

Animal Research for Type 2 Diabetes Mellitus, Its Limited Translation for Clinical Benefit, and the Way Forward

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Summary — Obesity and type 2 diabetes mellitus (T2DM) have reached pandemic proportions worldwide, and considerable research efforts have been dedicated to investigating disease pathology and therapeutic options. The two hallmark features of T2DM, insulin resistance and pancreatic dysfunction, have been studied extensively by using various animal models. Despite the knowledge acquired from such models, particularly mechanistic discoveries that sometimes mimic human T2DM mechanisms or pathways, many details of human T2DM pathogenesis remain unknown, therapeutic options remain limited, and a cure has eluded research. Emerging human data have raised concern regarding inter-species differences at many levels (e.g. in gene regulation, pancreatic cytoarchitecture, glucose transport, and insulin secretion regulation), and the subsequent impact of these differences on the clinical translation of animal research findings. Therefore, it is important to recognise and address the translational gap between basic animal-based research and the clinical advances needed to prevent and treat T2DM. The purpose of this report is to identify some limitations of T2DM animal research, and to propose how greater human relevance and applicability of hypothesis-driven basic T2DM research could be achieved through the use of human-based data acquisition at various biological levels. This report addresses how *in vitro*, *in vivo* and *in silico* technologies could be used to investigate particular aspects of human glucose regulation. We do not propose that T2DM animal research has been without value in the identification of mechanisms, pathways, or potential targets for therapies, nor do we claim that human-based methods can provide all the answers. We recognise that the ultimate goal of T2DM animal research is to identify ways to advance the prevention, recognition and treatment of T2DM in humans, but postulate that this is where the use of animal models falls short, despite decades of effort. The best way to achieve this goal is by prioritising human-centred research.

Key words: *animal research, human-relevant research, type 2 diabetes.*

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Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has reached pandemic status over the last three-and-a-half decades, and it continues to rise. Globally, in 2014, an estimated 422 million adults were living with diabetes (90–95% of it T2DM), compared to 108 million in 1980. The global age-standardised prevalence of diabetes in the adult population has increased, rising from 4.7% in 1980 to 8.5% in 2016, according to the World Health Organisation (WHO). The estimated global prevalence of diabetes for 2040 is 642 million, and the prevalence in the USA is expected to increase from 44.3 million in 2015 (one in seven Americans), to 60.5 million in 2040, according to the International Diabetes Federation. Therefore, it is critically important to advance the knowledge of T2DM pathophysiology, and to develop effective preventive and therapeutic measures for this complex, multifactorial disease, which is influenced by genetic, lifestyle and environmental risk factors.

The natural history of T2DM is characterised by a progression from normal glucose homeostasis to

a prediabetic state (impaired fasting glucose and impaired glucose tolerance), and then to diabetes (overt hyperglycaemia). The two hallmark features of T2DM are peripheral insulin resistance (impaired insulin sensing and action) and pancreatic β -cell dysfunction (impaired insulin secretion). The pancreatic β -cells produce, store and release insulin, and are located within the islets of Langerhans. It is widely acknowledged that β -cell dysfunction supersedes insulin resistance in the manifestation of T2DM, but the mechanisms by which hyperglycaemia can lead to further β -cell dysfunction and cell death are highly complex and remain to be characterised, as are the mechanisms that culminate in the morbidity (nephropathy, neuropathy, retinopathy, cardiovascular, cerebrovascular and peripheral vascular disease) and mortality of the disease (the seventh leading cause of death in the USA and the sixth worldwide). Most hypothesis-driven basic research in this field is dedicated to elucidating aetiopathogenic mechanisms that will advance our understanding of the disease and lead to effective preventive and therapeutic measures. In view of the important role of

islet β -cell dysfunction in the progression of the disease, research efforts are currently strongly focused on the human islets, investigating a range of aspects, such as the expression of voltage-gated channels, islet cell composition, basic mechanisms of β -cell division, and autonomic neural control (1).

The vast majority of basic scientific research on T2DM has been conducted in animal models, in species ranging from fruit flies to non-human primates, with rodents being the most widely studied. Decades of extensive research on animals has resulted in considerable advances in our understanding of glucose regulation, and species-specific variations in natural history, aetiopathogenesis, clinical manifestations, complications, and drug responses in the animal models used. However, these immutable species differences prevent the recapitulation of human T2DM in animal models, and thus frustrate the subsequent reliable translation of mechanistic and therapeutic knowledge for clinical benefit. There are numerous drugs in clinical use, of varying effectiveness and toxicity, for delaying the onset or treating T2DM. However, most of them have little or no impact on disease progression nor are they cures for T2DM, nor do they clearly prolong life.

As a result of species-specific differences at every level of glucose regulation (2), there is rising concern regarding the translatability of animal-based findings to human T2DM, and recognition of the need for more human-specific research. Given that the study of disease mechanisms remains a priority, and that the current animal-based hypothesis-driven research is unreliable for human translation, we examine how human-based investigative studies can be utilised to bridge this translational gap. We propose a framework for human-relevant studies, emphasising current and emerging methodologies, which will enable the human subject to serve as the quintessential animal model for 21st century diabetes research.

Understanding the Limitations of the Animal Models of Human T2DM

The regulation of glucose homeostasis, which maintains physiological levels of blood glucose concentrations (4–7mM fasting and 5–10mM postprandial; 3), involves complex interactions among a diverse range of cell types, tissues and organs (primarily the brain, liver, kidney, skeletal muscle, endocrine pancreas and adipose tissue). Detrimental biochemical changes that occur at the molecular, cellular, tissue and organ levels — further influenced by genetic, lifestyle and environmental risk factors — collectively culminate in the complex physiological manifestations observed in human T2DM. Therefore, the ideal disease model should recapitulate

human aetiology, pathogenesis, natural history, clinical manifestations, complications, and drug responses. However, no single animal model or combination of animal models can accurately replicate the human disease.

A comprehensive analysis of the most common animal models of T2DM has been documented elsewhere (4), and will not be discussed in detail here. Animal models differ significantly from humans, and even from other related animal models, at every level of molecular, cellular and physiological glucose regulation. This includes pancreatic cytoarchitecture, transcription factor binding sites, insulin signalling, neural control of glucose homeostasis, the expression and actions of species-specific β -cell receptors, and the insulin regulation of glucose transport, the rate-limiting step in human glucose metabolism (2, 5). These immutable species differences are further confounded by biological variation (e.g. age, sex and strain), technical limitations inherent in the methods of T2DM induction, e.g. surgical, chemical, dietary and genetic modification, and influences on experimental evaluation, e.g. assay type, and the effects of housing, husbandry practices and environmental control on the collected data (2, 6, 7).

A Human-based Framework

Recent publications on diabetes indicate that human-based methods can be utilised to assess various genetic, biochemical and physiological aspects of human glucose regulation. Since it is not possible to provide here a comprehensive list and assessment of publications from such a vast research field, we have focused on mechanism-based, hypothesis-driven basic science research. We highlight representative examples to demonstrate how *in vitro*, *in vivo* and *in silico* human-based methods can be utilised, to investigate the hallmark pathophysiological features of human T2DM. An integrative human-based research framework that takes into account human biological complexity at every level can form the foundation for translationally relevant mechanistic and therapeutic investigations.

Many hypotheses surrounding the mechanism of insulin resistance have been investigated, including inflammation, alterations in lipid metabolism, ectopic fat accumulation, impaired glucose transporter type 4 (GLUT4) trafficking, and mitochondrial and endoplasmic reticulum (ER) stress (8). Likewise, theories on the mechanism of β -cell death or functional impairment include oxidative stress (9), glucotoxicity (10), lipotoxicity (11, 12), amyloid deposition (13–15), β -cell apoptosis (16), inflammation-mediated mitochondrial and ER stress (17), pancreatic structural damage, and de-differentiation of β -cells (18). Such mechanistic aspects of T2DM can be studied in the laboratory,

whilst adhering to a human-based framework in the various investigations, as outlined below.

At the gene, protein and pathway level

Information on the molecular attributes of insulin resistance and β -cell dysfunction is best derived from the study of cells and tissues from the species of interest. For example, unlike rodents, humans carry only a single β -cell-specific and glucose-regulated insulin gene (19), the expression of which is tightly controlled at the transcriptional and post-transcriptional levels. Therefore, to obviate species specificity and increase human relevance, it is important to study the elaborate networks of gene regulation — involving such aspects as chromatin packing, *cis*- and *trans*-regulatory elements, autoregulation, and cell-type specific splicing — by using human specimens (20).

The human diabetic transcriptome, characterised from the study of human glucose regulatory organs and tissues under different conditions, can provide critical insight into the mechanisms governing T2DM pathological processes, including: the link between hyperglycaemia risk and islet cell maturation and function (21); changes in the T2DM islet leading to β -cell dysfunction (22); age-dependent and exercise-dependent skeletal muscle function (23); the role of hepatic and adipose tissue in insulin resistance (24); and complications such as diabetic kidney disease (25). Since comparative analyses have revealed notable species differences in protein-coding and non-coding RNA between rodents and humans (26), it is important to use exosomal RNA (mRNA, microRNA, and long non-coding RNA) isolated from human blood, urine, cerebrospinal fluid and cell culture supernatants, in order to obtain human-relevant information (e.g. biomarkers for early disease detection, disease risk, disease progression, risk of complications, and responses to therapeutics; 27, 28). Insights into the human T2DM phenotype can also be gained through nutrigenetics and nutrigenomics (29).

With CRISPR-Cas9 genome-editing technology, it is now possible to modify and functionally compare the genomes of patient and control pancreatic islet cells (30). Functional information encoded in the genome of T2DM patients can also be unravelled through proteomic methods (e.g. protein microarrays, immunofluorescence/high-content screening, multidimensional liquid chromatography, multiple reaction/high-sensitivity detection of serum and plasma proteins, mass spectrometry and magnetic resonance spectroscopy) to obtain mechanistic insight into the T2DM proteome, post-translational modifications, protein–protein interactions, and signalling pathways, among other features (31–33). Collaborative initiatives, such as

the Human Diabetes Proteome Project, will enable the identification of diabetes-related proteins (including the blood glycosylated proteome) and mechanistic details of protein dysregulation in T2DM (34).

Human obesity and the T2DM metabolic footprint (obtained through multiplatform analysis of carbohydrates, fatty acids, Krebs cycle intermediates, amino acids, choline and bile acids) can not only provide biomarkers for early detection of the disease in the subclinical stages, but also help in the understanding of disease pathogenesis at every stage (35). The signalling networks that control glucose regulation should be studied in human specimens rather than in animal specimens, due to species differences that can confound and limit data extrapolation (36, 37). Taken together, analysis of the human T2DM genome, epigenome, proteome, and the signalling networks that interconnect them, can provide in-depth mechanistic insight into human T2DM disease processes.

At the cell, tissue and organ level

Over the past few decades, a large amount of genetic, biochemical and physiological information on T2DM has been obtained from animal β -cell lines (38, 39). As we continue to uncover major species differences in factors affecting glucose biology — such as cell division, stimulus-secretion coupling and autocrine–paracrine interactions (2) — it is now becoming unquestionable that new information should be derived solely from human primary cells, tissues and organs, obtained from non-patient controls and patients in the various progressive stages of T2DM. Due to their robust replication and growth, heterologous cell lines also provide a convenient platform for research studies. However, human heterologous cell lines from many of the glucose regulatory organs are not yet widely available to researchers, and this situation must be remedied in order to maximise the value of human cell line research. The EndoC- β H1 human pancreatic β -cell line is currently available (40), but efforts should be significantly increased in order to generate a wider range of human heterologous cell lines from various organs, including the pancreas.

Commercially available immortalised primary cells offer a convenient, integrated platform for a range of research purposes. Isolated human primary cells are an important tool for basic research and preclinical studies, as they bridge the gap between discovery and translational science, in addition to eliminating the problems associated with species differences (40). Human primary cells are favoured for acute studies (41) and longer-term cultured cell lines are preferred for chronic studies (42). Even cryopreserved primary cells could serve

as suitable *in vitro* models for mechanistic and drug discovery research efforts (43), although the reliability of the results might decrease as the duration of cryostorage increased.

The complex, multifactorial aetiology of T2DM could be recapitulated in patient-specific and disease-specific human induced pluripotent stem cells. These cells are an invaluable platform for disease modelling, high-throughput drug discovery screening, preclinical toxicity assessment, and personalised medicine development (44) relevant for specific manifestations of T2DM. A plethora of human mimetic models can be developed from patient-specific cells at different clinical stages, and the cells can even be subjected to ageing techniques *in vitro* (45). It is crucial to use human stem cells rather than rodent stem cells, since species-specific molecular signatures, cellular signalling, growth rate, surface markers, developmental potential, satellite cell activation, and a host of other factors, will prevent accurate data extrapolation from rodents to humans (46, 47).

In addition to the types of cells used, it is important to employ physiologically relevant cell culture conditions, to closely simulate *in vivo* human glucose physiology. Compared to conventional static 2-D cell cultures, 3-D cell cultures permit the integration of several organ-specific cell types to create cytoarchitectures that more closely mimic the *in vivo* microenvironment. A prime example of this is 3-D primary liver cell culture methodology, which utilises hepatocytes and a range of non-parenchymal cells, such as fibroblasts, stellate cells and Kupffer cells (48). Co-culture systems with media exchange perfusion (e.g. Quasi-Vivo®) enable the further study of multi-organ interactions, mimicking the complex interplay that occurs *in vivo* (49). These types of study can be performed with cells and tissues from the various glucose regulatory organs, to investigate the systemic crosstalk networks relevant for T2DM disease mechanisms.

Human tissues are another useful platform for basic research and preclinical studies. Freshly isolated tissue samples are ideal, but even late-harvested *post-mortem* tissue (up to 24 hours of autolysis) can be a practical source for biologically relevant gene expression (50). Studies on *ex vivo* human pancreatic tissue — for example, islets isolated from such tissue that have been cultured for up to one week (42) — are useful for obtaining translatable mechanistic and toxicology information, given the substantial differences between rodent and human islet regulation (2). Many biochemical studies (including gene silencing) can be performed with small specimens from minimally invasive muscle biopsies. For instance, human skeletal muscle has been successfully used to study systemic glucose regulation, including the role of insulin and insulin resistance (51). Cell spheroids and groups of aggregated cells (generated by meth-

ods such as spontaneous cell aggregation, the hanging drop technique, or rotating wall vessel cultures), organoids and organotypic explant cultures, can better recapitulate *in vivo* cytoarchitecture and function, making it feasible to study the effects of *in vivo* microenvironments *in vitro*. These more realistic cell models provide a better platform to study various cell characteristics, such as metabolism and pharmacodynamics, by using, for example, electrophysiological and immunohistochemical methods (52, 53).

Engineered tissue is emerging as a functional substitute for various tissues, and is a versatile tool for basic research, preclinical studies, and toxicology studies. These surrogate tissues, derived from various organs and amenable to genetic modification, serve as physiologically relevant models (54). The use of human organs *ex vivo* could facilitate the validation and integration of human cell and tissue data into functional organ data in mechanistic investigations and preclinical toxicity assessments (55), further enhancing the predictive validity for humans. Microfluidic technologies, such as multiple organs-on-a-chip that emulate *in vivo* organ–organ interactions, are poised to revolutionise the way in which preclinical studies are conducted (56–58). These novel models are amenable to high levels of optimisation and modification, enabling the researcher to answer fundamental biological questions and assess new therapeutic approaches.

At the organism and population level

Insights into systemic glucose regulation can be obtained by studying human subjects with non-invasive or minimally invasive techniques. Conventional methods that are used in the diabetes clinic, such as the hyperinsulinaemic-euglycaemic clamp or homeostatic model assessment of insulin resistance, can also be applied in the laboratory, for mechanistic investigations — for example, to measure insulin action on glucose utilisation (59) or insulin resistance (60). Many non-invasive technologies, such as positron emission tomography (PET), computed tomography (CT), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), functional MRI, magnetic resonance spectroscopy (MRS) and advanced ultrasound techniques, can all be utilised successfully in basic research and preclinical studies (61–63). For example, PET analysis with ¹¹C-dihydrotrabenazine has been used to estimate β -cell mass (64), ¹³C-MRS can be used to evaluate hepatic mitochondrial oxidation (65), and functional MRI can be used to assess complications, such as cognitive impairment, in T2DM (66).

Pathological changes in β -cells contribute to the development of both type 1 and type 2 diabetes,

and there is considerable interest in the development and use of non-invasive imaging to monitor glucose regulation in real-time, over the course of the disease progression and after therapeutic intervention. Optimised and standardised molecular imaging techniques will not only improve clinical assessments, but also enable researchers to dissect T2DM mechanisms (e.g. structure, function and metabolism) in human subjects, under normal and pathological conditions in the presence and absence of therapeutic interventions.

Epidemiological studies have been instrumental in identifying lifestyle risk factors (diet, alcohol intake, tobacco use, exercise) and extrinsic factors, including gut microbiota and the intrauterine environment (67, 68). Such studies will continue to play a valuable role in defining the complex genetic and epigenetic factors, acting in conjunction with equally complex lifestyle and environmental factors, that culminate in the multifaceted human T2DM aetiology and natural history. The WHO and the International Diabetes Federation have emphasised the importance of lifestyle factors by confirming that: “In all the studies conducted so far in people at high risk, lifestyle changes have been substantially more effective than the use of drugs” (69).

T2DM predisposition, disease loci, and novel insight into disease mechanisms have all been assessed by studying single nucleotide polymorphisms (SNPs) (70) and by genome-wide association studies (71). Epigenetic regulation, which is characterised by the covalent modification of DNA or histone proteins (e.g. methylation, acetylation, phosphorylation, ubiquitination and sumoylation), has emerged as a species-specific and organ-specific factor in the pathogenesis of T2DM. Epigenetic variations, which exist prior to disease development, are widespread across the human genome. These modifications are associated with certain risk factors, such as age, body mass index, and HbA1c levels (72).

Genome-wide analyses of T2DM patients and non-diabetic cohorts have shown that both insulin resistance and insulin secretion are associated with altered DNA methylation patterns in the primary target tissues involved in glucose regulation, i.e. the pancreatic islets (73), skeletal muscle (74), adipose tissue (75) and the liver (76). Investigation of the human diabetic epigenome will continue to shed light on T2DM risk factors, aetiology and pathogenesis, and will provide insight into potential diagnostic and therapeutic targets. However, these analyses should be conducted in humans, since variable epigenomic regulation has been linked to genetic changes, transcriptional divergence, and disease genes, even in our closest genetic relative, the chimpanzee (77).

The influence of the gut microbiota on metabolic syndrome has recently emerged as a topic in T2DM research. However, the majority of the studies

involved have been conducted in rodents with very different diets, gut microbiota, physiological responses, and environmental conditions, compared to humans. Such studies can be conducted in humans, to define how human-specific exposures, such as the use of antibiotics and consumption of food additives, can provoke microbiota-mediated inflammation and the development of a metabolic syndrome leading to T2DM (78). The identification of reliable biomarkers to diagnose and phenotype specific manifestations of T2DM (e.g. neuropathy, nephropathy, retinopathy, etc.), when based on human clinical investigations (79, 80), will almost certainly generate human-relevant information that will lead to improved patient care.

The use of *in silico* systems biology

The complex pathophysiological interactions between the glucose regulatory organs and tissues, and the numerous genetic, lifestyle and environmental factors which modulate these interactions, contribute to the development and progression of T2DM. Therefore, the integration of complex human data obtained at every biological level, through a systems biology approach, will greatly enhance the translational potential of any results obtained (81). Disease diversity, captured from genome to phenome, can be merged in the discipline of systems biology, in order to define various aspects of T2DM pathology. Mechanistic insights can be obtained by integrating various collections of molecules, biological processes or physiological functions via ‘omics’ technologies.

For example, metabolomics (the detection and quantification of small metabolites, such as lipids, amino acids and hormones) is an effective approach to the elucidation of the intricate relationships between metabolism, obesity and T2DM, and as a clinical tool for risk evaluation and the monitoring of disease progression (82). Metabonomics (the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification) serves as a platform for the study of disease processes, drug toxicity, and gene function (83).

Since diet is a key risk factor influencing human T2DM, dietary patterns related to genetic variations, the role of gene–nutrient interactions, gene–diet–phenotype interactions, and epigenetic modifications caused by diet, can all be evaluated through nutrigenomics and nutrigenetics (29). In addition to basic science applications, these experiments offer clear potential for improved clinical diagnosis and treatment, especially in human patients at risk of developing, or in the early phase of, T2DM. For example, environmental risk factors that contribute to T2DM aetiology could be deter-

mined by examining the human exposome (which encompasses the totality of human environmental exposures from conception onward) through blood specimens (84).

Information derived from studies at different biological levels can be integrated through advanced *in silico* simulations and modelling. The complex interactions between multiple biochemical reactions that contribute to metabolic perturbations characteristic of human insulin resistance have been studied by using computational modelling. This has led to the first systems-level analysis of insulin resistance metabolism in fasted and fed states, and varying nutrient conditions (85). Easily accessible databases, such as the COM-BREX (COMputational BRidges to EXperiments) database — an online repository of information related to: a) experimentally determined protein function; b) predicted protein function; and c) relationships among proteins of unknown function and various types of experimental data, including molecular function, protein structure, and associated phenotypes — further facilitate the bench-to-bedside integration of human data (86).

At a systems level, computer modelling can also be used to integrate information derived from human-based methods. For example, the predictive value of an artificial pancreas control algorithms computational model led to the development of closed-loop glucose control via an implantable insulin pump, which was approved by the US Food and Drug Administration as a substitute for animal testing (87–89). This physiologically relevant model was generated by using quantitative information, such as plasma glucose concentration, and glucose and insulin fluxes, that were previously obtained from T2DM patients (87). Taken together, insights gleaned from human-based studies (from molecules to systems biology) will help overcome the species barrier and surely enhance translational success.

Future Perspectives: Incorporating the Human Context

Despite decades of extensive research efforts centred on animal models, the complex web of molecular mechanisms that lead to *human* T2DM pathogenesis, natural history, complications and therapeutic responses, still remains controversial and incompletely described. While we have a vast knowledge base for animal models, this has limited relevance for human T2DM, particularly with regard to its prevention and treatment. Since it is clear that preclinical research success has not been proportionally translated to preventive or therapeutic success, the T2DM research community is well-advised to switch its primary focus to human-based methodology that is capable of replicating human physiology.

The knowledge that we acquire through our research efforts over the next decade will have a significant impact on the way we shape the course of this pandemic disease. In the face of changing technologies, longer lives, and concerns over the relevance of animal-centred research, it is apparent that a paradigm shift is necessary. The scientific community will greatly benefit from focusing on the prioritisation of specific, multidisciplinary, human-based strategies that can greatly improve our understanding of T2DM disease mechanisms. This, in turn, should accelerate bench-to-bedside success. This will require collaborative efforts among basic and clinical researchers, as well as funding agencies, to better integrate clinical findings into hypothesis-driven basic research and to subsequently validate such basic research findings in the clinic.

Novel initiatives among different members of the diabetes research community (i.e. academia, medicine, industry and government) will be imperative, in order to prioritise human-based research. For example, biobanks could be established at research institutions and other facilities to enable the cost-effective distribution of human biological samples (e.g. biofluids, cells, tissues and organs) obtained from consenting patients. Researchers must be encouraged to utilise such human samples within a system that emphasises the greater usefulness and efficacy of human-specific research.

Human relevance must be at the forefront of the T2DM research effort. For example, funding agencies and journals must rebalance their grant review and manuscript review processes to reflect the need for human-based data acquisition, by requesting the inclusion of such data as an integral part of grant proposals and manuscripts. The current formula — requesting the validation of human data in animal models — has resulted in unreliable translation of the data to clinical medicine. While promoting the widespread use of existing human-based methods, it is equally important to fund the development of novel, next-generation technologies for use with human-centred research. If the ultimate goal of the diabetes research community is to understand disease mechanisms that will lead to better T2DM prevention and therapeutic outcomes for patients, then the best way to achieve that goal is by prioritising human-centred research.

Acknowledgments

The authors are affiliated with the Physicians Committee for Responsible Medicine, an organisation promoting best practices in medical research emphasising human relevance rather than animal use. The authors have no outside financial or other interests relevant for this report.

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