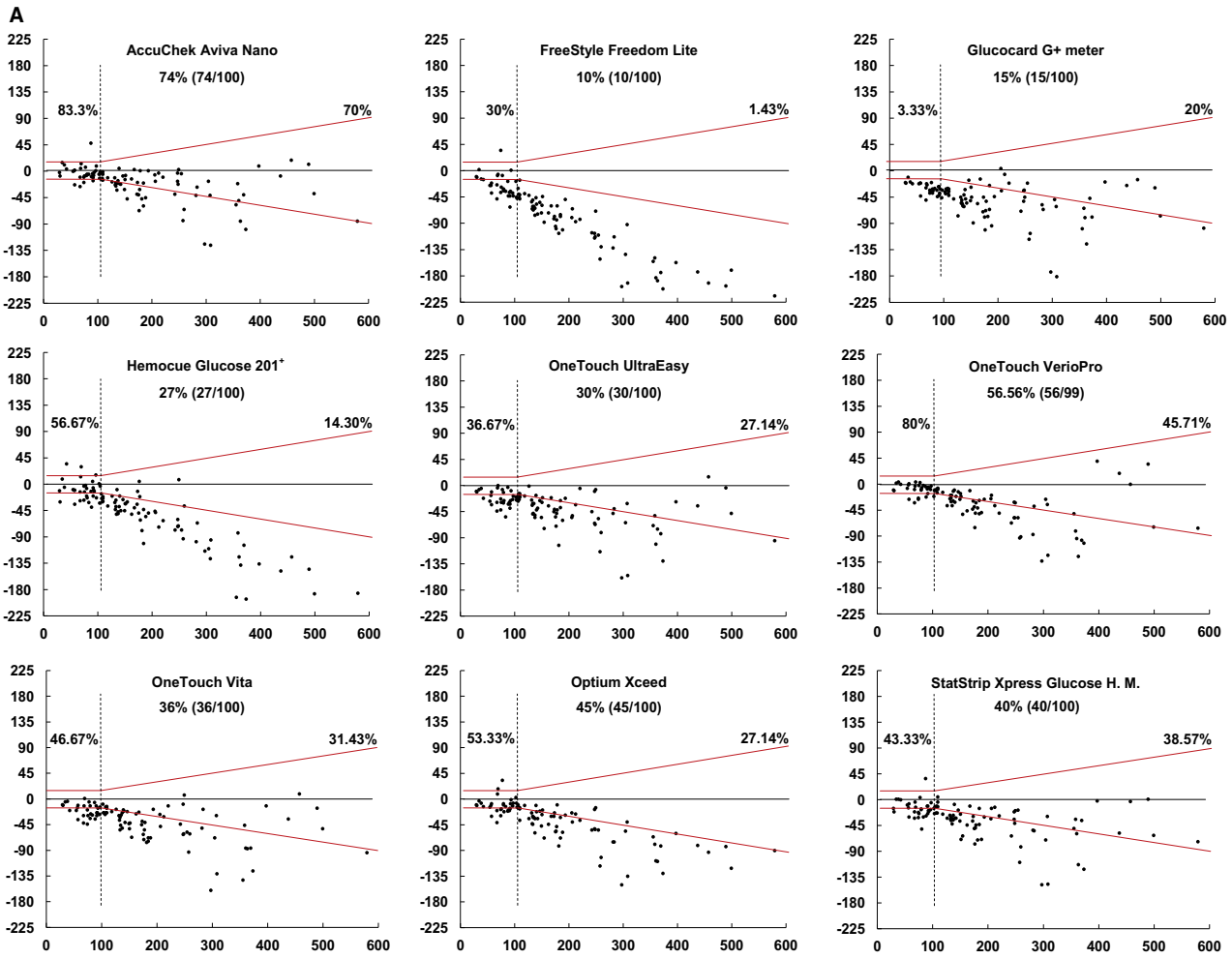

Fig 1. Bland-Altman plots representing accuracy of portable blood glucose meters for whole blood **(A)** and plasma **(B)** for ISO 15197:2013. On the *x* axis, are the reference glucose values, plotted against the absolute errors for each corresponding value. The standard required limits defined by the red symmetric lines: at ±15 mg/dL from the reference value for glucose determinations <100 mg/dL and at ±15% from the reference for glucose ≥100 mg/dL. Percentages express the number of samples within limits when reference was < or ≥100 mg/dL and for the total number of measurements (central% value).

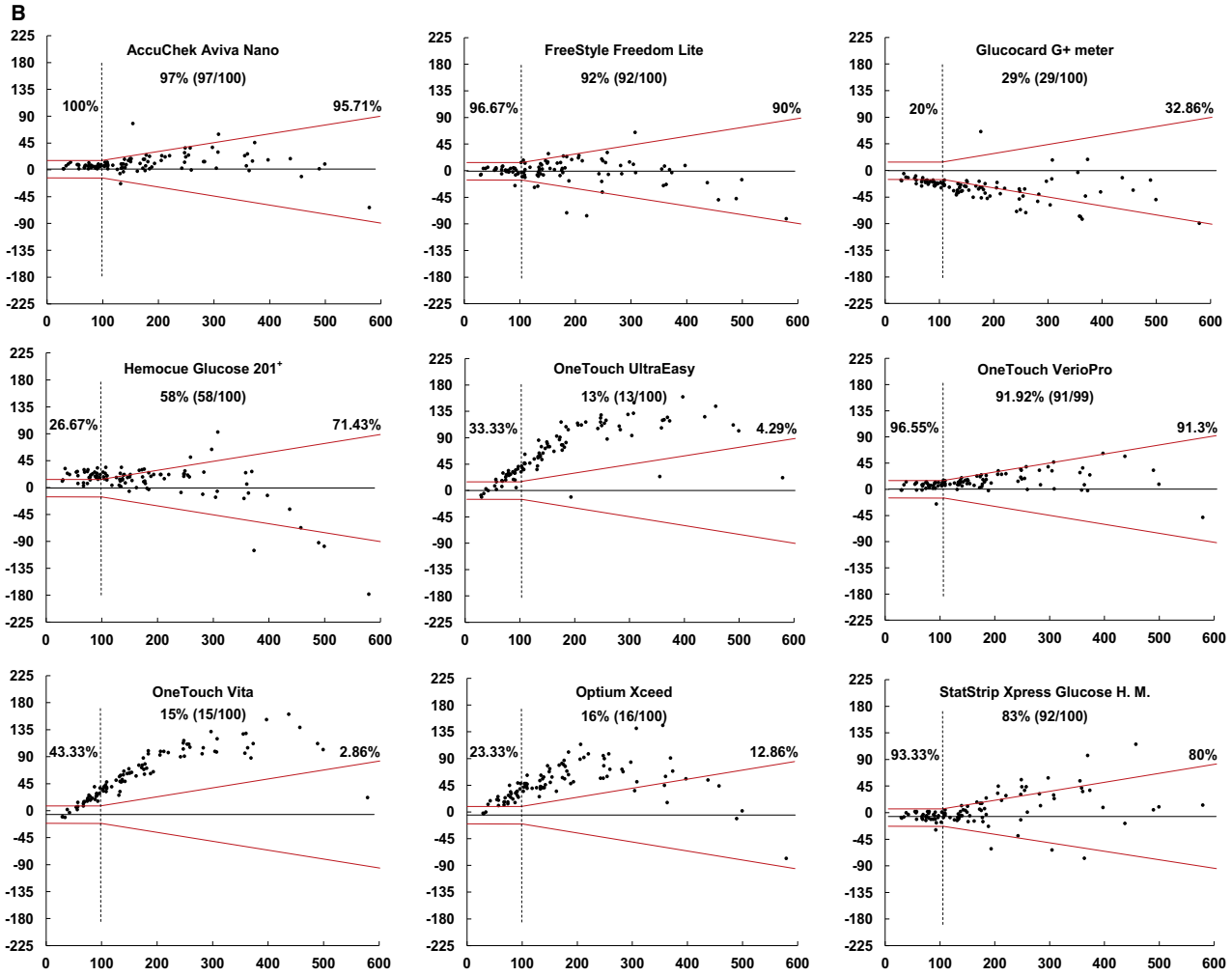

Fig 1. (Continued)

and Optium, and in plasma for Freestyle, Ultra, and Verio.

Additional accuracy analyses were performed and similar results were obtained regardless of whether the samples were pretreated (incubation at room temperature or addition of glucose) or not (data not shown).

Precision

Figure 3 summarizes the results of precision assessment. Aviva and Verio had the smallest coefficient of variation (CV) both in WB (CV = 4.5% [SD 2.7%] and CV = 4.8% [SD 2.3%] respectively, range 0.8–59.9%) and in plasma (CV = 2.7% [SD 1.2%] and CV = 2.9% [SD 2.4%], range 0.47–27.8%).

Hematocrit Interference

Hematocrit was assessed in 90 samples (glucose 34–489 mg/dL), with 17 in the low (35 [20–40]%), 71 in the normal (45 [37–54]%), and 2 in the high hematocrit range (59.5 [59–60]%). When mean differences were compared, only Hemocue showed smaller errors in the low hematocrit range (−29.53 [SD 54.92] mg/dL) when compared with the normal (−49.34 [SD 41.91] mg/dL) and high intervals (−64.5 mg/dL [SD 20.51]) ($P = .018$). Negative correlations of the error with hematocrit were found for Freestyle, Hemocue, Ultra, and Vita ($r = -0.243 - [-0.371]$; $P < .021$).

Discussion

In this assessment of PBGM in dogs, all devices showed significantly lower average WB glucose values than the reference and none fulfilled previous or current ISO accuracy requirements. Only Aviva achieved the requirements in plasma and was the most accurate of the devices tested. The EG analysis in WB was passed by 6 of the devices and, in plasma, by all. Likewise, Aviva was the most precise PBGM, followed by Verio, both with CV below 5% in WB. CV was reduced in most of the glucose meters by the use of plasma samples (Fig 3). Constant error, a type of bias that could potentially be corrected by calibration, was shown in WB for Aviva, Freestyle, and Optium, and in plasma for Freestyle, Ultra, and Verio.

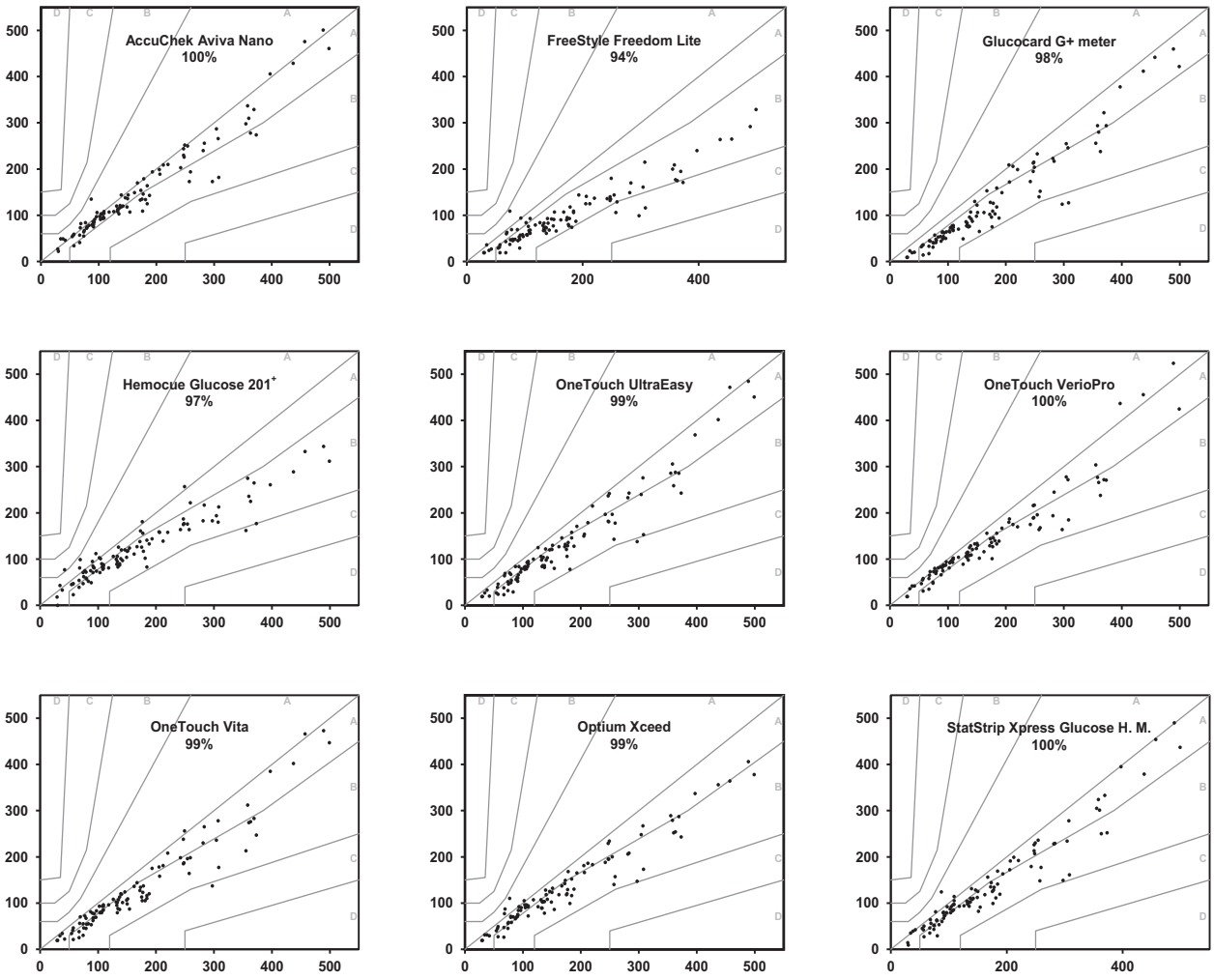

Fig 2. Error grid analysis representation for whole blood for each device with the percentage of values within A–B zones. The reference glucose values ("true" glucose value), on the *x* axis, are plotted against the blood glucose by the glucose meter (*y* axis). The different zones designate the magnitude of risk derived from the determination: no effect on clinical action (zone A), altered clinical action – little or no effect on clinical outcome (zone B), altered clinical action – likely to affect clinical outcome (zone C), altered clinical action – could have significant medical risk (zone D) and altered clinical action – could have dangerous consequences (zone E).[14] ISO 15197:2013 requires that 99% of the values fall within zones A–B for a device to be considered accurate.

These results, in agreement with previous studies, support the need to assess accuracy and precision in every PBGM before it is used in dogs for clinical or research purposes.[3,9] Not only inappropriate therapeutic decisions could be made when using inaccurate PBGM, but, when used for research, results might also lead to false conclusions. Indeed, most often, no information is given on the accuracy or precision of the PBGM in animal research studies.[16–18]

Portable blood glucose meters are often selected based on their brand or series of models, assuming that there are no differences in accuracy among them.[16,19] However, as interpreted by previous results,[2–6,8,9,20] and in accordance with this study, there is not a preferred brand to recommend and every PBGM should be evaluated independently (Fig 1a,b).

This study also assesses the effect of hematocrit on PBGM accuracy. Previous studies have shown that PBGM yield higher glucose concentrations than the reference method in euglycemic, anemic dogs and cats.[5,7,8,20] In this study, 90 samples with a wide glucose (34–489 mg/dL) and hematocrit range (20–60%) were included. Four PBGM (Freestyle, Hemocue, Ultra, and Vita) showed interferences by hematocrit.

Although clinical glucose control may not be as tight in dogs as in humans, the accuracy requirements for glucose meters should not be laxer.[7] This perception of permissive glucose control can lead PBGM to be judged as "acceptable" for clinical use,[7–9,20] especially if evaluations are mostly based on less stringent approaches, such as error grid analysis. The current 15197:2013 standard requires both analytical and EG criteria to be fulfilled, but the latter seem to be redundant in this study. Indeed, most of the devices fulfilled the EG criteria for WB without satisfying the analytical accuracy demands and, if a device fulfilled the analytical ISO requirements, it also passed the EG analysis. In fact, clinical consequences depend on the

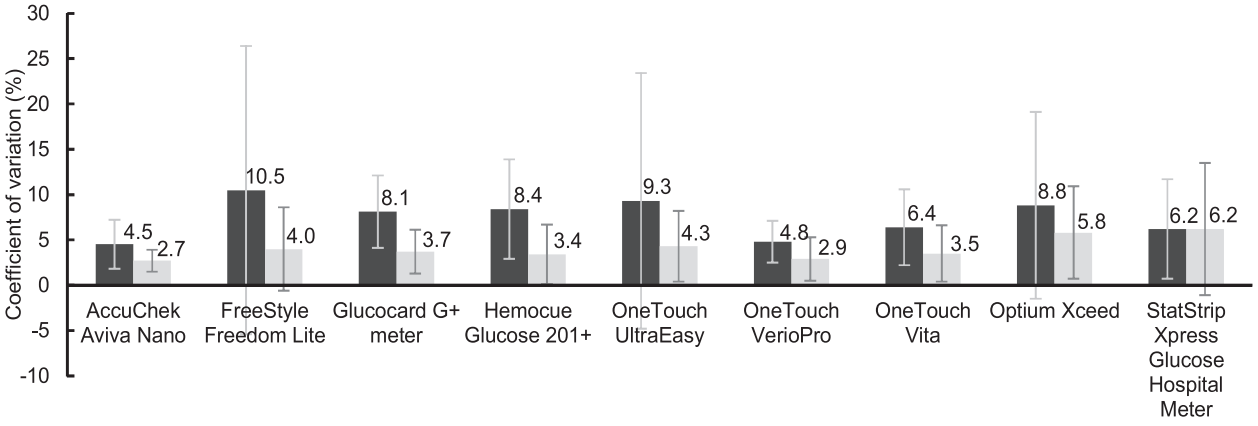

Fig 3. Precision evaluation: Coefficients of variation (%) for all devices in whole blood (dark) and plasma (light) with bars showing respective standard deviations.

specific needs of patients and their circumstances. Indeed, the amplitude of the acceptable limits can be crucial in some cases, such as in tight glycemic control protocols, where an imprecision and bias below 2% have been estimated to be needed to achieve 95% accuracy in insulin administration.[21] Thus, although optimal glucose control is less feasible in animals, accuracy should be strictly evaluated with rigorous analytical criteria.

The ISO 15197:2003 and 15197:2013 are standardized systematic evaluations approved by the CEN and followed by manufacturers that get the Conformité Européenne (CE) mark for their products.[12,13] The methodology and statistical tests in previous studies have been essentially the same[2–9] as those used here. In fact, the ISO includes these accepted analytical tests with tightening standards.[22] Its detailed methodology makes it a reliable tool also in veterinary medicine and allows direct comparison among studies. Of the 9 devices assessed in this study, only Optium had been previously evaluated in dogs,[5] with similar results to those here reported.

Most of the devices analyzed in this study fulfill the ISO 15197:2003 and 2013 for human capillary blood.[12,13] We are not aware of a known explanation for this interspecies gap in accuracy, but given the difference in performance between WB and plasma samples, we suggest that the cellular fraction must contain the source of error in canine samples. Furthermore, some interference with hematocrit was detected in 4 of the devices. Overall, when assessed in dogs, Aviva is the best option, regarding both accuracy and precision. Freestyle, Verio, and StatStrip were accurate in plasma samples, but were less precise.

There were several limitations in this study: the use of a single device of each PBGM, the use of venous (instead of capillary) blood, the incubation of or glucose addition to samples, the use of lithium-heparin anticoagulant for many samples and the lack of specific PBGM for animals.

To assess more than 1 device of the 9 different PBGM would not have been feasible with the resources available. Indeed, assessing all the devices simultaneously was already a challenge. Multiple potential sources of error such as temperature, altitude, humidity, sample volume, hemolysis, or pharmacological factors have been described to interfere with the measurements.[23,24] Although not all can be accounted for, in this study, we obtained and processed the samples in relatively stable conditions and in very similar conditions to those of routine clinical practice.[4] Finally, incubation of or glucose addition to the samples, despite being accepted by the ISO, might also be considered as sources of error. Nevertheless, after performing the additional accuracy evaluations for the untreated and treated samples, the similarity in the results obtained after stratification support that this was not the fact in this study.

In humans, small but significant differences have been found between capillary and venous glucose.[21,25] In fact, PBGM are calibrated to show venous-equivalent concentrations. Thus, some additional inaccuracy should be expected when venous samples are used instead of capillary blood.[25] Different ways to obtain capillary blood have been developed in small animals,[7,19] but venous sampling remains the most usual way to obtain blood in veterinary practice. This fact allowed us to obtain the necessary number of samples from routine clinical practice. The OneTouch devices were the only ones specifically recommended for capillary blood only (Table 1). Despite this, (OneTouch) Verio showed the second most accurate results for whole venous blood. Differences between capillary and venous samples in dogs have been evaluated before, proving negligible for most of the devices and glucose concentrations assessed (2–6 mg/dL).[5,7]

Blood collection with sodium-fluoride as a glycolysis inhibitor is considered the gold standard for glucose determinations.[26] However, we intended to reproduce what is most frequently done in our routine veterinary practice, where samples often are collected into lithium-heparin tubes before analysis. Early processing limited potential glucose consumption by the blood

cells. In fact, according to the manufacturers, lithium heparin is an appropriate anticoagulant for all the devices[27–35] and, in previous studies, it did not interfere with the results.[3,4,8]

Conclusions

When glycemic control is assessed, advantages of PBGM are to obtain easy, fast, and relatively cheap measurements with minimal volumes. However, even for humans, important variability in accuracy has been demonstrated. The disparity among devices in this study confirms the need of accuracy evaluations before its use in dogs and the ISO 15197:2013 is an excellent tool for this purpose in animals, as it is in humans. Although none of the PBGM fulfills the ISO requirements for whole venous blood, overall, AccuChek Aviva Nano is the best option among those evaluated, given its accuracy, precision, and lack of interference by hematocrit.

Footnotes

[a] Roche Diagnostics, Mannheim, Germany
[b] Abbott Diabetes Care Ltd., Witney, Oxon, UK
[c] Arkray-Menarini Diagnostics, Shiga, Japan
[d] Hemocue AB, Ängelholm, Sweden
[e] LifeScan Inc., Milpitas, CA
[f] Nova Biomedical, Waltham, MA
[g] IDEXX Laboratories, ME
[h] Siska WD, Rosen NK, Christian JA, Taddeo DA, DeNicola DB. Vet Clin Pathol. Proceedings of the 13th Annual Congress of the European Society for Veterinary Clinical Pathology; 2011 September 1–3; Dublin, Ireland. American Society for Veterinary Clinical Pathology, 2011
[i] Sigma-Aldrich Chemie Gmbh, Steinheim, Germany
[j] IBM SPSS Statistics Version 20; SPSS Inc., Chicago, IL

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3. Paper 3

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The repair enzyme protein isoaspartyl methyl transferase (PIMT) in type 1 diabetes

Short running title: The repair enzyme PIMT in type 1 diabetes

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Abstract

Post-translational protein modifications can elicit autoimmune response. The repair enzyme Protein-L-isoaspartate (D-aspartate) O-methyltransferase (PIMT/ *PCMT1*) was assessed in type 1 diabetes.

PCMT1 mRNA expression and PIMT protein concentration and activity were analysed in patients with type 1 diabetes, type 2 diabetes and controls. In diabetic dogs, blood *PCMT1* mRNA and pancreatic insulin and PIMT immunohistochemistry were evaluated. In *Pcmt* knock-out mice, oral glucose and insulin tolerance tests, insulin, glucagon, corticosterone and HbA1c concentrations and pancreatic insulin and glucagon immunostaining were assessed.

Patients with type 1 diabetes tended to show higher *PCMT1* mRNA expression than type 2 diabetes patients, but similar to controls, whereas PIMT protein levels tended to be higher in type 1 diabetes than in controls. PIMT protein concentration was significantly correlated with HbA1c and tended to be correlated with BMI and triglyceride. *PCMT1* mRNA expression was higher in patients with positive IA2-antibodies. In multivariate analysis, only age was significantly associated.

Diabetic dogs showed normal blood *Pcmt1* mRNA, but lower pancreatic insulin and PIMT. *Pcmt* knock-out mice displayed normal glucose tolerance, reduced insulin secretion, increased insulin sensitivity, impaired glucagon response to hypoglycaemia and lower pancreatic insulin (and glucagon?).

PIMT seems to be related to hyperglycaemia and islet cell function/development.

Posttranslational modifications (PTM) of proteins are needed for many of their functions, but can also result in the development of new antigenic epitopes. The latter can trigger autoantibody production and T-lymphocyte activation and could initiate autoimmune diseases where new, tissue-specific epitopes are targeted by the immune-system (Doyle & Mamula 2001; Størling et al. 2013; Nerup et al. 1994) . In type 1 diabetes, several modified auto-antigens have been shown to trigger a more intense immune response than the native protein. Indeed, antibodies from patients with type 1 diabetes bind to modified GAD65 with higher affinity than to native GAD65 (Trigwell et al. 2001; McGinty et al. 2014), and T-lymphocytes show increased recognition of the modified A-chain of human insulin (Mannering et al. 2005). Furthermore, insulin is prone to *in vitro* isomerisation, i.e, spontaneous conversion of asparagine (Asn) or aspartic acid (Asp) residues into isoaspartyl (IsoAsp) at susceptible protein sites (Brange 1992). The repair enzyme Protein-L-isoaspartate (D-aspartate) O-methyltransferase EC 2.1.1.77 (PIMT) (Clarke 1985) encoded by *PCMT1* (DeVry 1999) catalyses the conversion of IsoAsp back to normal aspartyl residues and prevents the accumulation of damaged proteins. In the human pancreas, PIMT is selectively and highly expressed in ß-cells (Wägner et al. 2007) and, in families with type 1 diabetes, a single nucleotide polymorphism (SNP) in *PCMT1* (rs4816, allele A), associated with increased PIMT enzyme activity, tended to be less frequently transmitted to the affected than to the unaffected offspring (Wägner et al. 2008). Pcmt knock-out mice exhibit hyperresponsive T cells, and transfer of their bone marrow to irradiated wildtype mice leads to autoimmunity (Doyle et al. 2003). Furthermore, in diabetes-prone BB/OK rats, the administration of PIMT-inducer CGP3466B delayed the onset and reduced the severity of diabetes and was associated with preserved ß-cell mass (Wägner et al. 2007).

The aim of this study was to delve further into the potential role of PTM and the repair enzyme PIMT in the pathogenesis of type 1 diabetes. In order to do so, *PCMT1* genotype and expression and PIMT protein concentration and activity were assessed in patients with type 1 diabetes and in

an animal model of the disease. In addition, a *Pcmt1 knock out (*KO) model was characterized for glucose tolerance and insulin sensitivity.

Research Design and Methods

The study was performed in the University's facilities and in the University Hospital. The protocols were accepted by the Hospital's ethics committee (CEIC Complejo Hospitalario Universitario Insular Materno-Infantil de Gran Canaria) and by the University's Animal Welfare Ethics Committee (CEBA-ULPGC).

Human participants: A total of 35 patients with short-duration (<5 years) of type 1 diabetes (GAD65- and/or IA2-antibody positive), 19 patients with type 2 diabetes (<5 years' duration, no complications), and 29 healthy subjects, matched for age and gender with type 1 diabetes patients, were included. Participants signed written informed consent before inclusion in the study. Clinical information was obtained from their clinical records (patients) or through an interview and examination (controls). In addition, all participants in the type 1 diabetes group and those in the type 2 diabetes group with an associated autoimmune disease were defined as having an autoimmune disorder. Participants were also stratified according to the number of autoimmune disorders (0, 1 and 2 or more). Routine laboratory assessment was performed with automated colorimetric analyses. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease-4 (MDRD4) equation (Levey et al. 1999), HbA1c was analysed by High-performance liquid chromatography (HPLC), using NSGP-DCCT standardisation and serum antibodies, by Radio-immune Assay (RIA).

Dogs: Canine diabetes has been proposed as a model of type 1 diabetes (Catchpole et al. 2005) with common susceptibility genes comparable to human autoimmune diabetes (Catchpole et al. 2013; Short et al. 2010; Kennedy et al. 2006). Because dogs share the same environment as humans, are both purebred and outbred and are phylogenetically closer to us than rodents, they represent a unique opportunity for the study of human disease.

Diabetic dogs were identified at the Veterinary Teaching Hospital (Universidad de Las Palmas de Gran Canaria). Their owners gave informed consent before the samples were obtained. Non-diabetic control dogs, matched for age, sex and breed, were identified among patients taken to the clinic for other reasons, after ruling out systemic disease. Clinical information was obtained by reviewing the dogs' records and by interviewing their owners. Blood was drawn from the yugular or cephalic vein, according to routine procedures. In addition, postmortem tissue samples were obtained from the Pathology Department, both from diabetic and non-diabetic dogs (see below).

PIMT KO mice: PIMT KO mice *(Pcmt1*$^{-/-}$) accumulate modified (IsoAsp-containing) proteins, show an enlargement of the brain and die of severe seizures at the age of 4-8 weeks(Kim et al. 2015)(Kim et al. 2015)(Kim et al. 2015)(Kim et al. 1997) and they show signs of autoimmunity (Doyle et al. 2003).

HeterozygousB6;129S4-*Pcmt1*$^{tm1Scl+/-}$mice, generated by inserting a *neo* cassette into exon one of the *pcmt* gene(Kim et al. 1997), were kindly provided by Dr. S. Clarke (University of California, Los Angeles, CA). Pcmt knock-out mice were obtained by intercrossing heterozygous mice. Presence of the *neo* cassette and the absence of the *pcmt* gene were assessed by standard PCR, from tail DNA. The following primer sequences were used: *pcmt*forward 5'-gcagcgacggcagtaacagc-3', *pcmt*reverse 5'-gcatcgagcgagcacgtactcgg-3', *neo* forward 5'-gcacgaggaagcggtcagcccattc-3', and *neo* reverse 5'-cgcatcgagcgagcacgtactcgg-3'(Ecogen, Stab Vida, Madrid, Spain).

Animals were housed in makrolon cages with top filters (Tecniplast, Buguggiate, Italy), in groups of four to six. Poplar woodchips were used as bedding and autoclaved paper (Lignocel Select Fine ® (Rettenmaier&Söhne, Rosenberg, Germany) and mouse homes and glass pots were provided as environmental enrichment. They were exposed to a 12-hour light:dark cycle, were given non-treated water from the public supply and fed a 18% protein 2018 Teklad Global Diet ® (Harlan Laboratories). The protocol followed National and European requirements (RD 1201/2005, Law 32/2007, EU Directive 2010/63/EU.

PCMT expression*: PCMT1/Pcmt* expression was analysed with relative quantitative Real-time (qRT)-PCR in both human and canine samples. For this purpose, mRNA was isolated from stored blood (DNA/RNA Stabilization Reagent and mRNA extraction kit for blood and bone marrow, both from Roche, Basel, Switzerland) and cDNA was synthesized (1st Strand cDNA Synthesis Kit for RT-PCR, Roche, Basel, Switzerland) from 66 human subjects (24 controls, 26 type 1 and 16 type 2 diabetes) and 16 dogs (8 diabetic and 8 control). qRT-PCR was carried out on cDNA using a Light-Cycler System and SYBR Green (Stratagene; Mx3000P QPCR System, Agilent, Santa Clara, CA). Transcription levels were normalised to ß-actin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* and analysed by comparative C_t.

To amplify human *PCMT1*, forward humPIMTF 5′- cgg.agc.taa.tcc.aca.atc.tcc-3′and reverse humPIMTR 5′-gct.tta.gct.cct.tca.tgc.aac-3′ were used. For human beta actin amplification primers designed by Primerdesign (Southampton, United Kingdom, Nº 5363) were used according to users instructions. For *GAPDH*, the forward primer humGAPDHF 5′-tgc.acc.acc.acc.tgc.tta.gc-3′ and reverse primer humGAPDHR 5′- ggc.atg.gac.tgt.ggt.cat.gag-.3′ were used (Vandesompele et al. 2002).

Canine *Pcmt* was amplified with forward primer canPCMTF 5′- cgg.tga.ccg.tgt.ggg.agg.tg-3′ and reverse primer canPCMTR 5′-gtg.gga.acg.gtc.tgt.agc.cag-3′ and canine beta-actin with forward primer canACTBF 5′- ccg.cac.cac.tgg.cat.cgt.ga-3′ and reverse primer canACTBR 5′-gct.cgg.cgg.tgg.tgg.tga.ag-.3′

PCMT1 **genotyping**: Human genomic DNA was extracted from venous blood with a commercial kit (Quiagen, Hilden, Germany). To detect the rs4816 variant at the *PCMT1* gene, 50 ng of genomic DNA were amplified using the PCR and the primers **FP1305** 5′- gcc.caa.tga.gct.act.gaa.ttg.t -3′ and **FP1306** 5′- ttg.gat.ctt.ctg.aaa.ata.aaa.atc.c -3′. PCR products were purified with MinElute PCR Purification columns (Quiagen), and subjected to Sanger sequencing using the 1306R oligonucleotide as primer (SECUGEN SL, Madrid, Spain).

Preparation of the erythrocyte lysate: Red blood cells (RBCs)**,** which lack the apparatus for protein synthesis and are thus unable to replace their damaged proteins, are rich in cytosolic PIMT, whose substrates are mainly located in membrane proteins, prone to IsoAsp formation (Kim et al. 1997). RBC fractions were obtained from whole blood, deprived of leukocytes and platelets by filtration through a nylon net and then washed with isotonic saline solution (0.9% NaCl). Samples were stored at -20ºC until lysis. To measure PIMT concentrations, the RBC fraction was diluted 3-fold in a hypotonic stabilizing solution (50 mM Sodium Phosphate, 1mM EDTA, pH 8, 25uM or phenylmethylsulfonyl fluoride (PMFS) and the cytosol was obtained after 5 rapid freeze–thaw cycles. Membranes were removed by centrifugation at 13000 g for 20 min. using a Beckman Rotor (Beckman Coulter L7-65 ultracentrifuge) and then washed again 5 times with the hypotonic solution. Both the membrane pellet and the cytosol were stored at -80ºC.

Isoaspartyl content: To evaluate IsoAsp residues and total protein content in erythrocyte membranes, the pellet was lysed in 1% Triton sodium dodecyl sulfate SDS-free Radioimmunoprecipitation Assay (RIPA)-buffer (prepared in-house) and then treated with PIMT according to the manufacturer's protocol (ISOQUANT Isoaspartate Detection kit; Promega, Madison, WI, USA). This method of quantitative detection is based on the use of PIMT to detect IsoAsp. PIMT catalyses the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to isoaspartic acid, generating S-adenosylhomocysteine (SAH), which can be measured by HPLC, as a surrogate of IsoAsp concentration. SAH levels were analysed in 68 subjects by reverse phase HPLC analysis (column Synergi™ Hydro-RP (Phenomenex)). Total protein concentration of the membrane-lysate was estimated with the Bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and SAH was normalized to protein levels.

PIMT protein: PIMT protein concentrations were measured in duplicates in erythrocyte cytosol (70 subjects) and in plasma (36 subjects), with a commercial Enzyme-Linked Immuno Sorbent

Assay (ELISA) kit (Uscn Life Science Inc, Wuhan, China). Erythrocyte cytosol was diluted 1:500 in PBS, whereas plasma was diluted 1:20 and ELISA was performed according to the manufacturer's protocol. Plasma PIMT levels were reported as relative optical density (at 450 nm) and cytosolic PIMT levels, in ng/ml. Protein concentration of the erythrocyte cytosol extracts was estimated with the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and PIMT levels were normalized to protein levels.

Glucose metabolism and insulin resistance: A total of 12 mice (50% KO/Wt; 50% females) were assessed at 60 +/- days of age. On two different days, separated by two-five days, after a 6-hour fast (8.00-14.00), an oral glucose tolerance test (OGTT; 2g/Kg of glucose (Glucose G7528, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)) (Andrikopoulos et al. 2008) and an intraperitoneal insulin tolerance test (ITT; 0.3 U/Kg (Actrapid, Novo Nordisk Pharma S.A., Madrid, Spain) (Heikkinen et al. 2007) were performed. Glucose was measured using a portable meter (Glucocard G+ meter, GT1280, Arkray-Menarini Diagnostics, Shiga, Japan), at 0, 15, 30, 60, 90 and 120 min during both tests. Blood samples were obtained by tail bleeding, directly onto the glucose strips or into EDTA tubes. Plasma was separated by centrifugation and stored at -20ºC until processing.

Effect of ageing: To assess the effect of age-dependent insulin resistance on the phenotype of mice lacking one copy of the *Pcmt* gene, at 120 and 360 days, OGTT and ITT were performed in heterozygous and wildtype mice. KO mice were not assessed at that age, due to short survival. HbA1c was analysed at 120 days in whole blood with a point-of care analyser, based on latex agglutination inhibition immunoassay (DCA, Siemens Healthcare Diagnostics, Deerfield, IL), following the manufacturer's instructions.

Insulin and counterregulatory hormones: Plasma insulin was measured at 0, 15, 30, 60 and 90 min, during the OGTT, by ELISA (Mercodia Ultrasensitive Mouse Insulin, Uppsala, Sweden), following the manufacturer's instructions. Plasma glucagon and corticosterone were measured at 0,

15, 30, 60 and 90 min (corticosterone only), during the ITT, with commercial ELISA kits (Quantikine glucagon immunoassay, R&D System, Minneapolis, MN, USA; Corticosterone EIA Kit, Cayman Chemical Company, Ann Arbor, MI, US). Areas under the curves (AUC) of glucose, insulin, glucagon and corticosterone were calculated using the trapezoid rule.

Necropsy and pancreatic studies: Canine pancreatic tissue samples were fixated in 10% buffer neutral formalin and processed to be paraffin embedded, or directly immersed in tissue embedding compound (Tissue-Tek®, O.C.T. Compund, Sakura Finetek) and stored at -80ºC. Five-µm sections were attached to glass slides (Superfrost®Plus, Thermo Scientific, Braunschweig), stained with hematoxilin-eosin and examined. Immunohistochemistry was performed using the following primary antibodies

Mice were sacrificed by isofluorane overdose. Blood was immediately extracted from the inferior vena cava (Microvette® 300, Sarstedt, city, country), and centrifuged (15 min. at 3000 rpm). Pancreas, liver and paragenital fat were weighed, macroscopically evaluated and collected in tissue embedding medium or snap-frozen in liquid nitrogen for further studies. Organ/body weight ratios were calculated and compared.

Beta cell mass was estimated by islet and beta cell (insulin-positive) area comparisons, and PIMT expression was evaluated on wildtype, heterozygous and KO pancreas by immunohistochemistry (antibodies: goat anti-insulin, Santa Cruz,Heidelberg, Germany; rabbit anti-PIMT, ProteinTech, Manchester, UK).

Statistical analyses: SPPS v.15L (SPSS Inc, Chicago, IL) and Excel (Microsoft Corporation) were used. Groups were compared using chi-squared for categorical variables, and Student's t (parametric) or Mann-Whitney's test (non parametric) for quantitative variables. To establish correlations between variables, Pearson's r (Gaussian distribution) and Spearman's Rho (non-Gaussian distribution) were used. In order to find predictors of *PCMT1* expression, multivariate, linear regression analysis was also performed. P was considered significant if <0.05.

Results

Human type 1 diabetes

A total of 83 patients (47% women) were included: 35 with type 1 diabetes (18.6 (18.2) months' duration), 19 with type 2 diabetes (24.2 (17.4) months' duration) and 29 controls. Their main features are displayed in table 1. In the type 1 diabetes group, all subjects were on multiple insulin injections (3 with metformin). In the type 2 diabetes group, seven people used insulin (five with oral agents), ten were on oral agents only (mostly metformin) and two managed their disease with diet and exercise alone.

Patients with type 1 diabetes tended to show lower *PCMT1* mRNA expression than type 2 diabetes, but similar to controls, whereas PIMT protein levels tended to be higher in type 1 diabetes than in controls, both in RBCs and in plasma. IsoAsp residues tended to be more abundant in type 1 diabetes than in type 2 diabetes, but similar to controls. No significant differences were found for any of the PIMT/*PCMT1*-related variables between patients with and without autoimmune disease (data not shown).

PCMT1 genotype distribution followed Hardy Weinberg equilibrium. No association was found between genotype (12 AA, 23 GA, 25 GG) and study group, nor with *PCMT1* expression, PIMT protein concentrations or IsoAsp levels. This was true both when all three genotypes were analysed separately and when the heterozygous genotype was grouped with each of the homozygous genotypes (AA+GA vs GG and GG+GA vs AA).

PIMT protein levels in plasma and RBCs were correlated (Pearson's r: 0.542, p=0.001), as were *PCMT1* mRNA expression levels normalized to β-actin and to *GAPDH* ($p<0.001$). A moderate correlation (Pearson: 0.360, p = 0.004) was found between erythrocyte PIMT and IsoAsp, but not between IsoAsp and *PCMT1* expression or plasma PIMT levels.

PIMT protein content was positively correlated with HbA1c (Rho 0.413, p= 0.015 for plasma PIMT and Rho 0.317, p= 0.008 for RBC PIMT), but not with glucose, and tended to be correlated with BMI (r 0.342, p=0.051 for plasma PIMT), triglyceride (Rho 0.222, p=0.071 with RBC PIMT) and

some hepatic transaminases (r -0.318, p=0.081 for AST with plasma PIMT and r 0.239, p=0.057 for RBC PIMT and ALT). IsoAsp was correlated with eGFR (Rho=0.324, p=0,008), whereas no correlation was found for any of the PIMT-related variables with age or cholesterol.

PCMT1 mRNA expression tended to be correlated with ALT (Rho 0.231, p= 0.076). However, in a multivariate analysis model including confounding variables, only age was significantly associated with *PCMT1* expression (p=0.021 for *GAPDH* and p=0.092 for β-actin adjusted)

Among the subjects with type 1 diabetes, *PCMT1* expression was significantly higher in anti-IA2 positive individuals (n=12) than in anti-IA2 negative individuals (n=8), for mRNA expression levels normalized to *GAPDH* (4.11 (2.88) vs 1.04 (1.15), p= 0.031) and almost significant for mRNA expression levels normalized to β-actin (0.33 (0.19) vs 0.17 (0.13), p=0.057). Furthermore, IA2-titer tended to be correlated with *PCMT1 mRNA expression* Rho 0.427, p=0.061). There was no correlation between duration of type 1 diabetes or the presence or number of associated autoimmune diseases and *PCMT1* mRNA expression, measures of PIMT content or IsoAsp. In a multivariate analysis model including age, sex, IA2- and GAD-antibodies, only age was significantly associated with *PCMT1* expression. In models including number of autoimmune diseases (0, 1 and 2 or more), this variable did not reach statistical significance.

Canine diabetes

Blood samples were obtained from 8 diabetic [(5 mongrels, 1 Yorkshire terrier, 1 Cocker spaniel, 1 Griffon), 7 female, mean age 9 (5-14) years] and 8 matched control dogs. *PCMT1* mRNA expression was similar in both groups (0.95 [0.31-3.89] and 0.92 [0.90-1.09], respectively, p>0.2). Pancreatic tissue samples showed fewer islets and less insulin-positive areas in the diabetic dogs than in the matched controls (see figure 1). PIMT was located in the cytoplasm of pancreatic cells, but its expression was not limited to insulin-positive cells, or to Langerhan's islets.

Pcmt knock-out mice

Although a certain degree of heterogeneity was present, KO mice showed growth-delay, kyphosis, macrocephaly, cachexia, and some signs of abnormal behaviour, such as anhedonia. Total body weight was lower in the 6 KO than in the 6 wildtype mice [19.92 g (±4.17) vs 21.33 (±5.72); p=0.59], though no differences were found in relative liver [5.15% (±0.72) vs 4.68 (±0.59); p=0.46], pancreas [1.06% (±0.17) vs 1.05 (±0.19)], p=0.77] or paragenital fat [1.20% (±0.46) vs 1.08 (±0.19); p=0.23)] weights. HOMA-IR index did not significantly differ between groups [KO 9.40 (±1.96) vs Wt 12.04 (±12.0); P = 0.62].

At 60 days of age, *Pcmt* KO mice showed normal glucose tolerance, but lower insulin concentrations than the wildtype mice after the glucose load: [1.33 (±0.18) vs 2.18 (±0.23) µg/L, *P*=0.01 at 30 minutes] (fig. 2a). During the ITT, KO mice showed a lower glucose nadir (32 mg/dl [13.5-71] vs 45 mg/dl [30-71]) and lower glucose at 120 min (83 mg/dl [44-131] vs 101 mg/dl [72-149]) ($p<0.05$) (see figure 1c). Furthermore, they showed lower baseline [440.9 (± 45.3) vs 593.2 (± 70.2) pg/ml; *P* = 0.043], AUC [496.6 (± 10.1) vs 623.9 (± 20.2), *P* = 0.002] and peak glucagon secretion [570.3 (± 54.8) vs 706.0 (± 32.9) pg/ml, *P* = 0.03] (fig. 3e), but similar corticosterone concentrations [205.9 (±84.6) vs 147.4 (±47.3) ng/ml; p=0.27] (figs. 3d and 3e). Heterozygous *Pcmt1*+/- and wildtype mice showed similar OGTT and ITT (fig. 3), both at 120 and 360 days of age, as well as similar HbA1c [4.12 (0.67)% [22mmol/mol (7.3)], vs 4.02 (0.45)% [20mmol/mol (4.9)], *p* = 0.66] at 120 days of age.

Histological examination of pancreatic samples showed Number of islets Pancreatic structure is being revised. Immunohistochemistry revealed co-localization of PIMT and insulin in the wildtype mice (fig. 3). Indeed, insulin expression was reduced in *Pcmt* KO pancreata. Glucagon expression is pending

Discussion

Type 1 diabetic patients and diabetic dogs showed *PCMT1* mRNA expression which was similar to their normoglycaemic controls. *PCMT1* genotype and accumulation of IsoAsp in blood were not different either, when the type 1 diabetic patients were compared with their hyperglycaemic and normoglycaemic controls. Nevertheless, PIMT protein concentrations (both in red cell cytoplasm and in plasma) tended to be higher in the group with type 1 diabetes than in age-matched healthy controls. Older, type 2 diabetic subjects showed significantly higher concentrations than healthy controls. This, together with the fact that PIMT protein levels were correlated with HbA1c, suggests that hyperglycaemia might play a role in PIMT protein expression. Furthermore, borderline correlations with BMI, triglyceride and hepatic transaminases suggest an association with markers of insulin resistance

Although the *Pcmt* KO mice showed normal glucose tolerance, they were relatively insulinopaenic and very sensitive to insulin, adding to the data supporting a relationship between PIMT and insulin resistance. Nevertheless, coexisting low glucagon concentrations (and low pancreatic glucagon by IHQ-to be checked) suggest that PIMT may play a role in islet development, and that increased insulin sensitivity and slow response to hypoglycaemia might be related to low insulin and glucagon production by immature/aplastic islets. Indeed, the parallel staining intensity of insulin and PIMT found in dogs and in mice (glucagon pending) support this hypothesis. No relevant clinical consequences were identified in mice lacking one copy of the *Pcmt* gene.

On the other hand, the support for an association between PIMT and autoimmunity is rather weak. Although increased *PCMT1* mRNA expression was seen in IA2-positive type 1 diabetes patients, as well as an almost significant correlation with IA2 titers, no association was found for any of the *PCMT1*/PIMT related variables and the presence/absence or number of autoimmune diseases.

Previous studies show a range of results regarding *PCMT1* mRNA and PIMT protein expression, depending on the species and tissues analysed and changes with age are not consistent, either (Desrosiers & Fanélus 2011). In the present study, no correlation was found between age and PIMT

protein concentrations, but it was higher in the older, more obese, type 2 diabetes group. To our knowledge, this is the first time PIMT concentrations were measured in plasma. In multivariate analysis, age was the only variable significantly associated with *PCMT1* expression.

Human, hepatic *PCMT1* mRNA is down-regulated in obese subjects (most with type 2 diabetes) (Pihlajamäki et al JCEM 2009). Indeed, its T3-induced expression is reduced by high-fat diet in C57Bl/6 mice (Pihlajamäki). Opposite to our positive correlation of PIMT with BMI and higher levels in T2D.

Examination of insulin signalling in *Pcmt* KO mice showed upregulation of the insulin receptor and activation of the Akt pathway (Farrar et al 2005). In fact, inhibition of with wortmannin, reduces this activation and prolongs their life expectancy (McKay et al 2012. PlosOne). Read these two articles carefully.

PCMT and apoptosis (Wägner et al 2007, Huebscher et al 1999,...) can also give explanation to some of the results.

Lack of concordance between *PCMT1* gene and PIMT protein results have been reported before (Wägner et al 2007) and regulation by miRNA has been shown in hepatoma cell lines (Amer et al. 2014) (Sambri et al. 2011), A genome wide methylation analysis in coronary artery disease detected *PCMT1* hypermethylation in cases compared to controls and consequences on *PCMT1* expression (Dick 2011).

In a study assessing the intrauterine effects of pregestational diabetes (Wägner et al -abstract Diabetologia 2015), *PCMT1* gene expression was lower in both the maternal and the foetal sides of the placenta of mothers with type 2 diabetes than in age- and gestational age-matched controls (unpublished results). No differences were found between mothers with type 1 diabetes and their healthy controls, but the former showed lower *PCMT1* expression in the foetal and maternal sides of the placenta than women whose partners had type 1 diabetes. No differences were found between type 1 and type 2 diabetes or between the foetal and maternal sides within each group. Interestingly,

mothers whose partners had type 1 diabetes showed lower *PCMT1* expression in the foetal than in the maternal side of the placenta.

The main strength of the present study is the multifaceted approach in the assessment of protein isomerisation and its repair by PIMT in type 1 diabetes, including human disease, an animal model of spontaneous diabetes and a mouse model lacking the protein of interest. In addition, both functional and histological studies have been performed in the animal models. Unfortunately, the results obtained are not conclusive. Some of the drawbacks include a relatively small number of patient samples, especially for correlations, the fact that all measurements were not available for all subjects and that the only type of human sample available was blood. Regarding the use of the dog as a model of type 1 diabetes, characterisation of our canine population indeed revealed that dioestrus diabetes (closer to human gestational diabetes) was the most common type (Brito-Casillas et al. ECVIM 2014). Short life expectancy in the Pcmt1 KO mice limited their full characterisation

In conclusion, in peripheral blood, PIMT expression seems to be associated with hyperglycaemia and other components of the metabolic syndrome, although a relationship with diabetes-specific autoimmunity cannot be ruled out. Locally, PIMT may play a role in islet cell development, both at the ß and the α-cell level.

Acknowledgements

Author Contributions paragraph

This paragraph should list each author's contributions as shown on the manuscript submission forms and should be placed in the Acknowledgments.

JCW: design of the project, coordination of the laboratory part of the project, erythocyte preparation, ELISA, preparation of manuscript

YBC: studies of PIMT KO and control mice (OGTT, ITT....), necropsy of mice and dogs, histological and immunohistochemical examination of pancreatic tissue, hormone level analysis by ELISA and HbA1c analysis.

LLR: human blood sample processing, DNA extraction, primer design, PCR, qRT-PCR, preparation of samples for HPLC, statistical analysis.

OPL: analysis of samples by HPLC

CM: coordination of the clinical veterinarian part of the project, identification and clinical characterisation of diabetic dogs

RMS: clinical data acquisition and analysis

FJN: identification of type 1 and type 2 diabetic patients and of healthy controls,

AMW: design of the project, organization of the clinical part of the project, inclusion of patients, analysis and interpretation of results, preparation of the manuscript

Brief statements of assistance:

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Part of the results of this study have been previously presented at the European Association for the Study of Diabetes annual meetings 2011 and 2013, as well as to the European Congress of Veterinary Internal Medicine 2011.

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Table 1: Patient characteristics and group comparison

Variables (n/n/n)	Controls	T1D	T2D	P value		
				T1D vs C	T1D vs T2D	T2D vs C
Age (years) (29/35/19)*	30.9 (10.2)	29.1 (10.1)	46.8 (17.8)	0.433	<0.001	0.001
Sex (N, % male)	16(55.2%)	18(51.4%)	10(52.6%)	0.770	0.934	0.866
rs4816 *PCMT1* Genotype (GG/GA/AA)	12/8/2	10/8/8	5/9/2			
Weight (Kg) (27/35/19)*	73.32 (15.28)	71.39 (15.63)	86.05 (14.83)	0.443	0.001	0.012
Height (m) (28/34/17)*	1.72 (0.099)	1.72 (0.092)	1.67 (0.073)	0.968	0.066	0.095
BMI (Kg/m^2)(27/34/17)*	24.81 (4.01)	24.21 (4.35)	31.37 (5.25)	0.450	<0.001	<0.001
Fasting glucose (mg/dl) (29/19/10)	88.3 (10.5)	159.5 (88.9)	106.6 (19.4)	<0.001	0.040	0.013
HbA1c (%, mmol/mol)(28/35/18)	5.27 (0.31) 34 (3.3)	7.67 (2.33) 60 (25.5)	6.64 (1.04) 49 (11.3)	<0.001	0.130	<0.001
Systolic blood pressure (Hg mm) (15/29/19)*	112.9 (13.6)	116.0 (11.7)	126.8 (17.3)	0.536	0.016	0.015
Diastolic blood pressure (Hg mm) (15/29/19)*	73.9 (6.6)	72.1 (7.6)	79.4 (12.1)	0.478	0.017	0.104
Creatinine* (mg/dl) (29/34/18)	1.03 (0.16)	0.91 (0.13)	1.00 (0.17)	0.003	0.117	0.554
eGFR	80.8(11.3)	94.1(18.6)	78.5(20.3)	0.001	0.007	ns
Total cholesterol (mg/dl) (28/33/19)*	177.9 (32.2)	172.1 (28.1)	177.7 (28.2)	0.455	0.464	0.986
HDLc (mg/dl) (28/32/19)*	57.9 (14.4)	53.3 (12.2)	45.0 (7.5)	0.245	0.019	<0.001
LDLc (mg/dl) (28/32/19)*	103.2 (25.1)	103.2 (26.6)	100.0 (22.8)	0.993	0.633	0.654
Triglyceride (mg/dl) (28/33/19)	83.5 (42.1)	78.3 (34.5)	148.9 (72.2)	0.988	<0.001	<0.001
ALT (U/l)(29/30/16)*	24.3 (13.3)	19.8 (8.6)	29.0 (17.8)	0.387	0.022	0.231
AST (U/l)(29/30/13)*	23.1 (7.5)	18.0 (4.9)	23.9 (7.3)	0.002	0.005	0.629
TSH (mIU/l) (28/33/12)*	2.02 (1.14)	1.97 (1.16)	2.85 (2.31)	0.701	0.268	0.273
PIMT mRNA expression[a] (24/26/16)	3.01 (3.10)	2.42 (2.59)	1.09 (1.75)	0.756	0.078	0.066
PIMT mRNA expression[b] (24/26/16)	0.26 (0.21)	0.27 (0.21)	0.15 (0.16)	0.854	0.092	0.149
Plasma PIMT (optical density at 450nm) (14/11/11)*	1.11 (0.30)	1.33 (0.32)	1.43 (0.44)	0.066	0.365	0.033
Cytosol PIMT (µg/ml) (27/26/17)*	13.83 (6.64)	17.69 (7.66)	22.80 (11.85)	0.055	0.320	0.002
SAH_BCA (IsoAsp) (ng/µl) (25/27/16)	5.97 (6.09)	5.76 (3.59)	4.15 (2.61)	0.558	0.085	0.500

T1D: Type 1 diabetes. **T2D:** Type 2 diabetes. **C:** Controls *normally distributed variables, a) normalized to *GAPDH*, b) normalized to *beta-actin*. eGFR was calculated by the MDRD4 equation: 186 x (creatinine) $^{-1.154}$ x (age) $^{-0.203}$ (x 0.7402 if female) (*Levey et al* 1999)

4. Paper 4

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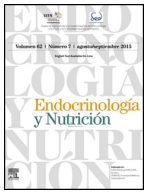

ORIGINAL ARTICLE

Uromastyx acanthinura as a natural treatment in a mouse model of type 2 diabetes

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KEYWORDS
Diabetes;
Uromastyx;
African traditional medicine;
Cross-over design;
Animal reduction;
Neuropathy

Abstract
Aims: Oral testimonies from North Africa attribute anti-diabetic effects to medicinal preparations of the lizard *Uromastyx acanthinura* (UA). No scientific evidence of such effects is currently available. The acute effects of oral administration of UA to C57Bl/6J mice with diet-induced diabetes were tested and, if effectiveness was shown, to assess the effect of subchronic UA administration in the same model.
Methods: Mice were fed a diet containing 60% fat for at least 12 weeks. To assess acute effects, different doses of UA or saline were orally administered with 2 g of glucose/kg during an oral glucose tolerance test (OGTT) on different days in a randomised crossover design. The most effective dose was then fed together with the high-fat diet for 90 days and compared to high-fat diet alone in a parallel design. Body weight (BW), food consumption, welfare, and external appearance were assessed weekly. HbA1c, OGTT, and intraperitoneal insulin tolerance tests (IPITT) were performed at baseline and after treatment. Severity of neuropathy was evaluated by cold allodynia response in the acetone test.
Results: UA significantly decreased glucose levels as compared to saline 15 min after administration. After 90 days of treatment, no differences were seen in OGTT or HbA1c between the groups, while IPITT showed higher glucose levels in UA-treated animals. Although weight increase in both groups, weight tended to be higher in the treated group, which had a significantly higher daily food consumption. Cold allodynia response improved in frequency and intensity in the UA group.

Abbreviations: UA, *Uromastyx acanthinura*; OGTT, oral glucose tolerance test; BW, body weight; IPITT, intraperitoneal insulin tolerance test; AUC, area under the curve.

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Conclusions: Orally administered UA acutely decreased blood glucose in diabetic mice. Paradoxically, long-term administration of UA increased food consumption, weight, and insulin resistance. Improved nociceptive response suggested an effect on pain and/or neuropathy. Although additional studies are needed to elucidate the properties and potential applications of UA, our results highlight the value of ethnomedical approaches to African traditional medicine as starting point to evaluate new bioactive components.

PALABRAS CLAVE
Diabetes;
Uromastyx;
Medicina tradicional africana;
Diseño cruzado;
Reducción animal;
Neuropatía

Uromastyx acanthinura como tratamiento natural en un modelo murino de diabetes tipo 2

Resumen
Objetivos: Testimonios orales Norteafricanos atribuyen efectos hipoglucemiantes a preparados medicinales del lagarto *Uromastyx acanthinura* (UA), para los que no existen evidencias científicas actualmente. El objetivo de este trabajo fue el de investigar los efectos agudos de UA administrado oralmente en ratones diabéticos C57Bl/6J inducidos por dieta grasa, y si se demostrase su efectividad evaluar el efecto de su administración subcrónica en el mismo modelo animal.
Métodos: Fue administrada una dieta a los animales con un contenido graso del 60% durante al menos 12 semanas. Para evaluar los efectos agudos diferentes dosis de UA o suero salino fueron administrados conjuntamente con 2 g/kg de glucosa durante sobrecargas orales de glucosa (SOG), en diferentes días, siguiendo un diseño cruzado aleatorizado. La dosis más efectiva en esta fase fue entonces administrada mezclada en la dieta durante 90 días y comparada con dieta solo en un diseño paralelo. El peso corporal y el consumo de alimento fueron evaluados semanalmente. HbA1c, SOG, y test de tolerancia intraperitoneal a la insulina (TTIPI) fueron realizados al inicio y tras el tratamiento. La gravedad de la neuropatía fue determinada mediante la evaluación de la alodinia al frío.
Resultados: El UA redujo significativamente las concentraciones de glucosa de manera aguda en comparación con el control a los 15 min tras su administración. Tras 90 días de tratamiento no se observaron diferencias en las SOG o HbA1c entre grupos, mientras que para los test de tolerancia intraperitoneal a la isulina valores más altos de glucosa fueron determinados en los animales tratados con UA. Aunque ambos grupos aumentaron su peso, este tendió a ser mayor en los tratados, que a su vez consumieron significativamente más comida por día. La respuesta a la alodinia al frío mejoró en frecuencia e intensidad en los tratados con UA.
Conclusiones: El UA administrado oralmente redujo de manera aguda la glucosa en sangre en ratones con diabetes. Paradójicamente, su administración crónica aumentó el consumo de alimento, el peso y la resistencia a la insulina. La mejora en la respuesta nociceptiva sugiere un efecto en el dolor y/o la neuropatía. Aunque son necesarios más estudios para aclarar las propiedades y posibles aplicaciones de este producto, nuestros resultados subrayan el valor de los enfoques etnomédicos hacia la medicina tradicional africana como origen para la evaluación de nuevos compuestos bioactivos.

Introduction

Metformin and exenatide, originally developed from natural sources, are widely used to treat diabetes.[1,2] Indeed, an ethnomedical approach can be used as the basis for drug discovery[3] and, once a bioactive product is identified, scientific screening and confirmation should follow. In this context, ancient, northern African, oral testimonies, reported by a patient, attribute glucose-lowering effects to a desert lizard [Uromastyx acanthinura, Bell, 1985; Black Spiny-tailed Lizard, Dab] (UA). Culinary testimonials described that the Uromastyx genus lizards are occasionally eaten by nomads,[4,5] but, to our knowledge, the only medicinal uses previously reported are otitis and earache, skin infections and burns.[6,7] The aim of this study was to assess the effect of an UA extract on glucose metabolism in an animal model of type 2 diabetes.

Materials and methods

An UA preparation, elaborated in Mauritania, was donated to our research group by a patient. Following an ethnomedical

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approach, an interview was performed and, after pharmacological information was obtained, UA's potential acute and chronic glucose-lowering bioactivity was evaluated in vivo in C57BL/6J mice with diet-induced diabetes.

In vitro testing was initially considered, but the lack of detailed pharmacological information, the heterogeneous nature of the product and the complexity of diabetes itself, made cell-assays a poor solution.

The interview was recorded and dissected to define: product acquisition and preparation, dosage and frequency and route of administration. The whole carcass of UA (head excluded) is grilled and dried, and then minced (0.3–3 cm) and incorporated into the diet. People traditionally take a handful of UA once or twice/day with food or water. The oral route was also used in the mice and doses were adjusted to body weight. A dose of 7.66 g was estimated for a 60 kg-person (0.13 g/kg), based on handful measurements of four different people and a range (0.13–1.56 g/kg) was tested in the mice.

The different doses of UA diluted in saline or saline alone (saline solution 0.9%, Braun Medical SA, Barcelona), were administered to each animal per body weight, from the starting dose 0.13 g/kg, with 2 g glucose/kg (Glucose G7528, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) during an oral glucose tolerance test (OGTT; fasting period from 8:00 h to 14:00 h) (Glucocard G+ metre, GT1280, Arkray-Menarini Diagnostics, Shiga, Japan),[8,9] on two different days, following a randomised, crossover design (Fig. 1a).[10] C57BL/6 mice were used fed a 60%-fat diet ($n = 13$, 8 males, 16 weeks of age) during the previous 12 weeks (D12492, Brogaarden, Lynge, Denmark), a well established model of type 2 diabetes.[11] Doses were increased until the desired effect was noted. In the case of absent effects, the highest dose which was feasible to be administered, based on density, concentration or volume, was selected as an endpoint. The same doses were evaluated simultaneously in the whole group. In the long-term evaluation, the dose which had been most effective in the short-term experiments, was administered daily for 90 days in the high-fat diet (UA group) and compared to high-fat diet alone (control group) ($n = 10$ per group: 50% males, 24 weeks of age, 20 weeks with 60%-fat diet). Body weight (BW), food and water consumption and welfare state were assessed weekly. Morning blood glucose was assessed every 1–2 weeks. HbA1c (DCA, Siemens Healthcare Diagnostics, Deerfield, IL), OGTT and intraperitoneal insulin tolerance tests (IPITT) were performed at baseline and after 3 months' treatment. Neuropathy was assessed at 3 months by cold allodynia response to acetone,[12] where higher nociceptive response scores indicate more severe neuropathy. Regarding sample size, to detect glycaemic variations of at least 30% ($\alpha = 0.05$) with a statistical power of 80%, nine animals per group were deemed necessary.[13] The crossover design applied in the acute phase allowed us to reduce this number. In the sub-chronic phase, ten animals per group were used, allowing for a 10% loss and to harmonise sex-distribution.

The area under the curve (AUC) of glucose was calculated by the trapezoid rule. For comparisons between groups, Wilcoxon's or Student's tests were used and results were presented as mean (standard deviation) or median [range]. A two-tailed $p < 0.05$ was considered significant (IBM SPSS Statistics Version 18, SPSS Inc., Chicago, IL). Animals were

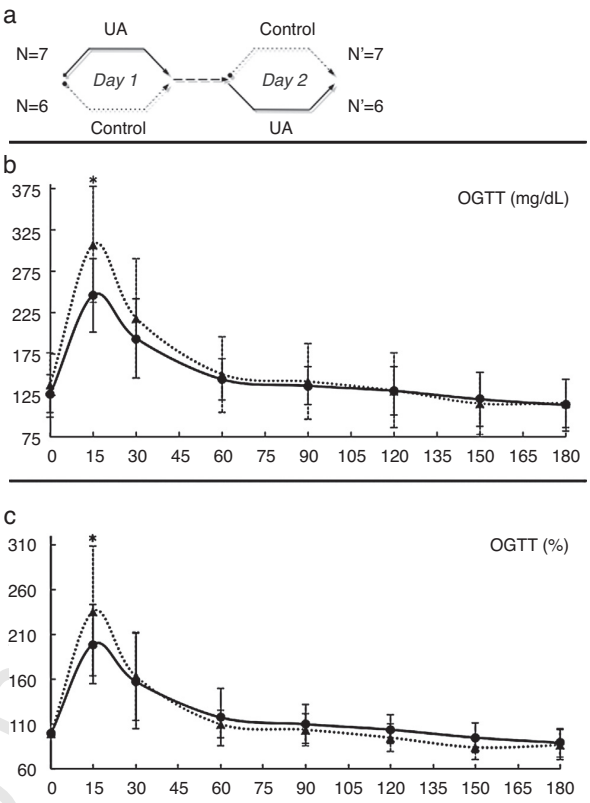

Figure 1 Results from the acute evaluation of *Uromastyx acanthinura* (UA) (solid line, UA; broken line, control). Crossover design of the experiment (a): on day 1, half of the group served as control while on day 2, after a 48 h rest, the same group received the treatment. The other half of the group received treatment on day 1 and served as control on day 2. Thus, each treated animal served as its own control. Glucose concentrations (mean and SD) during the oral glucose tolerance test (b) and percentage glucose change from baseline (mean and SD) during the oral glucose tolerance test (c) (* = $p < 0.05$).

housed in groups of four-five per cage and provided with environmental enrichment. The protocol was performed following National and European requirements (RD 1201/2005, Law 32/2007, EU Directive 2010/63/EU) and was approved by the Animal Welfare Ethics Committee (Comité Ético de Experimentación Animal de la ULPGC; 009/2011).

Results

Ethnomedical approach and short-term evaluation

Different traditional preparation methods of UA (basically, cooking methods) were described. Doses were rather approximate, since the person's hand is the most commonly used measure. The most effective dose was 0.048 g UA/mouse, which showed a glucose-lowering effect 15 min (′) after its administration [UA 246.15 (44.48) mg/dL vs. Control 307.39 (70.12) mg/dL; $p = 0.004$]. The AUC of glucose tended to be lower in the treatment arm [UA

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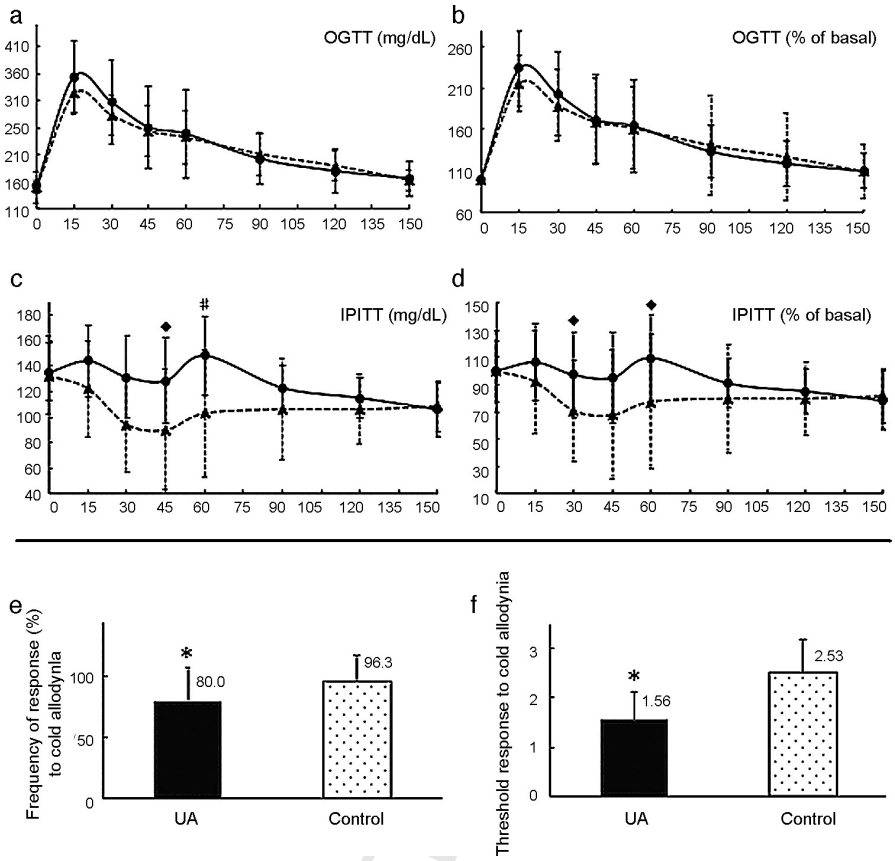

Figure 2 Results for the comparisons between the treatment (solid lines and columns) and control groups, after 3 months of *Uromastyx acanthinura* (UA): glucose concentrations during an oral glucose tolerance test (a) and percentage of baseline concentration during the OGTT (b); glucose values (mg/dL) per minute during the intraperitoneal insulin tolerance test (IPITT) (c) and percentage of baseline concentration during the IPITT (d); frequency (%) (e) and intensity threshold (f) of response to cold allodynia (*, $p < 0.05$; #, $p < 0.065$; ♦, $p \leq 0.093$).

203.27 (35.18) vs. 222.90 (54.48) mg/dL/min; p = 0.073] (Fig. 1b). Similar effects were observed at 15′ when results were defined as percentage variation from fasting glucose (Fig. 1c).

Long-term evaluation

In the post-treatment evaluation (UA $n = 8$, Control $n = 10$), no significant differences were observed for OGTT (Fig. 2a and b) or HbA1c [UA 5.06 (0.48), Control 4.78 (0.29) %; p = 0.134]. In the IPITT, there was a trend towards higher glucose values for UA at 60′ [148.12 (33.47) vs. 102.9 (52.40) mg/dL; p = 0.051], and 45′ [128.56 (36.29) vs. 89.95 (49.82) mg/dL; p = 0.086] (Fig. 2c and d). During follow-up, morning plasma glucose concentrations were significantly lower in the active treatment group only transiently: at weeks three (UA 132 [85–165], Control 167 [118–233] mg/dL; $p = 0.004$) and five (UA 129 [119–139], Control 150 [111–201] mg/dL; $p = 0.012$) after treatment start. Although both groups increased their BW, the increment tended to be higher in the UA group ΔBW 6.45 (8.18) vs. 5.06 (3.94) g, p = 0.064], which consumed more food [2.86 (2.83–2.86) vs. 2.52 (2.40–2.64) g/mouse/day, $p < 0.001$]. Cold allodynia response was less frequent [75 (37.5–100) vs. 100 (87.5–100)%; p = 0.034] and intense in the treated group [0.81 (0.35) vs. 1.22 (0.33); p = 0.022] (Fig. 2e and f). During long-term evaluation, idiopathic ulcerative dermatitis (pruritus, alopecia and ulcers) developed in both groups, associated with a certain level of distress (unplanned increment in light brightness). The source of distress was removed and animals were treated [enrofloxacin (7.5 mg/kg, 8 days); topic hydrogen peroxide and povidone-iodine]. All but two mice (2 females in the UA group, which were sacrificed due to their worsened condition) recovered their normal health status and stress level.

Discussion

Oral administration of UA acutely reduced blood glucose in diabetic mice. Paradoxically, long-term administration of UA tended to increase insulin-resistance, food-consumption and mean BW. Symptoms of neuropathy were significantly attenuated in the treated group.

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The acute glucose-lowering effects of UA might be explained by the persistence, in its carcass, of a bioactive compound, either synthesised by UA itself, as is the case of Heloderma suspectum (exendin),[2] or included in its diet and stored. Indeed, some of the plant species eaten by UA[14] have shown glucose-lowering effects.[15,16]

Food intake and BW increments could explain the discrepancy between the short- and long-term results and the lack of persistence of lower glucose values after the first 5 weeks of treatment. However, other causes can be suggested, including dose (insufficient for the long-term study), pharmacokinetics (short duration of action) or other, unknown metabolic effects.

Insulin-resistance significantly increased in the UA group when compared to controls. Therefore, the improved response to cold allodynia could be due to direct effects on pain or neuropathy itself, and not as side-effect from a better glucose control.

The effects detected in the acute evaluation indicate that the crossover design suited its purpose and allowed a reduction in the number of animals, which is one of the mandatory demands of the principles for laboratory animal handling.[17–20] Randomisation of treatment order reduced period bias, as well as bias from possible cumulative effects of UA. The homogeneity of results regardless of the treatment sequence (saline-product vs. product-saline) confirmed that the time left for clearance between treatments was long enough. The crossover design,[10] could be an answer to the claim to reduce animal numbers in the pre-evaluation of glucose-lowering and other bioactive compounds.

Only data obtained from animals that were healthy at the end of the study were considered. However, a confounding effect of the development of dermatitis and the distress level cannot be ruled out.[21] This dermatitis was not attributed to UA, since both groups were affected by what is a common disorder in C57Bl/6J.[21] Although a role of UA in the worse outcome (two losses) cannot be excluded, previous reports describe its topical effects as a treatment for, rather than a cause of dermatitis.[6]

Finally, our findings suggest direct effects of UA on glucose metabolism and appetite, as well as on pain and/or neuropathy, approaches to consider in further studies. They also support the value of African traditional medicine as a starting point for the screening of new bioactive components.

Conflict of interests

The authors are not aware of any conflicts of interest related to the contents of this article.

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5. Patent and paper 5

Patent pending (ref. number P201500700)

Draft to be sent to Diabetologia.

Abstract attached

'Acute and sub-chronic effects of *Aloe vera* in a mouse model of type 2 diabetes'

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Keywords: *Diabetes; Aloe; Traditional medicine; cross-over design; animal reduction*

Abbreviations:
UV: *Aloe vera*. OGTT: oral glucose tolerance test. BW: body weight. HbA1c: Glycated haemoglobin. IPITT: intraperitoneal insulin tolerance test. EIA: … AUC: area under the curve.

Abstract

Background and aims

Natural products are a potential source of new treatments for diabetes. Aloe vera (*AV*) has shown glucose-lowering effects in human and animal studies, but more accurate evaluations are required. Our aims were to assess acute and chronic effects of the administration of *AV* in a mouse model of type 2 diabetes.

Methods

Sixteen weeks old C57BL/6 mice (n=10/group, 50% males) were fed a 60% high-fat diet for at least 20 weeks. In order to assess its short-term effects, different doses of *AV* or saline were administered orally with 2 g glucose/Kg during a OGTT, on different days, in a randomised cross-over design. The most effective dose was administered daily in the diet, for 3 months, and compared with high-fat diet only, in a parallel group design (n=10/group, 50% males). Body weight, food and water consumptions and welfare state were assessed weekly. HbA1c (DCA, Siemens Healthcare Diagnostics, Deerfield, IL), OGTT and IPITT were performed before and after treatment. Glucose (Glucocard G+ meter, GT1280, Arkray-Menarini Diagnostics, Shiga, Japan) and insulin (Rat Insulin EIA Kit; Spi Bio, Cayman Chemical Montigny Le Bretonneux) concentrations were measured. AUCs were calculated by the trapezoid rule. Blood sampling and macroscopic organ evaluation, were performed post-mortem. For comparisons between groups, Wilcoxon's test or Student's test for paired data were used. A two-tailed $p<0.05$ was considered significant.

Results

Short-term experiments showed a glucose-lowering effect of *AV*, when compared to saline (at 15 min 240.1 ± 25.3 vs 311.9 ± 54.9 mg/dl, p=0.003; AUC 270.8 ± 34.6 vs 307.3 ± 61.7, p=0.016).

After chronic treatment, no significant differences were observed between groups overall, regarding OGTT, IPITT, HbAor the rest of blood tests.

However, when analysed by sex, in the treated female mice, final HbA1c was lower than in controls (4.76 ± 0.17 vs 4.84 ± 0.35, p=0.049), whereas glucose concentrations during the IPITT (at 15 min 100.7 ± 14 vs 75.9 ± 14 mg/dl; p=0.024) and insulinaemia during the OGTT (at 60 min, 0.41 ± 0.03 vs 0.10 ± 0.09 ng/ml, p=0.044) were higher.

Treated males showed a higher final BW (54.9 ± 2.1 vs 50.7 ± 3.4, p = 0.045), a relatively higher weight of the pancreas (pancreas/BW) (0.56 ± 0.003 vs 0.48 ± 0.004 %, p = 0.012) and a relatively lower left paragenital fat weight (0.76 ± 0.14 vs 1.4 ± 0.17 %, p = 0.001). A similar, non-significant trend was found in females.

Conclusion

AV acutely reduces blood glucose in diabetic mice. Longer-term treatment increases insulin secretion and improves glucose control in females.

6. Paper 6

Draft to be submitted to *Diabetologia*.

Abstract is attached

'Can we use HbA1c as a diagnostic tool in mouse models of diabetes?'

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Keywords: *Diabetes; HbA1c; mice; Animal Refinement; Glucose metabolism*

Abbreviations:
OGTT: oral glucose tolerance test. BW: body weight. IPITT: intraperitoneal insulin tolerance test. AUC: area under the curve

Abstract

Introduction

Glycated haemoglobin (HbA1c) has become a milestone in diabetes and is the best indicator for plasma glucose concentration over long periods in humans. In mice, there are no recommended devices or standards for HbA1c, and glucose metabolism characterisation is based on basal glucose and oral glucose and insulin tolerance tests (OGTT and ITT). Our aim was to establish a cut-off point for HbA1c measurements in diabetic and non-diabetic mice.

Methods

Data were collected from previous experiments with high-fat diet-induced diabetic C57Bl/6J mice and normoglycaemic B6;129S4-Pcmt1$^{tm1Scl+/-}$ and $^{+/+}$ mice. Body weight (BW), sex, age, fasting glucose, HbA1c, AUC and peak glucose concentrations during OGTT, AUC and nadir during ITT were assessed. Glucose measurements were obtained using a portable blood glucose meter (Glucocard-G+ meter). HbA1c was measured using an NGSP-standardised, point of care instrument (DCA, Siemens Healthcare Diagnostics, Deerfield, IL). Diabetes was defined as glucose concentrations above 200 mg/dL (11.1 mmol/L) during the OGTT.

Correlations with HbA1c (linear regression; Pearson's test) and ROC-curves were evaluated to establish a cut-off equivalent to a glucose concentration of 200 mg/dl, and assess the specificity/sensitivity for diagnosis.

Results

A total of 205 HbA1c measurements from 82 C57BI/6J and 24 B6;129S4-Pcmt1^{tm1Scl} mice were available. HbA1c (4.6% [3.4-7.3]) correlated positively with fasting glucose, OGTT peak and AUC ($p<0.007$) and negatively with ITT glucose nadir ($p<0.001$). For diabetic mice (OGTT glucose >200 mg/dL, n=173 HbA1c), BW, glucose nadir and fasting glucose were the parameters most strongly correlated with HbA1c and the diagnosis of the disease ($p<0.001$). Median HbA1c for diabetes was 4.6% [3.4-7.3] and for non-diabetes 4.0% [3.4-4.7]. ROC-curves showed an optimal cut-off for the diagnosis of diabetes at 4.2% (specificity 60%; sensitivity 75%). AUC

Conclusion

Based on the present results, an HbA1c cut-off of 4.2% is identified for the diagnosis of diabetes in this mouse model of diabetes, but both its specificity and sensitivity are rather low. Indeed, standardised, internationally accepted, diagnostic criteria are still to be established, and more studies are needed to explore the use in this and other models of the disease.

CONCLUSIONS

VI. CONCLUSIONS

The main conclusions achieved in the present Thesis on each section are:

1 Characterisation of canine diabetes in a population from the Canary Islands

a) The prevalence and incidence of canine diabetes in the Canary Islands is similar to those found in other regions

b) Dioestrus diabetes is the most frequent type of canine diabetes in our region, followed by idiopathic/autoimmune diabetes.

c) Autoantibodies against islet GAD65 and IA2 are present in a proportion of diabetic dogs from Canary Islands

d) Pancreata of diabetic dogs show severe reduction in the number of islets and β-cell mass. Time since diagnosis and cause of cDM determine the level of β-cell loss.

2 ISO-Based assessment of accuracy and precision of glucose meters in dogs

a) The disparity among devices in this study confirms the need of accuracy evaluations before its use in dogs

b) The adapted ISO 15197:2013 is an excellent tool for this purpose in animals, as it is in humans

c) Although none of the PBGM fulfilled the ISO requirements for whole venous blood, overall, AccuChek Aviva Nano is the best option among those evaluated, given its accuracy, precision, and lack of interference by hematocrit.

3 The repair enzyme protein isoaspartyl methyl transferase (PIMT) in type 1 diabetes

a) In peripheral blood, PIMT expression seems to be associated with hyperglycaemia and other components of the metabolic syndrome, although a relationship with autoimmunity cannot be ruled out.

b) Locally, PIMT may play a role in islet cell development, both at ß and α-cell level

4 *Uromastyx acanthinura* as a natural treatment in a mouse model of type 2 diabetes

a) Oral administration of UA acutely reduced blood glucose in diabetic mice.

b) Therefore, cross-over design applied to the detection of acute hypoglycaemic effects fit the purpose and allowed a reduction in the number of animals. This could be an answer to the claim to reduce animal numbers in the pre-evaluation of glucose-lowering and other bioactive compounds

c) Paradoxically, long-term administration of UA tended to increase insulin-resistance, food-consumption and mean BW. It also attenuated the symptoms of neuropathy
d) Our findings suggest direct effects of UA on glucose metabolism and appetite, as well as on pain and/or neuropathy, approaches to consider in further studies.
e) These results support the value of African traditional medicine as a starting point for the screening of new bioactive components.

5 Acute and sub-chronic effects of *Aloe vera barbadensis* in a mouse model of type 2 diabetes

a) Aloe vera acutely reduces blood glucose in diabetic mice.
b) Longer-term treatment increases insulin secretion and improves glucose control in females.

6 Can we use HbA1c as a diagnostic tool in mice?

a) HbA1c, measured with a point-of-care analyser, enables the assessment of long-term glucose metabolism in mice, improving research refinement
b) Although an HbA1c of 4.2% was the best cut-off point for the diagnosis of diabetes in our sample, low sensitivity and specificity limit its use for this purpose

APPENDIXES

VII. Appendixes

1. Additional *Curriculum Vitae* during this Thesis

National Congress Communications

López-Ríos L, Boronat M, Wiebe JC, Brito Y, Lorenzo M, Aguilar JA, García Y, Chirino R, Nóvoa J, Wägner AM. **Asociación de la razón ApoB/ApoA1 y la ApoA1 con la resistencia insulínica y el síndrome metabólico**. XX Congreso de la Sociedad Española de Diabetes (SED). Tenerife. 2009.

Wiebe Julia C, López-Ríos Laura, Brito-Casillas Yeray, Arin-Martínez Ainara, González Elisa, Tugores Antonio, Boronat Mauro, Nóvoa Javier, Wägner Ana M. **La expresión de la enzima reparadora Proteina-L-isoaspartato (D-aspartato) O-metiltransferasa está reducida en la diabetes tipo 1.** XXII Congreso de la Sociedad Española de Diabetes (SED). Málaga. Abril 2011.

L. López Ríos, Y. Brito Casillas, J. Wiebe, A. Wägner, J.F. Nóvoa, R. Chirino, M. Boronat-Cortés. **Medidas antropométricas y criterios diagnósticos de síndrome metabólico como herramienta para la identificación de individuos con resistencia a la insulina**. XXIII Congreso Nacional de la Sociedad Española de Diabetes (SED). Vigo. 2012.

Elisa González, Julia C. Wiebe, Laura López-Ríos, Yeray Brito-Casillas, Mauro Boronat, Javier Nóvoa, Carlos Melián4, Ana M. Wägner. **Papel de la enzima reparadora Proteina-L-isoaspartato (D-aspartato) O-metiltransferasa en la diabetes tipo 1**. XXIII Congreso Nacional de la Sociedad Española de Diabetes (SED). Vigo. 2012.

Yeray Brito-Casillas, Rosa M. Sánchez, Julia C. Wiebe, Laura López-Ríos, Mauro Boronat, Octavio Pérez, Javier Nóvoa, Ana M. Wägner. **Concentraciones de proteínas modificadas (IsoAsp) y de la enzima reparadora proteína L-Isoaspartato (D-aspartato) O-metiltransferasa (PIMT) en pacientes con diabetes.** XXIV Congreso de la Sociedad Española de Diabetes (SED). Sevilla. 2013.

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International Congress Communications

Laura López-Ríos, Julia C. Wiebe, Ana M. Wägner, Yeray Brito Casillas, Mercedes Lorenzo Media, José A. Aguilar Doreste, Nuria Pérez, Ricardo Chirino, Javier Nóvoa, Mauro Boronat. **The value of APOB/APOA1 ratio for the diagnosis of insulin resistance.** 78th European Atherosclerosis Society (EAS) Congress. Hamburg. 2010.

Yeray Brito, Julia Wiebe, Ainara Arin, Laura López, Grant McNaughton Smith, Javier Nóvoa, Nicolas Diaz chico, Ana Wägner. **Short term effects of aloe vera in a mouse model of type 2 diabetes: randomised, cross-over studies**. Congreso de Veterinarios de Canarias, XVIII Congreso Internacional de la Sociedad Española de Cirugía Veterinaria (SECIVE) y del XV Congreso Internacional de la Sociedad Española de Medicina Interna Veterinaria (SEMIV). Gran Canaria. 2010.

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Brito-Casillas Y, López-Ríos L, Wiebe JC, Nóvoa-Mogollón FJ, Ojeda A, Wägner AM. **Use of desert lizard as a natural product to treat diabetes in a mouse model of the disease**. Diabesity Working Group of the European Renal Association, European Dialysis and Transplant Association. Puerto de La Cruz, Tenerife. 2014.

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MJ Molina-Cimadevilla, T García-Robles, C Muñoz-Mediavilla), Y Brito-Casillas, AM Wägner, P Rey, A Sanchez. **Revisiting genitourinary problems in mice: treatment and re-characterization of Mouse Obstructive Genitourinary Syndrome (MOGS).** I Congreso Ibérico de las Ciencias del Animal de Laboratorio (SECAL-SPCAL). Cáceres. 2015.

Other Articles

'Revisiting genitourinary problems in mice: treatment and re-characterization of Mouse Obstructive Genitourinary Syndrome (MOGS)'

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Submitted to: *Comparative medicine*. IF 1.12 (JCR). Q2, Veterinary Sciences

Abstract
The current study aimed to characterize and to explore a treatment for a condition in which male mice presented with a solid bulge in the preputial area. Forty-two animals, from several animal houses in Spain, were included in the study for microbiological and pathological characterization. The condition mostly affected breeding animals (78.6%) and was associated with C57BL/6J genetic background. A solid, yellowish white substance was found inside the prepuce, displacing the penis cranially, avoiding its externalization and causing infertility. This blocking substance was completely amorphous, acidophilic and showed peripheral clusters of spermatozoa, a pattern almost identical to post-coital female plugs, suggesting an ejaculatory origin. Opposite to what has been suggested in previous publications, the penis was completely intact in all of the cases, with no signs of mutilation or wounds. The rest of the organs were apparently functional and the microorganisms found (Enterococcus spp 72.7%, Escherichia coli 54.5% and coagulase-negative Staphylococci 54.5%) seemed to be secondary contaminants from faecal saprophytic flora. Clinically and pathologically two clear conditions were differentiated: one with urinary obstruction and one without, depending on the capacity of the blocking substance to lock the urethra.
Based on these findings, an unblocking surgical technique was developed to recover breeding performance and tested in 15 mice. The blocking substance was eliminated by a longitudinal incision made cranially from the opening of the prepuce. Most of the animals recovered fertility, but the treatment did not work when urinary obstruction was present. This technique can be useful to safeguard fertility in important, transgenic stud males.

'Glucolipotoxicity is Necessary for the Induction of β-cell Damage by Calcineurin Inhibitors'

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Abstract
Insulin resistance is needed for the induction of diabetes mellitus by calcineurin inhibitors. Tacrolimus is specially diabetogenic in the presence of insulin resistance compared to cyclosporin-A, but the mechanisms involved in this interaction are unknown. Several studies have suggested that β-cell transcription factors like FoxO1, MafA, PDX-1 and NeuroD are involved in the transition towards diabetes. Our aim was to evaluate the effect of calcineurin inhibitors and metabolic stressors (glucose and palmitate) on these transcription factors. INS-1 cells were cultured for five days in the presence of 100μM palmitate and 22mM glucose to mimic the insulin resistance *milieu in vitro*. The effects of

calcineurin inhibitors were assessed by treating the cells with 250ng/mL cyclosporin-A or 15ng/mL tacrolimus for 48h. Glucose and palmitate increased nuclear FoxO1 and decreased nuclear MafA and both effects were magnified by tacrolimus. The addition of cyclosporin-A, however, did not produce relevant changes. Interestingly, after five days of tacrolimus withdrawal or switch to cyclosporin-A, nuclear levels of FoxO1 and MafA recovered. These results were confirmed in *in vivo* experiments. Thus, our data indicates that the higher diabetogenicity of tacrolimus may be due to its effect on transcription factors that maintain β-cell function.

To be submitted to: *Metabolism, Clinical and Experimental*. IF 3.894 (JCR). Q1.

'Outbreak and eradication of zoonotic *Ornythonissus bacoti*: report in a European animal facility'

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Abstract
A zoonotic opportunistic out-break involving *Ornythonissus bacoti*, capable of invading animal facilities despite their barriers, is described. An eradication protocol with ivermectin was tested and implemented. Immunocompetent mice and rat strains in a conventional health status facility developed dermatologic manifestations, followed by weight-loss and wounds in severe cases. Some litters showed lesions ressembling petequiae distributed over the whole body. Initially, only mild isolated cases were detected, but therafter, scratching was spread. Simultaneously, facility staff suffered from pruritic, erythematous papular lesions of varying severity. Direct observation of the cages showed fast, oval insects that moved around and from animals to bedding. Further examination of carcasses and bedding allowed the characterization of these accarii as *Ornythonisus bacoti*.
A solution of 0.1% ivermectin in propyleneglycol and distilled water was chosen to be administered as spray. Treatment efficacy tests showed 0% survival rate of the accarii. Ivermectin safety was assessed in each animal strain and no toxicity was identified. Hence, all animals and beddings were sprayed. Based on the parasite's lyfe-cicle, treatment was repeated two times, weekly. The frequency of cage-cleaning was increased and racks, walls and cages were also sprayed with ivermectin. Infestation was regularly monitored by examining the animals' fur and carcass. Seven weeks after initial application, samples remained negative for the presence of accarii. Eradication of the parasitosis was confirmed and at the time of this report, ten months after diagnosis, *O. bacoti* still remains undetected.

Submitted to: *Journal of the American Association for Laboratory Animal Science*. IF 1.78 (JCR). Q2 Veterinary Sciences

Research Stays

The Royal Veterinary College, University of London, Vet School. London, United Kingdom. April 2012-July 2012.

Unidad de Fisiología Celular y Nutrición, Instituto de Bioingeniería, Universidad Miguel Hernández. 9-15 July 2010

Research Projects

Title: **Cambios posttransduccionales de las proteínas y diabetes tipo 1: papel de la enzima reparadora PIMT**
Funding: Fundación Canaria de Investigación y Salud (FUNCIS)
Duration: 1 year (2008-2009)
Principal Investigator: AM Wägner

Title: **Cambios posttransduccionales de las proteínas y diabetes tipo 1: papel de la enzima reparadora PIMT**
Funding: European Foundation for the Study of Diabetes (EFSD) (EFSD/JDRF/NN Type 1 Diabetes Programme 2008)
Duration: 2 years (2008-2010)
Principal Investigator: AM Wägner

Title: **Cambios posttransduccionales de las proteínas y diabetes tipo 1: papel de la enzima reparadora PIMT**
Funding: Instituto de Salud Carlos III (PI08/01113)
Duration: 3 years (2009-2011)
Principal Investigator: AM Wägner

Title: **¿Modifican los cambios epigenéticos la expresión de *PCMT1* en la diabetes tipo 1? Estudios en pacientes, hijos/as de madres/padres diabéticos y en un modelo animal**
Funding: Instituto de Salud Carlos III (PI11/02441)
Duration: 3 years (2013- 2015)
Principal Investigator: AM Wägner

Title: **Caracterización de la hipercolesterolemia familiar en la isla de Gran Canaria**
Funding: Sociedad Española de Arterosclerosis (SEA)
Duration: 3 years (2014-2017)
Principal Investigator: RM Sánchez-Hernández

Grants and awards

Grant: **Patogenia de la diabetes tipo 1 mediante el estudio de modelos animales de diabetes autoinmune**
Funding: Fundación Canaria de Investigación y Salud (FUNCIS) (ID 41-2008)
Duration: 4 years (April 2009-April 2013)
Principal Investigator: AM Wägner

Grant: **Albert Renold Travel Fellowship for Young Scientists**
Funding: European Foundation for the Study of Diabetes (EFSD)
Aim: Research stay at the The Royal Veterinary College, University of London.
Duration: 3 months (April-July 2012)
Principal Investigators: AM Wägner and B Catchpole
http://www.europeandiabetesfoundation.org/programmes/reports/flipbook2012/index.html#p=20

Award: First prize for ***'Estudio de la diabetes mediante un modelo animal espontáneo: evidencias de autoinmunidad en perros diabéticos de Canarias'*** at the First Competition of **'My Thesis Research Project on a Poster'**. Jornadas de Doctorado de la Escuela de Doctorado de la Universidad de Las Palmas de Gran Canaria (EDULPGC). March-April 2015. ULPGC. http://hdl.handle.net/10553/13294

Contracts

Commercial collaboration contract with POLINAT S.L. to develop research studies based on *know-how* developed during this Thesis (2014-2015)

2. Resumen en español

La diabetes mellitus supone un importante problema de salud pública, tanto por su prevalencia como por sus complicaciones. Canarias tiene la incidencia de diabetes tipo 1 infantil más alta descrita en España y la tasa de complicaciones renales triplica la media española.

Los modelos animales de la enfermedad, tanto espontánea como inducida, pueden mejorar la comprensión de la patogenia de la diabetes y además, ser de utilidad en el estudio de nuevos tratamientos. En este sentido, la presente tesis tiene una doble vertiente, primero sobre el **estudio de la patogenia de la diabetes tipo 1** y segundo, sobre el **secreening de nuevos tratamientos para la diabetes**. Además, sobre el estudio intrínseco de los modelos de estudios, se desea **implementar mejoras sobre su monitorización, refinamiento o reducción**.

1. Diabetes Mellitus: definición, clasificación y epidemiología.

La diabetes mellitus (DM) es un desorden metabólico caracterizado por la existencia de hiperglucemia, que se origina por un defecto en la secreción de insulina, en la acción de misma o en ambos [1, 2].

El diagnóstico de la DM se realiza con la coexistencia de síntomas (poliuria, polidipsia) y una glucosa plasmática superior a 200 mg/dL o una hemoglobina glicosilada (HbA1c o A1c) ≥ 6.5%, entre otros criterios (ver tabla 1) [2]. Es crucial un diagnóstico precoz para reducir el riesgo de complicaciones. Se estima que entre un 22-50% de los pacientes con diabetes tipo 2 (DM2) tienen complicaciones crónicas en el momento del diagnóstico [3, 4].

Criterios diagnósticos de la Diabetes Mellitus	
A1c ≥ 6.5%	Este test debe realizarse en un laboratorio usando el método certificado NGSP y estandarizado según el ensayo DCCT*
Glucosa plasmática en ayunas ≥ 126 mg/dL	Ayuno de al menos 8 horas*
Glucosa plasmática a las 2h ≥ 200 mg/dL tras una sobrecarga oral de glucosa	El test debe realizarse como describe la OMS usando una sobrecarga de glucosa equivalente a 75 g de glucosa anhidra disuelta en agua*
Síntomas clásicos de hiperglucemia o crisis hiperglucémica y una glucosa plasmática en cualquier momento del día ≥ 200 mg/dL	
*En ausencia de hiperglucemia marcada, los resultados deben ser confirmados repitiendo el test.	

Table 1: Criterios diagnósticos de diabetes mellitus. Adaptados de *'Classification and diagnosis of diabetes'*, ADA [1]. NGSP: National Glycohemoblogin Standardisation Program. DCCT: Diabetes Control and Complications Trial. OMS: Organización mundial de la salud.

A pesar de que la DM se conoce desde hace milenios y se han hecho muchos avances en su diagnóstico y tratamiento, no existe actualmente cura para esta patología, siendo sus consecuencias para la salud pública cada vez mayores [5].

Esta enfermedad implica cambios en el estilo y la calidad de vida del paciente, con tratamientos orales o inyectados de por vida, así como un riesgo aumentado de complicaciones tales como ceguera, enfermedad renal crónica, posible amputación de miembros inferiores, enfermedad cardiovascular y un aumento general de la mortalidad [1, 6-10]. De hecho, cada siete segundos muere una persona a consecuencia de la diabetes [11], y en 2014, 4,9 millones de personas murieron en el mundo y 24.428 en España [11]. Desde un punto de vista económico, los costes de la diabetes mellitus y sus complicaciones suponen para el sistema europeo de salud un coste de 2291 € por paciente en el mismo año [11].

Las Islas Canarias no son una excepción y de hecho esta región tiene la mayor incidencia de enfermedad renal crónica secundaria a nefropatía diabética [12], además de una alta mortalidad relacionada con la enfermedad comparada con el resto de España [13, 14].

El diagnóstico y los criterios de clasificación de la diabetes mellitus se han revisado y modificado en un gran número de ocasiones [1, 2, 15-22]. Actualmente la clasificación más aceptada es la propuesta por la Asociación Americana de Diabetes (ADA), que diferencia cuatro tipos principales de diabetes: diabetes mellitus tipo 1 (DM1), tipo 2 DM2, y otros tipos específicos de diabetes (que incluyen defectos

genéticos en la secreción de la insulina o en su acción, desórdenes endocrinos como el síndrome de Cushing, enfermedades pancreáticas, entre otras) y diabetes mellitus gestacional (DMG) (ver tabla 2), aunque hay propuestas recientes para reconsiderar esta clasificación nuevamente [15]. La DM1 se caracteriza por destrucción de la célula beta que lleva a una deficiencia absoluta de insulina, mientras que la DM2 es una combinación de resistencia a la insulina (IR) y una deficiencia relativa de insulina, por último la DMG es aquella diabetes diagnosticada durante el segundo o tercer trimestre del embarazo [1, 23].

Clasificación de la Diabetes Mellitus	
Diabetes mellitus tipo 1 (DM1)	Debido a destrucción de la célula beta, que lleva normalmente a una deficiencia absoluta de insulina
Diabetes mellitus tipo 2 (DM2)	Debido a un defecto progresivo en la secreción de insulina secundario a un aumento de resistencia a la misma.
Diabetes mellitus gestacional (DMG)	Diabetes diagnosticada en el segundo o tercer trimestre del embarazo, sin ser una diabetes franca
Tipos específicos de diabetes	Debido a otras causas por ejemplo síndromes de diabetes monogénica (como diabetes neonatal, maturity-onset diabetes of the young [MODY]), enfermedades del páncreas exocrino (tales como fibrosis quísitica) y diabetes inducida por fármacos (por ejemplo en el tratamiento de VIH o tras un trasplante de órgano)

Table 2: Clasificación de la diabetes. Adaptado de *'Classification and diagnosis of diabetes'*, ADA [1].

La DM1 representa de un 5-10% de las causas de DM y es una de las enfermedades crónicas más frecuentes en la infancia [24]. La incidencia ajustada por edad varía entre las distintas poblaciones, con Finlandia en un extremo (>60 casos por 100.000 personas/año), seguida de Cerdeña y Suiza, y Venezuela, China o India (0,1 casos por 100.000 personas/año) en el otro extremo [25]. Los registros nacionales e internacionales muestran que la incidencia de DM1 está aumentando en un 3-5% por año [26-31] y se estima que se doblará el número de casos en los próximos diez años [26].

Los datos de las Islas Canarias muestran una incidencia de DM1 entre 23 y 32 casos por 100,000 habitantes/año, que podría ser similar a la incidencia de Finlandia, Cerdeña y Suiza [32-34].

La forma predominante de diabetes es la DM2, que representa el 90% de todos los casos de diabetes en el mundo [35, 36]. Este tipo de DM está directamente relacionada con el grado de obesidad, cuya incidencia está aumentando conforme los países son más ricos y adoptan un estilo de vida más occidental: una alta ingesta calórica combinada con una escasa actividad física [37]. De hecho, ya el 80% de los casos de DM se encuentra en los países en vías de desarrollo, cifra que continúa en aumento [5, 38]. En las Islas Canarias, la prevalencia de la diabetes mellitus tipo 2 es del 13,2%, similar a otras regiones de España [39, 40].

2. Mecanismos patogénicos en diabetes mellitus tipo 1 (DM1) y diabetes mellitus tipo 2 (DM2)

2.3 Patogénesis de la DM1

La DM1 es el resultado de una destrucción de las células beta pancreáticas de origen autoinmune mediada por células T, producido por interacciones complejas entre los genes y el medio ambiente [41, 42]. La mayoría de los genes asociados con DM1 o bien afectan a la regulación de la tolerancia frente a la respuesta inmune, a la intensidad de su respuesta, a los mecanismos de resistencia-defensa de las células β, o a la producción de insulina, de citokinas o quemoquinas [43].

Los genes que codifican las moléculas de clase II del complejo mayor de histocompatibilidad (*CMH*), de la región HLA [44, 45] comprenden el 50% de los genes asociados con el riesgo de padecer diabetes tipo 1 [45]. Otros genes, con menor efecto en el riesgo de padecer DM1, son el gen de la insulina (*INS*), el *CTLA-4, PTPN22* y el *IL2R*, aunque existen más de 50 locus de susceptibilidad identificados recientemente [43,46,47,48, 49,50].

Los autoanticuerpos están presentes en el 85-90% de los individuos diagnosticados recientemente de DM1 [2, 51]. Cuatro autoanticuerpos específicos contra el islote han sido identificados en contra de la insulina, de la descarboxilasa

del ácido glutámico 65 (GAD 65), de la proteína tirosina fosfatasa-2 (IA-2) y del transportador de zinc (ZnT8) [52-55]. Esos anticuerpos pueden ser detectados durante años, a veces décadas, antes del inicio de la enfermedad, pero no hay evidencia de su rol patogénico [56].

La razón de porqué se inicia este proceso autoinmune no está clara. En el modelo de Copenague, propuesto por Nerup en 1994 [57] y recientemente actualizado por Størling y colaboradores [58], se sugiere una combinación de factores ambientales en añadidura a una susceptibilidad genética predisponente que interacciona con un ataque a la célula beta, que ulteriormente provoca la liberación de componentes tales como la insulina o el GAD en formas que no son reconocidas por el sistema inmune [59]. Se plantea la posibilidad de que esta proteína haya sufrido cambios postraduccionales, generando nuevos epítopos antigénicos, que a su vez son capturados y presentados a las células dendríticas en el islote. Posteriormente serían transportados a los ganglios linfáticos pancreáticos donde los antígenos son procesados y presentados a las células T, de manera que la respuesta inmune es amplificada y perpetuada.

Los hallazgos histopatológicos típicos encontrados en el páncreas humano al diagnóstico de la DM1 incluyen una infiltración inmune en los islotes pancreáticos y la reducción del área de la célula beta [61-63]. Los criterios para la definición de insulitis propuestos son: ≥15 $CD45^{+}$ (common leukocyte antigen) en un mínimo de tres islotes aproximadamente de 150 μm de diámetro [62].

La mayoría del infiltrado celular es de naturaleza linfocítica, con predominancia de células T $CD3^{+}CD8^{+}$ en estadíos tempranos de la enfermedad que progresivamente proseguirá con un incremento de linfocitos B (CD20+) posterior [64, 65]

Con respecto a los factores ambientales con los que se han demostrado asociaciones con el desarrollo de DM1, éstos constituyen una lista amplia, la cual incluye desde infecciones por enterovirus [66, 67], a la propia polución y otros tóxicos ambientales (por ejemplo nitrosaminas) [68, 69], así como la exposición temprana a las proteínas de la leche de vaca, los cereales o el gluten [70-74], o incluso la deficiencia de vitamina D [75, 76].

Además, factores ambientales que han sido tradicionalmente relacionados con la patogénesis de la DM2, tales como un índice de masa corporal aumentado, el aumento del peso corporal y la reistencia a la insulina, han sido considerados también como predictores de DM1 [77-79].

2.4 Patogénesis de la DM2

La DM2 está causada por una combinación de resistencia a la insulina y disfunción de la función celular beta. En presencia de esta resistencia, las células beta incrementan su producción de insulina a modo de mecanismo compensatorio. Si esta situación se mantiene de manera sostenida en el tiempo, llevará a la disfunción total de la célula, especialmente en individuos predispuestos

genéticamente. Los mecanismos subyacentes en este proceso, aunque se han estudiado extensamente, aún siguen siendo desconocidos [80]. No obstante, los mecanismos más aceptados como causales de esta disfunción incluyen la glucotoxicidad, lipotoxicidad y deposición de amiloide [81].

La carga genética sobre la diabetes mellitus tipo 2 ha sido demostrada para más de 80 locus, todos asociados con variaciones en fenotirpos interindividuales de esta enfermedad, tales como los niveles de glucosa en sangre, insulina, proinsulina y valores de hemoglobina glicosilada [82].

La mayoría de los genes implicados son responsables sólo de un aumento pequeño del riesgo de la enfermedad, lo que refleja los múltiples mecanismos para la patogénesis de la misma. De hecho, aunque la mayoría de las variantes descritas están relacionadas con la función de la célula beta (*TCF7L2*, *HNF1A, GCK, PROX1*...), otros genes están asociados con la alteración de la sensibilidad a la insulina (*PPARG, IRS1, IGF1*) o con la obesidad (*FTO*) [81, 83-86].

Además de la predisposición genética, la edad avanzada, la obesidad y el sedentarismo son los factores de riesgo más importantes de la DM2 [5]. La adiposidad central y la circunferencia de la cintura, que están relacionados estrechamente con la resistencia a la insulina, son mejores predictores que el índice de masa corporal *per se* [87, 88].

Dado el elevado aumento en la prevalencia de obesidad (33% en 2005; estimado 57,8% de los adultos en 2030 [89]), las predicciones paralelas en el aumento de la prevalencia de DM2 por la IDF no son inesperadas [11].

3. Modelos animals de diabetes

La influencia que tiene para la vida de las personas la diabetes mellitus ha motivado la búsqueda de un entendimiento más profundo de su patogénesis, así como de nuevos tratamientos para combatirla [90].

Sobre esta búsqueda, aunque los diferentes abordajes científicos existentes constituyen todos herramiantes indispensables en esta proeza, y aunque la metodología tanto *in vitro* como *in silico* haya dado pasos agigantados y continúe ofreciendo, cada vez más, grandes aportaciones científicas en las últimas décadas, ninguna de estas alternativas puede aún hoy constituir un reemplazo para la información obtenida a partir del uso de los modelos animales de la enfermedad, lo que es debido a la complejidad etiológica y la afectación e interacciones multiorgánicas que toman lugar en la diabetes mellitus [91, 92].

Por todo ello, la mayoría de la investigación en diabetes se ejecuta sobre animales [93, 94] y las aclamadas 3Rs, sobretodo la reducción y el reemplazo [95] constituyen aún un sueño irrealizable.

Definitvamente, parece que la mejor respuesta para la investigación en este sentido es la combinación de múltiples abordajes entre la investigación humana, la *in vitro* y los estudios con modelos animales. Esta parece la vía que mejore nuestro entendimiento futuro sobre los mecanismos que subyacen en la patogénesis de la diabetes [94].

El uso moderno y estandarizado de los modelos animales proviene de estudios previos originados en las investigaciones de J.C. Brunner en el siglo XVII [96], no obstante ya el uso de animales como modelos de estudio anteriores han sido descritos [97].

Claude Bernard, el considerado como padre de la "vivisección", identificó la existencia de dos regiones pancreáticas, la endocrina y exocrina, mediante el uso de modelos animles [97].

Algunos de los más importantes hallazgos en la investigación biomédica y en la diabetes fueron realizados utilizando perros a los que se les disecó el páncreas (pancreatectomizados): el descubrimiento de la función pancreática por Von Mering y Minkowski (1890s) [98] y el descrubrimiento de la hormona insulina por Banting and Best (1920s) [99].

Acutalmente sin embargo, existe una amplia variedad de modelos animales que a su vez han aportado un número adicional de avances alrededor de la diabetes, entre ellos: el descubrimiento de la leptina, nuevos hallazgos en la patogénesis y las

complicaciones crónicas de la enfermedad, en el desarrollo de nuevos tratamientos, entre otros [100-102].

Estos modelos se clasifican mayoritariamente en base al tipo de diaebetes que padecen, o en la manera en la que esta se produce, siendo los dos grupos mayores de clasificación los modelos espontáneos o inducidos [92, 103-108].

Aunque los progresos en genética han llegado a ofrecer modelos transgénicos específicos, casi a la carta, lo que ha extendido el rango de modelos espontáneos o más susceptibles, o incluso resistentes, al desarrollo de la enfermedad [109]. En ocasiones, cuando desarrollan la diabetes de manera espontánea, la enfermedad es inconsistentemente clasificada como espontánea o inducida, en base a lo que estime el autor [92, 107], debido a la confusión contenida en que realmente hayan sido genéticamente inducidos, aunque los signos clínicos y el fenotipo asociado a la enfermedad aparezcan espontáneamente.

A continuación se describen brevemente algunos de los modelos más importantes de los usados en la investigación en diabetes.

3.2 Modelos espontáneos de diabetes

La diabetes mellitus aparece de manera espontánea en muchas especies de animales, incluyendo a los équidos, odontocetos, otros primates superiores e incluso el hipopótamo, entre muchos otros (*revisado por el Dr. E. Gale*) [93]. Algunas de estas especies han aportado importantes resultados al constituirse como auténticos pacientes dentro de la medicina veterinaria, o directamente como modelos animales, aportando un mejor entendimiento sobre los mecanismos subyacentes de la enfermedad.

La principal ventaja de los modelos animales espontáneos cuando se comparan con todos los otros modelos animales de diaebtes inducida, es que presumiblemente los mecanismos causales de su condición patológica serán mucho más similares a los que toman forma en la patogenicidad de las diabetes humanas, independientemente del tipo, especialmente en aquellos modelos que tienen un origen poligénico de enfermedad [93, 104, 105].

Especialmente en la última década han tomado forma importantes avances en la comprensión de la genética y la patofisiología de la diabetes espontánea en modelos de diabetes inmunomediada, así como la creación de un número elevado de nuevos modelos para esta enfermedad.

El modelo más comúnemente utilizado para el estudio de la diabetes tipo 1 es el ratón diabético no obeso conocido como NOD (Non Obese Diabetic), así como también la rata BBDP (Bio-Breeding-Diabetes-Prone) [103].

Otros modelos espontáneos incluyen a la rata Long Evans Tokushima lean (LETL) y al conejo de Neozelandés blanco. Estos modelos constituyen en sí mismos unas herramientas muy valiosas para el estudio de los procesos inmunomediados y la prevención de la diabetes tipo 1.

Para la diabetes tipo 2 en su caso, los modelos espontáneos más frecuentemente utilizados son las ratas Zucker Fatty rat (ZFR), la Zucker diabetic fatty (ZDF) y los ratones *ob/ob* y db/db.

Sin embargo, aunque su contribución haya sido considerable, el éxito logrado en el uso de los modelos animales ha sido bajo a la hora de extrapolar los resultados a la investigación en humanos [94, 110, 111].

Consecuentemente, y a raíz de estos fracasos, se recomienda que la elección del modelo sea lo más adecuada posible, atendiendo a la posibilidad de elegir no únicamente un modelo, si no quizá combinando varios en base a los objetivos perseguidos, y siempre procurándose que el modelo o los modelos en cuestión hayan sido validados para tal fin [92, 94].

a) El perro diabético

En 1921 la perrita Marjorie, una mestiza sobre la que se indujo diabetes, fue la primera receptora de la terapia insulínica, allanando el camino para el tratamiento de los pacientes humanos [99]. Este hito histórico tanto para la diabetes como para la

biociencia en general, convirtió a Marjorie en el animal más famoso en el mundo científico probablemente, hasta la aparición de la oveja Dolly.

Respecto a la diaebetes espontánea en perros, aunque los primeros casos clínicos fueron publicados desde hace más de medio siglo, en 1951 [170], la diabetes canina ha sido propuesta como modelo espontáneo de la enfermedad diabética autoinmune humana tan sólo recientemente, en 2005 [171].

La justificación de esta propuesta se basa en que su presentación clínica es similar a la diabetes tipo 1 humana, a que dentro de toda la población canina, al igual que en humanos, existen fenómenos genreralizados de mestizaje así como la existencia de etnias más puras con diferentes susceptibilidades o protecciones frente a la enfermedad. Además comparten nuestro medio ambiente y una patogénesis similar. Respecto a esta última, cabe destacar que el papel de la respuesta autoinmune y la influencia de ciertos genes en la predisposición/protección frente la enfermedad ha quedado demostrada [171, 172].

El hecho de que los perros compartan nuestro mismo entorno constituye una importante ventaja respecto a otros modelos animales, ya sean inducidos o espontáneos. Este valor añadido le otorga un especial interés en la medicina comparada para la realización de estudios donde se evalúe la interacción genética con el la influencia de factores medio ambientales. Más allá de la importancia que pueden

tener los roedores como modelos, los perros son genéticamente más parecidos al hombre que éstos.

i. Clínica, etiología y epidemiología de la diabetes canina

Los perros con diabetes comparten los signos clínicos clásicos de la tipo 1 humana, como son la polidipsia, poliuria y pérdida de peso, asociados a una marcada hiperglucemia y glucosuria [143].

El diagnóstico en perros se basa mayoritariamente en el hallazgo de estos y de otros signos clínicos como son las cataratas (del 10% al 20% de los casos), coexistiendo con hiperglucemia (>200 mg/dl) o con valores de fructosamina elevados (>350 micromol/l) y glucosuria. En conjunto, tanto la historia clínica, como el examen físico y los tests diagnósticos son cruciales para ayudar a diferenciar entre los diferentes tipos de diabetes canina presentes, que a su vez es necesario para poder proveer un adecuado tratamiento y determinar la prognosis más acertada.

El tratamiento de incluye la inyección de insulina en la mayoría de los casos, administrada de una a dos veces diarias, pero también el ejercicio, modificando las pautas de la dieta y aplicando esterilizaciones en las hembras no castradas [143].

Los diferentes tipos de diabetes quedan establecidos en base a la etiología [143, 171, 172], siguiendo la clasificación más aceptada actualmente, la cual constituye una

adaptación de la previamente mencionada clasificación humana propuesta por la ADA **(ver tabla 2)** [2].

Aunque existen ciertas diferencias entre las poblaciones caninas diabéticas existentes [173, 174], el tipo más común de diabetes está descrito como la forma Insulino-deficiente (IDD, insulin-deficient diabetes), la cual es similar a la diabetes tipo 1 humana. Otros tipos comunes de diabetes son la diéstrica, relacionada con el ciclo sexual de las hembras no castradas, o la secundaria a otros trastornos, como pancreatitis o hiperadrenocorticismo.

El papel de la inmunidad en la diabetes canina está soportado por la presencia de autoanticuerpos frente a las células de los islotes (ICA, islet cell antibodies), frente GAD65, IA2 y frente a proinsulina [171, 175-179]. No obstante, un estudio reciente pone en duda el papel de la autoinmunidad en este proceso en perros [180].

También ha sido demostrada una asociación entre el riesgo o protección frente a la diabetes canina y a haplotipos específicos en los genes de clase II del complejo mayor de histocompatibilidad (CMH), en los conocidos como *Dog Leukocyte Antigen (DLA)*, papel también en común con la diabetes humana [181].

La distribución de esto haplotipos del *DLA* entre diferentes razas de perros ha sido comparada con la distribución del HLA en diferentes etnias humanas [172,181]. La edad de comienzo oscila entre 5 y 12 años en la mayoría de los estudios [173], aunque raro, la formas juveniles de DMc también existen [|78]. El hecho de que sea un proceso autoinmune, la presentación clínica y el edad de diagnóstico en edad

adulta, sugieren que la DMc podría ser un modelo de diabetes autoinmune latente del adulto (LADA) [15,172].

La prevalencia de DMc oscila entre 0,0005-1,5%, dependiendo de las regiones geográficas y razas [171, 173, 174, 182-186], sugiriendo una interacción entre genes y ambiente. Sin embargo, la mayoría de estudios epidemiológicos han sido realizados en poblaciones del Norte de Europa y Norte América [173, 174, 182, 184, 185], con rasgos culturales y demográficos específicos. Atendiendo a esos datos, las razas más frecuentemente afectadas son Australian terrier, Keeshond, Schnauzer, Caniche Miniatura, Samoyedo y Cairn Terrier [173, 182, 183, 186]. Otras razas, como el Boxer y el pastor Alemán, parecen estar protegidas ante el desarrollo de DMc [181, 186].

El perro ha sido propuesto como modelo espontáneo de diabetes para la tipo 1 humana por B. Catchpole y colaboradores, ya que comparte características clínicas y etiológicas, como la presencia de autoanticuerpos frente a la célula beta. Con el objetivo de evaluar mecanismos asociados a la etiopatogenia de la diabetes mediante el uso de este modelo, emprendimos la ***caracterización de la población canina con diabetes espontánea de nuestra región***.

La monitorización de la glucosa en la diabetes canina es crucial para unos adecuados control y tratamiento. Existen glucómetros portátiles específicos de

pequeños animales, aunque desgraciadamente no están disponibles en cualquier lugar. De este modo se suelen usar glucómetros desarrollados para humanos en perros, aunque existe una marcada disparidad de resultados entre éstos y los métodos de referencia.

Durante la caracterización de la población canina antes descrita, esta disparidad se hizo evidente en la labor clínica. Por este motivo, con el fin de mejorar la monitorización de la enfermedad en el perro diabético, se realizó una ***evaluación de la exactitud y precisión de medida de 9 medidores portátiles de glucosa humanos en perros, en base a las normas ISO 15197:2003 y 15197:2013 adaptadas.*** Con esta evaluación se determinó la fiabilidad analítica de un amplio número de glucómetros humanos utilizados en el control en los ámbitos doméstico, clínico e investigador de la diabetes canina.

Sobre aquellos perros muestreados durante la caracterización de la población diabética canina, se realizaron estudios posteriores para la evaluación del ***papel de la proteína PIMT en la diabetes tipo 1***, ***en el perro como modelo de diabetes, en un modelo murino Knock out de la misma proteína y en pacientes humanos con diabetes tipo 1 y tipo 2.***

Las proteínas modificadas post-transduccionalmente y la enzima reparadora proteína-L-isoaspartato (D-aspartato) *O*-metiltransferasa (PIMT) podrían tener un papel en el desarrollo de la diabetes autoinmune. Los cambios post-transduccionales pueden generar sobre muchas moléculas biológicas modificaciones que son dañinas para el organismo, como cambios en su inmunogenicidad y la aparición de nuevos epítopos que condicionan la producción de auto-anticuerpos. La aparición de isoaspartilo (IsoAsp) en las proteínas es el resultado de una deamidación/isomerización espontánea no enzimática de los residuos de Asn o Asp, que se produce en condiciones fisiológicas. Algunas enfermedades autoinmunes, como el lupus eritematoso sistémico y la enfermedad celíaca muestran respuestas inmunológicas contra auto-antígenos modificados post-transduccionalmente.

Nerup *et al.* (Nerup, Mandrup-Poulsen et al. 1994), propusieron que los cambios post-transduccionales de las proteínas de los islotes desencadenan la respuesta específica de los linfocitos B y T frente al antígeno durante la fase iniciadora de la patogénesis de la DM1. De hecho, los autoantígenos principales de la DM1 son propensos a la deamidación/isomerización: la insulina puede ser modificada “in vitro”, la proteína GAD tiene cuatro sitios de Asx-Gly, propensos a la isomerización, de los cuales tres están localizados en epítopos reconocidos y la proteína IA-2 también contiene varios sitios propensos a modificaciones de este tipo.

En el perro con diabetes mellitus espontánea, se ha evaluado la expresión de PIMT en sangre mediante RT-PCR y su relación con la enfermedad. En las muestras post-mortem se han caracterizado histológicamente los islotes pancreáticos y su nivel de expresión de insulina y PIMT.

En el segundo modelo, el ratón *knock out* de PIMT, se han evaluado la tolerancia a la glucosa, la resistencia a la insulina, la expresión de insulina y hormonas contrareguladoras, y los cambios histológicos asociados a la carencia de esta proteína. En humanos, la expresión de ARNm de PIMT y la cantidad de proteína PIMT en sangre. En todos se ha correlacionado los distintos fenotipos diabéticos con el papel de PIMT.

3.2 Modelos inducidos de diabetes

Los modelos de diabetes inducida son probablemente aquellos que han sido más frecuentemente utilizados en la investigación, ya que son más fáciles de generar que los modelos espontáneos. Encajan bien con los objetivos de muchas líneas de investigación, menos centradas en el estudio de los procesos sutoinmunes y más sobre otros relacionados con la obesidad, el síndrome metabólico o la propia hiperglucemia.

Las dos formas más usadas para generar un modelo de diabetes inducida son los métodos quirúrgicos, en el que como principal ejemplo nos encontramos con la pancreatectomía parcial, y luego el grupo de la diabetes inducida por métodos no quirúrgicos, donde la inducción medianete toxinas o químicos, cuyo principal ejemplo lo consituyen los modelos generados con estreptozotocina, podrían ser el métodos más utilizado.

No obstante, también se utiliza la dieta hipercalórica con alto contenido graso o en sacarosa, la administración de inmunosupresores o incluso la infección vírica (por ejmplo por Coxsackie B virus [202-208].

Por otro lado, ambos métodos quirúrgicos y no quirúrgicos pueden ser combinados para reflejar situaciones expecíficas o más complejas dde la diabetes, como combinando una dieta en alto contenido graso junto con administraciones de sustancias químicas con especial tropismo por la célula beta, como el aloxano. La diabetes gestacional, por ejemplo, puede ser recreada realizando tanto estrategias quirúrgicas como de adminitración de streptozotocina, en una situación de embarazo del modelo [209].

Respecto al uso de los modelos animales de diaebtes inducida, en esta tesis se ha hecho uso del modelo C57Bl/6J alimentodo con dieta grasa, como modelo de síndrome metabólico y resistencia a la insulina.

El modelo modelo C57Bl/6J alimentodo con dieta grasa (HFDID) presenta obesidad marcada, RI, hiperinsulinemia, hiperglucemia e intolernacia a la glucose como caracterísiticas principals [202,236]. Este modelo también presenta resistencia periférica a la leptina [108], y una respuesta hiperglucémica prolongada al estrés [202]. Siguiendo a mayoría de protocolos con animales jóvenes (4-6 semanas de edad), el desarrollo de hiperglucemia ocurre durante largos periodos de tiempo, desde que la célula beta mantiene niveles altos de insulin para compensar la resistencia a la insulina que existe [237].

Al menos de diez a doce semanas son necesarias para obtener fenotipo evidente de obesidad y diabetes, y aunque la cepa está predispuesta a la obesidad existe una variabilidad individual importante [232, 238].

La principal ventaja de este modelo animal es que refleja la interacción compleja entre genetica y factores ambientales, comparado con el ratón $Lep^{ob/ob}$, que está extremadamente determinado genéticamente [105]. En este sentido, el ratón C57Bl/6J HFDID ha sido usado en el estudio de la alteración de la tolerancia a la glucosa [239], la DM2 y sus complicaciones [240-242] así como en estudios preclínicos con fármacos [241,243].

El uso de este modelo se ha centrado en la identificación de nuevos tratamientos para la diabetes.

La búsqueda y evaluación de nuevos posibles tratamientos para la diabetes mellitus, nos llevó durante el desarrollo de esta tesis a ***evaluar nuevos tratamientos de origen natural para la diabetes mellitus tipo 2***. Dos productos naturales fueron seleccionados para ser evaluados, uno de origen zoológico, derivado de un lagarto norte-africano (*Uromastyx achantinura*), y otro vegetal, preparado a partir de aloe de Canarias (*Aloe vera barbadensis*). Para ello utilizamos un modelo murino de DM2 (ratón C57Bl/6J inducido por dieta de 60% en contenido graso), sobre el que determinamos la actividad hipoglucemiante de nuevos posibles tratamientos de origen natural, tanto de manera aguda como crónica.

Finalmente, sobre el metabolismo glucosado en ratones, éste se evalúa mediante técnicas invasivas como son los tests de tolerancia a la insulina o las sobrecargas de glucosa, por vía oral o intraperitoneal. Sin embargo, la medición de HbA1c mediante una única extracción de un pequeño volumen de sangre, permite obtener mucha más información de una única determinación. Aunque suele

utilizarse para comparar entre grupos de animales, su estandarización es escasa y no existe un punto de corte para el diagnóstico de la enfermedad en ratones.

A raíz de la realización de los proyectos previamente descritos, se obtuvo un número elevado de determinaciones de HbA1c en ratones diabéticos y no diabéticos, a la vez que se realizaron evaluaciones de su metabolismo glucosado por las técnicas tradicionales. De este modo nos propusimos ***determinar un punto de corte para la HbA1c como diagnóstico de la enfermedad en el ratón C57Bl/6J con diabetes inducida por dieta grasa***.

Por tanto, los principales objetivos planteados durante el curso de la presente tesis han sido los siguientes:

1 Caracterizar la población diabética canina local:

a. Identificando y muestreando a los perros con diabetes espontánea

b. Analizando clínicamente, genotipando el DLA, midiendo la reactividad frente autoanticuerpos y realizando estudios histopatológicos en muestras de la población de estudio

2 **Evaluar el papel potencial de los cambios post-transduccionales sobre las proteínas y el papel reparador de PIMT en la patogénesis de la diabetes tipo 1:**

a. Realizando el genotipado de *PCMT1* y la expresión de las concentraciones de la proteína PIMT y su actividad en perros con diabetes espontánea y pacientes con diabetes tipo 1, tipo 2 (controles hiperglucémicos) y controles sanos

b. Fenotipando el metabolismo glucosado del ratón Pcmt1$^{-/-}$

3 **Evaluar nuevos tratamientos para la diabetes tipo 2, utilizando productos naturales de nuestra área geográfica:**

a. Evaluando los efectos agudos de ambos preparados zoológico y vegetal, en el ratón C57Bl/6J diabético inducido por dieta de alto contenido en grasa

c. Evaluando los efectos crónicos sobre el metabolismo glucosado de ambos productos sobre el mismo modelo animal

4 **Intervenir en la mejora de la monitorización de la diabetes en ambos modelos animales utilizados:**

a. Evaluando nueve glucómetros portátiles diseñados para humanos, en perros diabéticos espontáneos y no diabéticos, basándonos en la norma ISO 15197:2013

b. Evaluando el valor diagnóstico de la HbA1c en el ratón C57Bl/6J como modelo de diabetes inducida a través de la dieta rica en grasa

La metodología, resultados y discusión derivados de esta tesis se encuentran descritos en formato artículo, borrador de artículo, o resumen, en las sección V de este documento.

CONCLUSIONES:

A continuación, las principales conclusiones derivadas de la presente tesis:

1 Respecto a la caracterización de la población con diabetes canina de nuestra región:

a) Las prevalencia e incidencia de la diabetes canina en Canarias se encuentra dentro de los límites definidos para otras regiones.

b) La mayoría de los casos de diabetes canina están constituidos por hembras no castradas, dando como tipo de diabetes más común la diabetes diéstrica y en segundo lugar la idiopática o inmunomediada.

c) En una proporción de perros diabéticos de nuestra región se demuestra la presencia de autoanticuerpos contra GAD65 e IA2.

d) Los páncreas de perros diabéticos muestran una reducción severa del número de islotes y de masa celular β, llegando incluso a estar ausente en algunos casos. El tiempo de evolución y el tipo de diabetes parecen ser los factores que más influyen en esta pérdida de células β.

2 Respecto a la evaluación de la exactitud y precisión de glucómetros humanos en perros:

a) Es necesaria una evaluación de la exactitud y precisión de cualquier glucómetro previo a su uso, hecho que se demuestra en la disparidad de resultados entre los aparatos evaluados.
b) La norma ISO 15197:2013 adaptada a las muetras veterinarias, constituye un método adecuado para dicha evaluación.
c) Aunque ninguno de los aparatos logró los límites establecidos por la norma ISO, el AccuChek Aviva Nano es la mejor opción de los aquí evaluados.

3 Respecto al papel de la proteína PIMT en la diabetes:

a) En sangre periférica, la expresión de PIMT parece asociada a la hiperglucemia y otros componentes del síndrome metabólico, aunque su relación con la autoinmunidad no puede ser descartado.
b) Localmente, esta proteína podría desempeñar un papel en el desarrollo del islote pancreático, afectando tanto a los niveles de insulina como de glucagón.

4 Respecto al Uromastyx acanthinura como tratamiento natural en un modelo murino de diabetes tipo 2:

a) La administración aguda de *Uromastyx acanthinura* tiene efecto hipoglucemiante

b) El diseño cruzado del estudio permitió encontrar efectos hipoglucemiantes y simultáneamente nos dotó de una herramienta para la reducción del número de animales utilizados para este fin.

c) Paradójicamente, su administración crónica promovió una aumento de la resistencia a la insulina, del consumo de alimento y el peso corporal. También mitigó los signos de la neuropatía diabética.

d) Estos hallazgos determinan efectos directos del *Uromastyx acanthinura* en el metabolismo glucosado y el apetito, así como en el apetito y el dolor/neuropatía.

e) A su vez dan valor a considerar a la Medicina Tradicional Africana como un punto de partida para la búsqueda de nuevos compuestos bioactivos.

5 Respecto a la evaluación de los efectos hipoglucemiantes del Aloe vera barbadensis en un modelo murino de diabetes tipo 2.

a) Administrado de forma aguda, el preparado de aloe provocó un respuesta hipoglucemiante en ratones diabéticos.

b) Sin embargo, la administración crónica del producto sólo tuvo efectos en hembras, mejorando los niveles de HbA1c y aumentando su secreción insulínica.

6 Respecto a la HbA1c como método diagnóstico de la enfermedad en el ratón C57Bl/6J con diabetes:

a) La HbA1c, determinada con un analizador compacto, permite evaluar el metabolismo glucosado del ratón de manera crónica, mejorando el refinamiento animal.

b) El punto de corte establecido para el diagnóstico de la diabetes en este modelo animal es el de 4.2%, aunque su baja sensibilidad y especificidad limitan su utilidad clínica para este fin.

***Curriculum Vitae* adicional durante la tesis**

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Yeray Brito, Julia Wiebe, Ainara Arin, Laura López, Grant McNaughton Smith, Javier Nóvoa, Nicolas Diaz chico, Ana Wägner. **Short term effects of aloe vera in a mouse model of type 2 diabetes: randomised, cross-over studies**. Congreso de

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MJ Molina-Cimadevilla, T García-Robles, C Muñoz-Mediavilla), Y Brito-Casillas, AM Wägner, P Rey, A Sanchez. **Revisiting genitourinary problems in mice: treatment and re-characterization of Mouse Obstructive Genitourinary Syndrome (MOGS).** I Congreso Ibérico de las Ciencias del Animal de Laboratorio (SECAL-SPCAL). Cáceres. 2015.

Otros artículos:

'Revisiting genitourinary problems in mice: treatment and re-characterization of Mouse Obstructive Genitourinary Syndrome (MOGS)'

MJ Molina-Cimadevila (1), T García-Robles (2), C Muñoz-Mediavilla (3), Y Brito-Casillas (4), AM Wägner (4), P Rey (5) and A Sanchez (5).

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Enviado a: *Comparative medicine*. IF 1.12 (JCR). Q2, Veterinary Sciences

Abstract

The current study aimed to characterize and to explore a treatment for a condition in which male mice presented with a solid bulge in the preputial area. Forty-two animals, from several animal houses in Spain, were included in the study for microbiological and pathological characterization. The condition mostly affected breeding animals (78.6%) and was associated with C57BL/6J genetic background. A solid, yellowish white substance was found inside the prepuce, displacing the penis cranially, avoiding its externalization and causing infertility. This blocking substance was completely amorphous, acidophilic and showed peripheral clusters of spermatozoa, a pattern almost identical to post-coital female plugs, suggesting an ejaculatory origin. Opposite to what has been suggested in previous publications, the penis was completely intact in all of the cases, with no signs of mutilation or wounds. The rest of the organs were apparently functional and the microorganisms found (Enterococcus spp 72.7%, Escherichia coli 54.5% and coagulase-negative Staphylococci 54.5%) seemed to be secondary contaminants from faecal saprophytic flora. Clinically and pathologically two clear conditions were differentiated: one with urinary obstruction and one without, depending on the capacity of the blocking substance to lock the urethra.

Based on these findings, an unblocking surgical technique was developed to recover breeding performance and tested in 15 mice. The blocking substance was eliminated by a longitudinal incision made cranially from the opening of the prepuce. Most of the animals recovered fertility, but the treatment did not work when urinary obstruction was present. This technique can be useful to safeguard fertility in important, transgenic stud males.

'Glucolipotoxicity is Necessary for the Induction of β-cell Damage by Calcineurin Inhibitors'

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Abstract

Insulin resistance is needed for the induction of diabetes mellitus by calcineurin inhibitors. Tacrolimus is specially diabetogenic in the presence of insulin resistance compared to cyclosporin-A, but the mechanisms involved in this interaction are unknown. Several studies have suggested that β-cell transcription factors like FoxO1, MafA, PDX-1 and NeuroD are involved in the transition towards diabetes. Our aim was to evaluate the effect of calcineurin inhibitors and metabolic stressors (glucose and palmitate) on these transcription factors. INS-1 cells were cultured for five days in the presence of 100μM palmitate and 22mM glucose to mimic the insulin resistance *milieu in vitro*. The effects of calcineurin inhibitors were assessed by treating the cells with 250ng/mL cyclosporin-A or 15ng/mL tacrolimus for 48h. Glucose and palmitate increased nuclear FoxO1 and decreased nuclear MafA and both effects were magnified by tacrolimus. The addition of cyclosporin-A, however, did not produce relevant changes. Interestingly, after five days of tacrolimus withdrawal or switch to cyclosporin-A, nuclear levels of FoxO1 and MafA recovered. These results were confirmed in *in vivo* experiments. Thus, our data indicates that the higher diabetogenicity of tacrolimus may be due to its effect on transcription factors that maintain β-cell function.

Enviado a: *Metabolism, Clinical and Experimental*. IF 3.894 (JCR). Q1.

'Outbreak and eradication of zoonotic *Ornythonissus bacoti*: report in a European animal facility'

Brito-Casillas Y [1, 2,*], Díaz-Sarmiento M [1, 3], García-Arencibia M [1], Carranza C [1,4], Castrillo A [1,3], Fernández-Pérez L [1,5], Zumbado-Peña M [1, 6], González JF [7], Wägner AM [1, 2]

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Abstract

A zoonotic opportunistic out-break involving *Ornythonissus bacoti*, capable of invading animal facilities despite their barriers, is described. An eradication protocol with ivermectin was tested and implemented. Immunocompetent mice and rat strains in a conventional health status facility developed dermatologic manifestations, followed by weight-loss and wounds in severe cases. Some litters showed lesions ressembling petequiae distributed over the whole body. Initially, only mild isolated cases were detected, but therafter, scratching was spread. Simultaneously, facility staff suffered from pruritic, erythematous papular lesions of varying severity. Direct observation of the cages showed fast, oval insects that moved around and from animals to bedding. Further examination of carcasses and bedding allowed the characterization of these accarii as *Ornythonisus bacoti*.

A solution of 0.1% ivermectin in propyleneglycol and distilled water was chosen to be administered as spray. Treatment efficacy tests showed 0% survival rate of the accarii. Ivermectin safety was assessed in each animal strain and no toxicity was identified. Hence, all animals and beddings were sprayed. Based on the parasite's lyfe-cicle, treatment was repeated two times, weekly. The frequency of cage-cleaning was increased and racks, walls and cages were also sprayed with ivermectin. Infestation was regularly monitored by examining the animals' fur and carcass. Seven weeks after initial application, samples remained negative for the presence of accarii. Eradication of the parasitosis was confirmed and at the time of this report, ten months after diagnosis, *O. bacoti* still remains undetected.

Enviado a: *Journal of the American Association for Laboratory Animal Science*. IF 1.78 (JCR). Q2 Veterinary Sciences

Estancias:

The Royal Veterinary College, University of London, Vet School. London, United Kingdom. Abril 2012-Julio 2012.

Unidad de Fisiología Celular y Nutrición, Instituto de Bioingeniería, Universidad Miguel Hernández. 9-15 Julio 2010

Proyectos de investigación:

Título: **Cambios posttransduccionales de las proteínas y diabetes tipo 1: papel de la enzima reparadora PIMT**
Financiación: Fundación Canaria de Investigación y Salud (FUNCIS)
Duración: 1 año (2008-2009)
Investigador principal: AM Wägner

Título: **Cambios posttransduccionales de las proteínas y diabetes tipo 1: papel de la enzima reparadora PIMT**
Financiación: European Foundation for the Study of Diabetes (EFSD)
(EFSD/JDRF/NN Type 1 Diabetes Programme 2008)
Duración: 2 años (2008-2010)
Investigador principal: AM Wägner

Título: **Cambios posttransduccionales de las proteínas y diabetes tipo 1: papel de la enzima reparadora PIMT**
Financiación: Instituto de Salud Carlos III (PI08/01113)
Duración: 3 años (2009-2011)
Investigador principal: AM Wägner

Title: **¿Modifican los cambios epigenéticos la expresión de *PCMT1* en la diabetes tipo 1? Estudios en pacientes, hijos/as de madres/padres diabéticos y en un modelo animal**

Financiación: Instituto de Salud Carlos III (PI11/02441)

Duración: 3 años (2013- 2015)

Investigador principal AM Wägner

Title: **Caracterización de la hipercolesterolemia familiar en la isla de Gran Canaria**

Financiación: Sociedad Española de Arterosclerosis (SEA)

Duración: 3 años (2014-2017)

Investigador principal RM Sánchez-Hernández

Becas y premios:

Beca: **Patogenia de la diabetes tipo 1 mediante el estudio de modelos animales de diabetes autoinmune**

Financiación: Fundación Canaria de Investigación y Salud (FUNCIS) (ID 41-2008)

Duración: 4 años (Abril 2009-Abril 2013)

Investigador principal: AM Wägner

Beca: **Albert Renold Travel Fellowship for Young Scientists**

Financiación: European Foundation for the Study of Diabetes (EFSD)

Objetivo: Estancia investigadora en el Royal Veterinary College, University of London.

Duración: 3 meses (Abril-Julio 2012)

Investigadores principales: AM Wägner and B Catchpole

http://www.europeandiabetesfoundation.org/programmes/reports/flipbook2012/index.html#p=20

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