CHARACTERISATION AND PREDICTION OF CHECKPOINT INHIBITOR ASSOCIATED AUTOIMMUNE DIABETES MELLITUS

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A thesis submitted in fulfilment of the requirements of the Doctor of Philosophy degree.

Date: February 2025

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ABSTRACT

With the expanding use of immune checkpoint inhibitors in cancer therapy, there is growing interest in characterising and predicting the severe immune related adverse events that can arise as a complication of this therapy. Checkpoint inhibitor associated autoimmune diabetes mellitus (CIADM) is one such immune related adverse event and has been reported in case series to date as a novel form of fulminant type 1 diabetes. This thesis aims to characterise the clinical and immune phenotype of CIADM to improve identification and management of CIADM and investigate potential avenues for prevention of CIADM.

In a multicentre case series (Chapter 2) the clinical phenotype of patients with CIADM is detailed, along with the key hallmarks of absolute insulin deficiency, rapid pancreatic volume loss, exocrine insufficiency and variable degree of type 1 diabetes autoantibody positivity. A systematic review (Chapter 3) was subsequently conducted which demonstrated what features were most prominent in CIADM to allow for formation of clinical diagnostic criteria. Finally in Chapter 6, clinical guidance is provided based on multidisciplinary consensus to improve the identification and care of patients treated with immune checkpoint inhibitors who develop hyperglycaemia on treatment.

Using longitudinal blood, serum samples and radiological data in a case-control study, potential biomarkers for CIADM are identified in Chapter 4. Immune cell subsets, pancreatic volume, and Type 1 diabetes autoantibodies at baseline were found to be predictive of CIADM with a ROC curve AUC of 0.96. Whilst data on CIADM in humans has largely been limited to systemic markers, using a mouse model of CIADM, the islet immune infiltrate and endocrine cells are further profiled in Chapter 5 and potential key

local immune cell types in CIADM are identified. Together, these studies provide a foundation for future work in prediction and prevention of CIADM.

DECLARATION

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Signed,

Linda Wu

As primary supervisor, I confirm the above statement is accurate.

Signed,

Professor Jenny Gunton

AUTHOR ATTRIBUTION

I assess my contribution to the results described in each chapter to be:

- Chapter 1: 100%. Chapter 1 contains a published paper which I am the lead author of. I conceptualised, analysed the data and wrote the manuscript of this paper.
- Chapter 2: 100%. Chapter 2 comprises of a published paper which I am the lead author of. I conceptualised, collected and analysed the data and wrote the manuscript of this paper.
- Chapter 3: 100%. Chapter 3 comprises of a published paper which I am the lead author of. I conceptualised, collected and analysed the data and wrote the manuscript of this paper.
- Chapter 4: 80%. Chapter 4 comprises of a submitted paper which I am the lead author of. I conceptualised, retrieved patient samples and performed the flow cytometry, cell sorting, C-peptide assay, CT analysis, analysed the data and wrote the manuscript of this paper. I assisted with the Biolegend LegendPlex assay, RNA extraction, sequencing and analysis. I have acknowledged below the researchers who have contributed to this paper.
- Chapter 5: 100%. I conducted all the mouse work in this chapter including mouse care, injections, adoptive transfers, islet isolation and flow cytometry. I conducted all of the histology, immunofluorescence, immunohistochemistry and assays in this chapter. I analysed the data and wrote the chapter.
- Chapter 6:100%. Chapter 6 comprises of a submitted paper which I am the lead author of. I conceptualised, collected data, and wrote the manuscript.
- Chapter 7: 100%.

I hereby acknowledge the following researchers for their contributions to results included in this thesis:

- The Melanoma Institute of Australia Biobank for provision of patient samples used in Chapter 4
- Professor John Wentworth for assistance in conducting the ADAPS-PCR based autoantibody assay (Chapter 4).
- Professor Christopher Liddle for assistance in RNA-Seq data analysis (Chapter 4).
- Westmead Institute for Medical Research Genomics and Flow Cytometry teams for assistance in planning and troubleshooting for experiments for flow cytometry, cell sorting and assistance with RNA sequencing (Chapter 4 and Chapter 5).

I would like to thank University of Sydney and Australian Government for the financial support of the RTP scholarship I received during my time as a PhD candidate.
No artificial intelligence (AI) was utilised in any part of this thesis.

In addition to the authorship attribution statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Signed,

Professor Jenny Gunton

ACKNOWLEDGEMENTS

This thesis represents the most significant mountain I have ever challenged, and I have so many people I must acknowledge for their assistance along the way.

Firstly, thank you to my primary supervisor Professor Jenny Gunton. Thank you for always being so patient and resilient and supporting me through the many challenges thrown our way. I am so grateful you encouraged me to pursue such a fascinating novel area and entrusted me to freely explore scientific questions and grow as a scholar.

Thank you to my co-supervisors Alex, Sarah and Venessa. Thank you Alex, for your pragmatism and your wealth of clinical expertise. Sarah, I am so grateful for your wisdom and kindness, and for always being available and approachable with all my immune (knowledge) deficiencies. Thank you Venessa, for being my endless well of positivity and motivation.

I would like to thank the members of the CDOE laboratory in particular Josephine and Charmaine, for teaching me to be a better scientist, to be a more varied home cook, to be a more resilient human being. Thank you Nicole, for being kind enough to teach me nearly everything I know about flow cytometry.

Finally I would like to thank my friends and family. To my family, thank you for teaching me the value of hard work. I am so grateful for all the sacrifices you made to ensure I could have the best possible future. To my friends, thank you for supporting me unendingly and always being there to listen to my latest experimental dilemma. To James,

thank you for reminding me of who I am outside of work, and for supporting and loving me unconditionally.

Publications arising from this thesis

1. **Wu L**, Tsang VHM, Sasson SC, Menzies AM, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2021). Unravelling Checkpoint Inhibitor Diabetes: From Bench to Bedside. *Frontiers in Endocrinology*. 12:764138.

2. **Wu L**, Tsang V, Menzies AM, Sasson SC, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2023). Risk Factors and Characteristics of Checkpoint Inhibitor–Associated Autoimmune Diabetes Mellitus (CIADM): A Systematic Review and Delineation From Type 1 Diabetes**.** *Diabetes Care***.** 46(6): 1292–1299.

3. **Wu L**, Carlino MS, Brown DA, Mellor R, Moore K, Clifton-Bligh R, Long GV, Tsang V, Sasson SC, Menzies AM, Gunton JE (2023). Checkpoint Inhibitor Associated AutoImmune Diabetes Mellitus is Characterised by Rapid C-peptide Loss and Pancreatic Atrophy. *Journal of Clinical Endocrinology and Metabolism.* Volume 109, Issue 5, May 2024, Pages 1301–1307, https://doi.org/10.1210/clinem/dgad685

4. **Wu L**, Carlino MS, Brown DA, Mellor R, Moore K, Clifton-Bligh R, Long GV, Tsang V, Sasson SC, Menzies AM, Gunton JE (2024). Response to Letter to the Editor from Hao and Xue: "Checkpoint Inhibitor-associated Autoimmune Diabetes Mellitus Is Characterized by C-Peptide Loss and Pancreatic Atrophy", *The Journal of Clinical Endocrinology & Metabolism*, Volume 110, Issue 2, February 2025, Pages e552–e553, https://doi.org/10.1210/clinem/dgae555

Publications under review at time of submission

5. **Wu L,** Tsang V, Clifton-Bligh R, Carlino MS, Tse T, Huang YT, Oatley M, Cheung W, Long GV, Menzies AM, Gunton JE (2025). Hyperglycaemia in patients treated with immune checkpoint inhibitors: key clinical challenges and multi-disciplinary consensus recommendations. *Journal for Immunotherapy of Cancer.* Under review.

6. **Wu L,** Wentworth J, Liddle C, Fewings N, Carlino M, Brown DA, Clifton-Bligh R, Long GV, Scolyer RA, Sasson SC, Tsang VHM, Menzies AM, Gunton JE (2025). Conventional and novel biomarkers to predict checkpoint inhibitor associated autoimmune diabetes. *Nature Medicine*. Under review.

Oral presentations/ posters arising from this thesis

1. **Wu L**, Wentworth J, Liddle C, Carlino MS, Brown DA, Clifton-Bligh R, Long GV, Sasson SC, Tsang VHM, Menzies AM, Gunton JE (2024). Identifying conventional and novel biomarkers to predict checkpoint inhibitor associated autoimmune diabetes, *ENDO USA 2024 (Poster).*

2. **Wu L**, Tsang VHM, Sasson SC, Menzies AM, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2023). Checkpoint inhibitor related autoimmune diabetes: an Australian cohort and exploration of exocrine involvement, *Australian Diabetes Congress 2023. (Oral presentation)*

3. **Wu L**, Tsang VHM, Sasson SC, Menzies AM, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2022). Risk factors and characteristics of checkpoint inhibitor associated autoimmune diabetes (CIADM): a systematic review and meta-analysis. *Australian Diabetes Congress 2022 (Young Investigator Finalist) (Oral presentation)*

4. **Wu L**, Tsang VHM, Sasson SC, Menzies AM, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2021). Unravelling Checkpoint Inhibitor Diabetes Mellitus (Abstract #191). *Endocrine Society of Australia Conference 2021. (Oral presentation)*

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Chapter 1: **Introduction**

1.0 Diabetes Mellitus

The disease termed diabetes mellitus has afflicted humankind for millennia. With the evolution of modern medicine, our understanding of this disease has undergone numerous paradigm shifts.

The earliest descriptions of diabetes mellitus date back to 1500 BC, with Egyptian physicians describing a disease resulting in "too great emptying of urine" [1]. Aretaeus of Cappadocia was the first to coin the term 'diabetes', describing that a process by which "no essential part of the drink is absorbed by the body while great masses of the flesh are liquefied into urine" [2]. 'Mellitus', derived from 'mel' which is latin for honey, was adopted by Thomas Willis in 1675 in recognition of the role of hyperglycaemia and glycosuria in this disease [3]. These monumental discoveries have solidified the definition of diabetes mellitus and to this day the diagnosis of diabetes mellitus requires the presence of persistent hyperglycaemia. This is commonly demonstrated by an impaired response to a glucose load or elevated glycosylated haemoglobin (HbA1c) [4].

With time, the complications of hyperglycaemia were also recognised. In 1886 Professor Dreschfeld first described two forms of diabetic 'coma' which we know as the life-threatening acute complications of diabetes of diabetic ketoacidosis and hyperosmolar hyperglycaemic state [5]. From 1850, the effects of hyperglycaemia on atheromatous cardiovascular disease, neuropathy and foot ulceration were progressively identified [6].

Von Mering and Minkowski discovered the key role of the pancreas in diabetes, finding that removal of pancreata led to hyperglycaemia and glycosuria in dogs in 1889 [7]. In 1921, Fredrick Banting and Charles Best famously developed insulin-containing extracts, initially from dogs which had undergone pancreatic ductal ligation to 'degenerate' the non-endocrine tissue. The extracts were successfully used to treat diabetes in

pancreatectomised dogs [8]. In doing so they identified insulin, which has now become a cornerstone of treatment for diabetes.

It is now understood that insulin is an anabolic peptide hormone synthesised by the beta cells within the Islets of Langerhans in the pancreas [9]. Its primary function is down-regulation of circulating glucose by facilitating transport of glucose into insulin-responsive tissues [9].

1.0.1 Classifications and Diagnosis of Diabetes

As early as 1866 it was recognised by the British Physician Harley that "there are at least two distinct forms of the disease [diabetes] requiring diametrically opposing forms of treatment" [10]. Diabetes was initially described as juvenile-onset and adult-onset. The next major step in classification of diabetes mellitus took place in 1976, when Cudworth and Lister et al. introduced the terms 'type 1' and 'type 2' diabetes [11]. It was recognised that there were two broad groups of patients with diabetes, with 'type 1' attributed to the young, lean group with acute onset of illness, and 'type 2' attributed to the group who were overweight, older with a more insidious onset of disease [12]. Modern classifications of diabetes have not significantly altered from these initial determinations, although our understanding that type 1 diabetes can have onset at any age, and of the aetiology of these phenotypic differences has evolved. Many other rarer categories of diabetes have emerged, including genetic forms of diabetes (e.g. Maturity Onset Diabetes in Youth (MODY)), and secondary forms of diabetes, for example after pancreatitis.

The American Diabetes Association broadly classifies diabetes mellitus by aetiology into the following i) type 1 diabetes occurring due to autoimmune destruction of insulin-producing beta cells; ii) type 2 diabetes due to progressive loss of adequate insulin

secretion by beta cells in a setting of insulin resistance; iii) specific types of diabetes due to other causes such as maturity onset diabetes of the young and diseases of the exocrine pancreas; and iv) gestational diabetes [4]. Type 1 and type 2 diabetes remain by far the most common amongst these categories, with type 1 diabetes accounting for 5-10% and type 2 diabetes accounting for 90-95% of diabetes respectively [4]. Genetic causes of diabetes probably account for 2-5% of cases. Whilst the majority of type 2 diabetes is diagnosed in adulthood, peak incidence for new type 1 diabetes diagnosis is 10-14 years of age [13], and there is also a slightly smaller peak at 4-7 years.

A diagnosis of type 1 diabetes is made based on the presence of diabetes-range hyperglycaemia (HbA1c ≥6.5% and/or fasting glucose ≥7mmol/L and/or random glucose ≥11.1mmol/L) alongside evidence of autoimmunity towards the pancreatic islet detected via positive islet autoantibodies [4]. The antibodies typically used for diagnosis are anti-glutamic acid decarboxylase 65 (anti-GAD65), anti-insulinoma associated antigen 2 (anti-IA2), anti-insulin and anti-Zinc Transporter 8 (Anti-ZnT8).

Whilst type 1 diabetes is the broad term used for autoimmune diabetes, in recent years there has been increased recognition of the different 'subtypes' of autoimmune diabetes which carry a distinct clinical phenotype. This includes Latent Autoimmune Diabetes of Adulthood (LADA) which is typically defined as new onset diabetes in adulthood (>30 years) with islet autoantibody positivity and absence of insulin requirement 6 months post diagnosis [14]. Several studies have found that 4-14% of those diagnosed with type 2 diabetes in fact meet the criteria for LADA[15,16]. Fulminant type 1 diabetes (FTD) has also been recognised as a subtype of type 1 diabetes. FTD presents with sudden onset hyperglycaemia with ketoacidosis and rapid insulin deficiency, often with associated rise in pancreatic enzymes, absence of C-peptide and most have an absence of islet

autoantibodies [17]. FTD appears to be more common in Asian populations and accounts for up to 15-20% of new type 1 diabetes cases in a Japanese survey. Finally, with the introduction of immune checkpoint inhibitors in cancer therapy, checkpoint inhibitor associated autoimmune diabetes is a newly recognised immune related adverse effect arising from this therapy. This novel entity will be explored further later in this chapter.

1.0.2 Management

Management of diabetes is focused on attempting to restore normal blood glucose levels. In the case of type 2 diabetes, there are numerous therapeutic options to induce a reduction in insulin resistance and improvement in insulin secretion, ranging from lifestyle changes, tablet therapy to injectables and insulin[18]. In type 1 diabetes, replacement of insulin is required with either multiple daily subcutaneous insulin injections or insulin pump technology.

Maintenance of near-normal glucose in type 1 diabetes is often very challenging. In addition to regular glucose monitoring, constant consideration of factors that can impact glucose is required, including dietary intake, timing and doses of insulin and physical activity. Large prospective studies in type 1 diabetes have demonstrated that maintenance of tight glycaemic control reduces incidence of both macrovascular and microvascular complications [19]. Whilst recent technological advancements such as hybrid closed loop insulin pumps and continuous glucose monitoring have helped to reduce the strain of glycaemic monitoring and insulin administration, these technologies are not universally available nor are they able to fully alleviate the burden of diabetes management.

Despite the progress in our understanding of diabetes over the centuries, diabetes continues to exert a heavy burden of illness upon our society. As of 2021, diabetes affects 5.1% of adults in Australia, and the number of people living with diabetes has increased 2.8 fold in 20 years [13]. Diabetes contributed to 21,900 deaths (11% of all mortality) in Australia in 2022 [13]. Despite advancements in management, death rates for people with type 1 diabetes remains 2-5 times higher than the general population and expected life expectancy is 12.2 years lower than the general population [20,21].

1.1 Checkpoint inhibitor related autoimmune diabetes mellitus

This following section of the literature review contains a manuscript summarising the current clinical understanding of checkpoint inhibitor related autoimmune diabetes, including pathogenesis, diagnostic features and clinical management.

The manuscript has been published in Frontiers in Endocrinology after peer review. The text in this thesis is identical to the original paper. References for the manuscript are listed directly after the manuscript and the pdf version is available in the appendix.

Wu L, Tsang VHM, Sasson SC, Menzies AM, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2021). Unravelling Checkpoint Inhibitor Diabetes: From Bench to Bedside. *Frontiers in Endocrinology*. 12:764138.

Unravelling Checkpoint Inhibitor Associated Autoimmune Diabetes: from Bench to Bedside

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Abstract

Immune checkpoint inhibitors have transformed the landscape of oncological therapy, but at the price of a new array of immune related adverse events. Among these is β-cell failure, leading to checkpoint inhibitor-related autoimmune diabetes (CIADM) which entails substantial long-term morbidity. As our understanding of this novel disease grows, parallels and differences between CIADM and classic type 1 diabetes (T1D) may provide insights into the development of diabetes and identify novel potential therapeutic strategies. In this review, we outline the knowledge across the disciplines of endocrinology, oncology and immunology regarding the pathogenesis of CIADM and identify possible management strategies.

1.1.1 Introduction

The demonstrated successes of immune checkpoint inhibitors (ICIs) have resulted in a paradigm shift in the management of many malignancies. However, their association with novel immune related adverse effects (irAE), necessitates that a more detailed understanding of their pathogenesis is a major research priority. This will facilitate the early recognition and management of the autoimmune toxicities of ICIs that will become essential for many clinicians.

ICI-associated autoimmune diabetes mellitus (CIADM, also termed CPI-DM) is a novel form of autoimmune diabetes that arises as a rare complication of therapy, with an incidence between 0.21.4% (1–6). In contrast to many irAEs, CIADM often presents fulminantly with inexorable rapid progression (2–5). As with type 1 diabetes (T1D), the management is complex. We review the body of evidence across human and animal studies that add to our understanding of CIADM pathogenesis and islet autoimmunity in general. Finally, we highlight the clinical challenges in the management of patients with CIADM.

1.1.2 Immune related adverse events – an overview

ICIs augment adaptive immunity via blockade of immune checkpoints that can be upregulated on exhausted/anergised T cells and/or manipulated by cancer cells to facilitate immune evasion. In doing so, ICIs can induce a potent anti-tumour immune response. The key agents in current use are monoclonal antibodies targeting cytotoxic T-lymphocyte associated protein 4 (CTLA-4),

Programmed cell death protein 1 (PD-1) or its ligand Programmed cell death protein ligand 1 (PDL1). The dramatic efficacy of ICIs was first demonstrated in metastatic melanoma in 2011 with the FDA approval of ipilimumab, an anti-CTLA4 monoclonal antibody (7). A 1 year overall survival of 25-35% with previous standard of care chemotherapy (8), increased to 73% with use of combination ICI therapies nivolumab (anti-PD1) and ipilimumab (anti-CTLA4) (9). ICIs provide a robust long term benefit with a 52% 5 year overall survival in patients with advanced melanoma after combination ipilimumab and nivolumab treatment (9). ICIs are now used as first or second line treatment in 17 solid tumours with 57 FDA approved indications, with eligibility expanding in USA from 1.54% of malignancies in 2011 to 43.63% in 2018 (10). In addition to metastatic malignancies, adjuvant ICI therapy with anti-PD1 has also been demonstrated to reduce risk of relapse in resected stage III or IV melanoma and resected renal cell carcinoma (11,12).

One of the consequences of ICIs is the risk of developing irAEs. These can target virtually any organ system within the body and range in severity from mild to life-threatening. The incidence of grade 34 irAE (severe to life-threatening) is approximately 10-20% with anti-PD-1 monotherapy, 10-27% with anti-CTLA4 monotherapy, and 55% with combined anti-PD-1/CTLA-4 (9,12–19). Time to irAE onset varies depending on the ICI type and the organ involved (20,21). There does not appear to be a clear association between irAE and the underlying malignancy, with the exception of vitiligo which has a preponderance in melanoma patients, thought to relate to heightened reactivity between melanoma cells and melanocytic antigen targets in normal skin (22). Most (9,23–25) but not all (26) studies have demonstrated that development of irAEs are associated with better treatment response, suggestive of a link between autoimmunity and anti-tumour immune responses.

Interestingly, this association appears stronger in anti-PD-1 and anti-PD-L1 treated patients (27).

The mechanisms of irAE development remain ill-defined but appear specific to the target organ and ICI sub-type. Patterns of irAE seen with each class of ICI have revealed that specific immune checkpoints bear a more critical role in maintaining immune tolerance in certain organs. For example, ICI-related colitis had a 36% incidence in anti-CTLA4 treated patients in comparison to 1% in anti-PD1-treated patients (28). Furthermore, the key immune mediators appear to vary across irAE and vary compared to the matching classic *de novo* autoimmune diseases. One example is ICI related colitis, which is amongst the best studied of the irAE due to accessibility of tissue for histopathology.
Colonic biopsies from patients with ICI related colitis demonstrated high levels of activated CD8+ T cells and relatively lower proportions of Treg cells in comparison to ulcerative colitis affected patients, indicating distinct immunological differences between the two diseases and also highlighting a key pathogenic role for T cells in this disease (29,30). Contrastingly, histopathology from patients with ICI induced hypophysitis demonstrated both T and B cell infiltration with CTLA4 expression within pituitary cells and positive anti-pituitary antibodies in the circulation (31). Autoantibodies strongly associated with spontaneous autoimmune diseases such as T1D or myasthenia gravis are less commonly found in the irAE forms of disease (3,32). Antibodies in CIADM will be discussed in detail below.

Amongst irAE, the most common endocrinopathies are thyroid dysfunction, hypophysitis and less commonly CIADM and adrenalitis. Whilst irAE such as ICI related colitis have

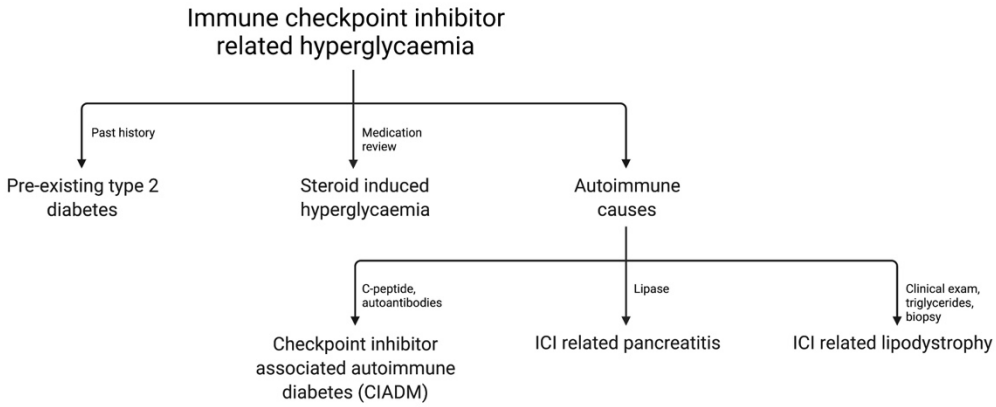
been demonstrated to respond to immunosuppression (26), endocrinopathies do not appear to respond and result in irreversible hormonal deficiencies in the vast majority (24,33,34).

1.1.3 ICI-related hyperglycaemia

With increased reports of ICI related hyperglycaemia and diabetes it is clear that a range of pathologies can contribute to elevated glucose. ICI-related autoimmune diabetes (CIADM) is the best described amongst these, largely due to its fulminant nature and thus high clinical importance. Other causes for ICI-related hyperglycaemia include exacerbation of type 2 diabetes, steroid-induced hyperglycaemia, pancreatitis with endocrine insufficiency, and autoimmune lipodystrophy (35–38). In one study of 411 patients receiving ICI therapy 27% had hyperglycaemia, 33.3% of whom had pre-existing hyperglycaemia, 39.5% had new-onset hyperglycaemia associated with steroid use, none had CIADM and the remainder had an unclear precipitant (37).

Common causes of hyperglycaemia should be excluded before making a diagnosis of CIADM, as the treatment varies widely. Similarly, readers should interpret with caution reported cases of CIADM patients to ensure the diagnosis was applied with definitive evidence of insulin deficiency or autoimmunity, rather than generic ICI-related hyperglycaemia which may occur in the context of other therapies such as corticosteroids. Figure 1 outlines the most common causes of ICI related hyperglycaemia and the key investigations to differentiate these.

Figure 1. Flowchart of common differentials for ICI related hyperglycaemia.



1.1.4 Pathophysiology of checkpoint inhibitor associated autoimmune diabetes

1.1.4.1 CIADM as a novel subtype of type 1 diabetes

Despite it being 100 years since the discovery of insulin and recognition of T1D, relatively little is known regarding the pathogenesis of islet autoimmunity. The commonly accepted theory is from Eisenbarth *et al* in 1986, proposing that in a genetically predisposed individual, exposure to environmental triggers can precipitate islet autoimmunity and progressive β-cell destruction (39). Diagnosis of T1D requires demonstration of hyperglycaemia and is supported by evidence of autoimmunity and insulin deficiency, with 90% of patients being positive for islet autoantibodies at some point in their clinical course (40,41). The common antibodies in T1D are to GAD (glutamic acid decarboxylase), insulin, ZnT8 (zinc transporter 8) and IA-2 (insulinoma-associated-2).

Over time distinct clinical phenotypes have been recognised within T1D. While traditional T1D is most commonly diagnosed in children and young adults, it can be diagnosed at any age. In addition, there is a milder, more slowly progressive phenotype in older patients termed latent autoimmune diabetes of adulthood (LADA) (41). ‘Fulminant T1D’ is

increasingly reported in Asian populations, usually presenting with diabetic ketoacidosis. It is often associated with a lack of islet autoantibodies (42).

CIADM is considered a novel form of T1D, triggered specifically by ICI use. There is increasing evidence that the risk factors, pathophysiology and clinical phenotype of CIADM differ from traditional T1D.

1.1.4.2 Defining key immune mediators

There is robust evidence for the role of T cells as a key culprit in the development of T1D. T1D can be transferred by bone-marrow transplantation in humans, rats and mice. Pancreatic histopathology from patients with T1D typically demonstrates hallmark insulitis with immune infiltrates of predominately CD8+ T cells, and to a lesser extent macrophages, B cells and CD4+ T cells (43,44). Islet immune infiltrates are typically limited in number and decline in frequency after diagnosis. Older patients and those with LADA display less insulitis than their classic younger T1D counterparts (45).

In contrast to T cells, the role of B cells in T1D is less well defined. Islet autoantibodies can predate onset of overt T1D by years (40). In genetically susceptible individuals the number of detectable islet autoantibodies directly correlates with risk of development of T1D (46). Whilst this evidence suggests islet antibody-producing B cells have some role in the pathogenesis of autoimmunity, islet autoantibodies do not display direct cytotoxicity to β-cells *in vitro* and they are not absolutely required for T1D development. That is demonstrated by a case of T1D in a person with X-linked agammaglobulinemia, lack of vertical transmission in autoantibody positive mothers with T1D and evidence that patients with hereditary B cell deficiencies may still develop T1D (47,48).

The evidence delineating key immune mediators in CIADM is limited. To date only one patient with CIADM has had pancreatic histopathology reported, a 63 year old man with

renal cell carcinoma and pre-existing type 2 diabetes whom developed diabetic ketoacidosis and low C-peptide after treatment with combination anti-CTLA4/PD-1 therapy (49). He was islet-antibody negative and his pancreas was resected due to tumour involvement. Histopathology demonstrated T cell infiltration throughout both endocrine and exocrine pancreas, with $CD8^+$ T cell predominant insulitis. This finding suggests that CIADM may result as an off-target effect of ICI, given $CD8^+$ T cells are the major cellular target of these drugs. Interestingly, few β-cells remained and PD-L1 was not expressed in those residual cells, despite their PD-L1 expression in classic T1D human pancreas (50). Although it is not possible to draw definitive conclusions, it is plausible that any PD-L1-positive β-cells were previously targeted for autoimmune destruction and thus absent by time of surgery.

The argument for T cell mediated β-cell destruction in CIADM is further supported by a small case series utilizing flow cytometry and tetramer assays on peripheral blood mononuclear cells from recently diagnosed CIADM patients (51). Hughes *et al* identified an increased population of islet antigen specific $CD8^+$ T cells in four CIADM patients, consistent with expected findings in new onset T1D patients (52,53). The majority of these cells were $CD45RO^+$ effector memory cells (51). Further studies are required to more clearly delineate the differences in immune changes between the fulminant process likely to be active in CIADM and T1D, where the autoimmune attack is thought to have preceded diagnosis by years (39).

Compared to traditional T1D where islet autoantibodies are present in 90% (40), autoantibody positivity is lower in CIADM, ranging from 0-71% (2–5,54). The largest review thus far of CIADM patients reported anti-GAD positivity in 43% of the 151 cases

tested (55). The relative paucity of traditional autoantibodies supports the theory that ICI triggered diabetes involves divergent immune pathways to those in traditional T1D islet antigen and B cell interactions. Six cases with CIADM have had retrospective testing of autoantibodies on pre-ICI treatment samples (2,56–58). Of these patients, 3 patients (50%) had traditional T1D autoantibodies present on pre-treatment samples, 2 patients seroconverted to autoantibody positivity, and 1 patient remained negative for islet autoantibodies throughout (2,56–58). This is a much higher incidence of autoantibody positivity than the general population, with the most common T1D autoantibody anti-GAD being present in 1.7% of the general population (59). These findings suggest that in a proportion of patients with CIADM, islet autoimmunity may predate ICI therapy. It may be tolerised via normal immune checkpoints but be unmasked by use of ICIs. Thus, traditional T1D autoantibodies do harbour some potential as biomarkers for CIADM, albeit limited in sensitivity by their low prevalence.

1.1.4.3 PD-1/PD-L1 axis

Exposure to ICI therapy involving the PD-1/PD-L1 axis is by far the strongest predictor for development of CIADM. Pharmacovigilance data from the FDA Adverse Events Reports System (FAERS) suggests that the incidence (as a proportion of all adverse events reported) is highest in those exposed to combination anti-CTLA4 plus either anti-PD-1 or anti-PD-L1 therapy (2.60%), followed by anti-PD-1 therapy alone (1.18%), anti-PD-L1 therapy alone (0.73%) and anti-CTLA4 therapy (0.33%) (60). The rare reports of CIADM with anti-CTLA4 monotherapy do not present clear evidence of either insulin deficiency or islet autoantibodies (60–62). This makes the diagnosis of CIADM less certain and the non-autoimmune differentials for hyperglycaemia still plausible in these cases.

The PD-1/PD-L1 axis has a well-established role in immune tolerance and maintenance of T cell anergy (63). PD-1 is an inhibitory molecule within the CD28 and CTLA4 superfamily and can be expressed on T cells, B cells, activated monocytes and dendritic cells. It interacts with two ligands, PD-L1 which is distributed across leukocytes, lymphoid and other tissues including pancreatic islets, and PD-L2 which is found on dendritic cells and monocytes. Polymorphisms in PD-1/PD-L1 genes in humans have been associated with a range of autoimmune diseases including type 1 diabetes, systemic lupus erythematosus and multiple sclerosis (64).

The development of CIADM after PD-1/PD-L1 inhibition highlights the critical role of this axis in the maintenance of self-tolerance towards pancreatic islets. The role of PD-L1 as a 'defensive' immunomodulator is supported by studies showing that β-cells from patients with T1D or autoantibody positive individuals express higher levels of PD-L1 compared to normal controls (50,65), and this expression was further induced *in vitro* by type I and II interferons (65). Notably, PD-L1 expression was only found in islets containing β-cells and correlated with $CD8^+$ T cell infiltration, implying PD-L1 expression in β-cells is upregulated in response to autoimmune attack (65). Similarly, peripheral blood findings have supported a role for PD-1 in T1D pathogenesis. Whole blood RNA analyses have demonstrated PD-L1 upregulation in a cohort of newly diagnosed patients with T1D (66). $CD4^+$ expression of PD-1 was reduced in T1D patients, and $CD4^+$ $CD25^+$ T reg cells of patients with T1D have impaired upregulation of PD-1 in response to stimulation in comparison to normal controls, suggestive of a role for PD-1 in defective immune regulation even in traditional T1D pathogenesis (67–69). Higher frequency of $CXCR5^-$ $PD\text{-}1^{hi}$ CD4+ T peripheral helper cells is also present in T1D, a T-cell subtype implicated in chemotaxis and activation of B cells in autoimmune disease (70).

In the non-obese diabetic (NOD) model for autoimmune diabetes, mice null for either PD-1 or PD-L1 developed accelerated diabetes and significantly greater numbers of insulin specific T cells (71,72). Interestingly, knockdown of PD-1/PD-L1 does not induce diabetes in other strains of mice such as C57BL/6, but instead induce lupus like disease and autoimmune cardiomyopathy, suggesting that PD-1/PD-L1 is not the only factor required to maintain islet tolerance and the relative importance of PD-1 may vary across both target organ and species (73,74). Furthermore, PD-1/PD-L1 blockade broke islet tolerance and result in diabetes in NOD mice maintained on tolerising therapy with antigen specific splenocytes, whilst anti-CTLA4 and anti-PD-L2 did not (75). Similar to human studies, NOD mice showed increased PD-L1 expression in β-cells in the presence of IFN-gamma, insulitis and overt diabetes (50,76). Conversely, loss of PD-1 in $CD4^+$ T cells led to increased islet antigen specific immune infiltrate within islets, pancreatic lymph nodes and the spleen, as well as increased destructive insulitis (77).

1.1.4.4 Genetic risk

The role for genetic predisposition in T1D is well defined, with a 65% concordance in monozygotic twins diagnosed with T1D by age 60 (78). HLA polymorphisms are the strongest genetic risk factor, with class II haplotypes HLA-DR3-DQ2 and DR4-DQ8 seen in 90% of T1D patients (79,80). In Asian populations DR4-DQ4 and DR9-DQ9 confer a high risk of T1D and fulminant diabetes (81). Genome-wide association studies have identified more than 50 further non-HLA susceptibility loci for T1D and these have contributed to the creation of genetic risk scores to aid in prediction of T1D and differentiation from other forms of diabetes (82).

Although the significance of HLA haplotypes has been pursued in CIADM, the findings have thus far been heterogenous. A recent review of 200 patients with CIADM showed that of the 78 patients with HLA genotyping reported, there was a pooled incidence of 51.3% that carried the HLA-DR4 haplotype, 14.1% had HLA-DR3 haplotype, whilst 10.3% had protective haplotypes (55). Metaanalysis demonstrated that presence of protective haplotypes was associated with a later median onset of CIADM (18 vs 9 weeks, p= 0.017). Only one case to date has had a T1D genetic risk score applied (58) and found a GRS score was below the 5th percentile, indicating a lack of known genetic predisposition to T1D. Overall, HLA susceptibility haplotypes for classic T1D appear to have some bearing in CIADM, albeit a much weaker association than that seen in classic T1D, indicating other risk factors are in play.

1.1.4.5 Role of the exocrine pancreas

Whilst T1D has traditionally been considered to involve isolated β-cell loss, there is increasing evidence that changes also occur in other islet cells and exocrine pancreatic tissue. Although β-cells only constitute 1-2% of pancreatic volume, even in recently diagnosed patients with T1D pancreatic volumes are smaller by approximately a third (83–85). Presence of these changes even in presymptomatic autoantibody positive patients suggests that the exocrine changes may play a role in disease pathogenesis rather than being purely secondary to hyperglycaemia. In fulminant T1D, elevations in lipase and amylase have also been reported at presentation (42). Conversely, in the classic T1D phenotype serum lipase and trypsinogen have also been shown to be significantly lower in patients with both T1D patients and patients positive for multiple autoantibodies in comparison to controls (86). Histology of pancreata from T1D patients shows immune cell infiltrate and fibrosis within exocrine tissue (43,87), C4d complement deposition within exocrine ducts (88) and reduced acinar cell numbers (87). Although these changes do not

result in overt exocrine insufficiency, patients with T1D have been shown to have lower faecal elastase values, in particular in those with established disease (89). It has been theorised that the development of these exocrine changes may be due to loss of the insulinotropic effect on acinar tissue, but this remains unproven. Another possible mechanism for damage is via either direct or bystander autoimmune attack, although the evidence for the former is limited to small scale studies of exocrine antigen targeted autoantibodies (90,91).

Exocrine pancreatic injury after ICI therapy is common, with meta-analysis reporting 2.7% incidence of asymptomatic pancreatic enzyme elevation, and 1.9% incidence of overt pancreatitis (92). The true incidence of asymptomatic pancreatic enzyme elevation is likely even higher, with reports from a centre performing routine lipase and amylase finding 8.4% had a grade 3 or higher elevated amylase and 26.9% grade 3 or higher lipase level (CTCAE v4) (93). Unlike CIADM, meta-analysis suggests pancreatitis is more common with anti-CTLA4 therapy (3.98%) compared to anti-PD-1 therapy (0.94%) (92). Abu-Sbieh *et al* reported that of 2279 patients treated with ICIs, 4% developed pancreatitis (defined in this study by lipase with or without clinical symptoms) and of these, 7% developed diabetes – although the precise phenotype of diabetes in these patients is not clearly delineated (94).

Exocrine involvement in ICI related diabetes varies across a spectrum from overt pancreatitis with exocrine and endocrine insufficiency, through to a T1D-like phenotype with no features of pancreatic inflammation. The primary differentiating factor is likely to be the immune target of the irAE, which we postulate will be acinar tissue in the pancreatitis related diabetes phenotype and β-cells in the T1D-like phenotype. Overlap

between phenotypes appears to be substantial, with a recent metaanalysis reporting that 51% of patients diagnosed with CIADM had an elevation in either lipase and/or amylase at time of diagnosis, a value disproportionately higher than that seen with ICI use in general (55). Rapid pancreatic atrophy has been reported in all CIADM patients whom have had pancreatic volumetry analyses, with significant decline from baseline pre-treatment volumes on imaging, through to CIADM diagnosis and follow-up (54,95). The increased prevalence of exocrine pancreatic inflammation in CIADM patients raises the possibility of immune triggering, where the exposure of pancreatic epitopes through pancreatic inflammation and destruction increases immune sensitization and risk of islet autoimmunity.

Whilst it is apparent there exists a degree of overlap where patients may manifest features of both pancreatic inflammation and T1D as defined by sensitive biochemical parameters such as lipase, the extent to which these patients manifest clinical features of chronic pancreatitis and exocrine insufficiency remains unclear. One case series to date reported faecal elastase values in CIADM, with 2 of 5 patients demonstrating values consistent with pancreatic exocrine insufficiency (95).

1.1.4.6 Enteroendocrine involvement

Whilst the majority of T1D research focuses on β-cells, alpha cell mass is reduced in patients with longstanding T1D (96). This has correlated with findings of reduced glucagon responses in those with established T1D (97,98), although glucagon responses in early T1D have been mixed (99,100). The clinical implications of this are significant, as loss of glucagon from alpha cells compromises physiological defenses against hypoglycaemia and increases morbidity and mortality (101,102).

The impact of CIADM on alpha cell function is not well defined. Several case series have measured random glucagon levels in new onset CIADM patients and found no abnormalities (2,3,54). To further explore glucagon responses Marchand *et al* performed mixed meal tests on 4 patients with fulminant presentations of CIADM, with 2 showing more blunted glucagon responses in comparison to 15 C-peptide negative longstanding T1D controls (95).

Given that incretins such as GLP-1 have roles in stimulating insulin secretion and suppressing glucagon, it is possible that dysregulation of incretins can contribute to dysglycaemia in CIADM. Bastin *et al* demonstrated that patients with fulminant diabetes after ICI therapy have reduced GLP-1 and GIP levels at baseline and post oral glucose tolerance test in comparison to those with nonfulminant and type 2 diabetes (103). Small study numbers limit the conclusions that can be drawn on mechanisms and implications of impaired enteroendocrine function in CIADM.

1.1.4.7 Putting it together

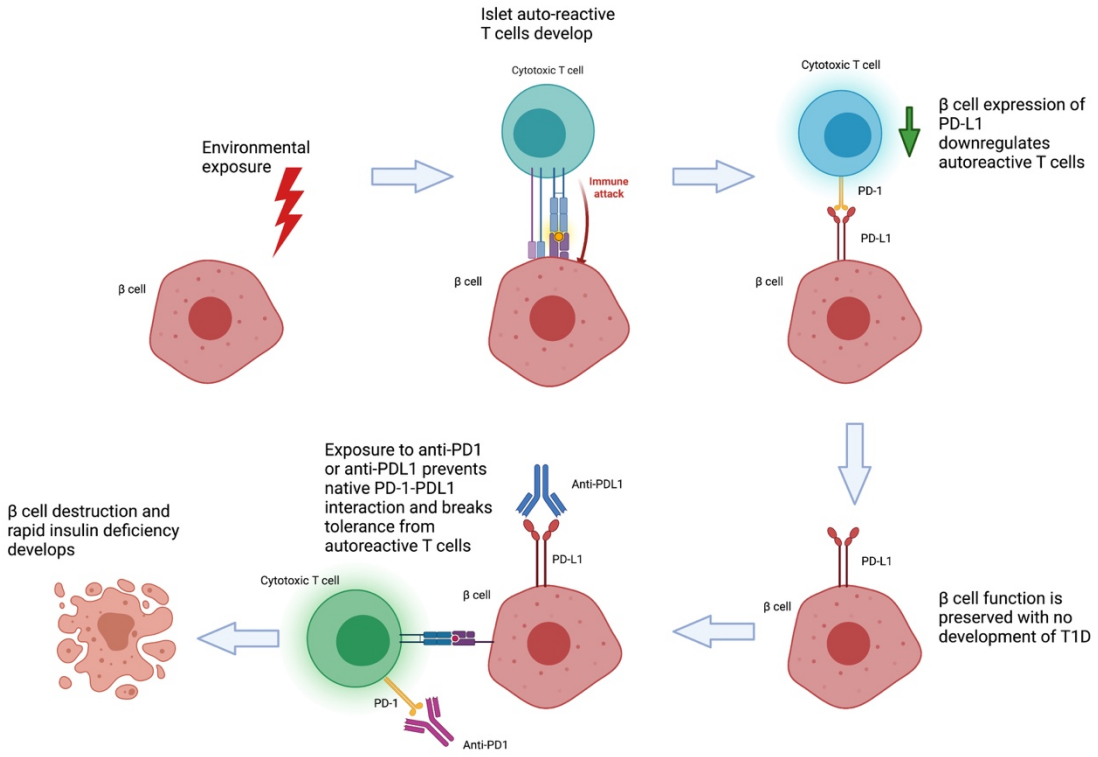
Table 1 summarises the clinical and pathophysiological differences between CIADM and T1D.

Table 1. Comparison of the disease phenotype of checkpoint inhibitor associated autoimmune diabetes (CIADM) to traditional type 1 diabetes (T1D). DKA = diabetic ketoacidosis, irAE = immune related adverse event

	CIADM	T1D
Presentation	DKA in 67.5% at presentation (55). 47.5% have a comorbid irAE, most common being thyroid (24.5%) (55).	DKA in 39% children at presentation (104), 6% in adults (105)
Clinical course	Fulminant presentation, median 9 weeks after ICI treatment No spontaneous remission phase or "honeymooning". Overt insulin deficiency and low C-peptide at presentation in most (<0.3ng/ml in 63.4%) (55)	Progressive development of islet autoantibodies → overt hyperglycaemia at presentation 'Honeymooning' in 68.9% of children with T1D with partial recovery of β-cell function (106) Progressive decline in C-peptide, 48% maintain stimulated C-peptide >0.2nmol/L at 5 years (107)
Autoantibodies	Anti-GAD autoantibodies + in 43% (overall islet autoantibody positivity 20-71%) (55)	Islet autoantibodies + in 90% (40)
Genetic predisposition	65.4% with T1D susceptibility haplotype, 10.3% with T1D protective haplotype (55)	T1D susceptible haplotypes in 90% (80)
Exocrine pancreas involvement	Pancreatic enzymes elevated in 51% (55), pancreatic atrophy on imaging (54, 95)	Lower lipase vs normal controls except in fulminant phenotype (86), reduced pancreatic volumes (83-85)
Proposed pathophysiology	Prior exposure to environmental trigger leading to islet specific autoimmunity, tolerised by PD-L1 Exposure to anti-PD1 or anti-PD-L1 unmasks autoimmunity and triggers β-cell destruction	Genetic predisposed individual exposed to an environmental trigger, leading to autoimmune β-cell destruction

On balance, the current literature supports a model of CIADM developing in genetically predisposed individuals who develop autoreactive T cells to beta-cells in response to an environmental trigger (Figure 2). These autoreactive T cells are generally controlled by immune checkpoints but result in pathology following their activation by anti-PD-1/PD-L1 therapy. Specific at-risk alleles for CIADM likely differ from classic T1D. Whilst patients with genetic susceptibility to impaired islet self-tolerance would have developed classic T1D earlier in life, populations with a particular reliance on the PD-1 axis for pancreatic tolerance may be at increased risk of CIADM specifically after antiPD1/PD-L1 exposure. This may explain why such patients are able to remain free of T1D throughout adulthood until exposure to ICI therapy.

Figure 2. Proposed pathogenesis of checkpoint inhibitor associated autoimmune diabetes (CIADM).



1.1.5 Checkpoint inhibitor associated autoimmune diabetes

1.1.5.1 Detection and diagnosis

From case series to date, CIADM has an incidence of 0.2-1.4% in those treated with ICIs (1–6). In a FAERS database of 57,683 patients treated with ICIs with reported adverse events, a progressive increase in the proportion of cases of CIADM has been reported each year, likely reflective of the increased use of ICIs in general (60). As previously discussed, exposure to anti-PD-1 therapy is the greatest risk factor. No significant differences have been noted on meta-analysis when adjusting for age or sex (60), with the largest review reporting a median age of 64 years and male predominance (62.5%), reflective of the populations treated for melanoma and non-small cell lung cancer where ICIs are most commonly used (55). BMI is reported in a minority of cases. 50% (26 of 52) of subjects being of normal/low BMI, a value likely confounded by concurrent malignancy and toxicity. No strong link with a family history of diabetes has been noted, with 13% having a family history of either type 1 or 2 diabetes (55). Reflective of current ICI use, melanoma was the most common malignancy amongst subjects (50.5%), followed by lung cancer (26.0%) and renal cell carcinoma (7%) (55). In these patients, 47.5% were associated with another irAE, of which thyroid dysfunction was most common (24.5%) (55).

Diabetic ketoacidosis is a common presentation for CIADM, with incidence varying from 45.9967.5% based on large cohort analyses (55,60,61). The hyperosmolar hyperglycaemic state has been reported in 1% (55). The largest systematic review cohort to date of 200 patients reported a median time from ICI commencement to CIADM onset of 9 weeks and found this interval to be significantly shorter in patients presenting with DKA (8 weeks vs 15 weeks) (55). The most common symptoms at presentation are polyuria and polydipsia

(48%), followed by gastrointestinal symptoms (vomiting, abdominal pain, diarrhoea) in 41.7% and fatigue (40.6%) (55).

In terms of laboratory findings at presentation, hyperglycaemia was common with 94.3% having values ³300mg/dL (³16.7mmol/L) and a median HbA1c at presentation of 62mmol/mol (7.8%) (55). HbA1c was lower in those with shorter time from ICI commencement to CIADM onset, indicative of fulminant disease development in which the HbA1c is not a good indicator. In those with C-peptide testing within 1 month of diagnosis, C-peptide was overtly low in 63.4%. Islet autoantibodies were positive in 45%, with 43% being anti-GAD positive (55). HLA-DR4 or DR9 was identified in 65.3% (44 of 78) whilst 10.3% had traditionally protective alleles. Elevated pancreatic enzymes were present at diagnosis of CIADM in 51% and acute renal failure in 55% (55).
The interpretation of the above pooled data is limited by the heterogeneity in CIADM definition as determined by each case/series, and by reporting bias. To aid clinicians in the identification of the highest risk group of patients with ICI related diabetes and refine the future data that emerges for this disease we recommend the following diagnostic criteria for CIADM. Firstly presence of hyperglycaemia is required, either by random blood glucose ³11.1mmol or HbA1c ³6.5% (as per American Diabetes Association criteria for all forms of diabetes (41)), acknowledging that in fulminant presentations of CIADM HbA1c may not yet be elevated. Secondly, the suspicion of β-cell destruction needs to be demonstrated by presence a low C-peptide (<0.4nmol/L) soon after diagnosis.

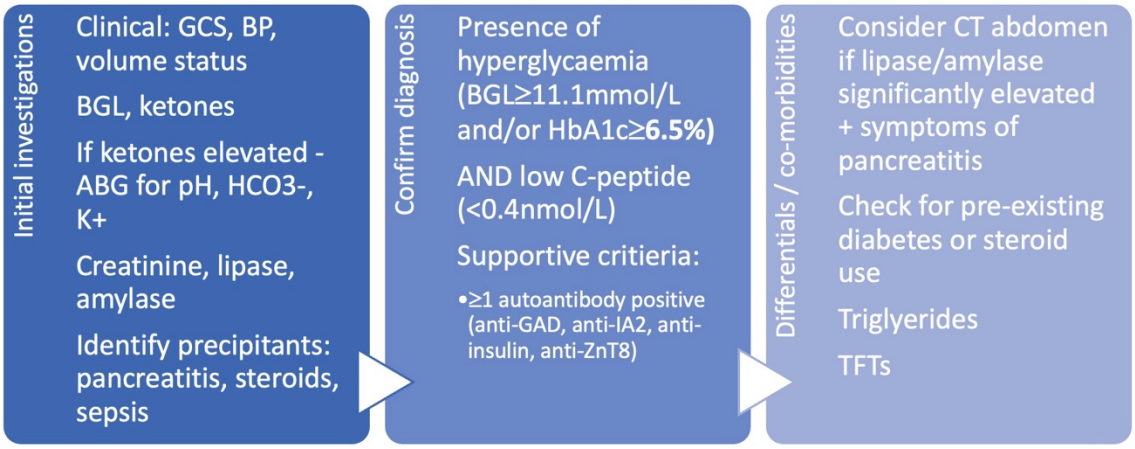
Seropositivity for 1 or more islet auto-antibodies (anti-GAD, anti-IA2, anti-insulin, anti-ZnT8) is supportive but not sufficient in isolation due to low overall prevalence in the CIADM population. Given the logistic challenges in performing a formal mixed meal test

in CIADM patients we recommend a post-prandial C-peptide as an alternative, assessing for inappropriately low insulin production in a setting of relative hyperglycaemia. C-peptide adds value in the capture of those with rapidly progressive insulin deficient diabetes.

Trials in T1D demonstrate that presence of a detectable C-peptide is associated with improved outcomes and thus identifying patients with low C-peptide at presentation may capture a higher risk population that benefit from closer management (104). Case series suggest that C-peptide may not always be overtly low at diagnosis with CIADM (3,54) and certainly in classic type 1 diabetes 48% of patients maintain a mixed meal stimulated C-peptide >0.2nmol/L in the first 5 years from diagnosis (105). Although even a normal C-peptide is considered inappropriate physiologically during hyperglycaemia, other forms of diabetes can also present with a normal C-peptide due to a pancreatic stunning effect with glucose toxicity (106) as well as reduced clearance of C-peptide in the setting of renal impairment. Thus, in those with a suspicion for CIADM diabetes but not yet manifesting an overtly low C-peptide at presentation we recommend repeating at 1 month to reduce the effect of these confounders.

As depicted in Figure 3, in addition to the aforementioned tests for diagnosis of CIADM we recommend ancillary investigations to stratify the severity of the presentation, assess for need for intensive care support, identify precipitants and exclude differentials for ICI induced hyperglycaemia.

Figure 3. Proposed diagnostic criteria and initial investigations in patients presenting with hyperglycaemia after immune checkpoint inhibitor use. GCS = Glasgow coma scale, BP = blood pressure, BGL = blood glucose level, ABG = arterial blood gas, HCO3- = serum bicarbonate, K+ = serum potassium, TFTs = thyroid function tests.



We acknowledge that these criterion will include patients with autoimmune pancreatitis related diabetes. There is significant overlap in the two populations and given the propensity of both towards diabetic ketoacidosis, they will require similar management with insulin. Those with concurrent significant pancreatitis require assessment for exocrine insufficiency with faecal elastase.

A further area of diagnostic challenge is the recognition of CIADM in patients with pre-existing type 2 diabetes. Various case reports have reported CIADM in patients with type 2 diabetes (5,107). However, heterogenous criteria have been used to define CIADM in this setting, such as new insulin requirement, sudden worsening of HbA1c, positive islet auto-antibodies or loss of C-peptide. The challenge is in distinguishing the acuity and severity with which this occurs after ICI administration in comparison with the natural history of type 2 diabetes where eventual loss of C-peptide and insulin dependence may also occur.

We have not sought to define diagnostic criteria for CIADM arising in patients with type 2 diabetes, as we feel this is an area where clinical judgement is paramount and ultimately both groups will benefit from insulin therapy.

A different ICI related cause of diabetes which should not be classified as CIADM is autoimmune lipodystrophy (35,36). Acquired generalised lipodystrophy is characterised by autoimmune loss of adipose tissue, leading to severe insulin resistance, hypertriglyceridaemia and non-alcoholic steatohepatitis. The two reported cases presented with severe hyperglycaemia and weight loss with notably elevated insulin, C-peptide and triglyceride levels. Both patients had been treated with antiPD1 inhibitors. Diagnosis was confirmed with gluteal fat biopsy in both patients demonstrating panniculitis with extensive lymphoid infiltrate and fibrosis within adipose tissue (35,36,108–111). Triglycerides are included in routine ancillary investigations in ICI treated patients with hyperglycaemia and will assist in differentiating this condition, especially before clinically apparent changes in fat distribution are present.

1.1.5.2 Clinical course and management

Management of CIADM requires insulin therapy, with all but two cases reporting a persistent and irreversible deficit in insulin production (2,3,54,61). Two case reports have described spontaneous return to normal C-peptide levels and successful cessation of insulin therapy in patients with hyperglycaemia and positive islet autoantibodies. However, neither had documented low C-peptide at diagnosis so alternate diagnoses are possible (95,112). There has been one case report of in a patient with newly diagnosed CIADM who required infliximab for treatment of concurrent oligoarthritis, with subsequent improvement in glycaemic control and insulin cessation. This patient's C-peptide levels were never overtly low and this case is confounded by steroid use (113).

Use of corticosteroids with the intent to halt CIADM or other concurrent irAE is not effective (114–117). Overall, there is no current evidence to support use of immune suppression in CIADM.

As described by our group previously, patients with new-onset CIADM continuous glucose monitoring demonstrates similar patterns in glycaemic variability to patients with T1D with no evidence of a 'honey-moon' period (3). This may reflect rapid β-cell loss as suggested by rapid decline in C-peptide levels. For this reason, we advocate that all patients should be managed akin to patients with T1D with use of basal bolus insulin or insulin pump therapy. Given the correlation between loss of C-peptide and hypoglycaemia risk (118), we recommend early consideration of adjuncts like continuous glucose monitoring in those with low C-peptide to reduce hypoglycaemia.

In contrast to traditional T1D, the oncological history and progress of a patient with CIADM bears significant implications both in prognosis and management goals. There is insufficient data available to draw conclusions on the impact of CIADM on oncological response. A recent review of 87 CIADM patients with reported oncological outcomes found a partial or complete response in 58.0%, which given 50.5% of the cohort had melanoma, is similar to the general ICI treatment cohort ((55). Given the irreversibility of CIADM once it is diagnosed, cessation of ICIs for this reason is unlikely to be of benefit. Unlike T1D, CIADM affects a large spectrum of the adult population ranging from fit patients receiving adjuvant therapy to frailer patients already burdened by multiple lines of therapy and advanced disease. The risks of hypoglycaemia are higher in frail populations, and more conservative glycaemic targets are appropriate in those with poorer functional status and advanced progressive disease, with the individual in mind according to

American Diabetes Association recommendations (41). In people who have short life-expectancy, insulin therapy should be simplified to target symptom control only. Conversely, it is also important to bear in mind that impressive survival outcomes offered by ICIs also signifies a larger population of patients will be cured and thus benefit from managing their diabetes with tighter glycaemic targets to prevent long term glycaemic complications. Clear communication regarding the expected cancer prognosis between oncologist and endocrinologist is key to setting safe management goals in this instance.

1.1.6 Future directions

Like all irAEs, the scope for further research into CIADM is broad. Further studies will help define the exact role of the exocrine pancreas and the extent to which acinar and other islet cells are affected. It is also unclear what predisposes a small subset of patients treated with anti-PD1/PD-L1 to this disease nor what other triggers may be required. Biomarkers to predict whom amongst those treated with ICIs will develop CIADM would have high clinical utility in particular as indications for ICI use expand. Several biomarkers have shown utility in predicting irAE, ranging from autoantibodies (119–121), single nucleotide polymorphisms (122,123), cytokines (124), lymphocyte count indices (125,126) to microbiome analyses (127). Whilst autoantibodies can reliably predict T1D onset in traditional T1D, the relatively lower prevalence of autoantibodies suggests this is not the case in CIADM (2,56–58). Given the unique immune trigger in CIADM, it is possible that novel autoantibodies to islet epitopes may exist that are yet undiscovered.

1.1.7 Conclusion

As the use of ICIs continues to increase, the prevalence of CIADM will accordingly rise. Given the irreversible nature of the disease, further research to understand the pathophysiology and identify early biomarkers will be key to potentially preventing

CIADM. Closer understanding of the presentation and initial investigations for CIADM amongst treating clinicians is essential to further reduce the incidence of fulminant DKA presentations and morbidity from this disease.

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1.2 Predicting Checkpoint Inhibitor Associated Diabetes Mellitus

Checkpoint inhibitor associated diabetes mellitus (CIADM) is an uncommon but highly morbid immune related adverse event that results in a high incidence of diabetic ketoacidosis and a life-long requirement for multiple daily insulin injections. As indications for ICIs broaden, it would be valuable to identify potential biomarkers to predict who will develop CIADM prior to onset of disease.

1.2.1. Biomarkers for type 1 diabetes

Type 1 diabetes has well established biomarkers that are validated and have been used to identify at risk individuals to target in clinical prevention trials[22]. HLA haplotyping for HLA-DR and DQ loci in combination with family history can stratify type 1 diabetes risk in an individual between <0.01% up to 50%[23]. Furthermore, genetic risk scores combining single nucleotide polymorphisms have also been shown to improve prediction of type 1 diabetes[24].

As mentioned previously, islet autoantibodies are the most well-established biomarkers for type 1 diabetes and form part of the pre-clinical staging system. The number of autoantibodies detected is associated directly with risk of diabetes development with a 0.4% risk of diabetes in children with no autoantibodies at 10 year follow up, up to 14.5% with 1 islet autoantibody and 69.7% risk in those with multiple islet autoantibodies [25]. Each islet autoantibody has slightly differing predictive value, with anti-GAD being associated with latent adult onset type 1 diabetes [26]. Differing epitopes of autoantibodies also appear to carry varying affinity and associated risk of diabetes, with high affinity anti-GAD being associated with diabetes, whilst low affinity anti-GAD is rarely found in those with diabetes [27].

Immune cell subsets have also been pursued as potential biomarkers for type 1 diabetes. Several studies have noted increased prevalence of T regulatory cells in individuals who progressed to overt type 1 diabetes compared to at risk controls[28–30], however the published data is conflicting here, with other reports finding no difference or reduced prevalence of T regulatory cells[31–33] and with reduced suppressive capacity[33,34]. Multimer assays have been used to identify antigen specific T cell subsets for characterisation, which offer precision in antigen specificity but are limited by the requirement for specific HLA subtypes (e.g. HLA-A2) and a larger volume of blood due to the lower prevalence of specific T cells in peripheral circulation[35]. Several studies have confirmed presence of memory CD45RO+ T cell responses to GAD in patients with type 1 diabetes that was not present in controls suggestive of a primed immune response [36,37].

Pancreatic volume has been identified to be significantly lower by 25% in patients with type 1 diabetes than controls at time of diagnosis [38,39], and in the ENDIA Study both pancreatic volume and pancreatic exocrine function were lower in children with recent onset type 1 diabetes [40]. Lower level of pancreatic exocrine enzymes lipase and amylase have also been found in adult patients with type 1 diabetes and at risk individuals compared with controls [41]. These changes suggest that the pancreatic exocrine tissue is also impacted in type 1 diabetes and may serve as a potential avenue for biomarker development, as islets make up only 2% of pancreatic mass and cannot solely account for this degree of volume change [42].

Several studies have also utilised novel biomarkers including circulating demethylated insulin DNA[43], metabolomic[44,45] and transcriptomic[46,47] biomarkers to predict type 1

diabetes. The TEDDY study in particular identified transcriptomic differences with controls having more robust enteroviral immune responses compared to children who developed type 1 diabetes and a distinct activated NK cell profile associated with progression [47,48].

1.2.2. Predictors of immune related adverse events

Research into biomarkers to predict immune related adverse events (irAEs) is a rapidly expanding and heterogenous area due to the vast range of ICIs, patient populations, cancers and immune related adverse events under investigation.

Patient factors that have been associated with development of irAEs in the literature include younger age[49], smoking history[50], history of autoimmune disease[51,52], higher BMI[53,54] and sarcopenia[55]. Presence of tumours with a higher mutational burden has also been associated with irAE[56]. Combination therapy with anti-PD1/PDL1 and anti-CTLA4 classes[57] or in combination with chemotherapy has been linked with higher risk of irAEs[58].

Circulating blood markers have been extensively researched as potential biomarkers for irAE. Neutrophil to lymphocyte ratios [51,53] and baseline and early on treatment dynamic changes in cytokines including IL-6, IL-17, TNFα [59–62] have been associated with irAE development and severity. Looking more specifically at T cell phenotyping, single cell RNA-Seq revealed baseline and early on treatment expansion in CD4+ T effector memory subsets to be associated with severe irAE development in a cohort of patients with melanoma[63]. IrAE development has also been associated with lower baseline T regulatory cell prevalence and greater rise in activated CD4+ HLADR+ CD38+ cells on ICI treatment

in a cohort of melanoma patients with predominantly GI toxicity[64] as well as greater T cell diversification as studied in a cohort of prostate cancer patients[65] .

Several studies have identified that flow cytometry based biomarkers appear to also vary based on the underlying organ involved in the irAE. For example, Bukhari et al's single cell RNA-Seq study identified that those with arthritis had lower baseline CD8+ T central memory cells, those with pneumonitis had more CD4+ Th2 cells and those with thyroiditis had higher baseline Th17 cells [66]. In a study of melanoma patients with severe irAE, it was identified that some of the dynamic changes such as increased frequency of activated T cells were only observed with certain irAE types (gastrointestinal, hepatobiliary) but not others (pneumonitis or type 1 diabetes)[64].

Autoantibodies including antinuclear antibody (ANA), rheumatoid factor (RF) and extractable anti-nuclear antigens have been investigated in relation to irAE development, with one study finding lower autoantibody levels [67] were associated with irAE, and 2 studies identifying early rise in autoantibody titres were associated with increased risk of irAE[68,69]. Studies in thyroid autoimmunity have shown baseline thyroid autoantibody prevalence was associated with increased risk of development of thyroid irAE[70,71]. Whether T1DM autoantibodies can predict CIADM has not previously been studied.

1.3 Aims and hypotheses

From the literature presented above it is evident that although checkpoint inhibitor related diabetes and type 1 diabetes both are autoimmune in pathogenesis, there are underlying differences that are not yet well understood with regards to pathogenesis, predictors and clinical phenotype. The overarching aim of this thesis is therefore to improve our understanding of clinical phenotype, risk factors and identify potential predictors of checkpoint inhibitor related autoimmune diabetes, by studying both primary human clinical samples and mouse models of disease.

Aim 1. To characterise the clinical phenotype of checkpoint inhibitor associated autoimmune diabetes (CIADM).

Hypothesis: CIADM will have a distinct clinical phenotype compared to other forms of diabetes.

- **Aim 1.1** To recruit and analyse a local multicentre case series of patients with CIADM and assess their presentation, risk factors, immunologic, endocrine and exocrine characteristics.
- **Aim 1.2** To conduct a systematic review of all published cases of patients with CIADM to assess their presentation, risk factors, immunologic, endocrine and exocrine characteristics.
- **Aim 1.3** To determine appropriate diagnostic criteria for CIADM based on the above data.
- **Aim 1.4** To develop clinical guidance tools to aid in the clinical diagnosis and management of CIADM.

Aim 2. To identify potential predictors of checkpoint inhibitor associated autoimmune diabetes.

Hypothesis: Radiologic, transcriptomic and immunologic analysis will reveal distinct features that predate the onset of CIADM and increase an individual's risk of development of CIADM after immune checkpoint inhibitor (ICI) exposure.

- **Aim 2.1** To determine pancreatic volume changes between patients with CIADM and matched controls using CT based pancreatic volumetry prior to ICI, on ICI and after development of CIADM.
- **Aim 2.2** To identify differentially expressed genes and isoforms from CD8+ cells biobanked from patients with CIADM and matched controls, prior to ICI, on ICI and after development of CIADM.
- **Aim 2.3** To compare the immunophenotype of biobanked lymphocytes from patients with CIADM and matched controls using flow cytometry on samples prior to ICI, on ICI and after development of CIADM.
- **Aim 2.4** To identify differences in potential serum biomarkers (type 1 diabetes autoantibodies, C-peptide, inflammatory cytokines) between patients with CIADM and matched controls prior to ICI, on ICI and after development of CIADM.

Aim 3. To develop a mouse model for checkpoint inhibitor associated autoimmune diabetes and determine the key immune mediators for CIADM in comparison to other mouse models of type 1 diabetes (NOD, streptozotocin induced) and controls.

Hypothesis: CIADM will have a distinct immunophenotype from other mouse models of autoimmune diabetes.

- **Aim 3.1** To develop a mouse model of CIADM using anti-PD1/anti-PDL1 based therapy in a mouse.

- **Aim 3.2** To compare the islet infiltrate between CIADM and other mouse models of type 1 diabetes using histology, flow cytometry and mass cytometry.
- **Aim 3.3** To determine the endocrine characteristics of mouse models of CIADM in comparison to other mouse models of type 1 diabetes (alpha cell mass, beta cell mass, serum insulin, glucose).
- **Aim 3.4** To compare the exocrine characteristics of mouse models of CIADM in comparison to other mouse models of type 1 diabetes (serum lipase, faecal triglycerides).
- **Aim 3.5** To identify determinants of pathogenicity in immune cells subsets via adoptive transfer of splenocytes from diabetic mice models (NOD and CIADM) to NOD-SCID immunocompromised hosts.

Chapter 2: **CIADM in humans: a multi-centre case series**

2.0 Preface

The following manuscript contains a multicentre case series conducted with local ethics approval. It provided detailed clinical, biochemical, radiological and immunologic profiling of patients with checkpoint inhibitor associated autoimmune diabetes. The manuscript was published in *Journal of Clinical Endocrinology and Metabolism* in 2024 with the text in the thesis identical to the paper. The full pdf version is available in the appendix.

Wu L, Carlino MS, Brown DA, Mellor R, Moore K, Clifton-Bligh R, Long GV, Tsang V, Sasson SC, Menzies AM, Gunton JE (2023). Checkpoint Inhibitor Associated AutoImmune Diabetes Mellitus is Characterised by Rapid C-peptide Loss and Pancreatic Atrophy. *Journal of Clinical Endocrinology and Metabolism.* Volume 109, Issue 5, May 2024, Pages 1301–1307, https://doi.org/10.1210/clinem/dgad685

A letter to the editor written by Lu et al response to this article and our subsequent reply to this letter are also enclosed in PDF form in the appendix.

Title Page

Full title:

Checkpoint inhibitor associated autoimmune diabetes mellitus is characterised by C-peptide loss and pancreatic atrophy

Short running title:

Checkpoint inhibitor associated autoimmune diabetes

Authors:

Linda Wu[1-4], Matteo Salvatore Carlino[2,4,5], David Alexander Brown[1,2,4,6], Georgina Venetia Long[4-5,7], Roderick Clifton-Bligh[3,4], Rhiannon Mellor[2], Krystal Moore[2], Sarah Christina Sasson[2,4*], Alexander Maxwell Menzies[4-5,7*], Venessa Tsang[3,4*], Jenny Elizabeth Gunton[1,2,4*]

Structured Abstract

Objective

To conduct a multi-centre case series characterising the clinical characteristics at presentation and pancreatic volume changes of patients with checkpoint inhibitor associated autoimmune diabetes (CIADM).

Research Design and Methods

Electronic medical records were reviewed with 36 consecutive patients identified with CIADM, as defined by: i) previous immune checkpoint inhibitor (ICI) therapy; ii) new onset hyperglycaemia (BGL>=11.1mmol/L and/or HbA1c >= 6.5%); and iii) insulin deficiency (C-peptide <0.4nmol/L or diabetic ketoacidosis (DKA)) within 1 month of presentation. Pancreatic volume was available and measured using CT volumetry for 17 patients with CIADM, and 3 sets of control patients: 7 with ICI-related pancreatitis; 13

with asymptomatic ICI -related lipase elevation; and 11 ICI treated controls for comparison.

Results

All patients had either anti-PD1 or anti-PD-L1 therapy. Median time from ICI commencement to CIADM diagnosis was 15 weeks. At presentation, 25 (69%) had DKA, 27 (84%) had low C-peptide and by 1 month, 100% had low C-peptide. Traditional type 1 diabetes (T1D) autoantibodies were positive in 15/35 (43%). Lipase was elevated in 13/27 (48%) at presentation. In 4 patients with longitudinal lipase testing, elevated levels peaked 1 month prior to CIADM diagnosis. Pancreatic volume was lower pre-ICI in CIADM patients compared to controls, and demonstrated a mean decline of 41% from pre-treatment to 6 months post CIADM diagnosis.

Conclusions

Pronounced biochemical and radiologic changes occur during CIADM pathogenesis. Rapid loss of C-peptide is a distinct characteristic that can be used to aid diagnosis as autoantibodies are often negative.

ABBREVIATIONS LIST

BGL = blood glucose level

BMI = body mass index

CIADM = checkpoint inhibitor associated autoimmune diabetes CT = computed tomography

CTLA4 = cytotoxic T-cell-associated antigen 4

DKA = diabetic ketoacidosis

HLA = human leukocyte antigens

ICI = immune checkpoint inhibitor

irAE = immune related adverse effect

PD1 = programmed cell death protein 1

PDL1 = programmed cell death protein ligand 1

T1D = type 1 diabetes

2.1 Introduction

In recent years, immune checkpoint inhibitors (ICIs) have not only transformed the landscape of oncological therapy but also enhanced our understanding of mechanisms that can result in autoimmunity. ICIs block critical immune checkpoints such as programmed cell death protein 1 (PD-1) and cytotoxic T-cell-associated antigen 4 (CTLA-4). These agents show significant anti-tumour immune efficacy in a range of malignancies including melanoma and non-small cell lung cancer, but also carry a risk of triggering novel autoimmune toxicities known as immune related adverse events (irAE) [1–3].

Checkpoint inhibitor associated autoimmune diabetes mellitus (CIADM), also known as immune checkpoint inhibitor -induced diabetes mellitus (ICI-DM), is an increasingly prevalent novel form of diabetes occurring subsequent to ICI therapy with presumed autoimmune pancreatic β-cell destruction. CIADM is a relatively uncommon irAE with an incidence of 0.2-1.4% [4–9]. However, when considered in the context of 43.6% of all cancer patients in USA being eligible for ICI therapy [10], this translates to thousands of new CIADM patients per year in the USA alone.

There is a lack of consensus on the diagnostic criteria for CIADM, and the reporting of phenotypical data such as type 1 diabetes (T1D) autoantibodies, serum C-peptide and exocrine pancreatic inflammation has been variable. In a previous meta-analysis of all reported CIADM cases conducted by our group [11], we have demonstrated that low C-peptide is a universal feature of CIADM that correlates with a lifelong insulin dependence, whereas T1D autoantibodies are less reliable. On these grounds we have applied our proposed diagnostic criteria to our case series which includes 1) new onset hyperglycaemia

and 2) evidence of insulin deficiency with rapid loss of C-peptide at presentation or by 1 month post-diagnosis.

It has been suggested in small case series that pancreatic volume rapidly reduces during the course of progression to overt CIADM [12,13]. No previous comparisons have been made to patients on ICIs alone, or patients with pancreatic irAE but without diabetes. Furthermore, measures of exocrine pancreatic disease such as lipase or faecal elastase have not been extensively analysed.

Whilst awareness of checkpoint inhibitor associated autoimmune diabetes (CIADM) is growing, there remain large knowledge gaps in our understanding of the condition. In particular, it is unclear what factors may predict CIADM development and there is yet to be a clear consensus on diagnostic criteria. We conducted a multi-centre case series characterising the typical presentation of patients with CIADM, as well as the associated exocrine pancreatic changes during the course of disease pathogenesis.

2.2 Research Design and Methods

Patients with new onset diabetes after ICI treatment were identified during the period January 2015 to March 2023 from four quaternary oncological facilities in Sydney, Australia. Human Research Ethics approval was obtained for review of electronic medical records in multi-centre retrospective study (2021/PID00003). Ten patients from this cohort had previously been published[6] although data on further clinical parameters including lipase, glucose and pancreatic volumetry have been obtained and analysed.

Patients were included as having a diagnosis of CIADM if they met the following criteria detailed in our recent meta-analysis [11]. These were i) previous ICI exposure, and ii) new onset hyperglycaemia (BGL≥11.1mmol/L and/or HbA1c ≥6.5%) and iii) evidence of insulin deficiency (C-peptide <0.4nmol/L or DKA) within 1 month of presentation. Patient demographics, biochemistry, oncological characteristics, antibody status and HLA haplotyping was recorded. Antibodies were tested via hospital laboratory using RSR® anti-GAD ELISA (RSR Limited Cat# GDE/96, RRID:AB_2910239), RSRÒ anti-IA-2 ELISA (RSR Limited Cat# IAE/96/2, RRID:AB_2910240), RSR® anti-insulin antibody radioimmunoassay (RSR Limited Cat# IAA/100, RRID:AB_3073785) and EuroImmun anti-ZnT8 ELISA(EUROIMMUN Cat# EA 1027-9601, RRID:AB_3073786). HLA types were defined as "susceptible", "protective" or "not associated" based on previous studies in patients with type 1 diabetes [14]. A complete case analysis approach was used to address missing data and patients with inadequate data to confirm a diagnosis of CIADM were excluded.

Pancreatic volumetry was performed using Vitrea® software (Tochigi, Japan). Volume was calculated using the in-built summation of area technique as previously published [15], with pancreas parenchyma manually outlined on each section. CT scans were obtained for three groups of patients 1) patients with CIADM, 2) patients treated with ICIs that developed autoimmune pancreatitis, 3) patients treated with ICIs that developed asymptomatic lipase elevation and 4) ICI-treated controls with normal lipase values. Control patient groups were obtained through retrospective review of the ICI treated patient database at Melanoma Institute Australia with diagnoses as recorded on medical records by the treating physician (Royal Prince Alfred Hospital Research Ethics Committee Protocol No. X10-0305 and HREC/10/RPAH). For each group, CTs were

obtained from time points prior to ICI commencement, whilst on ICI therapy (timepoint >6 months on therapy), and at time of diagnosis of CIADM, pancreatitis or lipase elevation, and 6 months post diagnosis, if available.

R software was used for statistical analysis (version 4.1.1). Normal distribution was tested with Shapiro-Wilk testing. One-way ANOVA analysis was performed to determine the significance of the volumetry changes across time periods and detect differences between patient groups. Difference in time to CIADM between groups was compared via log-rank testing. Student's T-test and chi-square testing were used to compare between groups for continuous and categorical variables respectively for parametric variables and Wilcoxon signed rank test for non-parametric variables.

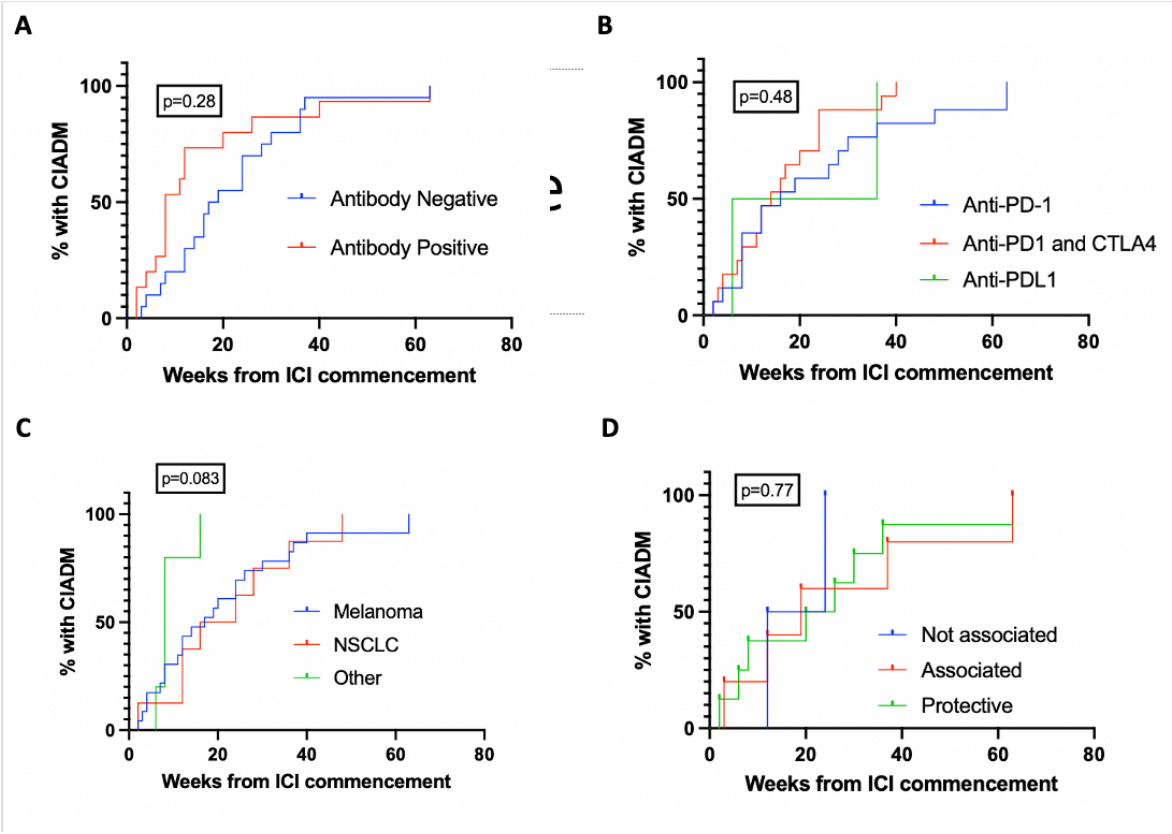
2.3 Results

Thirty-six (36) patients met the inclusion criteria for CIADM. Median age at diagnosis was 64 years, and 58% of patients were male (n=21). Mean baseline BMI was 24.7 kg/m^2 and 14% had pre-existing type 2 diabetes (n=5), requiring diet control alone in 2 patients and oral hypoglycaemic agents in 3.

Melanoma was the most common malignancy (n=23, 64%), followed by non-small cell lung cancer (n=7, 19%). All patients were exposed to either anti-PD1 or anti-PDL1 class ICIs, with 51% combination anti-PD1 and anti-CTLA4 therapy, 43% receiving anti-PD1 monotherapy and 6% anti-PDL1 monotherapy. Many patients (78%, n=28) experienced other irAE; the most common were thyroid disease (42%, n=15), hepatitis (14%, n=5), hypophysitis (14%, n=5), pancreatitis (11%, n=4), and colitis (8%, n=3).

Median time from the start of ICI to diagnosis with CIADM was 15 weeks (interquartile range 8-26.5 weeks). Figure 1 depicts the time to diabetes diagnosis from initial exposure to immunotherapy, stratified by autoantibody status at diagnosis of CIADM, type of ICI, malignancy and HLA typing if performed. These factors were not found to be significantly associated with a difference in time to diagnosis.

Figure 1. Cumulative incidence curves from ICI exposure to development of CIADM. Cumulative incidence curves are stratified by A) autoantibody positivity; B) ICI type ; C) type of malignancy; D) results of HLA typing for T1D susceptibility. PD-1 = programmed cell death protein 1, CTLA4 = cytotoxic T-cell-associated antigen 4, PD-L1 = programmed cell death ligand 1, NSCLC = non-small cell lung cancer.



Biochemistry and autoantibodies at presentation are summarised in Table 1. Of the 14 patients tested, 8 (57%) had HLA susceptibility haplotypes for T1D and 2 (15%) had protective haplotypes. No significant difference in C-peptide values or DKA incidence was found between groups based on subanalyses stratified by HLA susceptibility, cancer type, ICI type or lipase levels.

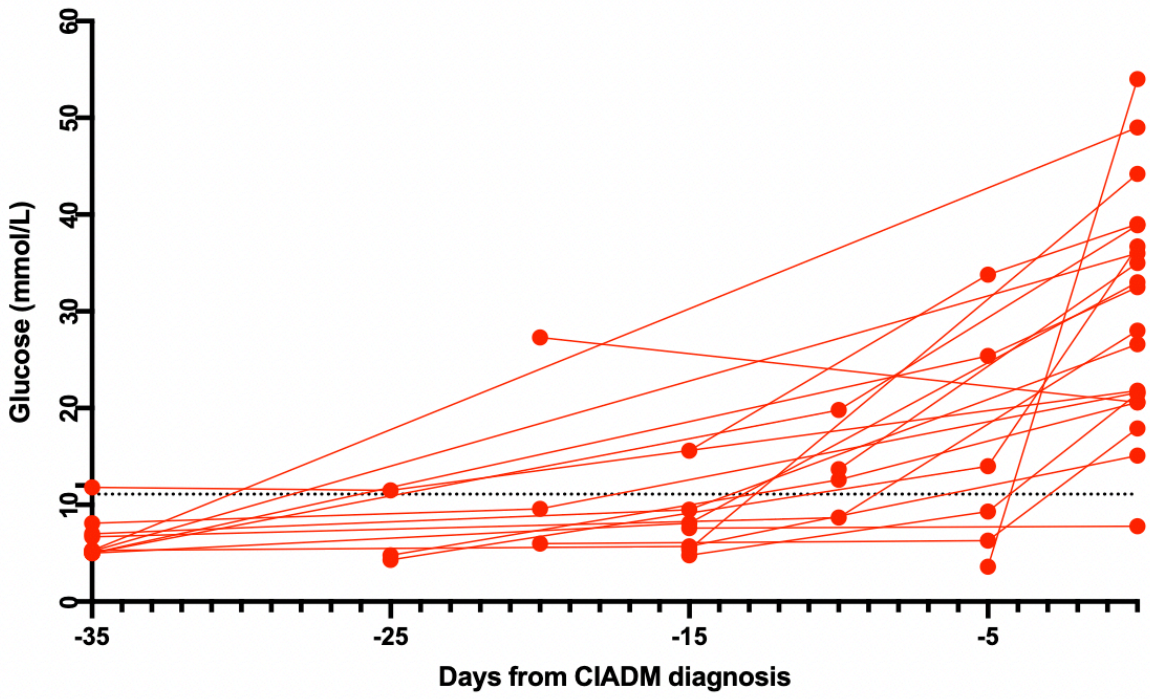
Table 1. Biochemistry and autoantibodies at presentation with CIADM.

		N (%)
DKA at presentation		25 (69)
DKA/HHS overlap at presentation		6 (17)
Mean glucose (mmol/L) ± SD		34.5 ± 15.1
Mean ketones (mmol/L) ± SD		4.3 ± 3.0
Mean C-peptide (nmol/L) ± SD		0.21 ± 0.19
Low C-peptide (<0.4 nmol/L)		27/32 (84)
HbA1c (%)		8.2 ± 1.7
Autoantibody positive	Any	15 (43)
	Anti-GAD (n=35)	15 (43)
	Anti-IA2 (n=35)	1 (3)
	Anti-ZnT8 (n=35)	2 (6)
	Anti-insulin (n=8)	0 (0)
	Antibody negative	20 (57)
Elevated lipase (normal range <60u/L, n=27)		13 (48)
Low faecal elastase (normal range <200ug/g, n=10)		9 (90)

HHS= hyperosmolar hyperglycaemic state, SD = standard deviation, HbA1c = glycosylated haemoglobin, anti-GAD = anti-glutamic acid decarboxylase antibodies, anti-ICA = anti-islet cell antigen antibodies, anti-ZnT8 = anti-Zinc transporter 8 antibodies.

Figure 2 depicts serum glucose levels detected on routine biochemistry prior to formal CIADM diagnosis for the 20 patients who had glucose measurements preceding diagnosis. 8/20 patients had an abnormal random glucose level meeting criteria for diabetes (≧11.1mmol/L) preceding CIADM diagnosis.

Figure 2. Serum glucose prior to CIADM diagnosis (N=20). The dotted black line shows the cut-off (≥11.1mmol/L) for a diabetic-range random glucose.

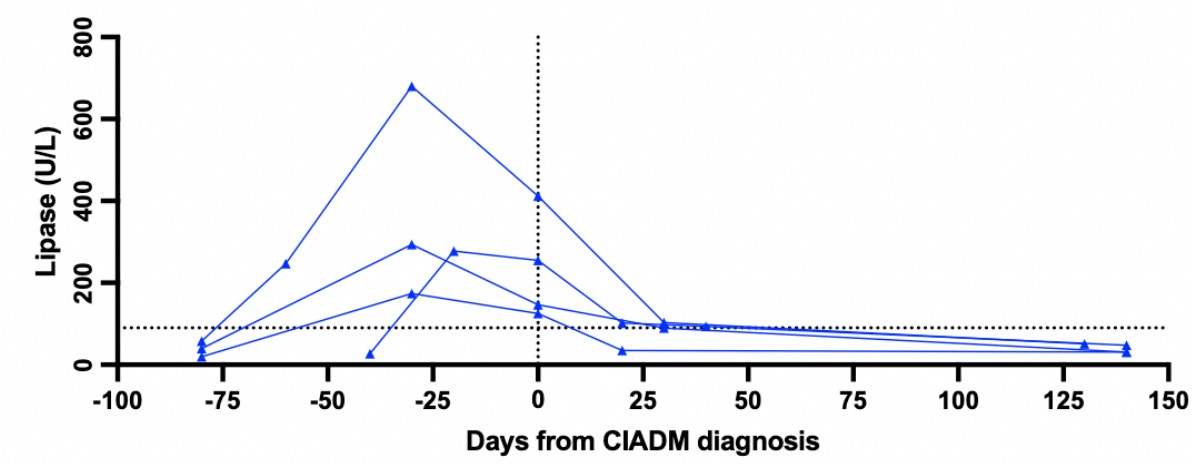


2.3.1 Serum lipase and pancreatic exocrine function

Of the 27 patients tested for lipase, values were elevated in 13 (48%), and 4 patients had overt ICI related pancreatitis. Figure 3 depicts lipase levels at the time of presentation for patients who had elevated lipase and had serial measurements (n=4), showing a distinct rise in lipase that precedes the onset of CIADM by roughly 1 month. Ten patients had faecal elastase performed to further evaluate the extent of pancreatic insufficiency, which

was low (<200ug/g) in 9 patients (90%). Two of the 9 patients with low faecal elastase had concurrent ICI related colitis which may also lower faecal elastase values. Two patients had follow up testing 5 years after initial testing at CIADM diagnosis demonstrated a low faecal elastase, with one patient returning to normal range and the other having persisting low values in the range of exocrine insufficiency.

Figure 3. Lipase level during time of diagnosis for CIADM patients (n=4). Dotted line represented upper limit normal range (60U/L).

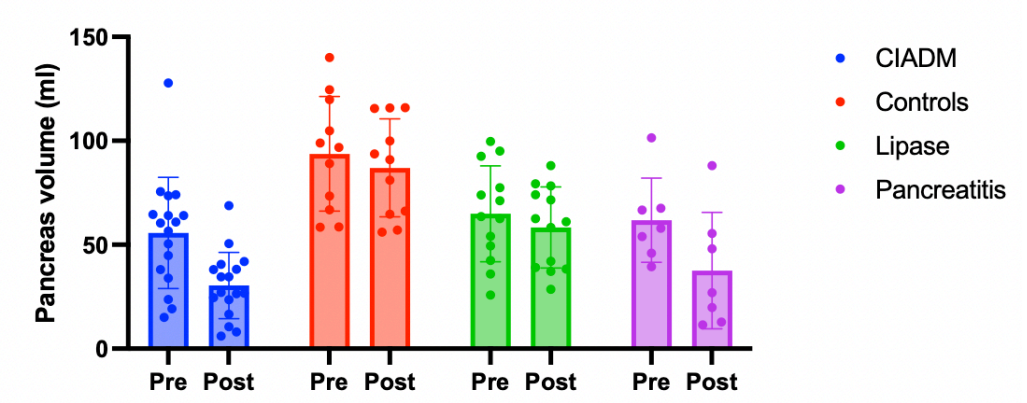


Baseline pancreatic volume was significantly different between the groups (Figure 4b)– CIADM group, ICI treated control group, ICI-related lipase elevation group and ICI-related pancreatitis group (F=5.4, p=0.003), with the CIADM group baseline pancreatic volume being significantly lower than the control group (p=0.002). There was a progressive decline of 41% (mean) comparing the pre-treatment to 6 months post diagnosis of CIADM (Figure 4c). An example of this progressive decline in pancreatic volume is portrayed in Figure 4d with representative CT slices through the pancreas of a CIADM patient. This decline in the CIADM group is similar to the 39% decrease in

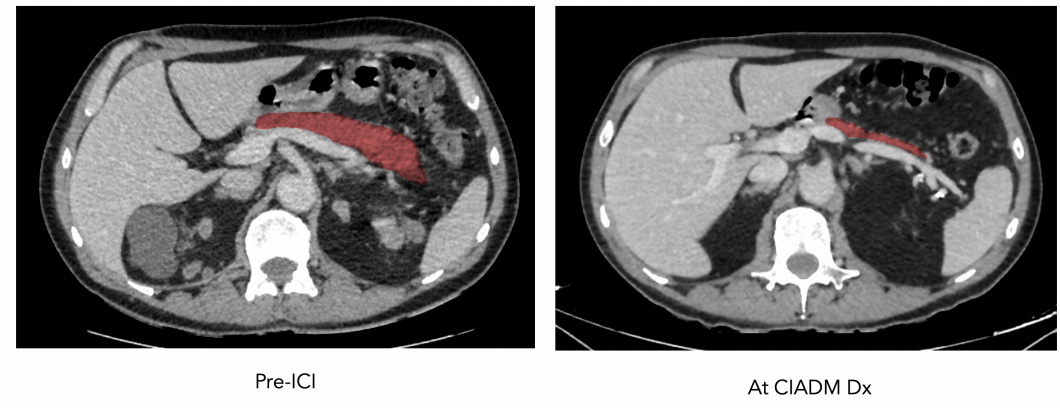
pancreatic volume noted in the ICI related pancreatitis cohort and significantly greater than the 6% reduction in ICI treated controls and 6% decrement in those with isolated lipase elevation (F=8.0, p<0.001).

Figure 4. Pancreatic volumetry in ICI treated patients.

A.



B.



A) Pancreatic volumes for the 4 patient groups prior to ICI and post diagnosis of CIADM/6 months of ICI use/lipase elevation/pancreatitis respectively. Dots indicate individual values, columns indicates mean and error bars indicate standard deviation. B) Baseline pancreatic volumes for the 4 patient groups. C) % change in volume over time for the 4 patient groups. D) Representative CT slices from 1 patient taken at the level of the splenic vein. Pancreas highlighted in red.

There was no significant difference between baseline pancreatic volumes of autoantibody positive and negative groups (p=0.54), or HLA susceptible and non-susceptible groups (p=0.18). There was also no significant difference between these groups when comparing percentage decline in pancreatic volume (p=0.79 and p=0.17 respectively).

2.3.2 Follow up

Oncological response was assessable in 32 patients, and 22 (69%) patients responded to ICI therapy (4 with complete response, 18 with partial response), 2 had stable disease and 8 (25%) had progressive disease as best response. At a median followup of 35 months 13/22 responses were ongoing. 8/36 patients were deceased by time of data collection, and all deaths were due disease progression. Of the 26 melanoma patients with oncological response reported, 20 (77%) responded (4 with complete response, 16 with partial response) and 6 had progressive disease as best response.

All of the CIADM patients remained insulin dependent on follow up. All had low C-peptide at diagnosis or within 1 month (<0.4nmol/L) and 6/9 had undetectable levels of C-peptide on ultrasensitive assay. Mean HbA1c on follow up was 8.1% (range 5.9-10.1%). 32/36 patients were managed with multiple daily insulin injections and 4/36 used insulin pump therapy. 8/36 (22%) patients were deceased by time of followup due to melanoma.

2.4 Discussion

The prevalence of autoantibodies in our cohort was 43%, and all antibody positive patients were positive for anti-GAD. Given that the rate of autoantibody positivity in classic T1D patients is 90%[16], this significant discrepancy suggests that CIADM pathogenesis may be less contingent on humoral immunity, or potentially may involve different epitopes in immune triggering. The survival curves in Figure 1 showed a trend to earlier diagnosis in antibody positive people that is consistent with our meta-analysis[11]. In that series, we found that a serum C-peptide of <0.4nmol/L on an ultrasensitive assay had 100% sensitivity in detecting patients with a long term requirement for insulin, again consistent with our case series findings. We support the use of a rapid loss of C-peptide (<0.4nmol/L by 1 month post CIADM diagnosis) in conjunction with new onset hyperglycaemia (HbA1c ≥6.5% and/or BGL≥11mmol/L) as diagnostic criteria for CIADM.

All patients in our CIADM cohort had exposure to either anti-PD-1 or anti-PD-L1 therapy, highlighting that inhibition of the PD-1/PD-L1 axis is critical to the pathogenesis of CIADM. This aligns with previously published cases of CIADM [5–9,11,17,18]. Published case reports of anti-CTLA4 checkpoint inhibitor associated diabetes do not provide clear evidence of insulin deficiency or thorough exclusion of other forms of diabetes [18–20]. The situation is reversed with checkpoint-inhibitor hypophysitis, where the rate of hypophysitis is higher with CTLA-4 therapy, alone or in combination with PD1 [21], suggesting important pathophysiological differences between the two irAEs.

The role of genetics appears different, or potentially less important in CIADM in comparison to traditional T1D. Of the patients tested for HLA haplotypes, only 46% had a traditional T1D susceptibility haplotype (similar to 59.3% in the meta-analysis), and 15%

in our case series and 7.6% in the meta-analysis had protective haplotypes[11]. In comparison, amongst classic T1D patients 90% have a HLA susceptibility haplotype[14,22]. The lower prevalence of T1D susceptibility haplotypes in the CIADM population may reflect that in surviving to the mean age of 66 years without T1D the highest risk groups have already been selected out. Furthermore, the additional exposure of anti-PD1 in this context skews the balance of genetics versus environment greatly and may trigger other mechanisms of autoimmunity.

Pancreatic volumetry demonstrated rapid decline in pancreatic volume well beyond what would be expected with insulitis alone, which was consistent with other case series reporting pancreatic volumetry data in CIADM patients[12,13]. The degree of pancreatic volume loss was on par with those with overt ICI related pancreatitis, whilst a lipase elevation in isolation did not lead to such significant volume loss. Interestingly, baseline pancreatic volume for CIADM patients was significantly lower compared with controls. This generates the hypothesis that CIADM patients have a lower pancreatic reserve and/or prior insult that puts them at increased risk of CIADM. Further studies with larger sample sizes will be needed to determine whether smaller pancreatic volume reliably predicts CIADM development.

This is the first study to assess and compare the extent of pancreatic volume loss between different pancreatic phenotypes including CIADM, pancreatitis, lipase elevation and ICI treated controls. Previously, it has been demonstrated both by radiologic analyses and by postmortem examination that pancreatic weight for patients with type 1 diabetes is significantly lower than normal controls and even patients with type 2 diabetes, although was not significantly affected by duration of diabetes [23,24]. Further analyses of pancreatic

volume in patients with recent onset type 1 diabetes shows a 26% reduction in volume compared to healthy controls even at this early stage of disease[25], suggesting that reduction in pancreatic volume may not be a unique feature to CIADM but a feature of autoimmune diabetes progression. Whilst ICI related pancreatitis volume has not previously been characterised, it is known that chronic pancreatitis is associated with pancreatic volume reduction of approximately 21% [26].

Combined with the relatively high incidence of elevated lipase at time of presentation (48%) and low faecal elastase values in 90% of those tested, exocrine pancreatic involvement seems to be a common feature in CIADM. Further follow up data would be of interest to determine the extent to which exocrine function recovers if lost, as was the case in one of our patients with follow up faecal elastase testing.

Our longitudinal lipase data also is the first to demonstrate a lipase rise that precedes the onset of diabetes, presumably signifying the onset of autoimmune exocrine pancreatic damage. Furthermore, treatment of patients on ICIs with an elevated lipase with steroids (as was done in two of these four patients) precipitated fulminant hyperglycaemia and DKA. Thus, CIADM should be closely monitored for if treating people with elevated lipase with steroids. Moreover, CIADM was not prevented by steroids in these two patients.

Analysis of glucose values prior to formal CIADM diagnosis suggested that 40% of patients had abnormal glucose values preceding formal CIADM diagnosis. This indicates that improved clinical awareness could have led to earlier detection of CIADM and potential avoidance of DKA in these patients.

Our follow up data on HbA1c values suggests that long term glycaemic management in these patients is challenging, and HbA1c targets are likely set relatively higher in view of older age, more comorbidities and risks associated with hypoglycaemia. Lower use of insulin pump therapy is due to limitations within health care systems and more limited capacity to facilitate the steep learning curve required.

2.5 Conclusions

CIADM is an uncommon but highly morbid complication of ICI therapy which requires distinct diagnostic criteria from traditional T1D. Our results are consistent with CIADM co-occurring with significant pancreatic volume loss (with or without other features of pancreatic exocrine deficiency). Identifying the pre-morbid predictors of CIADM may allow targeted trials of treatments and earlier detection of this complication of ICI.

Data Availability

Data from this study are available from the corresponding author on reasonable request.

Acknowledgements

AMM is supported by a NHMRC Investigator Grant, Nicholas and Helen Moore, and Melanoma Institute Australia. JEG is supported by NHMRC Program Grant Complexity in Nutrition. GVL is supported by a NHMRC Investigator Grant and the University of Sydney Medical Foundation.

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Chapter 3: **Understanding CIADM in humans: a systematic review**

3.0. Preface

This chapter contains a systematic review characterising all published cases and case series of checkpoint inhibitor associated diabetes. The manuscript was published in *Diabetes Care* in 2023 and the text in the thesis is identical to published paper. A pdf version is supplied in the appendix.

Wu L, Tsang V, Menzies AM, Sasson SC, Carlino MS, Brown DA, Clifton-Bligh R, Gunton JE (2023). Risk Factors and Characteristics of Checkpoint Inhibitor–Associated Autoimmune Diabetes Mellitus (CIADM): A Systematic Review and Delineation From Type 1 Diabetes***. Diabetes Care.*** 46(6): 1292–1299.

Full Title: Risk factors and characteristics of checkpoint inhibitor associated autoimmune diabetes (CIADM): a systematic review and delineation from type 1 diabetes

Short Title: CIADM: a systematic review

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Abstract

Background

Checkpoint inhibitor associated autoimmune diabetes (CIADM) is a distinct form of autoimmune diabetes that is a rare complication of immune checkpoint inhibitor therapy. Data regarding CIADM is limited.

Purpose

To systematically review available evidence to identify presentation characteristics and risk factors for early or severe presentations of adult patients with CIADM.

Data sources

Medline and PubMed databases were reviewed.

Study selection

English full text articles from 2014 to April 2022 were identified using a pre-defined search strategy. Patients meeting diagnostic criteria for CIADM with evidence of hyperglycaemia (blood glucose level >11mmol/L or HbA1c >= 6.5%) and insulin deficiency (C-peptide <0.4nmol/l and/or diabetic ketoacidosis) were included for analysis.

Data extraction

The search strategy identified 1206 papers. From 146 papers 278 patients were labelled with “CIADM”, with 192 patients meeting our diagnostic criteria and included in analysis.

Data synthesis

Mean age was 63.4years (±12.4). All but one patient (99.5%) had prior exposure to either anti-PD1 or anti-PDL1 therapy. Of the 91 patients tested (47.3%), 59.3% had susceptibility haplotypes for type 1 diabetes. Median time to CIADM onset was 12 weeks (IQR 6-24). Diabetic ketoacidosis (DKA) occurred in 69.7%, and initial C-peptide was low in 91.6%. Type 1 diabetes autoantibodies were present in 40.4% (73/179) and were significantly associated with DKA (p=0.0009) and earlier time to CIADM onset (p=0.02).

Limitations

Reporting of follow up data, lipase and HLA haplotyping was limited.

Conclusions

CIADM commonly presents in DKA. While T1D autoantibodies are only positive in 40.4%, they associate with earlier, more severe presentations.

Article Highlights

· Checkpoint inhibitor associated autoimmune diabetes (CIADM) is a novel form of autoimmune diabetes of which little is known.

· This systematic review aims to apply novel diagnostic criteria to review reported cases of CIADM, characterise clinical features and identify factors associated with earlier and more severe presentations.

· The incidence of diabetic ketoacidosis at presentation of CIADM is high (69.7%) and traditional T1D autoantibodies are less frequent than in T1D (40.4%). Positive autoantibodies are associated with earlier presentations and higher rates of diabetic ketoacidosis.

· Differences in presentation characteristics and autoantibody prevalence between CIADM and T1D necessitates distinct diagnostic criteria and further research.

3.1 Introduction

Immune checkpoint inhibitors (ICIs) have resulted in a paradigm shift in the treatment of many cancers and as a result the indications for their use continue to expand. These agents inhibit specific regulatory immune pathways such as programmed cell death 1 (PD-1) and cytotoxic T lymphocyte activating factor 4 (CTLA4) to increase anti-tumour immune activity with impressive efficacy across a number of malignancies, most commonly melanoma and non-small cell lung cancer (1–3). A consequence of ICI use is the risk of triggering immune related adverse events (irAEs) - a broad and heterogenous group of autoimmune diseases affecting virtually any system within the body. Endocrinopathies are different from other irAEs in that resultant hormonal deficits are usually irreversible, except for some thyroid changes (4–6).

Similar to type 1 diabetes, checkpoint inhibitor associated autoimmune diabetes (CIADM; also termed ICI-DM, CPI-DM) is thought to occur secondary to autoimmune destruction of pancreatic beta cells. Amongst irAE, CIADM is relatively uncommon with an incidence of 0.2-1.4% (7–12). Despite this, it is of significant clinical concern due to the high incidence of diabetic ketoacidosis at presentation as well as the lifelong persistent insulin deficiency, and the associated risks of diabetes complications and decreased life-expectancy (7–13). Furthermore, as indications for use of ICIs expand into adjuvant and neoadjuvant settings and patient survival improves (2,3), the overall prevalence of CIADM will correspondingly increase.

There is limited data available on CIADM. The relatively uncommon nature of the diagnosis compounded by the heterogenous diagnostic criteria applied thus far has made it difficult to draw conclusions from the case reports, small series and limited number of systematic reviews published on CIADM to date.

The primary aim for this review is to define clear, easily applied criteria for the diagnosis of CIADM, as well as analyse the presentation characteristics, kinetics, associations and risk factors for earlier and severe presentations of CIADM.

3.2 Methods

3.2.1 Data sources

A systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines (Supplemental Figure 1) to address the aims stated above. The review was registered with PROSPERO prior to commencement (CRD42021276016). MEDLINE and PubMed databases were reviewed by LW for full text English articles published from 1st January 2014 through to April 2022. The applied search terms were [“immune checkpoint inhibitor” OR “checkpoint inhibitor” OR “ipilimumab” OR “nivolumab” OR “pembrolizumab” OR “atezolizumab” OR “durvalumab” OR “avelumab”] AND [“diabetes mellitus” OR “type 1 diabetes” OR diabetes*] limited to English language and humans.

3.2.2 Case selection

Studies were then reviewed by LW and all cases of reported CIADM in adults with evidence of ICI exposure and diabetes were included in initial analysis. Where individual patient data was not provided, corresponding authors were contacted via email to request this.

We defined diagnostic criteria for CIADM as evidence of new onset hyperglycaemia in the diabetes range (BGL>11mmol/L or HbA1c >= 6.5%) (criteria 1) and insulin deficiency

(C-peptide <0.4nmol/l and/or diabetic ketoacidosis) (criteria 2) without known treatment with an SGLT2 inhibitor, in patients who have received checkpoint ICIs.

To avoid 'double reporting', one review of Vigibase, a World Health Organisation database of individual case safety reports, was not included in this review as no individual data was provided, and it would be impossible to determine if the treating physician had reported the case in another publication (14).

Supplemental table 2 includes all cases described in the literature as "CIADM" and any reasons for inclusion or exclusion of each patient in our final cohort.

3.2.3 Data extraction

Data was collected including patient baseline demographics, oncological characteristics, CIADM presentations and follow up data. Patient data was age, gender, ethnicity, body mass index, pre-existing type 2 diabetes and medications associated, history of autoimmune disease, family history of autoimmune diabetes, and HLA haplotyping. Oncological characteristics were cancer type, ICI type, ICI response, associated irAE, and previous systemic therapy. Presentation characteristics were time to onset from initial ICI exposure, diabetic ketoacidosis (DKA) at presentation, pH, blood glucose, beta-hydroxybutyrate levels, HbA1c, C-peptide, lipase, presence of acute kidney injury, type 1 diabetes autoantibody status, steroid use and immunosuppression. Follow up data was further C-peptide measurements, insulin use, and exocrine insufficiency. Results that were not definitively reported (e.g. "C-peptide was low") were reported as missing data and excluded from analysis.

3.2.4 Quality assessment

An adapted Newcastle-Ottawa scale was utilised to assess the quality of included studies. The modified criteria for quality assessment were as follows:

1) Is the case definition adequate – meeting diagnostic criteria for hyperglycaemia and insulin deficiency? (Score 0/1)

2) Do the reported cases represent all eligible cases over a defined time period, in a defined catchment area, or an appropriate sample of those cases? (Score 0/1)

3) Have other diagnostic possibilities been excluded, including demonstration that the outcome of interest was not present at the start of the study? (Score 0/1)

4) Is the duration of follow up adequate to confirm/exclude the diagnosis (minimum 1 month if not meeting criteria at presentation)? (Score 0/1)

3.2.5 Data synthesis

Statistical analyses were conducted using R software (v4.1) and GraphPad Prism (v9.4.0). Continuous variables were compared using Mann Whitney U and/or ANOVA analyses and categorical variables compared using Chi-squared test. Multivariate logistic regression with ANOVA was performed to determine risk factors occurrence of DKA.

3.3 Results

There were 1206 papers meeting the search criteria (Supplemental Figure 1). Of these, a total of 278 patients were identified in the 146 included studies that had reported

"CIADM". These patients were reported as "CIADM" by their authors on the basis of new onset diabetes after ICI use, with only some authors applying additional criteria such as autoantibody status or C-peptide levels.

From these, 192 patients met both of our proposed diagnostic criteria for CIADM. Forty-five patients were excluded due to lack of individual data, and 31 for insufficient results to determine if they met criteria and thus were excluded. Ten patients did not meet our criteria for CIADM but had been reported as CIADM in the literature: five patients did not meet criteria for C-peptide (i.e. serum C-peptide ≧0.4nmol/L at presentation or follow up); two patients had concurrent use of SGLT2 inhibitors at time of DKA confounding C-peptide measurements and no demonstration of low C-peptide after cessation of SGLT2 inhibitor; two patients had type 2 diabetes which was insulin-requiring prior to diagnosis of "CIADM"; and one patient was excluded due to prior partial pancreatectomy. Details of the quality assessment and exclusion criteria are available in Supplemental Table 2.

3.3.1 Baseline characteristics

Table 1 summarises the key baseline characteristics for the CIADM patients (n=192). Mean age was 63.4 years (+/- 12.8) and 62.3% of patients were male, likely reflecting the profile of patients treated with ICIs (e.g. melanoma is most common in older males). Type 2 diabetes was a pre-existing condition in 5.2%, which is within the usual range for that age group. The mean HbA1c of the subgroup with pre-existing type 2 diabetes was 6.6% (range 5.9-8%), reported in 6/17 patients. Mean BMI was 24.7kg/m2, with 32/48 (67%) patients having a normal range BMI at presentation. Melanoma was the most common cancer type (47.8%). All but one patient had exposure to either anti-PD1 or anti-PD-L1 therapy as monotherapy or combination with anti-CTLA4. ICI was first line therapy for malignancy in 49.7% of patients, whilst 35.2% had been treated with chemotherapy

previously, and 8.5% treated with a targeted therapy. Concurrent other irAE at/before time of presentation were present in 43.8%, with thyroid involvement being the most common followed by pituitary. HLA haplotyping was reported in 91 patients (47%) with 54 (59%) having a typical HLA susceptibility haplotype for T1D, while 28 (31%) were neutral and 7 (8%) developed CIADM despite having a haplotype that typically protects against T1D.

3.3.2 Presentation characteristics

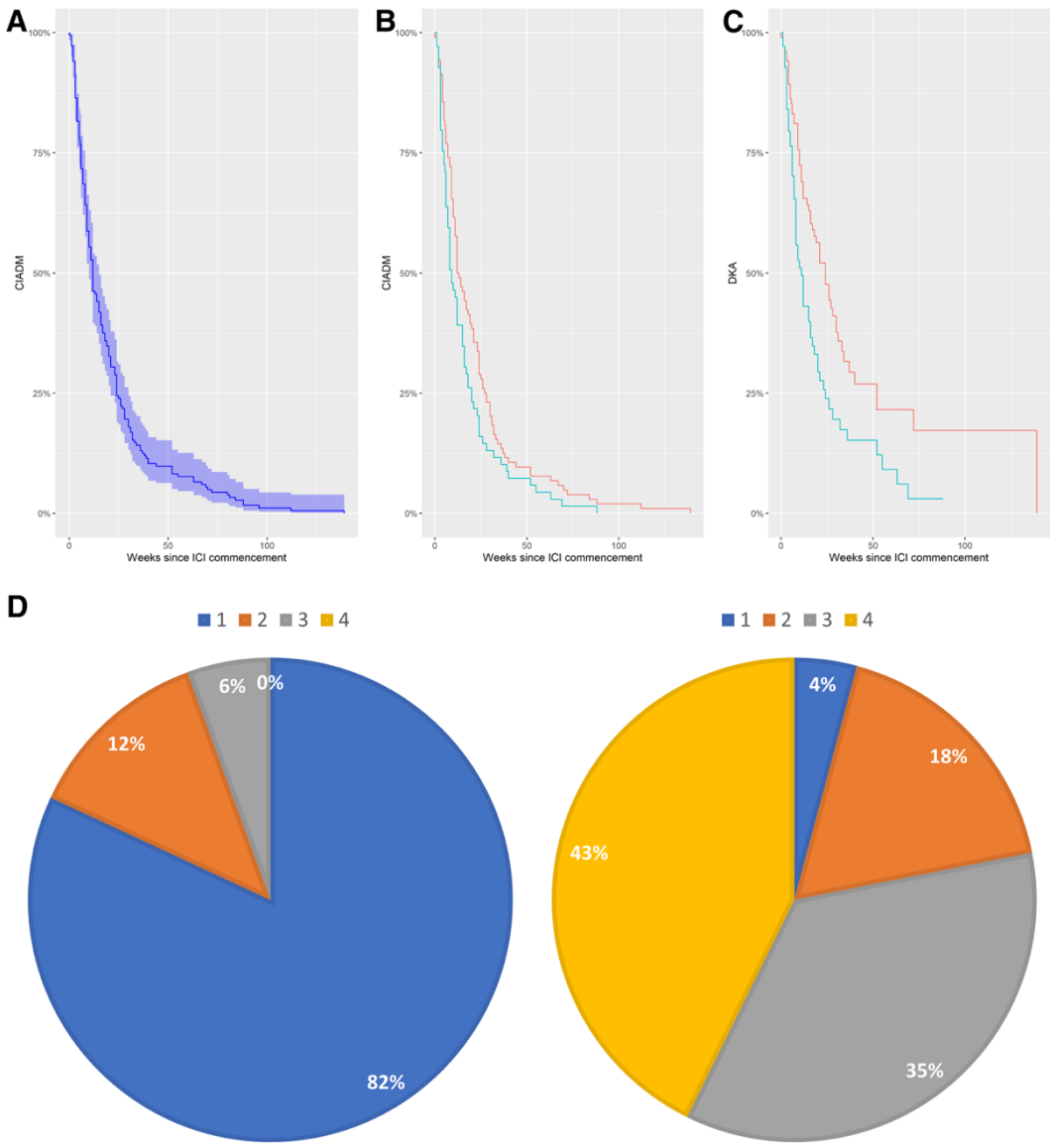
Table 1 summarises the characteristics at presentation for patients with CIADM. Median time to onset of CIADM from initial ICI exposure was 12 weeks (IQR 6-24) (Figure 1a).

Table 1. Baseline, presentation and follow up characteristics of checkpoint inhibitor associated diabetes mellitus (CIADM) patients (n=192). SD = standard deviation, BMI = body mass index, NSCLC = non-small cell lung cancer, ICI = immune checkpoint inhibitor, PD-1 = programmed death 1, PD-L1 = programmed death ligand 1, CTLA4 = cytotoxic leukocyte activated factor 4, irAE = immune related adverse event, HLA = human leukocyte antigen. IQR = interquartile range, ICI = immune checkpoint inhibitor, TKI = tyrosine kinase inhibitor, SD = standard deviation, HbA1c = glycosylated haemoglobin A1c, type 1 diabetes = type 1 diabetes mellitus, GAD = glutamic acid decarboxylase, IA2 = islet antigen 2, ZnT8 = zinc transporter 8, ICA = islet cell antigen.

	CIADM patients (n=192)
Baseline demographics	
Mean age (yrs +/- SD)	63.4 (12.8)
Male (%)	114/183 (62.3)
BMI (kg/m2 +/- SD) Reported in n=48	24.7 (6.6)
Pre-existing type 2 diabetes (%)	17 (5.2)
Underlying malignancy	
Melanoma (%)	88 (47.8)
NSCLC (%)	51 (27.7)
Other (%)	45 (24.4)
ICI type	
PD-1	128 (67.0)
PD-L1	21 (11.0)
PD-1/PD-L1 and CTLA-4 combination	41 (21.5)
CTLA4	1 (0.0)
Previous non-ICI therapy N=153	
Nil	76 (49.7)
Chemotherapy	54 (35.2)
TKI	13 (8.5)
Other	10 (6.5)
Concurrent irAE (%)	**71 (43.8)**
Thyroid	40 (20.8)
Pituitary	17 (8.9)
Skin	13 (6.8)
Colitis	4 (2.1)

Adrenal	3 (1.6
Other	20 (10.4)
HLA haplotyping performed (%)	**91 (47.3)**
HLA susceptible	54 (59.3)
HLA protective	7 (7.6)
HLA neutral	28 (30.7)
HLA mixed	2 (2.1)
Presentation characteristics	
Median time to onset (weeks; +/-IQR) N=184	12 (6-24)
DKA (%)	134 (69.7)
pH (mean +/- SD) N= 102	7.17 (0.16)
HbA1c (%/% mean +/- SD) N= 146	8.1 (1.5)
HbA1c >=6.5% (%)	123/146 (84.3)
Serum glucose (mmol/L +/- SD) N= 172	35.3 (14.6)
Beta-hydroxybutyrate (mmol/L +/- SD) N=87	5.5 (3.5)
C-peptide (nmol/L +/- SD) N=154	0.19 (0.74)
C-peptide < 0.4nmol/L at presentation	141/154 (91.6)
Follow up C-peptide (nmol/L mean +/- SD) N=30	0.02 (0.04)
Type 1 diabetes autoantibody positive (%)	72/178 (40.4)
Anti-GAD	66/166 (39.7)
Anti-IA2	14/101 (13.9)
Anti-Insulin	4/45 (8.9)
Anti-ZnT8	1/34 (2.9)
Anti-ICA	2/33 (6.0)
Steroids	9 (4.6)
Exocrine pancreatic assessment	
Elevated lipase (%)	25/36 (69.4)
Mean lipase (IU/L +/- SD)	292(361)
Low faecal elastase	2/2 (100)
Insulin requirements (mean units/kg/day +/- SD) N=11	0.51 (0.25)

Figure 1. *A*: Kaplan-Meier curve depicting time to onset of CIADM from initial ICI commencement. *B*: Kaplan-Meier curve depicting time to onset of CIADM from initial ICI commencement, with stratification by those who were positive for T1D autoantibodies (blue) and those who were negative (red). *C*: Kaplan-Meier curve depicting time to DKA from initial ICI commencement, with stratification by those who were positive for T1D autoantibodies (blue) and those who were negative (red). *D*: Pie charts depicting, at left, proportion of seropositive patients with CIADM (n = 192) positive with 1, 2, 3, or 4 autoantibodies and, right, proportion of patients with T1D (n = 256) previously reported by Bingley (23) as being seropositive with 1, 2, 3, or 4 autoantibodies.



Diabetic ketoacidosis was present at diagnosis in 69.7%, and the remainder presented with hyperglycaemia without acidosis. Mean HbA1c at presentation was 8.1% and HbA1c was overtly elevated (>6.5%) in 84.3%. T1D autoantibodies were present in 40.4% of the 178 patients tested, with anti-GAD in 66 of 72 tested.

Acute kidney injury was present in 27/47 (57.4%) patients that had assessment of renal function reported. Steroid use was ongoing and deemed a potential contributor to CIADM presentation in 9 (4.6%) of patients. Lipase was tested in 36/192 patients with 69.4% having levels above the reference range, and 55 % having lipase levels >2x ULN (CTCAE v4 grade 3). Two patients had faecal elastase testing performed, both with low values consistent with exocrine insufficiency.

3.3.3 C-peptide threshold analysis

C-peptide was less than 0.4nmol/L in 141/154 (91.6%) at presentation (n=154). Of the 13 patients with initial C-peptide ³0.4nmol/L, 12 had follow-up C-peptide <0.4nmol/L and 1 patient was diagnosed with CIADM based on DKA alone without C-peptide reported. Follow-up C-peptide was reported in 30/192 patients with a mean value of 0.02nmol/L (+/- 0.04; range 0 – 0.19nmol/L). The time of repeat C-peptide varied from 1 week to 10 months, but all reported values were low (<0.4nmol/L).

Taking the 141 patients identified using our CIADM criteria as “true positives”, using lower thresholds of C-peptide would reduce the sensitivity of detection of CIADM to 97.2% for a cut off of 0.3nmol/L (4 patients excluded) and 93.6% for a cut off of 0.2nmol/L (9 patients excluded). This is largely due to the lack of follow up C-peptide testing.

3.3.4 Follow up

All patients included in this review were managed with insulin therapy alone which was not able to be ceased during follow up. Of the 83/192 patients with details regarding their insulin regimen reported, 75 were managed with multiple daily injections, 5 were on pre-mixed insulin, and 4 were managed insulin pump therapy. Insulin requirements were reported in 11 patients with a mean requirement of 0.51 units/kg/day (+/- 0.25). All patients (4/4) that did not meet our C-peptide criteria for CIADM diagnosis (C-peptide>=0.4nmol/L at presentation and followup) were able to cease insulin on follow up.

Eight patients were given a course of steroids as a trial of therapy for new onset diabetes and/or concurrent irAE, and 8 patients were given GLP-1 agonists. None of these patients were able to be weaned off insulin and ultimately, they were taken off these medications.

One hundred and five patients had their response to ICI therapy reported. The overall response rate was 64% (n=31, 30% complete and n=36, 34% partial), 18 had stable disease and 20 patients had progressive disease.

Median follow up duration was 6 months (IQR 3-9 months), reported in 76/192 patients.

3.3.5 Time to CIADM onset

Univariate analysis was performed initially to determine associations for each variable with time to onset of CIADM (Supplemental Table 1). Patients with positive T1D autoantibodies (N=72) presented a mean 3.5 weeks earlier (p=0.021) than those that were seronegative (N=106), and other variables (age, sex, cancer type, ICI type, irAE, HLA haplotype, elevation in lipase) were not significant on univariate analysis. Figure 1b depicts the time to onset of autoantibody positive patients with CIADM in comparison to

those without T1D autoantibodies. A one-way ANOVA analysis comparing time to onset of CIADM by type of immune checkpoint inhibitor (anti-PD1, anti-PDL1, or combination therapy anti- PD-1/PD-L1 with anti-CTLA4) was not significant ($p=0.780$, $F=0.25$).

3.3.6 Diabetic ketoacidosis

Multivariate logistic regression analysis with ANOVA analysis found that T1D autoantibody positivity was associated with significantly increased risk of presenting in DKA (OR 3.4, 95% CI 1.6-7.5, $p<0.001$) after adjusting for sex, age, cancer type and ICI type. Figure 1c depicts the increased incidence, and earlier onset of DKA in autoantibody positive patients.

3.3.7. Autoantibody positivity

Patients who had positive autoantibodies were more likely to have elevated lipase ($p=0.022$) and a higher prevalence of pre-existing type 2 diabetes ($p=0.013$). No significant differences between seropositive and seronegative groups were found in serum C-peptide, sex, age, HLA haplotype, presence of other irAE, cancer or ICI type. As depicted in Figure 1d, 82% of T1D autoantibody positive CIADM patients had a single autoantibody positive only, which is in contrast to typical findings in patients with type 1 diabetes.

3.3.8. Lipase positivity

Subgroup analyses compared patients with elevated lipase values above the reference range (n=36) to those that had normal lipase at CIADM presentation. Patients with elevated lipase had significantly lower pH values (mean difference 0.2, 95% CI 0.06-0.32, p=0.012), and higher glucose levels at presentation (mean difference 16.7mmol/L, 95% CI 5-27, p=0.005). Age, sex, cancer, ICI type, presence of irAE, BMI, presence of type 2 diabetes, HbA1c, C-peptide and ketone levels were not significantly different between the groups.

3.3.8. Data quality and risk of bias

Supplemental Table 2 presents the modified Newcastle Ottawa Scores for the studies identified during initial literature review. Overall the quality of the data was poor, with a mean score of 2.6/4. Case definition criteria was met in 92% of cases and exclusion of other causes in 89%, however representation criteria was met in only 12%, and follow up was adequate in 75%.

3.4 Discussion

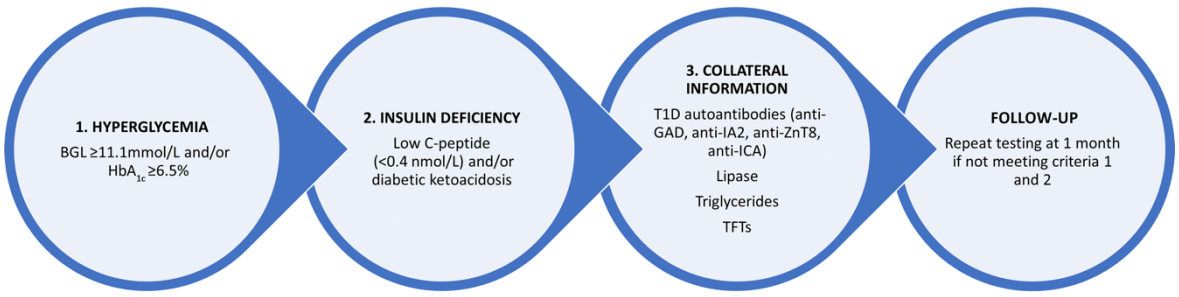
We have systematically reviewed reported cases of checkpoint inhibitor associated diabetes mellitus (CIADM) and filtered these cases on an individual level based on a combination of hyperglycaemia and insulin deficiency. In doing so we have presented for the first time and on the largest possible scale, a phenotype of diabetes that is clinically, biochemically and immunologically distinct (Table 2).

Table 2. Comparison of key characteristics of checkpoint inhibitor associated diabetes mellitus (CIADM) in comparison to classic type 1 diabetes mellitus (type 1 diabetes).

	CIADM	**Classic type 1 diabetes**
Triggers/ Risk factors	Anti-PD1/Anti-PDL1 targeted checkpoint inhibitors; Autoantibody positivity significantly associated with earlier time to CIADM andgreater risk of DKA	Unclear
Presentation	DKA in 69.7%	DKA in 39% children at presentation, 6% in adults [53]
Clinical course	Median 12 weeks after ICI treatment; Low C-peptide (<0.4nmol/L) at presentation in 91.6%; follow up C-peptide low in 100%	'Honeymooning' in 68.9% of children with T1D [54]; 36% adults and 7% children with type 1 diabetes have C-peptide >0.2nmol/L 3-5yrs from diagnosis [55]
Autoantibodies	Present in 40.4%	Present in 90% [36]
Genetic predisposition	T1D susceptible haplotypes in 59.3%	T1D susceptible haplotypes in 90% [42]
Exocrine pancreatic involvement	Pancreatic enzymes elevated in 69.4%; low faecal elastase in 2/2; Pancreatic atrophy on imaging [32]	Lower lipase vs normal controls except in fulminant phenotype; Reduced pancreatic volumes [56]

The application of our diagnostic criteria (Figure 2) was used to determine with confidence the characteristics of patients with CIADM. The pre-existing diagnostic criteria for CIADM are heterogenous, varying from all patients with new onset diabetes after ICI use, to those that are autoantibody positive, and those that are also insulin deficient. The inclusion of all patients with new onset diabetes after ICI use creates a heterogenous population that includes patients with type 2 diabetes and steroid induced hyperglycaemia, which as shown in one study is the most common aetiology of new onset hyperglycaemia after ICI use (15). Furthermore, autoantibodies cannot be relied upon in the diagnosis of CIADM as only 40.4% of our cohort was antibody positive, and in the literature seropositivity ranges from 0-71% (7–12). Therefore, we sought to use evidence of insulin deficiency (diabetic ketoacidosis and/or low C-peptide <0.4nmol/L) to define our cases and exclude differentials.

Figure 2. Proposed diagnostic criteria for CIADM. Key criteria are presence of hyperglycemia and insulin deficiency, and testing can be repeated at 1 month in those with clinical concern for CIADM but not meeting criteria at presentation. Collateral information includes assessment of T1D autoantibodies, screening assessment of the exocrine pancreas with lipase, exclusion of autoimmune lipodystrophy with serum triglycerides, and screening for the most common comorbid immune-related adverse event (thyroiditis) with thyroid function tests (TFTs). BGL, blood glucose level.



C-peptide has been used extensively in type 1 diabetes to aid in the diagnosis of autoantibody negative T1D and to risk-stratify patients with latent autoimmune diabetes of adulthood, and thus makes a widely available and useful diagnostic tool (16). As our review highlights, serum C-peptide is generally low in patients presenting with CIADM (91.6%) but also all patients who had follow up testing after initially "normal" C-peptide levels progressed to overtly low C-peptide levels. Early insulin therapy should be considered in all patients with a presumptive diagnosis of CIADM even if initial C-peptide is not low, until subsequent C-peptide testing can be performed on followup to confirm the diagnosis and guide ongoing use of insulin (Figure 2).

Type 1 diabetes consensus guidelines from ADA/EASD recommend a threshold of <0.2nmol/L to confirm a diagnosis of type 1 diabetes, whilst C-peptide 0.2-0.6nmol/L is considered a gray area (17). One key point of difference in CIADM is that the expected prevalence of T1DM antibodies is significantly lower than T1DM, and thus we feel that C-peptide cut offs need to be set at a higher, more sensitive threshold. In the right clinical context, a C-peptide <0.4nmol/L collected at the time of hyperglycaemia appears to be specific for CIADM; we identified no cases of recovery from diabetes when below this level. Furthermore, all patients that maintained on followup C-peptide levels above this threshold, went on to insulin independence, suggestive of a different form of diabetes. However, whether a C-peptide level greater than this has any clinical utility in predicting future onset of diabetes remains unclear.

Patients with CIADM have distinct clinical differences to those presenting with classic T1D, as summarised in Table 2. Our results confirm previous associations in case series and reports that CIADM is a condition nearly exclusively associated with anti-PD1 and

anti-PDL1 therapy, and 50% of patients will present by 12 weeks of ICI commencement. Not only is the disease onset more rapid, but it also more fulminant, with a high incidence of diabetic ketoacidosis (DKA) and rapid loss of endogenous insulin production. Meanwhile in classic T1D no unifying precipitant has been identified, and the onset of disease is commonly much more gradual with lower incidence of DKA and with autoantibodies preceding clinical disease by years (18,19).

The prevalence of typical T1D susceptibility haplotypes is lower in the CIADM cohort compared to classic T1D patients (20). In particular, a significant proportion of patients developed CIADM despite protective haplotypes. This is likely to reflect that CIADM patients are derived from a population that lack a strong propensity for T1D, as demonstrated by the protection from T1D until a mean of 63.4 years of age.

T1D autoantibody prevalence is substantially lower in the CIADM cohort, perhaps reflective of differing autoimmune responses to ICI therapy. Studies in humans and animals indicate that ICIs predominantly lead to T cell activation responses (21–24), and it is possible that traditional T1D associated B cell mediated autoantibody production is bypassed in CIADM. Similar to T1D anti-GAD was the most commonly positive autoantibody, but interestingly the prevalence of other autoantibodies was significantly lower than T1D as well (Figure 1d).

A key finding of this work is patients who were seropositive for classic type 1 diabetes T1D autoantibodies exhibited more rapid and severe onset of diabetes. This is a confirmatory finding consistent with the two previous systematic reviews in this area (25,26). This difference may be a result of differential immune pathway activation

between these two groups, or the presence of a "primed" immune system leading to a more fulminant response to checkpoint inhibition. Of the six patients with CIADM with autoantibody results reported from pre-treatment timepoints, 3 patients had positive autoantibodies prior to ICI exposure, 2 seroconverted to autoantibody positivity, and 1 patient remained seronegative throughout (8,27–29). Unfortunately, these numbers are too limited to draw strong conclusions and likely skewed towards retrospective testing of autoantibody positive individuals.

Understanding the immunology of CIADM is challenging, not only due to the rarity of the disease but also the limited accessibility of the human pancreas and challenges in identifying an islet-specific immune cell population in peripheral blood. Studies of human peripheral blood mononuclear cells in 4 HLA-A2+ CIADM patients found that only 2 had increased levels of classic T1D antigen specific CD8+ T cells, of which the majority were effector or memory T cells (30). In the 2 patients with CIADM that have had post-mortem pancreatic tissue available for histological analysis, both have shown inflammatory infiltrate within the pancreas, in both endocrine and exocrine compartments (24,31). Additional staining in a recent case reported by Perdigoto et al also showed beta cell expression of PD-L1 and IDO1 ligands in a similar manner to pancreatitis controls, suggestive of inhibitory immune ligand expression in response to inflammation (24). Interferon-gamma and TNF-alpha expression was also high in the peri-islet inflammatory infiltrate.

Akin to classic type 1 diabetes, of the myriad of treatments with demonstrated efficacy in CIADM prevention in NOD mice (anti-CD3, repeated antigen exposure, anti-IFN-gamma, anti-TNF-alpha, JAK1/2 inhibitor), none have yet been used successfully in a patient with

definite CIADM. Although 2 patients labelled as CIADM have become insulin independent after infliximab treatment, neither demonstrated a low C-peptide or manifest diabetic ketoacidosis to suggest overt insulin deficiency, which makes it difficult to determine the cause for hyperglycaemia (32,33).

The cohort of CIADM patients reported exhibited higher prevalence of lipase elevation than the general ICI treated cohort (55.2% vs 26.9% with grade 3 lipase elevation) (34). Lipase elevations in T1D otherwise have been reported in the Asian predominant fulminant phenotype (35), but generally are lower at presentation than normal controls by time of presentation of classic T1D patients (36). Studies of pancreatic volumetry in CIADM patients have shown rapid loss of pancreatic volume by time of presentation beyond what can be attributed to beta cell mass alone (33,37). Volume loss has also been reported on MRI scans of newly diagnosed patients with classic T1D, although to a lesser extent (36). More research would be required to determine whether presence of pancreatic inflammation increases risk of subsequent CIADM, which would suggest that pancreatic inflammation may expose pancreatic epitopes that promote pancreatic autoimmunity. Furthermore, studies in T1D have shown that loss of endogenous insulin production directly impacts on acinar exocrine function, and that subcutaneous insulin administration delivers much lower concentrations of insulin to acinar cells than local production by beta cells (38).

This review not only represents the largest number of confirmed CIADM cases reviewed in literature, with the inclusion of a stringent diagnostic criteria to better define the clinical phenotype of CIADM and exclude other differentials. Although previously, Wright et al. has reported 283 cases of new onset diabetes after ICI use, as the authors acknowledge

there was insufficient data available to distinguish between different forms of diabetes mellitus (14). Two other systematic reviews in the area have included all patients with new onset diabetes after ICI but acknowledged the limitations of including a heterogenous population. Akturk et al. found that 100% of patients with C-peptide measured in their cohort had low values consistent with insulin deficiency (25), and Lo Preiato et al found that “C-peptide levels were usually and permanently compromised” (26).

A further strength of this review is in the exclusive use of individual level data, which allows for more informative analysis. We believe these findings will allow clinicians to assess patients exposed to ICI that present with hyperglycaemia with a greater degree of confidence. The implication of a missed diagnosis of CIADM is serious given the high prevalence of DKA at diagnosis, and thus increased awareness of the defining characteristics of this condition is important.

The main limitation of this review is in the fragmented nature of reporting found in collated articles. Certain variables such as HLA haplotyping, cancer response, lipase, faecal elastase and follow up data were infrequently reported. We acknowledge that this review is subject to language bias (inclusion of English articles only) thus potentially skewing the representation of other populations, and publication bias. Given the rare nature of CIADM diagnoses there is also no data on a relevant control population. Future prospective studies in this area would be useful in clarifying this condition.

3.5 Conclusions

Our review analyses the individual characteristics of patients presenting with CIADM and finds that CIADM patients have many areas of distinction from T1D. In particular, the prevalence of T1D autoantibodies is significantly lower than classic T1D and autoantibody positive CIADM patients present earlier with diabetes and with significantly greater risk of DKA at presentation. This necessitates the development of a separate set of diagnostic criteria and further reporting of characteristics such as exocrine pancreatic function to better understand this novel form of diabetes.

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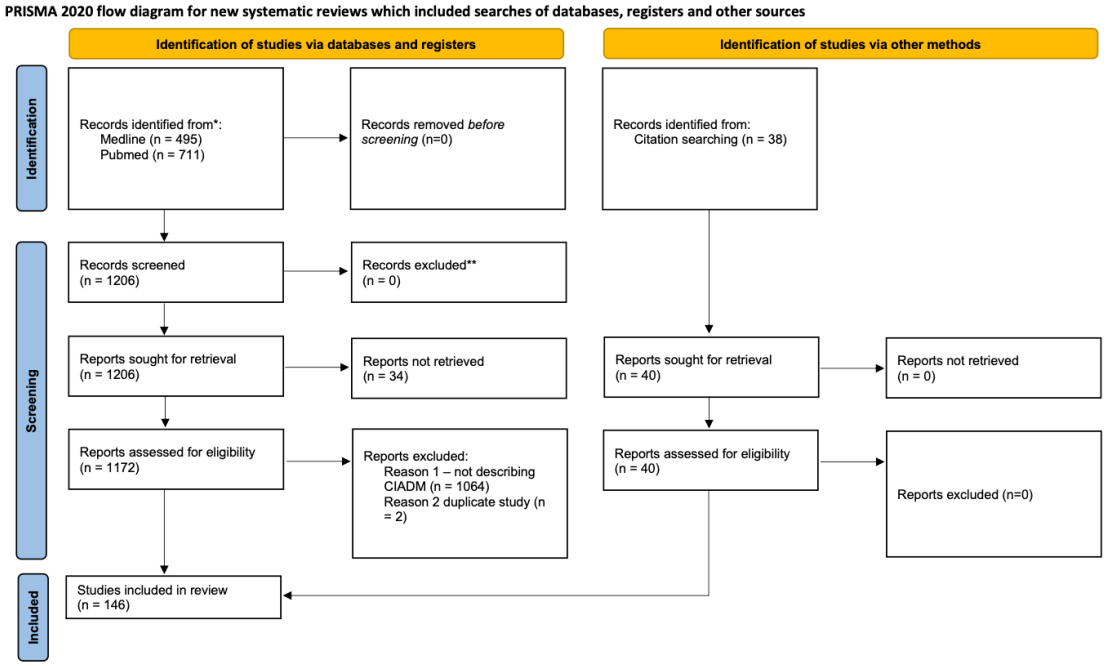
3.7 Supplemental material

Supplemental Figure 1. PRISMA 2020 flow diagram for systematic reviews. CIADM = checkpoint inhibitor associated autoimmune diabetes.



Supplemental Table 1. Univariate analysis of associations between baseline variable and time to onset of CIADM using Mann Whitney U testing and one-way ANOVA.

Variable		Mean time to onset (weeks)	SD	Mean difference	p
Age	Under 60yr	22.1	24.1	2.0	0.20
	60yr or older	18.2	20.6		
Sex	Female	18.0	23.4	-3	0.05
	Male	20.7	21.0		
Cancer type	Melanoma	20.3	23.1		0.76
	NSCLC	20.1	25.2		
	Other	17.9	14.5		
ICI type	Anti-PD1	20.3	22.8		0.78
	Anti-PDL1	18.7	18.0		
	Combination	17.7	21.1		
Presence of irAE	Yes	22.1	25.3	2.0	0.23
	No	18.4	19.9		
HLA haplotyping	Susceptible	25.3	34.5		0.39
	Neutral	18.1	19.3		
	Protective	39.3	26.7		
	Mixed	34.5	40.3		
Type 1 diabetes autoantibodies	Positive	15.6	17.2	3.5	0.02*
	Negative	21.5	23.2		
Elevated lipase	Present	16.0	17.8	2.0	0.50
	Absent	14.9	8.3		

Supplemental Table 2. Details of included and excluded papers, modified Newcastle Ottawa (N-O) scores and reasons for exclusion.

Modified Newcastle Ottawa Criteria: Each scored 0/1 for total out of 4.

1) Is the case definition adequate – meeting diagnostic criteria for hyperglycaemia and insulin deficiency?

2) Do the reported cases represent all eligible cases over a defined time period, in a defined catchment area, or an appropriate sample of those cases?

3) Have other diagnostic possibilities been excluded, including demonstration that the outcome of interest was not present at the start of the study?

4) Is the duration of follow up adequate to confirm/exclude the diagnosis (minimum 1 month if not meeting criteria at presentation)?

Ref	First Author	Year	Cases	Modified N-O Scale					Included Y/N	Reasons if excluded
				1	2	3	4	Total		
1	Abdullah	2019	1	1	0	1	1	3	Yes	
2	Abu-sbieh	2019	6	0	1	0	1	2	No	Results to support diagnosis of diabetes not provided
3	Akturk	2018	1	1	0	1	1	3	Yes	
4	Aleksova	2016	1	1	0	1	1	3	Yes	
5	Alhusseini	2016	1	1	0	1	1	3	Yes	
6	Alrifai	2019	1	1	0	1	1	3	Yes	
7	Alzenaidi	2017	1	1	0	1	0	2	Yes	
8	Araujo	2017	1	1	0	1	1	3	Yes	
9	Asif Humayun	2016	1	1	0	1	0	2	Yes	
10	Atkins	2018	1	1	0	1	1	3	Yes	
11	Banatwalla	2021	1	1	0	1	0	2	Yes	
12	Bastin	2020	1	1	0	1	1	3	Yes	
13	Boswell	2021	1	1	0	1	1	3	Yes	
14	Boyle	2018	1	1	0	1	0	2	Yes	
15	Byun	2020	18	1	1	1	1	4	No	Lack of individual data
16	Caccioti	2020	1	0	1	0	0	1	No	Not an adult; no results to support diagnosis provided
17	Cao	2021	1	1	0	1	1	3	Yes	
18	Capitao	2017	1	1	0	1	1	3	Yes	
19	Chae	2017	1	1	0	1	1	3	Yes	
20	Chan	2017	1	1	0	1	1	3	Yes	
21	Changizaddeh	2017	1	1	0	1	1	3	Yes	
22	Chokr	2018	1	1	0	1	1	3	Yes	
23	Clontz	2021	1	1	0	1	1	3	Yes	
24	Clotman	2018	1	1	0	1	0	2	Yes	
25	Cuenca	2020	1	1	0	1	0	2	Yes	
26	de Filette	2019	1	1	0	1	0	2	Yes	
27	Delasos	2021	1	1	0	1	0	2	Yes	
28	Edahiro	2019	1	1	0	1	1	3	Yes	
29	Fu	2021	1	1	0	1	1	3	Yes	
30	Fukui	2016	1	1	0	1	1	3	Yes	

31	Galligan	2018	9	1	1	1	1	4	Yes	
32	Gauci	2017	1	1	0	1	1	3	Yes	
33	Gaudy	2015	1	1	0	1	1	3	Yes	
34	Godwin	2017	1	1	0	1	1	3	Yes	
35	Gunawan	2018	1	1	0	1	1	3	Yes	
36	Gunjur	2019	1	1	0	1	1	3	Yes	
37	Hakami	2019	1	1	0	1	1	3	Yes	
38	Hansen	2016	1	0	0	0	1	1	No	No DKA and C-peptide >0.4nmol/L
39	Hao	2017	1	1	0	1	0	2	Yes	
40	Haque	2020	1	1	0	1	1	3	Yes	
41	Harsch	2018	1	1	0	1	1	3	Yes	
42	Hatakeyama	2019	1	1	0	1	1	3	Yes	
43	Hickmott	2017	1	1	0	1	1	3	Yes	
44	Ho	2018	1	0	0	0	0	1	No	Results to support diagnosis of diabetes not provided
45	Hofmann	2016	3	1	1	1	1	4	Yes	
46	Hong	2020	4	1	1	1	0	3	Yes	
47	Honoki	2020	1	0	0	0	0	0	No	Pre-existing type 2 diabetes on insulin
48	Huang	2021	1	1	0	1	1	3	Yes	
49	Hughes	2015	5	1	0	1	1	3	Yes	
50	Inaba	2022	7	1	1	1	1	4	Yes	
51	Ishi	2021	2	1	0	1	1	3	Yes	
52	Ishikawa	2017	1	1	0	1	1	3	Yes	
53	Jouneghani	2022	1	1	0	1	0	2	Yes	
54	Kahler	2020	5	1	1	1	1	4	Yes	
55	Kapke	2017	2	1	0	1	1	3	Yes	
56	Kedzior	2021	1	1	0	1	1	3	Yes	
57	Keerty	2020	1	1	0	1	1	3	Yes	
58	Kethireddy	2021	1	0	0	0	1	1	No	Results to support diagnosis of diabetes not provided
59	Kichloo	2020	1	1	0	1	0	2	Yes	
60	Kikuchi	2021	1	1	0	1	1	3	Yes	
61	Knight	2021	4	0/1	1	0/1	0	3	2/4	2 patients excluded (No DKA, no C-peptide reported)
62	Kong	2016	1	1	0	1	1	3	Yes	

63	Kotwal	2019	12	0/1	1	0/1	0	3	8/12	4 excluded (3 no C-peptide/ DKA report; 1 had C-peptide >0.4nmol/L)
64	Kumagai	2017	1	1	0	1	1	3	Yes	
65	Kurihara	2020	1	1	0	1	1	3	Yes	
66	Kusuki	2020	1	1	0	1	1	3	Yes	
67	Kyriacou	2020	1	1	0	1	1	3	Yes	
68	Lanzolla	2019	1	1	0	1	0	2	Yes	
69	Lee	2018	1	1	0	0	1	2	No	DKA whilst on SGLT2 inhibitor
70	Leonardi	2017	1	1	0	1	1	3	Yes	
71	Li, Li	2017	1	1	0	1	1	3	Yes	
72	Li, Sicheng	2018	1	1	0	1	1	3	Yes	
73	Li, Wei	2020	1	1	0	1	1	3	Yes	
74	Lopes	2020	1	1	0	1	0	2	Yes	
75	Lowe	2016	1	1	0	1	1	3	Yes	
76	Maamari	2019	1	1	0	1	0	2	Yes	
77	Mae	2020	1	1	0	1	1	3	Yes	
78	Maekawa	2019	1	1	0	1	1	3	Yes	
79	Magis	2018	5	1	1	1	1	4	Yes	
80	Makiguchi	2022	1	1	0	1	0	2	Yes	
81	Marchand	2019	6	0/1	0	0/1	1	3	4/6	2 patients excluded (C-peptide >0.4nmol/L and no DKA)
82	Marshall	2020	1	1	0	1	1	3	Yes	
83	Martin-Liberal	2015	1	1	0	1	1	3	Yes	
84	Matsumura	2017	1	1	0	0	1	2	No	Confounding factors - partial pancreatectomy
85	Matsuura	2018	1	0	0	0	1	1	No	Pre-existing type 2 diabetes on insulin
86	Mellati	2015	2	1	0	1	0	2	Yes	
87	Mengibar	2019	1	1	0	1	1	3	Yes	
88	Meyers	2018	1	0	0	0	0	0	No	Results to support diagnosis of diabetes not provided
89	Miyauchi	2020	1	1	0	1	1	3	Yes	
90	Miyoshi	2016	1	1	0	1	1	3	Yes	
91	Mellah	2017	1	1	0	1	0	2	Yes	
92	Munakata	2016	1	1	0	1	1	3	Yes	

93	Nikouline	2021	1	1	0	1	1	3	Yes	
94	Nishioki	2020	1	1	0	1	0	2	Yes	
95	Ohara	2020	1	0	0	0	1	1	No	C-peptide >0.4nmol/L with no DKA
96	Okahata	2019	1	1	0	1	1	3	Yes	
97	Oldfield	2021	1	1	0	0	1	2	No	DKA whilst on SGLT2 inhibitor
98	Omodaka	2018	1	1	0	1	1	3	Yes	
99	Patel	2019	1	1	0	1	1	3	Yes	
100	Patti	2018	1	1	0	1	1	3	Yes	
101	Peyrony	2020	1	1	0	1	0	2	Yes	
102	Pornatharukchareon	2020	1	1	0	1	1	3	Yes	
103	Presotto	2019	1	1	1	1	0	3	Yes	
104	Rahman	2020	1	1	0	1	1	3	Yes	
105	Ramos-Levi	2018	1	1	1	1	0	3	Yes	
106	de Vera Gomez	2021	1	1	0	1	1	3	Yes	
107	Sakaguchi	2019	1	1	0	1	1	3	Yes	
108	Sakai	2017	1	1	0	1	1	3	Yes	
109	Sakurai	2018	1	1	0	1	1	3	Yes	
110	Shah	2016	1	1	0	1	0	2	Yes	
111	Shiba	2018	1	1	0	1	1	3	Yes	
112	Shibayama	2019	1	1	0	1	1	3	Yes	
113	Smith-Cohn	2017	1	1	0	1	1	3	Yes	
114	Sothornwit	2019	1	1	0	1	1	2	Yes	
115	Stamatouli	2018	27	1	1	1	1	4	No	Data for individual patients not available
116	Sum	2018	1	1	0	1	1	3	Yes	
117	Takahashi	2018	1	1	0	1	1	3	Yes	
118	Takata	2021	1	1	0	1	0	2	Yes	
119	Tassone	2018	1	1	0	1	1	3	Yes	
120	Telo	2016	1	1	0	1	0	2	Yes	
121	Teramoto	2016	1	1	0	1	0	2	Yes	
122	Tittel	2021	15	0	1	0	0	1	No	Results to support diagnosis of diabetes not provided
123	Thoreau	2017	1	1	0	1	1	3	Yes	
124	Tohi	2019	1	1	0	1	1	3	Yes	
125	Trinh	2019	1	0	0	0	1	1	No	C-peptide >0.4nmol/L with no DKA
126	Tsang	2019	10	1	1	1	1	4	Yes	
127	Tsiogka	2017	1	1	0	1	1	3	Yes	

128	Tzoulis	2018	1	1	0	1	1	3	Yes	
129	Usui	2016	2	1	0	1	1	3	Yes	
130	Venetsanaki	2019	1	1	0	1	1	3	Yes	
131	Villareal	2018	1	1	0	1	1	3	Yes	
132	Way	2017	2	1	0	1	1	3	Yes	
133	Wen	2020	1	1	0	1	0	2	Yes	
134	Wong	2020	1	0	0	0	0	0	No	C-peptide >0.4nmol/L with no DKA
135	Wright	2017	2	1	0	1	1	3	1/2	One patient excluded as no C-peptide available, no DKA
136	Wu	2021	1	1	0	1	1	3	Yes	
137	Yamaguchi	2020	1	1	0	1	1	3	Yes	
138	Yamamoto	2019	1	1	0	1	0	2	Yes	
139	Yaura	2021	1	1	0	1	1	3	Yes	
140	Yilmaz	2021	1	1	0	1	1	3	Yes	
141	Yoneda	2019	1	1	0	1	1	3	Yes	
142	Yun	2020	5	1	1	1	1	4	Yes	
143	Zagouras	2020	1	1	0	1	1	3	Yes	
144	Zaied	2018	1	1	0	1	1	3	Yes	
145	Zand Irani	2022	1	1	0	1	1	3	Yes	
146	Zezza	2019	2	1	0	1	1	3	Yes	

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Chapter 4: Conventional and novel biomarkers to predict checkpoint inhibitor associated autoimmune diabetes

4.0 Preface

This manuscript presents original research investigating potential biomarkers for prediction of checkpoint inhibitor associated diabetes mellitus in humans prior to commencement of immune checkpoint inhibitors. The references for this manuscript can be found directly at the end of the manuscript.

This manuscript has been submitted to *Nature Medicine* and is currently under review.

Title: **Conventional and novel biomarkers to predict checkpoint inhibitor associated autoimmune diabetes**

Short Title: Predicting checkpoint inhibitor diabetes

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Keywords: Diabetes; Immune Checkpoint Inhibitors; Autoimmunity; Type 1 diabetes; Melanoma

Abbreviations:

CCL = C-C motif chemokine ligand

CIADM = Checkpoint inhibitor associated autoimmune diabetes

CXCL = C-X-C motif chemokine ligand

ICI = Immune checkpoint inhibitor

IL = interleukin

IRAE = immune-related adverse event

PBMC = peripheral blood mononuclear cell

PD-1 = programmed cell death protein 1

PD-L1 = programmed cell death ligand 1

T1D = Type 1 diabetes

TGF = Transforming growth factor

TNF = tumour necrosis factor

Abstract

Introduction

Checkpoint inhibitor-associated autoimmune diabetes (CIADM) is a rare but life-altering complication of immune checkpoint inhibitor (ICI) therapy. Biomarkers that predict type 1 diabetes (T1D) are unreliable for CIADM prediction. As indications for ICIs expand, ability to predict CIADM is of increasing importance.

Aim

To identify biomarkers for prediction of CIADM prior to ICI-therapy.

Methods

From our prospective biobank, 14 ICI-treated patients (anti-PD-1 ± anti CTLA4) with metastatic melanoma who developed CIADM were identified. Controls were selected from the same biobank, matched 2:1. Pre-treatment, on-ICI and post-CIADM serum and peripheral blood mononuclear cells (PBMCs) were analysed. Serum was analysed for T1D autoantibodies, C-peptide, glucose and cytokines. PBMCs were profiled using flow cytometry. Pancreatic volume was measured using CT volumetry.

Results

Before treatment, CIADM patients had smaller pancreatic volume (27% reduction, $p=0.044$) and higher anti-GAD antibody titres (median 2.9 versus 0, $p=0.01$). They had significantly higher baseline proportions of Th17 helper cells ($p=0.03$), higher CD4+ central memory cells ($p=0.04$) and lower naïve CD4+ cells ($p=0.01$). With ICI treatment, greater declines in pancreatic volume were seen in CIADM patients compared to controls ($p<0.0001$). Activated CD4+ subsets increased significantly in CIADM and controls with immune-related adverse effects (IRAE) but not controls without IRAE.

Using only pre-treatment results, pancreatic volume, anti-GAD antibody titre and baseline immune flow profile were highly predictive of CIADM development, with an area under the curve (AUC) of >0.96.

Conclusions

People who develop CIADM are immunologically predisposed and have antecedent pancreatic and immunological changes measurable in blood and by imaging that accurately predict disease. These biomarkers could be used to guide ICI use in the clinic, particularly when planning treatment in the adjuvant setting for low-risk tumours.

4.1 Introduction

Immune checkpoint inhibitors (ICIs) have transformed the treatment for many malignancies since their initial introduction in melanoma therapy. Eleven ICIs are now FDA-approved for at least 43 indications in a wide range of malignancies [1]. Whilst primarily used in the setting of metastatic cancer, recent studies also demonstrate benefits in the adjuvant and neoadjuvant settings [2,3].

As ICI use increases, the corresponding incidence of immune-related adverse effects will also rise. Amongst these, checkpoint inhibitor related autoimmune diabetes mellitus (CIADM; also termed ICI-DM) is of particular interest due to the major, life-long physical and psychosocial impacts of insulin requiring diabetes and the propensity for fulminant onset with high risk of diabetic ketoacidosis. We have previously demonstrated that CIADM bears similarities to its *de novo* counterpart type 1 diabetes (T1D) with respect to insulin deficiency and lifelong insulin dependency. However, there are also distinct differences including a high prevalence of T1D antibody negativity and fulminant beta cell failure, thus warranting separate diagnostic criteria and evaluation [4].

The ability to estimate an individual's risk of developing serious immune-related adverse effects prior to starting ICI would inform treatment decisions, especially in the adjuvant setting and where effective alternative treatments strategies are available. Studies show that the overall risk of other immune-related adverse effects is associated with higher baseline CD4+ counts [5], early T regulatory cell expansion [6], increased CD8+ clonal responses [7], more diverse T cell repertoire [8], higher cytokine levels at baseline and early in treatment [6,9,10], neutrophil to lymphocyte ratio [11] and genetic variants [12].

In T1D, anti-islet autoantibodies predict risk of disease with high accuracy [13]. HLA haplotypes are strongly linked to T1D risk and genetic risk scores are available to further delineate risk [14]. A decline in pancreatic volume is associated with risk of progression from pre-clinical to overt T1D [15]. Flow cytometry shows differences in CD4+ T follicular helper cells, T regulatory cells, naïve and Th17 cell subsets associated with T1D onset [16–20]. Islet-specific autoreactive T cells are a promising T1D biomarker but assays are subject to HLA type restrictions [21,22].

The aim of this study is to identify potential biomarkers for CIADM risk prior to commencement of ICI therapy and early during treatment. A secondary aim is to identify biomarkers for risk prediction after ICI-commencement but before CIADM onset. We compare CIADM cases to controls receiving ICI pre-treatment, early during treatment and after CIADM diagnosis.

4.2 Methods

Sample selection

Fourteen patients with CIADM and 28 ICI treated controls that had longitudinal biospecimens were identified from the prospectively collected Melanoma Institute of Australia medical record database (MRD2) and biospecimen bank. All patients gave written informed consent (Royal Prince Alfred Hospital Research Ethics Committee Protocol No. X10-0305 and HREC/10/RPAH).

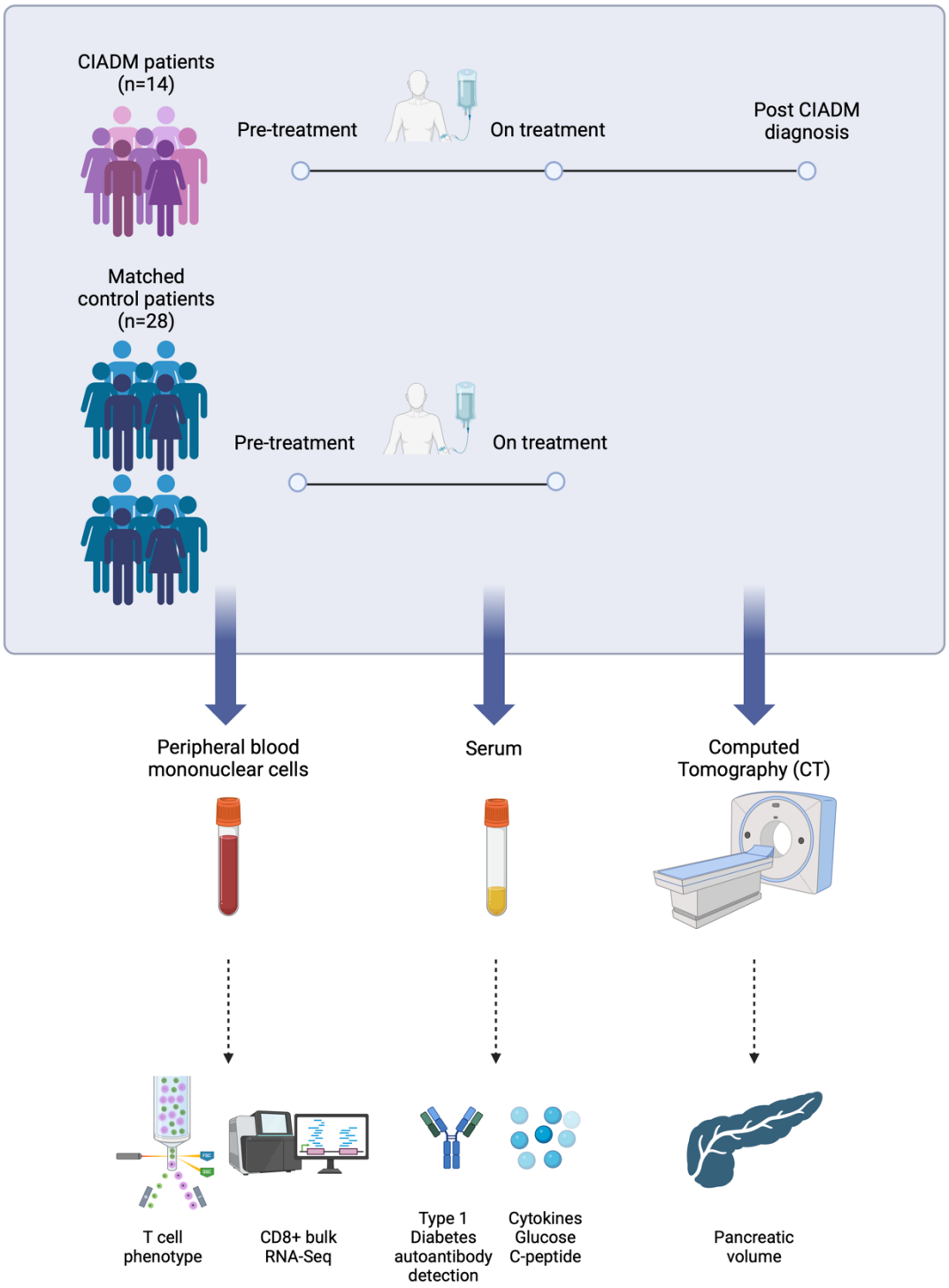
Two controls were selected for each CIADM patient, matched as closely as possibly for age (±5 years), gender, type of immune checkpoint inhibitor therapy (single agent anti-

PD1 versus combined anti-CTLA4 plus anti-PD1), time on therapy, treatment response and concurrent other irAEs. If CIADM patients had no other immune-related adverse events, they were matched to controls without irAE. If CIADM patients had other immune-related adverse events, they were matched to controls with those same irAEs where possible.

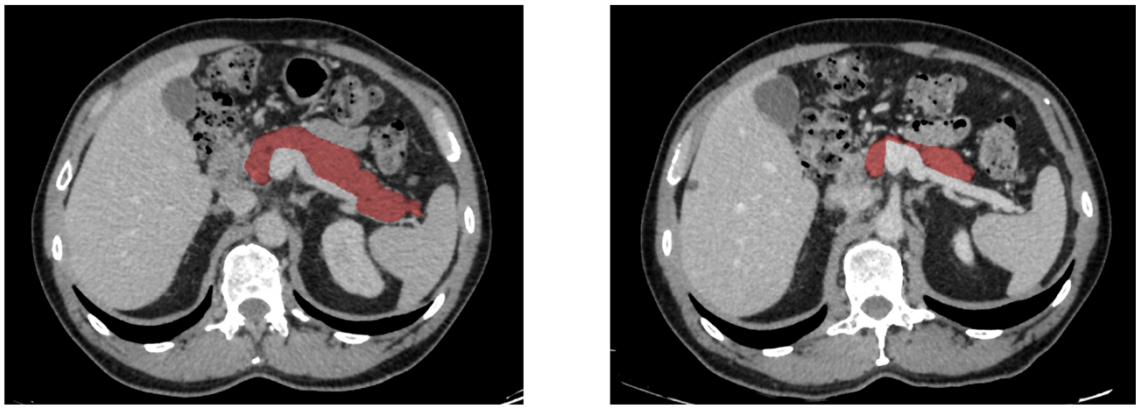
Control patients had prospectively collected pre-ICI and on-ICI PBMC (~3 months after treatment initiation to best match time of CIADM onset) and serum samples analysed. CIADM patients additionally had post-CIADM diagnosis samples analysed. A subgroup of control patients did not develop any immune-related adverse effects and they were also separately compared to assess the effect of general ICI related immune changes on various parameters. A summary of the methods is depicted in Figure 1.

Figure 1.A. Summary of methodology. B. Representative CT scans of a patient with CIADM prior to ICI therapy and at time of CIADM diagnosis (red = pancreatic area).

A.



B.



Pre-ICI

At CIADM Dx

Autoantibody analysis

Type 1 diabetes autoantibodies (anti-GAD, anti-IA2, anti-ZnT8 and anti-IA2) in serum samples were determined using agglutination PCR assay as previously described [23]. Thresholds for each autoantibody were set at the 98th percentile of results obtained from testing between 60 and 84 negative serum samples included in the 2023 International Islet Autoantibody Standardization Program [24].

Cytokine expression

Serum cytokine expression was measured using the Biolegend LEGENDplex^{TM} Human Essential Immune Response Multiplex Assay (Catalogue. No. 740930). This measures interleukins IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL17A, tumor necrosis factor α (TNFα), CCL2, CXCL8 (IL-8), CXCL10 and free transforming growth factor β1 (TGFβ1). The assay was conducted in accordance with manufacturer's instructions with samples run in duplicate.

C-peptide assay

Serum C-peptide was measured using human C-peptide ELISA assay (CrystalChem, Catalog #80954) as per manufacturer's instructions.

Glucose levels

Serum glucose was measured directly using Abbott Freestyle Libre glucometer and glucose test strips.

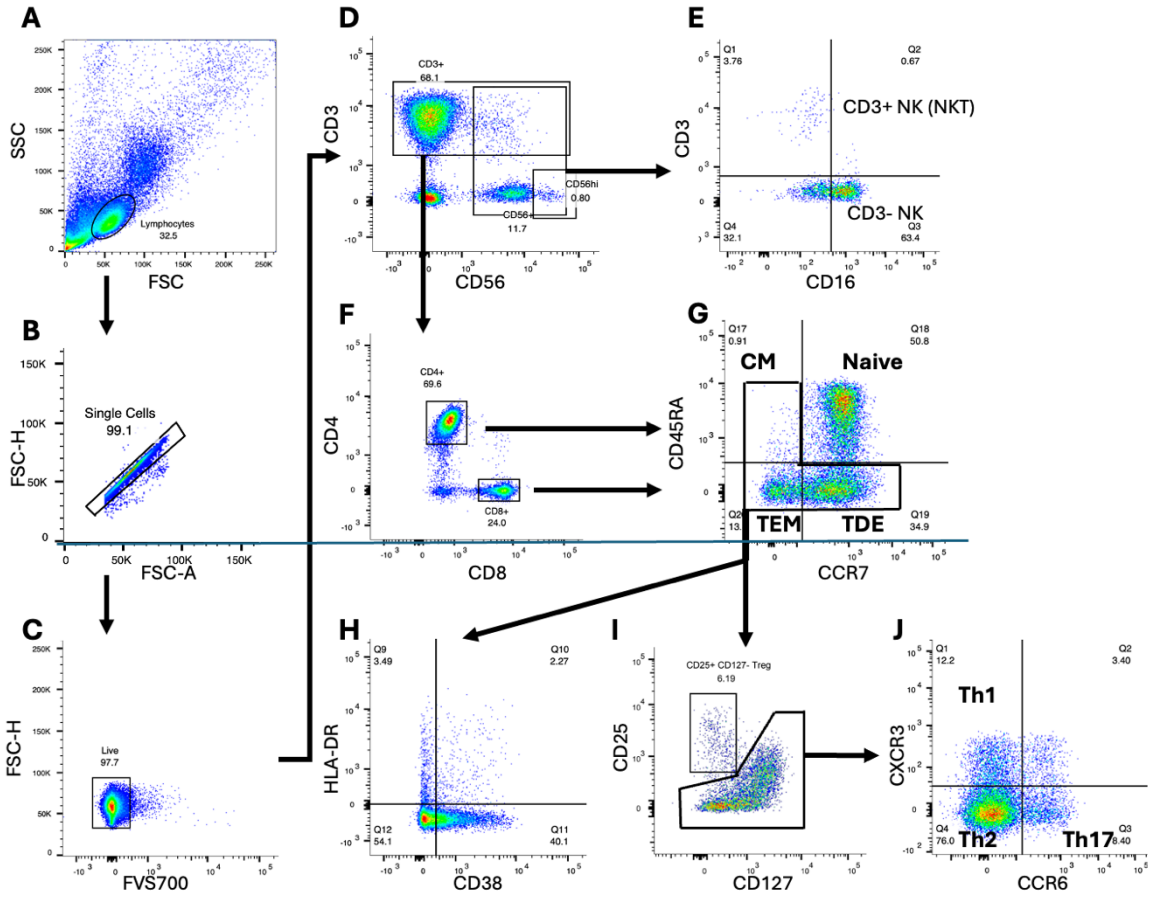
CT pancreatic volumetry

CT pancreatic volumetry was conducted as previously published using Vitrea® software (Figure 1B). CT scans were obtained within 6 months of each blood collection timepoint. Pancreatic cross sectional area was manually. Some of those results have previously been published [25] and the expanded cohort is presented in results below. CT scans were not available for one patient with CIADM and two controls. CIADM had a median onset after 13 weeks of ICI treatment, so CIADM patients did not have protocol CT scans on-ICI pre-CIADM, so data is not available for that timepoint.

Flow cytometry

Cryopreserved PBMCs were thawed in media and washed in FACS buffer prior to staining. Samples were stained first with FVS700 Viability Dye (Cat no. 564997) in dark for 10 minutes, followed by human AB serum for 10 minutes. Cells were then stained with CCR6 BV480 (Cat no. 556130), CXCR3 PE-CF596 (Cat no. 562451), CCR7 BB700 (Cat no. 566438), from BD Biosciences and CXCR5 PE-Cy7 (Cat no. 356923) from Biolegend at 37°C for 15 minutes. Surface staining was then performed with CD45RA APC-H7 (Cat no. 560674), CD8 BUV496 (Cat no. 612943), CD127 BV786 (Cat no. 563324), CD3

BUV661 (Cat no. 612965), CD25 BB515 (Cat no. 564467), CD56 BUV737 (Cat no. 612767), CD16 BUV563 (Cat no. 741449), CD4 BUV805 (Cat no. 612887), CD38 BV421 (Cat no. 562445), HLA-DR BUV395 (Cat no. 565972) from BD Biosciences at 4°C for 30 minutes. Cells were analysed using the BD Symphony Analyser with gating strategy as shown in Supplementary Figure 1. A minimum of 30,000 cells were analysed per sample.



Supplementary Figure 1. Gating strategy for T and NK cell phenotyping in PBMC. Cells were gated on A. Lymphocytes. B. Single cells. C. Live cells. D. CD56+ cells were distinguished from CD3+ cells. E. CD56+ cells were gated on CD3 and CD16+ status to identify CD3+ and CD3- NK cells. F. CD3+ cells were gated by CD4+ or CD8+. G. CD4+ and CD8+ were separately gated on CD45RA versus CCR7 to identify central memory (CM), naïve T cell, T effector memory (TEM) and terminally differentiated effector (TDE) status. H. Activation markers HLA-DR and CD38 were used to gate % activated T cells in the CD4+ and CD8+ subsets I. CD4+ CD25+ CD127- T regulatory cells were gated. J. CD4+ cells were gated against CXCR3 and CCR6 to identify Th1, Th17 and Th2 status.

Cell sorting

Cryopreserved PBMCs were thawed and washed in FACS buffer prior to staining with FC block, CD45+, CD3+ CD8+, CD4+ and DAPI. CD8+ cells were identified via gating for CD45+ CD3+ CD8+ CD4- and DAPI-ve subsets via the BD Influx cell sorter. 1000 CD8+ cells per samples were sorted per well into a 96 well plate and frozen down as per manufacturer's instructions.

RNA extraction

Total RNA was extracted using Ultra Low Input Takarabio® kit. RNA was extracted and sequenced using a NovaSeq X with approximately 10 million 150bp paired end reads.

Data analysis

RNA-Seq analysis was performed using R, using edgeR for differential gene analysis and STAR, RSEM, Tximport and DESeq2 with a Gencode 45 (latest) annotation for isoform analysis.

Flow cytometry data were analysed using FlowJo® Statistical analysis was performed using R or GraphPad Prism version 10.

Most serum, cytokine and flow cytometry data were not normally distributed, and were compared with Mann-Whitney where only 2 datasets were compared, or Kruskall-Wallis testing with Dunn's correction for multiple comparisons where >2 sets were examined. Normally distributed data were compared using one-way ANOVA with correction for multiple comparisons. Multiple-comparison adjusted p values of <0.05 were considered statistically significant.

Receiver operating curve (ROC) analysis was performed to calculate predictive value of the selected biomarkers. P values of <0.05 were taken as significant. Illustrations were made using Biorender or GraphPad Prism.

4.3 Results

Fourteen patients with CIADM and 28 matched controls treated with ICI were included. All patients had metastatic melanoma. Of the total samples sought, 2 PBMC samples were not available for CIADM patients at the pre-treatment timepoint. Baseline characteristics are shown in Table 1. Prior exposure to other anti-cancer treatment was predominantly dabrafenib and trametinib therapy.

Table 1. Baseline characteristics.

	CIADM (n=14)	Control (n=28)
Mean age (years; +/- SD)	71.2 (12.3)	67.2 (12.1)
Type of ICI therapy		
Anti-PD1	7 (50%)	14 (50%)
Anti-PD1 plus anti-CTLA4	7 (50%)	14 (50%)
Prior exposure to other anti-cancer therapy (e.g. TKI, chemotherapy)	7 (50%)	14 (50%)
Response to ICI therapy		
Complete response	7 (50%)	14 (50%)
Partial response	4 (29%)	8 (29%)
Stable disease	0	0
Progressive disease	3 (21%)	6 (21%)
Immune-related adverse effects	14 (100%)	17 (60%)
Thyroiditis	8	6
Colitis	3	2
Pancreatitis	3	0
Hepatitis	2	
Skin	2	
HLA haplotyping	9 of 14	0
High-risk N=5 of 9, High-risk+Protective N=1, Neutral N=2, Protective N=1		N/A

SD = standard deviation. TKI = tyrosine kinase inhibitor. HLA haplotyping positive = increased risk of type 1 diabetes, negative = no association with type 1 diabetes, protective = reduced risk of type 1 diabetes.

Subclinical anti-GAD and anti-IAA antibody levels are associated with CIADM

Before ICI-treatment, glutamic acid decarboxylase auto-antibody (Anti-GAD) titres were significantly higher in CIADM cases than controls (Figure 2A, p=0.002, Mann-Whitney). Despite the significantly higher levels with this sensitive assay, only 2 patients had levels above the reference range for anti-GAD before ICI exposure.

Figure 2B shows that anti-insulin autoantibodies (IAA) titres were also significantly higher in pre-treatment CIADM patients than in pre-treatment controls (p=0.048, Mann-Whitney). As insulin exposure is expected to provoke IAA development, it should be noted that no patients had exposure to insulin prior to ICI treatment and the rise of IAA seen in CIADM patients after diagnosis is expected due to insulin commencement.

Pancreatic volume is lower in CIADM patients before ICI treatment

Pancreatic volume was measured using computed tomography scans. Pancreatic volume on computed tomography scans was lower before ICI exposure in people who went on to develop CIADM than in controls (median 60 versus 73mls, Figure 2C, p=0.019).

Antibody levels and pancreatic volume change with ICI treatment

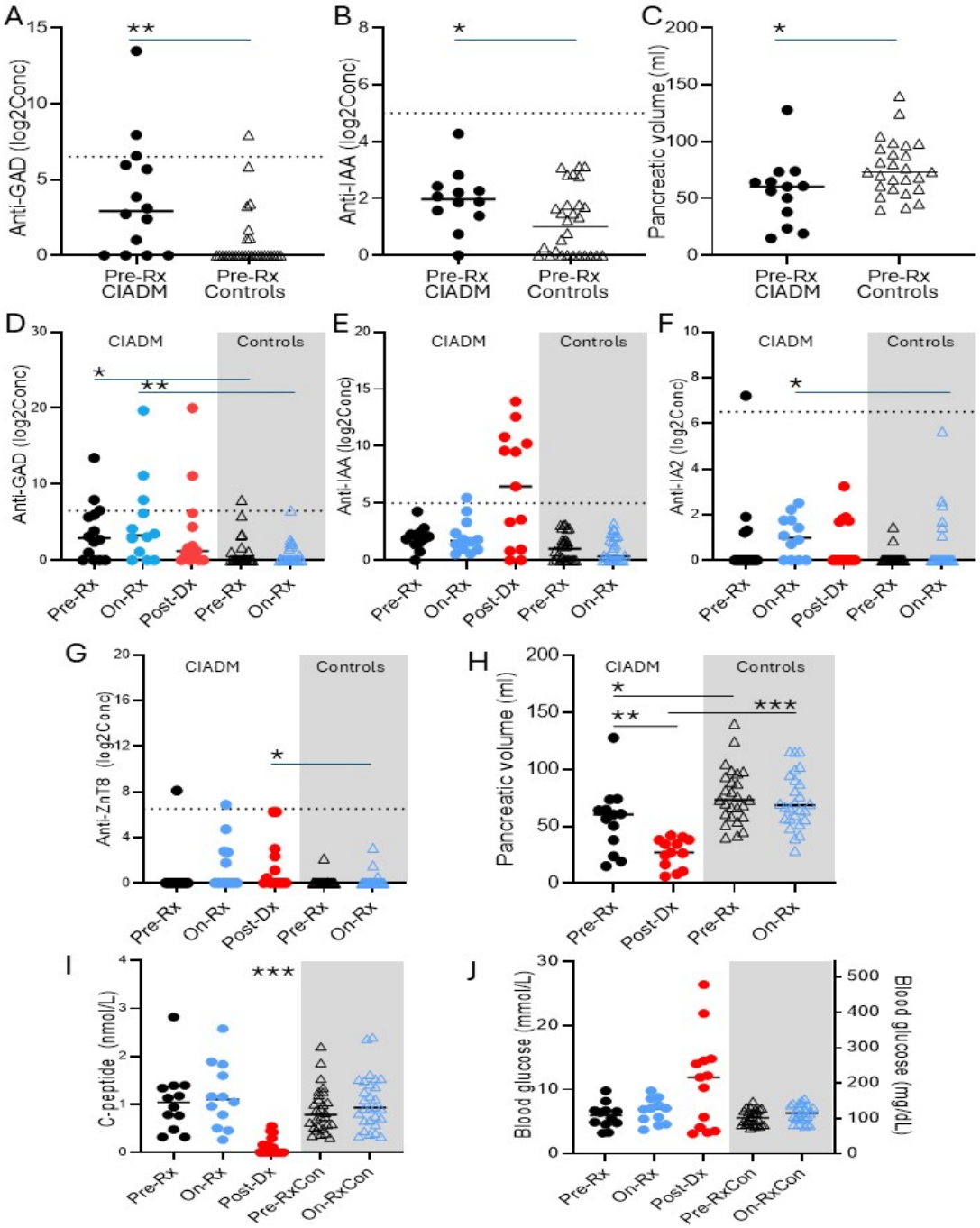
After ICI exposure, anti-GAD titres were significantly higher in people who went on develop CIADM than in on-treatment controls (p=0.008, Kruskall-Wallis with Dunn's correction for multiple comparisons, Figure 2D). Anti-IAA titres tended to be higher on-ICI in those who developed CIADM than in on-ICI controls, but this did not remain significant after correction for multiple comparisons (p=0.09, Figure 2E).

Figure 2F shows that anti-insulinoma antigen 2 (IA2) titres were higher on-ICI in CIADM patients than in on-ICI controls (p=0.045). In Figure 2G, anti-zinc transporter 8 (ZnT8)

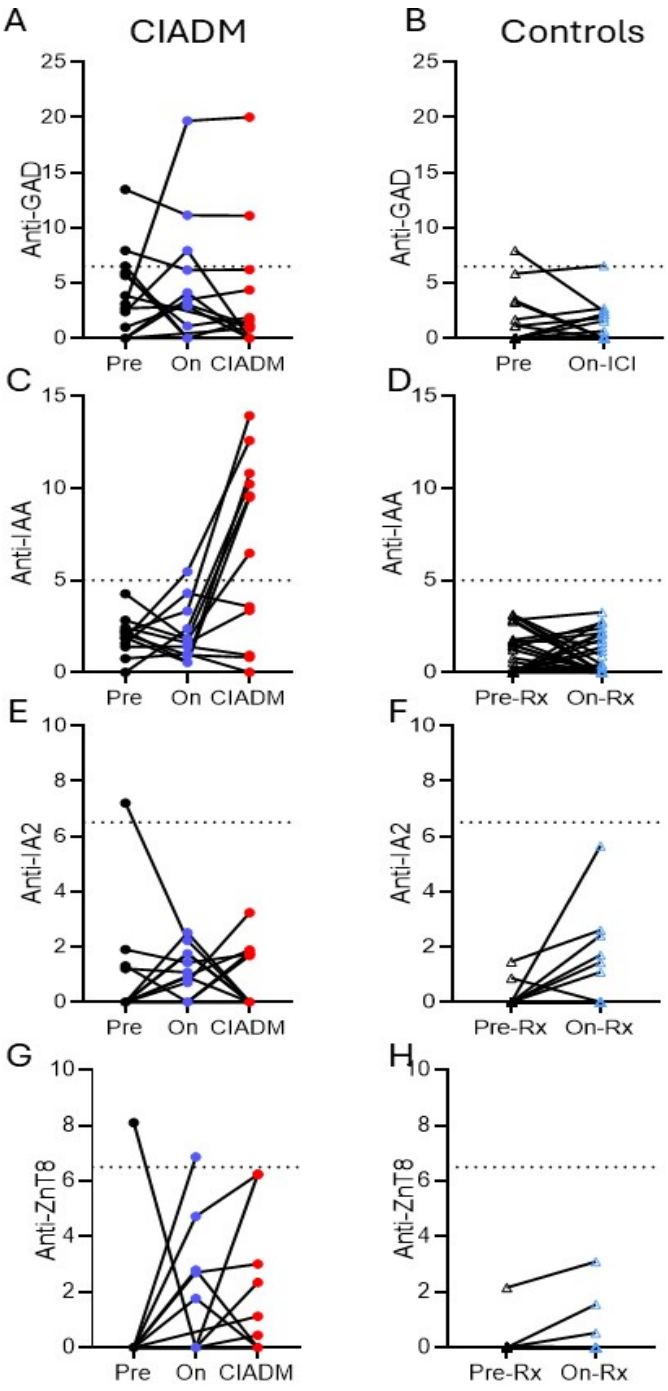
titres were higher at CIADM-diagnosis than in on-ICI controls ($p=0.04$) but did not differ before diabetes-onset.

Pancreatic volume was profoundly reduced at CIADM diagnosis compared to before-ICI ($p<0.01$) and was substantially lower than controls on-ICI ($p<0.0001$, Figure 2H). With the short time to CIADM diagnosis, CIADM patients did not have on-treatment scan prior to onset of CIADM.

Figure 2. Antibody levels, pancreatic volume, C-peptide and glucose. A) Anti-GAD antibodies before ICI in control and CIADM. B) Anti-IAA pre-ICI. C) Pancreatic volume pre-ICI. D) Anti-GAD before and on-ICI. E) Anti-IAA before and on-ICI. F) Anti-IA2 before and on ICI. G) Anti-ZnT8 before and on-ICI. H) Pancreatic volumes before an on-ICI. I) C-peptide before and on ICI. J) Blood glucose before and on ICI. Lines indicate median. Dotted lines at A, B, D, E, F and G indicate thresholds for positive. *p<0.05, **p<0.01. ***p<0.0001 for indicated comparison. For A-C, Mann-Whitney tests. For D-J Kruskall-Wallis corrected for multiple comparison with Dunn's test.



Changes in antibody titres with ICI-treatment within individuals were examined to assess whether this may be an independent predictor of CIADM. There were no significant differences in antibody-change between control and CIADM patients (Supplementary Figure 2).



Supplementary Figure 2. Antibody titres over time in individual patients. Please note, for each antibody, most control subjects had 0 to 0 levels. In most cases, the CIADM samples were taken after insulin was commenced.

Pre-treatment and pre-diabetes glucose and C-peptide levels do not predict future CIADM

Insulin secretion was assessed by measuring C-peptide and glucose. People who went on to develop CIADM did not have lower C-peptide before ICI treatment, or on-ICI before CIADM.

C-peptide fell from a median of 1.0 (IQR 0.6-1.4) nmol/L pre-ICI and 1.1 (0.6-1.8) to 0.05 (0-0.3) nmol/L post diagnosis for CIADM patients. In controls it remained normal (Figure 2I).

Formal blood glucose was not available for all CIADM patients after diagnosis, before commencement of insulin, and the available glucose levels did not differ significantly (Figure 2J). Overall, neither C-peptide nor serum glucose are predictive for future CIADM.

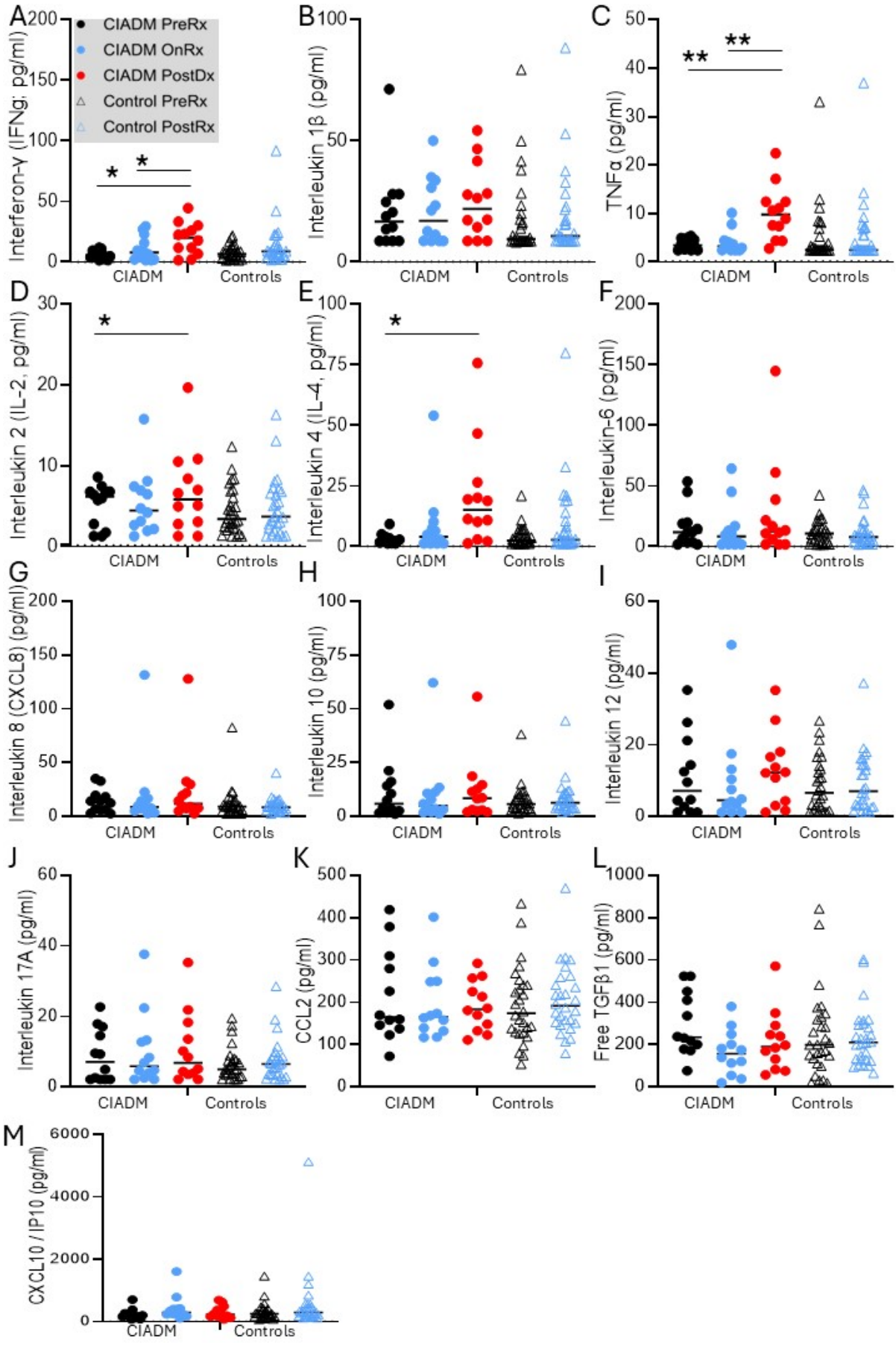
Altered circulating cytokine levels are associated with CIADM

Figure 3 depicts circulating cytokine concentrations at the different timepoints in CIADM patients and controls. No cytokines showed differential expression before ICI therapy. Interferon-γ (IFNγ), interleukin 1β (IL-1β), and tumor necrosis factor-α (TNFα) are the cytokines most classically associated with T1D. IFNγ was elevated at CIADM diagnosis compared to before or on-ICI (Figure 3A, $p<0.05$). IL-1β did not show any significant differences (Figure 3B). However, Figure 3C shows that TNFα also rose significantly at the time of CIADM diagnosis compared to baseline or to on-ICI in CIADM patients.

Interleukin 2 (IL-2) and IL-4 were both also significantly higher at CIADM diagnosis than at baseline (Figures 3D and 3E, $p<0.05$). Interleukins 6, 8 (also called CXCL8), 10, 12,

and 17A did not differ between groups (Figures 3E-J), nor did CCL2, free TGFβ or CXCL10 (Figures 3K-M).

Figure 3. Circulating cytokines. * p<0.05, **p<0.01 for the indicated comparison, Kruskall-Wallis with Dunn's correction for multiple comparisons. Lines indicate median.

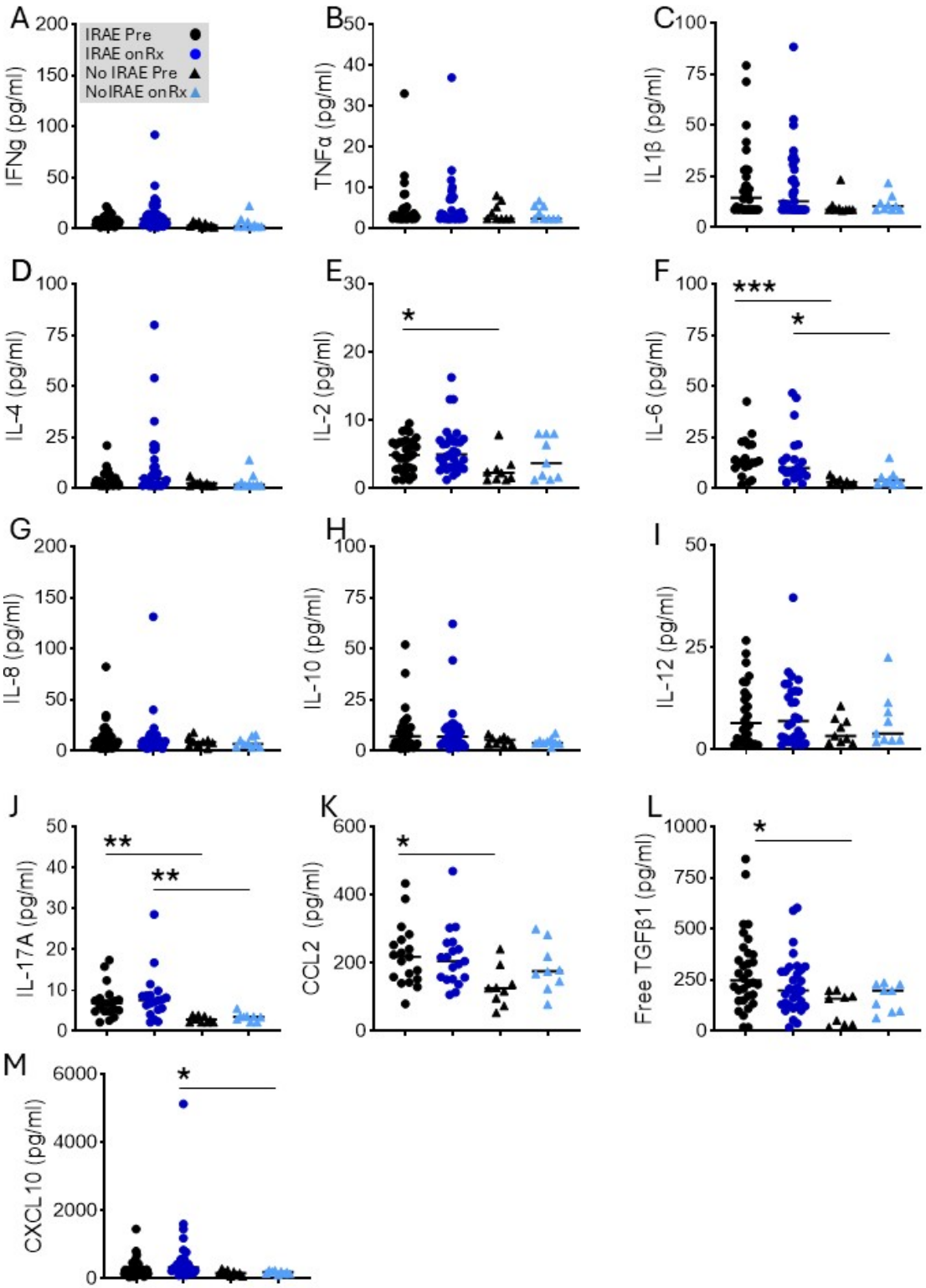


Cytokine levels predict immune related adverse effects

The 9 controls who did not develop any known immune related adverse event were compared to people who developed CIADM plus controls who developed an IRAE to test whether circulating cytokine levels may be predictive of developing any IRAE (Figure 4). When compared to controls without IRAE, before ICI treatment, IRAE patients had had significantly higher levels of IL-2, IL-6, IL-17A, CCL2, and free TGFβ before commencing ICI therapy (all $p<0.05$; Figures 4E, 4F, 4J, 4K and 4L, all Kruskall-Wallis with Dunn's correction for multiple comparisons).

IRAE patients after ICI exposure had higher IL-6 (Figure 4F), IL-17A (Figure 4J) and CXCL10 (Figure 4M) than on-ICI levels in people with no IRAE.

Figure 4. Cytokine levels in patients without immune related adverse effects (IRAE) pre and on-ICI and in people with IRAE pre and post-ICI. *p<0.05 and ** p<0.01.

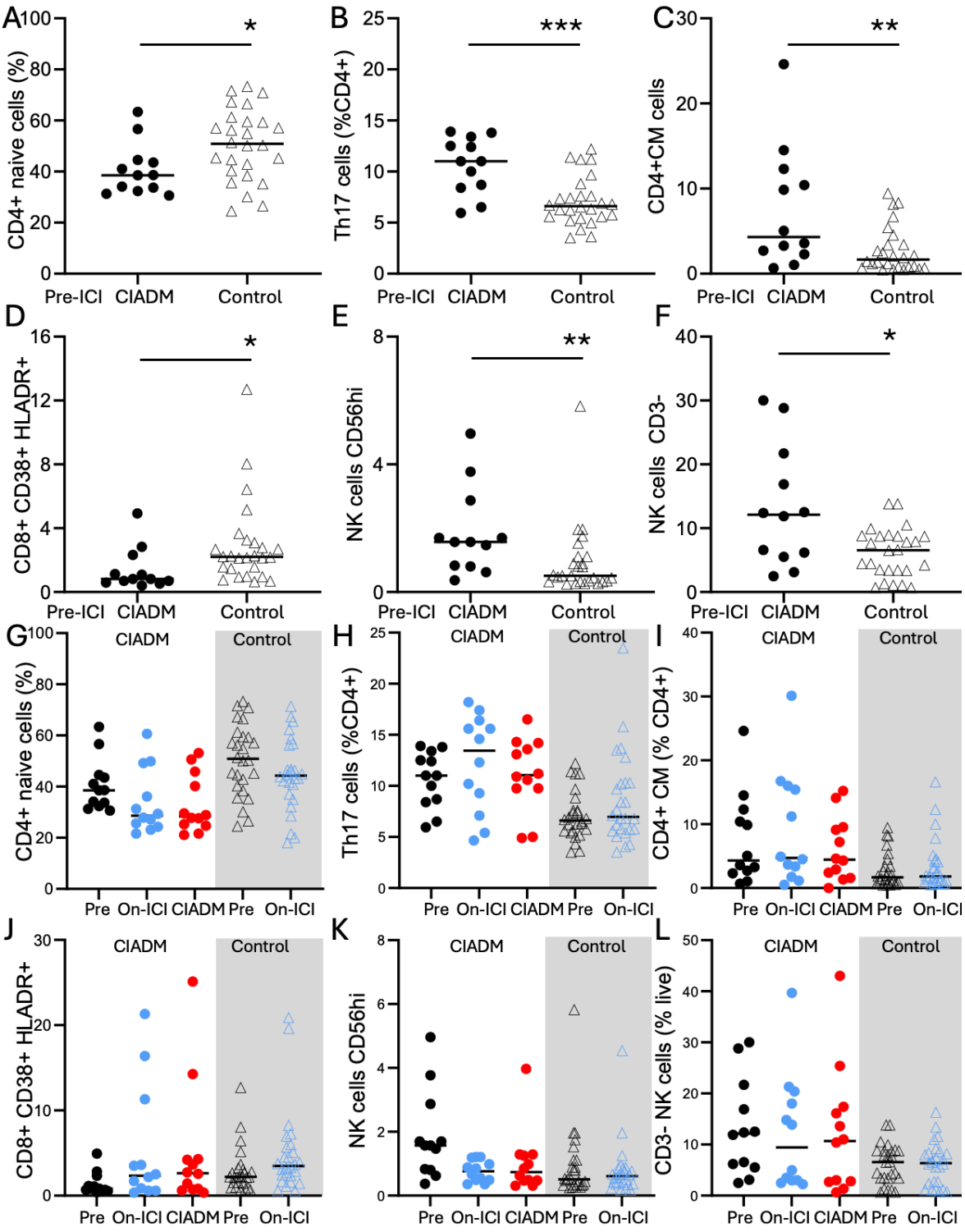


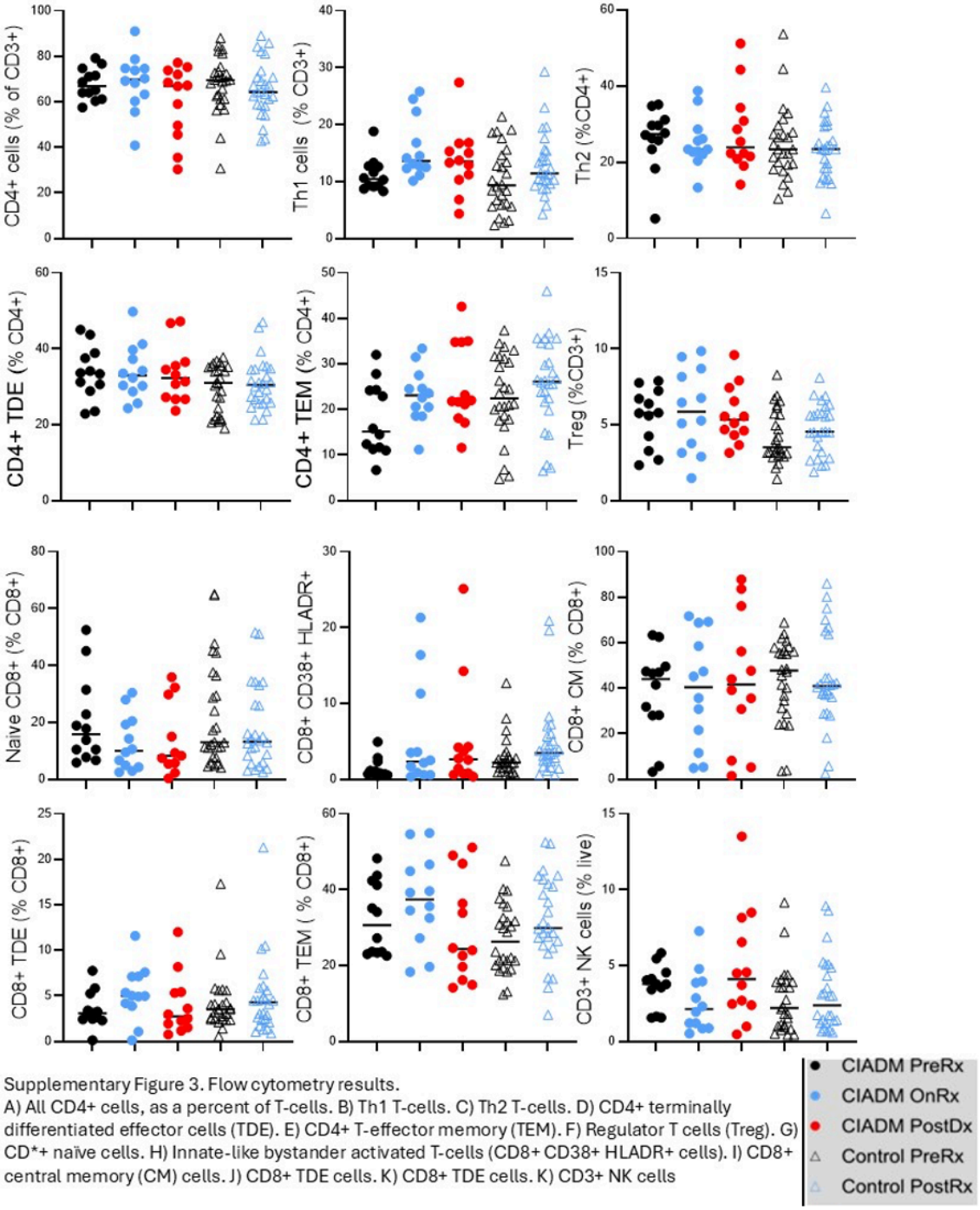
People who developed CIADM have a more activated immune system at baseline

Immuno-phenotyping of circulating PBMCs reveals significant differences at baseline between patients who developed CIADM and controls. CIADM patients, before ICI-exposure, had fewer naïve CD4+ T-cells (Figure 5A, $p<0.05$), and more Th17 cells (Figure 5B, $p=0.001$) and more CD4+ central memory cells (CM, $p<0.01$, Figure 5C). Pre-ICI CIADM patients also had fewer CD8+ CD38+ HLADR+ T cells (Figure 5D, $p<0.05$). Interestingly, given the fulminant phenotype of diabetes in many CIADM patients, there were also differences in baseline natural killer (NK) cells, with more CD56hi NK cells (Figure 5E, $p<0.01$), and more CD3- NK cells (Figure 5F, $p<0.05$).

After ICI treatment, there were no further significant changes in these cells (Figures 5G-L). Other cell subsets were not significantly altered (Supplementary Figure 3).

Figure 5. Flow cytometry immune cell subsets. Figures A-F depict pre-ICI differences between CIADM and control patients, Figures G-L depict data across all time points. * $p<0.05$, ** $p<0.01$ for the indicated comparison, Kruskall-Wallis with Dunn's correction for multiple comparisons. Lines indicate median.



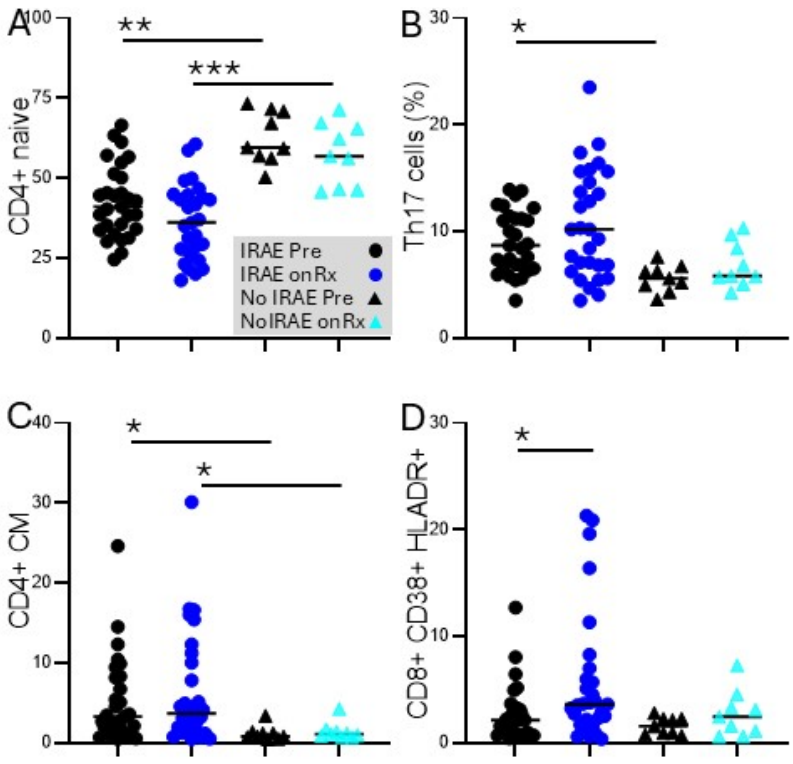


Supplementary Figure 3. Flow cytometry results.
A) All CD4+ cells, as a percent of T-cells. B) Th1 T-cells. C) Th2 T-cells. D) CD4+ terminally differentiated effector cells (TDE). E) CD4+ T-effector memory (TEM). F) Regulator T cells (Treg). G) CD*+ naïve cells. H) Innate-like bystander activated T-cells (CD8+ CD38+ HLADR+ cells). I) CD8+ central memory (CM) cells. J) CD8+ TDE cells. K) CD8+ TDE cells. K) CD3+ NK cells

Immune cell phenotypes also differ with IRAE

Flow cytometry parameters were compared in people with IRAE were compared to those with no IRAE (Supplementary Figure 4). At baseline, people who went on to develop an IRAE had fewer naïve CD4 cells at baseline, more Th17 cells, and more CD4+ central memory cells.

After ICI treatment, people with IRAE showed increased CD8+ CD38+ HLADR+ cells (Supplementary Figure 4D).



Supplementary Figure 4. Immune cell subtypes in people with and without IRAE

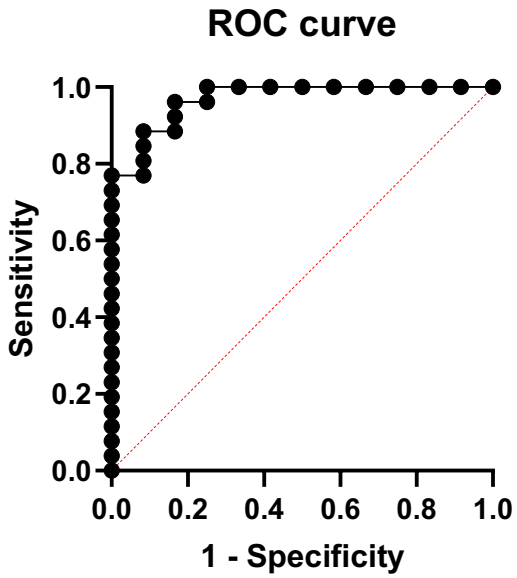
Differential gene expression in CD8+ T cells in CIADM

CD8+ T-cells are thought to be the major mediator of beta-cell death in T1D. Circulating CD8+ T-cells were collected and RNA expression was profiled with RNA-sequencing. Surprisingly, before ICI therapy, there were no differentially expressed genes that passed a false discovery rate (FDR) of <0.05 comparing CIADM and control patients.

ROC Curve analyses of key predictors

The baseline variables that were significantly associated with CIADM development (anti-GAD, anti-IAA, pancreatic volume, CD4+ central memory, CD4+ naïve, Th17 cells, CD8+ HLA-DR+CD38+, and NK CD56hi) were combined in a multiple logistic regression model. Figure 7 shows that this gave a receiver operated characteristic (ROC) curve with an area under the curve of 0.968 (95% CI 0.919-1.0, p<0.0001). This was associated with positive predictive value of 92.6% and a negative predictive value of 90.91%.

Figure 7. ROC curve for multiple logistic regression predicting CIADM diagnosis from combining baseline anti-GAD, anti-IAA, pancreatic volume, CD4+ central memory, CD4+ naïve, Th17 cells, CD8+ HLA-DR+CD38+, and NK CD56hi.



The data were separately analysed using only antibodies and pancreatic volume, as clinical flow-cytometry testing may not be available in all centres in a clinically meaningful timeframe. This including anti-GAD, anti-IAA and pancreatic volume gave an ROC curve

with AUC of 0.891 (p=0.0001), with negative predictive value of 77.8% and positive predictive value of 82.8%.

4.4 Discussion

This paper is the first to report a number of predictors of CIADM which are present before checkpoint inhibitor therapy. Using serial samples, patients and controls were tested using a combination of flow cytometry, cytokine expression, autoantibody analysis, RNA-Sequencing and imaging analysis. We identify that CIADM patients have higher levels of anti-GAD and anti-IAA at baseline and lower baseline pancreatic volume compared to matched controls. CIADM patients had higher baseline Th17+, higher CD4+ central memory cells and lower naïve CD4+ cells than controls. CIADM patients also exhibited differences in lymphocyte expansion early on treatment with higher activated CD4+ CD38+ HLA-DR+ subsets and lower naïve CD4+ subsets compared to controls.

In humans, limited data are available regarding the immunophenotype of CIADM. Hughes et al reported a case series of five patients with CIADM and amongst the four patients that had HLA-A2+ haplotyping, two had increased diabetes antigen specific T cells, which were predominantly effector or memory cells [26]. A mass cytometry based study of 28 patients with melanoma treated with ICI included two patients with new onset T1DM, which we would term CIADM. This study identified higher activated CD4+ cells in those with severe irAE of all types on treatment similar to our study, but conversely to our findings found higher naïve CD4+ T cells to be associated with more severe irAE [27]. However, of the two CIADM patients included, no significant differences in comparison to controls were found.

When looking at immune-related adverse effect studies in general, Lozano *et al's* study of T cell phenotyping in patients treated with ICIs for melanoma, using single cell RNA-Sequencing revealed baseline and early on treatment expansion in CD4+ T effector memory subsets to be associated with severe immune-related adverse effects of all types, whereas in our study CD4+ central memory subsets defined by flow cytometry had the strongest association with CIADM at baseline [28]. Bukhari *et al* previously identified on single cell sequencing of PBMCs from patients with thyroiditis was associated with higher baseline Th17 subsets [29]. Kim *et al* similarly found higher baseline Th17 subsets to be associated with development of severe immune-related adverse effects of all types in a cohort of patients treated for non-small cell lung cancer and thymic epithelial tumours with ICIs [30].

In type 1 diabetes, both Th1 and Th17 pathways are acknowledged as direct drivers of disease pathogenesis in human and animal studies [31–33]. A recent study found that Ustekinumab which binds IL-12 and IL-23 to target Th1 and Th17 cells was able to preserve beta cell function in adolescents with recent onset type 1 diabetes[34]. We find increased baseline Th17 cell numbers in CIADM patients and associated significant increases of cytokines associated with the Th17 pathway including IL-6, TGFβ, TNF-α and IFN-γ in CIADM patients compared to controls.

In comparison to other immune-related adverse effects, one of the unique aspects of CIADM is that its *de novo* counterpart type 1 diabetes has well established biomarkers in the form of islet autoantibodies, especially anti-GAD, anti-IA2, anti-IAA and anti-ZnT8. It is known that CIADM patients at diagnosis have lower prevalence of those autoantibodies than in type 1 diabetes [4]. We and others have reported pre-treatment anti-GAD positivity in

a small proportion of CIADM patients but this has not been extensively tested or compared with controls [35–38]. Anti-GAD positivity is present in a small proportion of the general population, with a median specificity of anti-GAD in the Islet Autoantibody Accreditation Program of 98.9% [39]. A Norwegian study of over 4000 individuals found anti-GAD has a prevalence of 1.7% in the non-diabetic Norwegian adult population [40] where it was associated with thyroid autoimmunity. Anti-GAD titre was higher in individuals in individuals with prediabetes than those with normal metabolic parameters. Our study used the highly sensitive ADAP assay to detect subclinical levels of anti-GAD which were significantly higher than controls and were associated with progression to CIADM. It is plausible that patients with CIADM have a subclinical degree of anti-islet autoimmunity as evidenced by low titres of anti-GAD and anti-IAA reflecting subclinical islet autoimmunity that places them at risk once exposed to ICIs.

The use of pancreatic volumetry as a biomarker for type 1 diabetes and CIADM is established. Previous studies [15,41,42] in individuals at high risk of type 1 diabetes have shown reduced pancreatic volume with progression to type 1 diabetes. Several studies have corroborated that CIADM is associated with a decline in pancreatic volume, however comparisons with controls receiving ICI has not been previously reported [43–45].

The strengths of this paper lie in the inclusion of longitudinal case control matched samples obtained and use of a diverse range of biomarker methodology. The biomarkers we have utilised in our final prediction model are all non-invasive, scalable and easily accessible clinically though peripheral blood collection and CT scans that are already being conducted as part of routine care. Automated pancreatic volumetry methodology has previously been validated [46].

The limitations of this study include relatively low sample size, due to the incidence of CIADM at 0.9% of PD1/ PD-L1 ICI treated patients [47]. Even so, this is the largest series of CIADM patients with longitudinal sample analyses. The lack of significant differentially expressed genes by RNA-sequencing of CD8+ cells was surprising, but consistent with the results showing most of the flow cytometry identified differences were in CD4+ cells. After the CD8+ results were analysed, the CD4+ cells were no longer available to sequence. Inclusion of a Type 1 diabetes Genetic Risk Score [12] may further improve the ability to predict CIADM but this test is not routinely available.

The detection of subclinical anti-GAD titres and lower baseline pancreatic volume in our CIADM cohort suggests that CIADM patients have prior anti-islet immune responses that is poised under permissive conditions to cause disease. That these patients have not developed type 1 diabetes prior to the introduction of an anti-PD1 or anti-PDL1 inhibitor indicates that this immune pathway plays an important role in islet autoimmunity in these patients. The findings of higher Th17 helper cells, CD4+ central memory cells and lower CD4+ naïve cells at baseline with more activation on ICI introduction gives the impression of a more experienced and autoreactive immune system in CIADM patients compared to controls without immune-related adverse effects. Combined, these findings suggest that CIADM patients have a distinct immune profile that can be detected prior to ICI use.

The ability to predict irAE has unique potential when the clinical indication for ICI is not definitively superior to alternatives. For example in stage III melanoma, ICIs are currently considered alongside targeted therapy such as dabrafenib plus trametinib as effective adjuvant therapy and specific contraindications to ICIs such as autoimmune disease,

immunosuppressive treatment guide choice of therapy[48]. In this scenario, the ability to identify individuals at high risk of severe irAE could further guide therapeutic choices in this area and reduce irAE related morbidity.

4.5 Conclusions

Immune-related adverse events are common in those treated with immune checkpoint inhibitors and can vary in severity from mild to fatal. Prediction of immune-related adverse effects prior to therapy has the potential to inform clinical decisions, allow for earlier detection and open a potential window for prevention. Combining biomarkers from the fields of type 1 diabetes and immune-related adverse effects research, we have identified biomarkers that have potential to predict checkpoint inhibitor related autoimmune diabetes from baseline and on treatment characteristics. Prospective validation of these biomarkers is a crucial next step but a challenging prospect due to the relative low incidence of CIADM.

Acknowledgements

We would like to thank Westmead Institute for Medical Research Flow Cytometry department and Genomics department for assistance and advice. We would like to thank Westmead Hospital Department of Radiology for allowing use of Vitrea®.

Disclosures

DAB receives royalties from Roche for GDF15 assays, which have been used as a biomarker of diabetes development.

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Chapter 5: **Comparison of animal models of type 1 diabetes and CIADM**

5.1 Introduction

In earlier chapters we have detailed the discovery of checkpoint inhibitor related autoimmune diabetes mellitus (CIADM) in humans and the differences between CIADM and *de novo* type 1 diabetes in terms of risk factors, clinical course, peripheral blood immune markers, endocrine and exocrine function. One of the biggest limitations to improving our understanding of CIADM has been very limited access to pancreatic tissue in humans for further analysis of local immunologic changes.

NOD mice are the most commonly used mice model for type 1 diabetes[72]. NOD mice have a predisposition to develop insulitis and islet autoantibodies from an early age [73,74], and spontaneous autoimmune diabetes by 30 weeks in 70-90% of females and 30-40% of males [75]. Models for CIADM have been developed previously, with use of anti-PD1 and anti-PDL1 antibodies in NOD mice to precipitate rapid onset diabetes [76,77]. Anti-PD1 or anti-PDL1 administered to NOD mice led to rapid onset diabetes, with increase in expression of chemotactic markers such as CXCR3+, CXCL10+ in insulin specific CD4+ cells [77] and increase in insulin-specific follicular T cell and B cells [78] in comparison to B6.g7 mice (C57BL/6J mice expressing the NOD MHC class II molecules I-A^{g7}, which are known to be diabetes resistant). A comparison of the endocrine and immunologic features of spontaneous type 1 diabetes in NOD mice to CIADM in NOD or other mice has not previously been made.

In this chapter, we sought to characterise the endocrine, exocrine and tissue lymphocyte immunologic phenotype of CIADM in comparison to the traditional model of type 1 diabetes in NOD mice. Additionally, we compared CIADM to young NOD mice which have little to no insulitis and aged NOD mice that were resistant to diabetes by 40 weeks of

age to provide insight into age related changes and changes associated with apparent immune tolerance and resistance to diabetes development. We additionally studied an additional comparison group of streptozotocin (STZ) induced diabetes. Streptozotocin in high doses causes direct beta cell death. Multiple low dose STZ is used more commonly in type 1 diabetes models as repeated exposure to STZ triggers partial beta cell damage and leads to priming of auto-immune responses that are thought to somewhat mimic type 1 diabetes [79]. Thus, multiple low doses of streptozotocin is used as a model of type 1 diabetes triggered by a beta cell toxin [79,80].

5.2 Aims

To compare the endocrine and immunologic phenotype of different models of type 1 diabetes in NOD mice, specifically 1) spontaneous autoimmune diabetes, 2) checkpoint inhibitor induced diabetes, 3) streptozotocin induced diabetes and 4) control mice both aged (nondiabetic) and young.

5.3 Methods

5.3.0 Chemicals, reagents and equipment

Table 5.1 Chemicals, reagents and equipment

Chemicals, reagents and equipment	Source
Abcam Triglyceride Assay kit	Abcam, Waltham, Massachusetts, USA (Ab65336)
Antigen Decloaker 10X	Biocare Medical, Pacheco, California, USA (CB910M)
Bovine Serum Albumin	Bovogen Biologicals Australia Pty Ltd, Keilor East, Victoria, Australia (BSAS1.0)
Coverslip 24x50mm	Epredia, Kalmazoo, Michigan USA
Crystal Chem Ultra Sensitive Mouse insulin ELISA kit	Crystal Chem Inc., Downers Grove, Illinois USA (90080)
Cusabio Mouse Pancreatic Lipase ELISA Kit	Cusabio, Houston, Texas, USA (CSB-E16930m)
DAKO antibody diluent	Dako, Carpinteria, California, USA (S0809)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St Louis, Missouri, USA (D2653)
Eosin	Sigma-Aldrich, St Louis, Missouri, USA (230251)
Ficoll-Paque Premium	Cytiva, Malborough, Massachusetts, (17544203)

Fluoromount Mounting Medium	Sigma-Aldrich, St Louis, Missouri, (F4680)
Freestyle Lite glucose test strips	Abbott Diabetes Care, Macquarie Park, NSW, Australia
Glutamine (200mM)	Thermo Fisher Scientific, Waltham, Massachusetts, USA (25030-81)
Hamilton syringe	Hamilton Company, 500 µL, USA (Model 1750 LT SYR, #81201)
Liberase TL Research Grade	Roche, Basel, Vaud, Switzerland (05401020001)
Liquid DAB + Chromogen substrate	Dako, Carpinteria, California (K346811-2)
M199 (with Hanks salts, without L-glutamine and sodium bicarbonate)	Sigma-Aldrich, St Louis, Missouri USA (M9163)
Neutral buffered formalin 10% (NBF)	Sigma-Aldrich, St Louis, Missouri USA (HT50-1-1)
Pap pen	Daido Sangyo, Tokyo, Japan
PBS tablets	Thermo Fisher Scientific, Waltham, Massachusetts, USA (18912014)
Pen-strep	Life Technology, Carlsbad, California, USA (15070-063)
Peroxidase	BioCare Medical, Pacheco, California, USA (PX968)
Protein Block, serum free	Dako, Carpinteria, California (X0909)
Shandon Harris Haematoxylin	Thermo Fisher Scientific, Waltham, Massachusetts, USA (6765015)

Sodium bicarbonate (7.5%)	Thermo Fisher Scientific, Waltham, Massachusetts, USA (25080-094)
Trypan Blue	Thermo Fisher Scientific, Waltham, Massachusetts, USA (15250061)
0.25% Trypsin + EDTA	Thermo Fisher Scientific, Waltham, Massachusetts, USA (25200-056)
Tween 20	Sigma-Aldrich, St Louis, Missouri USA (P1379)
Ultraplus Slides	Thermo Fisher Scientific, Waltham, Massachusetts, USA
UltraPure EDTA 0.5M	Invitrogen, Waltham, Massachusetts, USA (15575020)

Table 5.2 Drugs for injection

Drug name	**Source**
Anti-PD1 (J43)	Invivomab, BioXcell, Lebanon, North Hampton, USA (#BE0033-2)
Anti-PDL1 (RMP1-14)	Invivomab, BioXcell, Lebanon, North Hampton, USA (#BE0146)
Detemir Insulin (Levemir) 100IU/ml	Novo Nordisk, Bagsvaerd, Denmark
Dextrose	Sigma-Aldrich, St Louis, Missouri, USA(D9434)
Ketamine (Ketamav) 100mg/ml	Mavlab, Slacks Creek, Queensland, Australia
Streptozotocin	Sigma-Aldrich, St Louis, Missouri, USA (S0130); Lot WXBD1402V,WXBD0304V
Xylazine hydrochloride (Xylazil-20) 20mg/ml	Troy Laboratories PTY LTD, Glendenning, New South Wales, Australia

Table 5.3 Buffers and media

Buffer/media name	Recipe
Acid ethanol	75% Ethanol plus 1% by volume of 3%HCl
Freezing media (mouse)	90% FBS 10% DMSO
Insulin (for treating diabetic mice)	Detemir or Glargine insulin was diluted in phosphate buffered saline (PBS) with 0.1% bovine serum albumin
InvivoPure pH 7.0 Dilution buffer	BioxCell, Lebanon, North Hampton, USA (IP0070)
Islet isolation media	10% heat inactivated FCS, 3.3mM sodium bicarbonate made up with M199
Molecular Biology Grade Water	Accugene Lonza, Basel, Switzerland (51200)
Nonidet P-40	Thermo Fisher Scientific, Waltham, Massachusetts, USA (J19628.K2)
Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific, Waltham, Massachusetts, USA (14190-144)
PBST	Phosphate buffered saline (PBS) plus 1% Tween 20
RPMI-1640 Medium	Sigma-Aldrich, St Louis, Missouri, USA (R0883)
Streptozotocin-administration buffer	Solution A: 2.1g citric acid anhydrous in 100ml distilled water Solution B: 2.92g sodium citrate in 100ml distilled water Fresh buffer made mixing 28ml of solution A with 25.5ml of solution B, pH 4.2

Table 5.4 Antibodies for histology

Antigen	Source	Catalogue
Anti-Rabbit EnVision+ system-HRP labelled polymer	Dako, Carpinteria, California	K4003
Anti-Rabbit HRP/DAB (ABC) Detection IHC Kit	Dako, Carpinteria, California	K4011
Anti-rat IgG, AF647 secondary antibody (goat)	Cell Signalling Technology, Danvers, Massachusetts, USA	4418S
Foxp3 (rat, FJK-16s)	eBioscience, San Diego, California, USA	14-5773-82
Glucagon (rabbit, polyclonal)	Cell Signalling Technology, Danvers, Massachusetts, USA	2760
Insulin (rabbit, polyclonal)	Cell Signalling Technology, Danvers, Massachusetts, USA	4590S
PD-L1 (rabbit; E1L3N)	Cell Signalling Technology, Danvers, Massachusetts, USA	13684T

Table 5.5 Other flow related reagents

Name	Company	Catalogue
BD Brilliant Stain Buffer	BD Biosciences, Franklin Lakes, New Jersey, USA	566349
DAPI	Novus Biologicals, Toronto, Ontario, USA	NBP2311561
Mouse BD Fc Block	BD Biosciences, Franklin Lakes, New Jersey, USA	553141
Newport Green DCF	Thermo Fisher Scientific, Waltham, Massachusetts, USA	N7991

5.3.1. Animal studies

5.3.1.1 Animal ethics approval and mouse strains

All mice work was approved by the Western Sydney Local Health District Animal Ethics Committee (Protocol number 4349.06.21).

Mice were obtained from Animal Resources Centre (ARC), Perth, WA, Australia. NOD/ShiLtJ (NOD) mice were used as model mice for type 1 diabetes, checkpoint inhibitor induced diabetes, and streptozotocin induced diabetes. NOD-scid mice were used as recipients for NOD splenocytes for adoptive transfer of autoimmune diabetes. NOD-scid mice are NOD mice in genetic background but also homozygous for the severe combined immunodeficiency mutation ($Prkdc^{scid}$) and thus have defects in innate and adaptive immunity and do not have functional T or B cells[81]. Mice were issued at 4-6 weeks of age and acclimatised for 1 week prior to any experiment. Diabetes was defined as two consecutive BGL readings ≧ 15mmol/L.

5.3.1.2. Animal numbers and experimental design

NOD mice:

The target number of mice per group was 8, with additional mice used for pilot experiments which established the CIADM drug efficacy and streptozotocin dosing. Female NOD mice were used for a higher incidence of spontaneous diabetes.

- 8 mice were administered anti-PDL1 for the CIADM group. Mice were culled on development of diabetes or monitored for up to 8 weeks post drug completion with glucose tolerance testing (GTT) prior to cull
- 8 mice were administered streptozotocin for the streptozotocin induced diabetes group. Mice were culled on development of diabetes or monitored for up to 8 weeks post drug completion with GTT prior to cull

- 28 NOD mice were not treated with any medication. 4 mice were culled at <10 weeks for a young control cohort, the remainder were aged and monitored for spontaneous autoimmune diabetes. Mice that developed diabetes (n=18) were culled and added to the 'type 1 diabetes' cohort. Mice that did not develop diabetes by 12 months (n=6) were culled and added to the 'aged control' cohort.

NOD-SCID mice:

NOD-SCID mice were used as recipients of adoptive transfers, with a minimum of $5x10^6$ splenocytes transferred to each mouse.

- 5 mice received splenocytes from 6 week old non-diabetic NOD mice
- 6 mice received splenocytes from 9 week old non-diabetic NOD mice
- 6 mice received splenocytes from 12 week old non-diabetic NOD mice
- 20 mice received splenocytes from NOD mice that developed CIADM
    - 4 recipient mice were pretreated with anti-PDL1 prior to transfer of splenocytes and then monitored for 2 months prior to further anti-PDL1 treatment
    - 4 mice were treated with anti-PDL1 3 months after transfer of splenocytes only
    - 12 mice received CIADM splenocytes without additional anti-PDL1 therapy
- 12 mice received adoptive transfer of subsets of sorted CIADM splenocytes from NOD mice that developed CIADM
    - 3 mice received CD8+ cells
    - 4 mice received CD4+ cells
    - 5 mice received B220+ cells
- 2 mice received anti-PDL1 therapy only with no adoptive transfer

- 4 mice received no anti-PDL1 therapy and no adoptive transfer

All mice were monitored at least twice weekly post adoptive transfer for development of diabetes. Mice were monitored up to 3 months post adoptive transfer and culled at time of confirmation of diabetes or at 3 months post adoptive transfer if they did not develop diabetes.

5.3.1.3. Animal housing and routine mouse care

All mice were housed in the Westmead Bioresources Facility (WBF) in the Westmead Institute for Medical Research. NOD-SCID mice were housed in the immunocompromised mouse room with sterilised cages, a closed and filtered air circuit, autoclaved bedding, food and water. NOD mice were housed in the standard mouse room. Mice were housed up to 4 per cage. Bedding was changed weekly.

Routine mouse care included minimum weekly visual check, weight and tail nick blood glucose measurement using a Freestyle Lite glucometer (Abbott Diabetes Care Ltd, UK) for those that had risk of diabetes (i.e. NOD mice, NOD-scid post adoptive transfer of splenocytes). Mice that developed diabetes were then monitored daily and culled within 72hrs. Long-acting insulin (Levemir diluted in PBS with 1% albumin) was administered once daily at a dose of 0.1units/kg via intraperitoneal (IP) injection if BGL was over 20mmol/L.

5.3.1.4. Glucose tolerance testing

Glucose tolerance testing (GTT) was performed on mice that did not have overt diabetes at the end of the protocol, prior to sacrifice. Mice were fasted for 5 hours. Mice were weighed and baseline BGL was measured, followed by administration of 2g/kg of glucose

as 20% dextrose via IP injection. Blood glucose levels were re-measured via tail nick using a glucometer at 15, 30, 60, 90, and 120 minutes after dextrose injection.

5.3.1.5. Anti-PD1/PDL1 administration

Anti-PD1 and anti-PDL1 drugs were administered via intraperitoneal injection to NOD mice for induction of checkpoint inhibitor associated autoimmune diabetes as previously described [76]. Dosing was 0.25mg in 250µl of manufacturer-specified buffer (InvivoPure pH 7.0 Dilution buffer) given on Day 1, 3, 5 and 7. Weight and glucose measurements were taken on each day prior to injection. If a mouse had developed overt diabetes prior to administration of a planned dose no further doses were given. Mice were subsequently monitored twice weekly with visual check, weight and BGL measurement until cull at diabetes or 3 months.

5.3.1.6. Streptozotocin dosing and administration

Streptozotocin (STZ) is a beta cell toxin commonly used in literature to induce beta cell apoptosis and emulate insulin deficient diabetes. The compound is an analogue of glucose, and uses GLUT2 transporters specifically to enter cells, leading to poly-ADP ribosylation, DNA alkylation and subsequent cell death. GLUT2 is highly expressed in beta cells, therefore, streptozotocin is an effective agent for inducing diabetes. GLUT2 is also expressed in hepatocytes and some renal cells, which is responsible for the hepatotoxicity and renal toxicity seen at high doses of streptozotocin. Streptozotocin was administered to NOD mice via IP injection over 5 consecutive days as previously published [82,83]. Doses of 20mg/kg, 30mg/kg and 40mg/kg were compared to assess for optimal induction of diabetes.

Streptozotocin was dissolved in acetate buffer and filtered through a 20µm syringe filter. The required aliquot then diluted in 250ul 0.9% saline and administered via IP injection. If a mouse had developed overt diabetes prior to administration of a planned dose no further doses were given. This occurred in one mouse receiving 40mg/kg streptozotocin.

5.3.1.7 Termination and tissue harvest

Prior to cull mice were weighed and Ketamine and Xylazine was administered. Fresh stool samples were collected during cull and stored on dry ice prior to transfer to -80°C. Mice were exsanguinated via cardiac puncture and blood was collected in EDTA for further analysis. Two mice from each group had pancreata collected, weighted and fixed in 10% formalin for histology. The remainder of mice from each group underwent islet isolation. Whole spleen from each mouse was collected in PBS for splenocyte cryopreservation.

5.3.1.8. Islet isolation and processing

Prior to islet isolation the required aliquot of Liberase (0.625mg per pancreas) was dissolved in water (0.25ml per pancreas). M199 with 10% fetal calf serum (FCS) was then added to make up a final volume of 3ml per pancreas for perfusion.

After anaesthesia was achieved with Ketamine and Xylazine, cardiac puncture was completed, and laparotomy was performed to expose the stomach, pancreas and common bile duct. Clamps were placed at the superior origin of the common bile duct arising from the liver and at the gastroduodenal bulb within the duodenum. The upper end of the common bile duct was then cannulated with a 30G needle and 3ml of perfusion media was gradually administered via syringe. The perfused pancreas was resected and placed in a 50ml Falcon tube on ice prior to further processing.

The perfused pancreas was then placed in a 37°C water bath for 11 minutes. M199 with FCS was added to wash, and then the tubes were shaken, vortexed and centrifuged. This step was repeated twice prior to filtering the mixture through a metal sieve to remove undigested tissue. The filtrate was then spun down, decanted and then resuspended in Ficoll. After vortexing, further Ficoll was added and then M199 media is layered slowly over the top. The mixture was then centrifuged again with slow acceleration and deceleration for islet isolation. After spinning, the pellet was discarded and the supernatant is topped up with further M199 with FCS and washed twice more. Finally the pellet after the final centrifuge was resuspended in 5ml of M199 with FCS and islets are assessed visually for quality, patency of structure and counted. The islet mixture was then spun down again, resuspended in 5ml of Trypsin with EDTA at 37°C. The mixture was passed through a 21G needle and syringe 5 times for mechanical and enzymatic dissociation into a single cell mixture. The suspension was then spun down, washed and resuspended in freezing media. The islets were then aliquoted into cryovials and stored in a Mr Frosty Freezing container at -80°C prior to transfer to liquid nitrogen for long term storage.

5.3.1.9. Splenocyte cryopreservation

Spleens were passed through a 70µm filter using a sterile 2ml syringe to macerate the tissue and washed with PBS. The suspension was then passed through a 40µm filter and then spun down to a pellet. The pellet was resuspended in ACK Lysis buffer and stored in the dark on ice for 20 minutes for red cell lysis. The suspension was then spun down, resuspended in PBS for cell counting and then spun down to a pellet. The pellet was resuspended in freezing media and aliquoted into cryovials for transfer to a Mr Frosty Freezing container at -80°C and then liquid nitrogen.

5.3.1.10. Adoptive transfers

Cryopreserved splenocytes were thawed in a 37°C water bath and resuspended in warm RPMI-1640 media in 10% fetal calf serum. Cells were washed twice prior to resuspending in normal saline at a final concentration of 10^8 cells/ml for adoptive transfer.

Adoptive transfers were conducted via tail vein injection. Mice were anaesthetised under isofluorane with 5% induction and 2% isofluorane maintenance in oxygen using a Bains paediatric Y circuit. Anaesthesia depth was confirmed with footpad reflex testing. Once fully anaesthetised the tail region was cleaned with alcohol and approximately 10^7 cells in suspension were injected into the tail vein using a Hamilton syringe. Mice were then transferred to their regular cage and observed for recovery.

5.3.2. Histology

Pancreas was fixed in 10% formalin for 24-48hrs prior to transfer to ethanol and embedding in wax. Sections were cut at 4µm thickness and fixed to Ultraplus glass slides. For purposes of alpha and beta cell quantification, 5 consecutive sections were fixed out of every 20 sections and this process was continued until the entire pancreas had been cut. Slides were baked at 60°C for 2 hours. Prior to any staining, slides were dewaxed and rehydrated using xylene for 3 x 3 minutes, 100% ethanol for 3 x 3 minutes and then 70% ethanol for 1 minute followed by tap water for 2 minutes.

5.3.2.1. Haematoxylin and eosin staining

Slides were submerged in Haematoxylin for 60s, then placed under warm running tap water for 1 minute. They were then dipped in acid alcohol, placed back under running

water for 1 minute, 70% ethanol for 1 minute and 95% ethanol for 1 minute. Slides were then dipped in eosin twice and then placed in 100% ethanol for 1 minute, and 100% ethanol again for 1 minute and then 95% ethanol. They were air dried and then placed into xylene for 3 x 5 minutes prior to application of mounting media and sealed using a coverslip.

5.3.2.2. Insulin immunostaining

After dewaxing and rehydrating as described above, a hydrophobic barrier was drawn around each section using a pap pen (Daido Sangyo, Tokyo, Japan). 0.3% Hydrogen peroxide (DAKO, USA) was applied to each section for 5 minutes and then the slide was washed in PBST for 2 x 2 minutes. Sections were then blocked for 15 minutes with 2% BSA in PBS and then excess was tapped off.

Sections were then covered with primary antibody (rabbit anti-insulin antibody, Cell Signalling, 1:100 dilution with DAKO antibody diluent) and incubated at room temperature for 1 hour. After washing in PBST 2 x 2 minutes, secondary antibody was then applied (anti-rabbit IgG labelled polymer-HRP, DAKO Cytomation En Vision+ System Kit) and slides were incubated at room temperature for 1 hour. Slides were then washed in PBST for 2 x 2 minutes again and then DAB was applied for 10 minutes (DAKO Cytomation En Vision+ System Kit). Slides were then washed with running water for 2 minutes, prior to counterstaining with haematoxylin and mounting and sealed with a coverslip as described above.

5.3.2.2. Glucagon immunostaining

The protocol for glucagon immunohistochemistry is analogous to that for insulin, however uses a rabbit anti-glucagon (Cell Signalling) primary antibody at 1:200 concentration with

antibody diluent. Slides were incubated with primary for 1hr at room temperature and then washed 2x 2 minutes in PBST. Secondary antibody (anti-rabbit IgG labelled polymer-HRP, DAKO Cytomation En Vision+ System Kit) was applied and slides were incubated at room temperature for 1 hour. Slides were then washed, incubated with DAB, counterstained and sealed with a coverslip as previously described.

5.3.2.3. Anti-PDL1 immunostaining

Anti-PDL1 staining was performed via immunohistochemistry similar to insulin and glucagon. Slides were dewaxed and rehydrated as above. Antigen retrieval was performed in a Biocare NxGen Decloaking Chamber at 95°C for 20 minutes in Tris-EDTA antigen retrieval buffer. Slides were cooled in buffer under running water for 2 minutes prior to washing in PBST for 2 minutes. A Pap pen was used to draw a hydrophobic barrier around each section and 0.3% Hydrogen peroxide (DAKO, USA) was applied for 5 minutes. Slides were washed in PBST for 2 x 2 minutes and then DAKO Protein Block was applied for 30 minutes. After tapping off excess protein block, anti-PDL1 antibody (rabbit anti-mouse PDL1) diluted 1:200 in DAKO antibody diluent was added to each section. Slides were kept in a humidified slide box in 4°C refrigerator for 16 hours.

Slides were washed in PBST 2 x 2 minutes and the secondary anti-rabbit HRP (anti-rabbit IgG labelled polymer-HRP, DAKO Cytomation En Vision+ System Kit) was applied for 1 hour. They were then washed, incubated with DAB for 1 minute, counterstained and sealed with a coverslip.

5.3.2.4. Anti-Foxp3 immunostaining

Pancreatic sections were stained for Foxp3 and detected by immunofluorescence microscopy. Slides were first dewaxed and rehydrated as described above. They were then

incubated in a Biocare NxGen decloaking chamber immersed in DAKO pH 9 antigen retrieval buffer at 95°C for 30 minutes. The chamber containing the slides was cooled under running water for 2 minutes prior to transfer to PBST for 2 minutes. As above, a hydrophobic barrier was drawn around each section using a Pap pen prior to application of 0.3% Hydrogen peroxide (DAKO, USA) for 5 minutes. Slides were then washed twice for 2 minutes in PBST and then DAKO protein block was applied for 30 minutes. After tapping off excess protein block, the primary anti-FoxP3 antibody 1:200 (rat anti-mouse FoxP3) in antibody diluent was applied to the sections, and slides were kept in a humidified slide box in 4°C refrigerator for 16 hours.

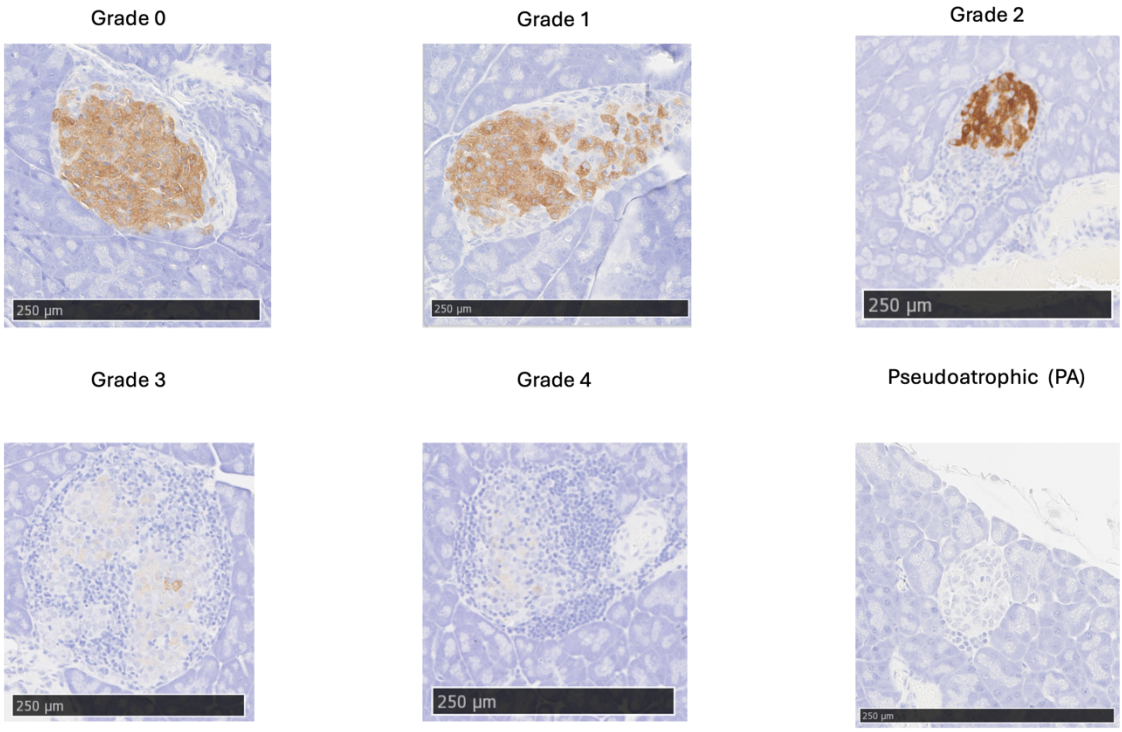
Slides were then washed in PBST 2 x 2 minutes prior to application of anti-insulin 1:100 (guinea pig anti-insulin primary antibody) in antibody diluent for 1 hour whilst protected from light, to assist in islet localisation. Slides are then washed again in PBST 2 x 2 minutes. A secondary antibody cocktail containing anti-rat Cy3 antibody 1:400, anti-guinea pig AF488 antibody 1:400 and DAPI 1:1000 along with antibody diluent was made and applied to the sections and covered for 45 minutes. Slides are then washed 2x2 minutes in PBST, before mounting in aqueous mounting medium and sealed with a coverslip. They were dried in the cover slide box. Slides were imaged on the Olympus BX53 Fluorescence microscope.

5.3.2.5. Insulitis scoring

Pancreatic sections were stained with glucagon via DAB based immunohistochemistry and counterstained with haematoxylin as described above. Glucagon sections were used to identify islets as post insulitis islets may be difficult to recognise in morphology and can lack beta cells. Fifty islets were randomly picked from these slides per pancreas and scored from 0-4 for insulitis (Figure 5.1). Islets were scored as follows: 0 for islets with normal

appearance and no inflammatory infiltrate, 1 for mild mononuclear infiltrate (<25%) of islet at the islet periphery (peri-insulitis), 2 for moderate infiltration (25-50%), 3 for severe infiltration (50-75%) and 4 for destructive insulitis (>75%). Pseudoatrophic (PA) islets were separately quantified. PA islets are those that are devoid of inflammatory infiltrate but also no longer have insulin positive staining represent chronic burnt out diabetes and are well described in the literature pertaining to humans [84].

Figure 5.1. Insulitis grading examples using insulin DAB stained with haematoxylin counterstain on mouse pancreatic sections.



5.3.2.6. Beta and alpha cell mass calculation

Two pancreata were cut per group for quantification. Every 20th section was stained with insulin and the same protocol was conducted for glucagon.

Slides were scanned at 40X magnification using the NDP Nanozoomer Slide Scanner (Hamamatsu). ImageJ (Fiji) Software was used to trace the area of pancreatic tissue and the percentage of insulin or glucagon positive tissue (based off a set DAB+ threshold) was calculated. Insulin positive area was calculated as insulin DAB+/pancreatic section area *100%. Glucagon content was calculated both as % pancreatic section area similar to insulin, but also as a % of the total area of 50 measured islets as alpha cell mass was significantly lower. Glucagon content was calculated as glucagon DAB+/pancreatic section area *100% using the former method and glucagon DAB+/total islet area*100% in the latter.

5.3.3. Flow cytometry

5.3.3.1. Panel set up and titration

Flow Cytometry was performed using the BD Symphony cytometer. Each antibody was tested at 1, 2, 4 and 6ul/10^6 cells on mouse splenocytes to determine the optimal stain providing clearest separation between positive and negative groups. Newport green DCF (NPG) was also used in the panel as a marker for viable beta cells. NPG binds to intracellular zinc, which is found at high concentrations in viable beta cells[85]. NPG+ cells were identified on the AF488 channel. DAPI was included in all experiments as a cellular viability dye.

Table 5.6 Antibodies for flow cytometry

Antigen	Fluorophore	Company	Clone	Catalogue
CD3	BUV737	BD Biosciences, Franklin Lakes, New Jersey, USA	17A2	612803
CD4	R718	BD Biosciences, Franklin Lakes, New Jersey, USA	GK1.5	567311
CD8	BV650	BD Biosciences, Franklin Lakes, New Jersey, USA	53-6.7	563234
CD335 (NKp46)	APCCy7	Biolegend, San Diego, California, USA	29A1.4	137645
CD25	PECY7	BD Biosciences, Franklin Lakes, New Jersey, USA	PE61	557741
CD69	APC	Biolegend, San Diego, California, USA	H1.2F3	104513
CD103	BV421	BD Biosciences, Franklin Lakes, New Jersey, USA	M290	562771
CD45	BUV395	BD Biosciences, Franklin Lakes, New Jersey, USA	30-F11	565967
CD44	BV750	BD Biosciences, Franklin Lakes, New Jersey, USA	IM7	747255
CD45R B220	PECF594	BD Biosciences, Franklin Lakes, New Jersey, USA	RA3-6B2	562313
F4/80	BV480	BD Biosciences, Franklin Lakes, New Jersey, USA	T45-2342	565635
CD11b	BB700	BD Biosciences, Franklin Lakes, New Jersey, USA	M1/70	566416
Ly6g	PECy5	eBioscience, San Diego, California, USA	1A8	15-9668-82
CD274 (PD-L1)	PE	BD Biosciences, Franklin Lakes, New Jersey, USA	MIH5	558091

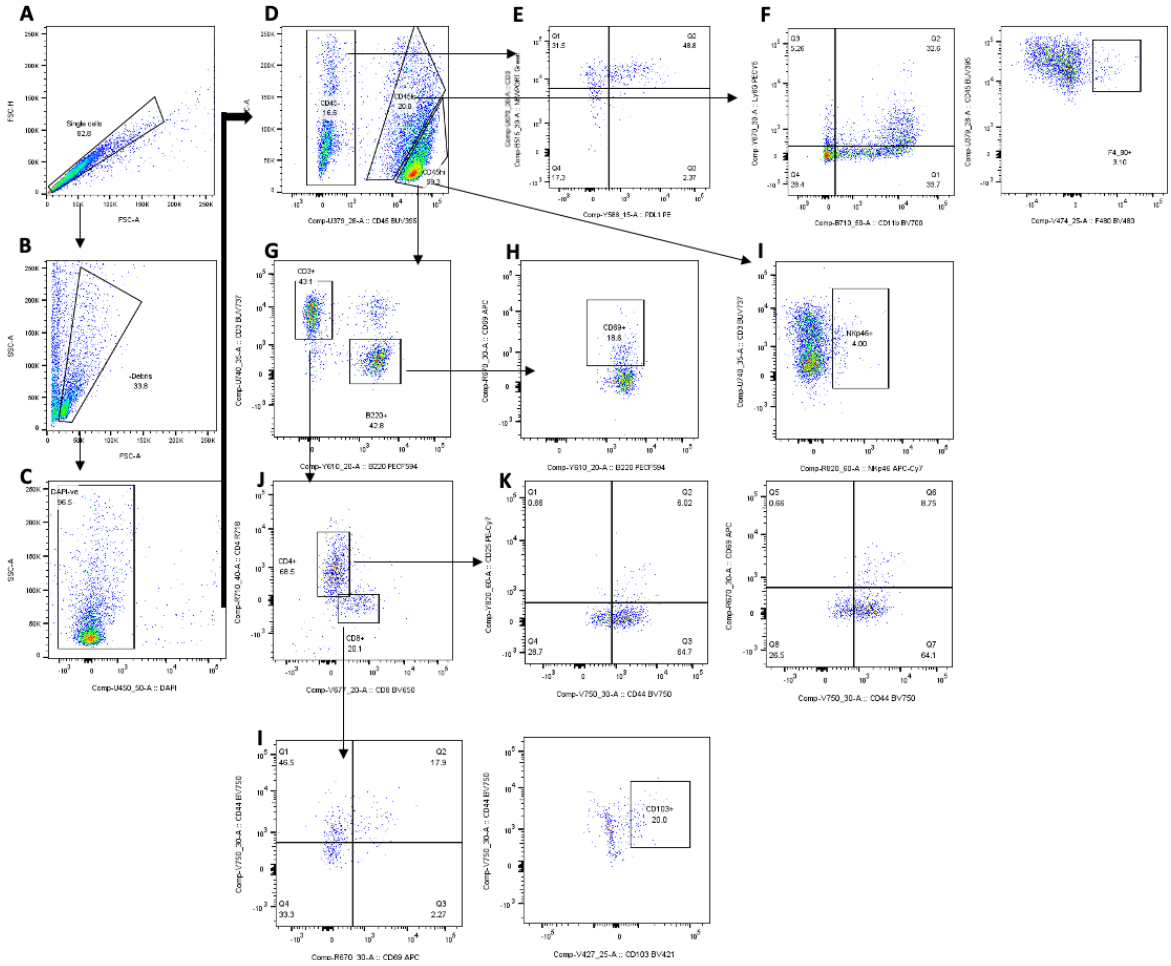
5.3.3.2. Flow cytometry protocol

Islets and associated infiltrate were dispersed into a single cell suspension as described above. The suspension was filtered through a 35um cell strainer into a round bottom 5ml tube (FACS tube). Cells were washed with FACS buffer twice, spun down and supernatant decanted. 50ul Newport green (10ul of 1mM stock solution in 1ml FACS buffer to make 10uM working stock) was added to each tube before covering with parafilm to avoid evaporation and incubating at 37°C for 20 minutes. Cells were washed in FACS buffer and then incubated in the dark on ice for 10min with rat anti-mouse Fc Block (CD16/32; 2ul per 10^6 cells) to reduce nonspecific binding. 50ul of Brilliant Stain buffer was added followed by the antibody cocktail. The suspension was mixed gently and then placed on ice in the dark for 15 minutes to incubate. The cells were then washed twice with FACS buffer prior to analysis.

5.3.3.3. Flow cytometry analysis

Flow samples were run on the BD Symphony at medium flow rates, with fluorochrome compensation performed on the day of analysis and standard CST settings in place. A minimum of 30,000 events were analysed per sample. Gating strategy was used as shown in Figure 5.2. All concentrations and voltages were maintained as constant throughout the experiments. Data analysis was performed using FACSDiva (BD Biosciences) and FlowJo (BD Biosciences) and graphed using GraphPad Prism 10.0 (San Diego, California, USA).

Figure 5.2. Gating strategy for islet single cell suspension.

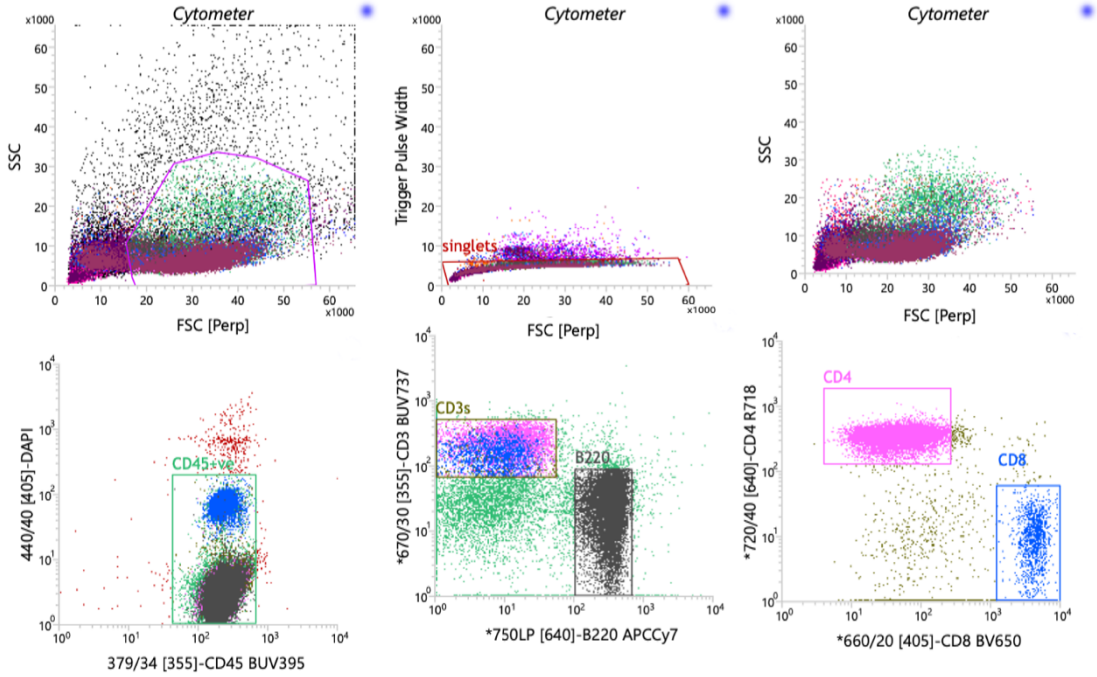


A. Single cells were gated. B. Debris was gated out. C. Live/dead stain gating applied. D. Live cells were gated into CD45-, CD45lo and CD45hi. E. CD45- cells were gated based on Newport Green for beta cells and PD-L1 expression. F. CD45lo cells were gated for CD11b vs Lg6G to identify neutrophils (left), and F4/80 to identify macrophages (right). G. CD45hi cells were gated to identify CD3+ T cells and B220+ B cells. H. B220+ cells were gated for CD69+ activation status. I. NK cells were identified by gating CD45hi cells for Nkp46+ status. J. CD3+ T cells were gated into CD4+ and CD8+ subsets. K. CD4+ T cells were gated by CD25+, CD44+ (left) and CD69+ (right) status to identify activated and T regulatory cells. L. CD8+ T cells were gated by CD44+, CD69+ (left) and CD103+ (right) status to identify activated and tissue resident memory cells.

5.3.3.4. Cell sort protocol

Cell sort was performed on NOD mouse splenocytes using a subset of antibodies from the flow panel above (CD45+, CD3+, CD4+, CD8+, B220+, DAPI). Staining was performed with a protocol similar to the above. First splenocytes were thawed in a 37°C water bath, and then warm RPMI-1640 media was added. Cells were then washed in FACS buffer twice, prior to incubation with FC block for 10min in the dark on ice. Brilliant stain buffer and the antibody cocktail was then added and the suspension was incubated for 15 min in the dark on ice. The cells were then washed twice more with FACS buffer prior to sorting on the BD Influx. Cells were sorted into CD45+CD3+CD4+, CD45+CD3+CD8+ and CD45+CD3-B220+ subsets as per Figure 5.3. Cells were washed, resuspended in normal saline and adoptively transferred to NOD-scid recipients as described above.

Figure 5.3. Gating strategy utilised for cell sorting of mouse splenocytes.



5.3.4. Assays

Insulin

Mouse serum insulin concentration was determined from 5µl of serum via enzyme linked immunosorbent assay (ELISA) using the CrystalChem Ultrasensitive Insulin ELISA kit according to the manufacturer's instructions. Standard curve (linear fit) and interpolated values were calculated using GraphPad Prism 10.0 (San Diego, California, USA).

Lipase

Mouse pancreatic lipase concentration was determined from 50µl of serum using the Cusabio Mouse Pancreatic Lipase ELISA kit as per manufacturer's instructions. Standard curves (4 parameter logistic fit) and interpolated values were generated using GraphPad Prism 10.0 (San Diego, California, USA).

Faecal fat assessment

Faecal triglycerides were determined as a measure of exocrine function as previously published [86] and as performed in humans. Faecal samples were thawed to room temperature. Each pellet was weighed prior to homogenisation using a Rotor-stator Homogeniser in 200µl of 5% Nonidet P-40 (NP-40); a detergent to keep lipids in solution. 25µl of lysate was then added with 25µl of assay buffer to each sample well of the plate. The assay was then conducted as per manufacturer's protocol and triglyceride concentration was calculated using GraphPad Prism 10.0 (San Diego, California, USA).

5.3.5. Statistical analysis

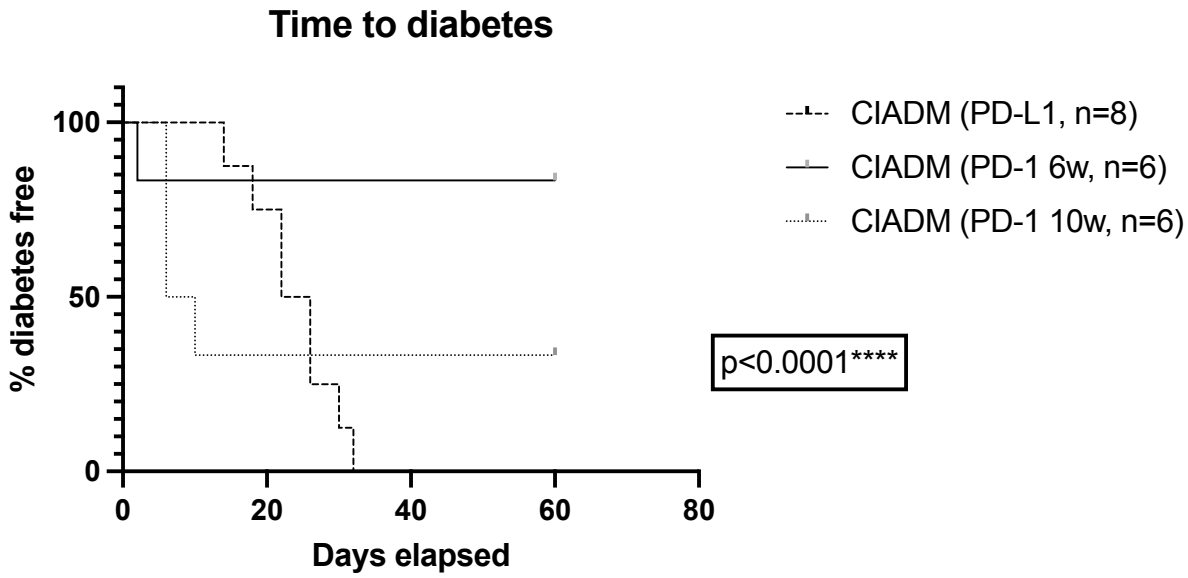
Statistical analyses were conducted using GraphPad Prism 10.0 (San Diego, California, USA). Unpaired T-test and one way ANOVA was used to compare parametric normally distributed variables between groups. P values of <0.05 were considered statistically significant. Post hoc testing for one-way ANOVA was only conducted where the original p value was <0.05.

5.4 Results

5.4.1. Anti-PDL1 administration for development of CIADM

Figure 5.4 depicts the time to onset of diabetes for each mouse group after checkpoint inhibitor administration. Anti-PD1 (J43) administration led to diabetes development in only 1 of 6 (17%) 6 week old female NOD mice and 4 of 6 (67%) 10 week old female NOD mice. In comparison, Anti-PDL1 (RMP1-14) administered to 6 week female NOD mice led to 100% incidence of diabetes with a median time of onset 3 weeks from first injection. It was therefore utilised for the remainder of experiments.

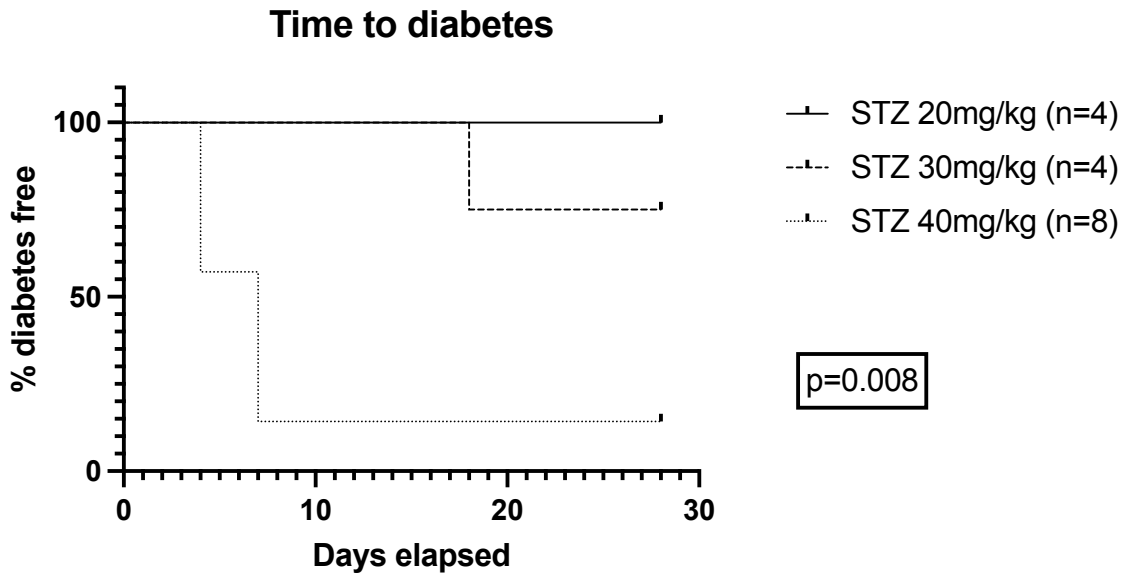
Figure 5.4. Time to diabetes in each group administered checkpoint inhibitors. Different age at administration and different type of checkpoint inhibitor (anti-PD1 vs anti-PDL1) led to significantly different rates of diabetes (Log-rank test, p<0.0001).



5.4.2. Streptozotocin administration for development of diabetes

Low dose streptozotocin (STZ) administered at 20mg/kg over 5 days did not induce diabetes in any of the 4 mice tested. 30mg/kg STZ led to diabetes in 1 of 4 mice (25% incidence) and 40mg/kg STZ led to diabetes in 7 of 8 mice (87.5% incidence). 40mg/kg dosing was therefore utilised for the remainder of experiments as it provided reliable induction of diabetes.

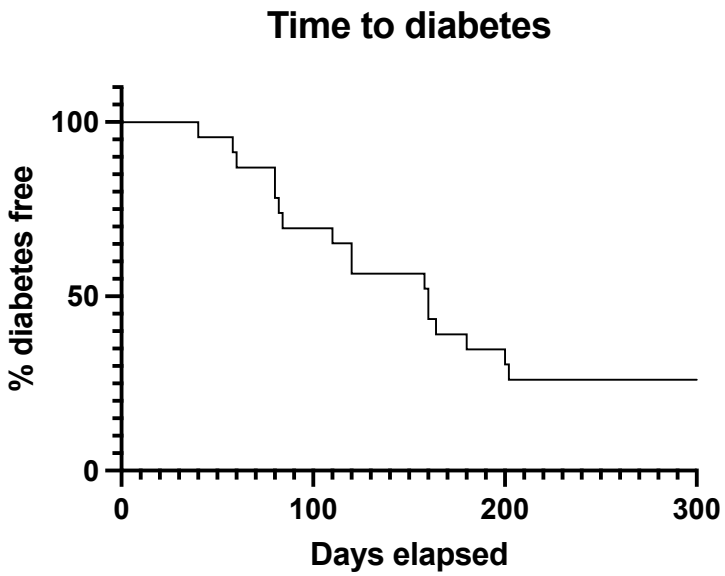
Figure 5.5. Time to diabetes onset with varying doses of repeated low dose STZ. 40mg/kg STZ led to significantly higher rates of diabetes development compared to 20mg/kg or 30mg/kg (Log-rank test, p=0.008).



5.4.3. Incidence of spontaneous type 1 diabetes in NOD mice

Twenty-four female NOD mice were aged and monitored for diabetes. They achieved an overall diabetes incidence of 75% (18 mice) with median onset of spontaneous diabetes at 17 weeks.

Figure 5.6. Time to onset of diabetes in 6 week old NOD female mice (n=24).

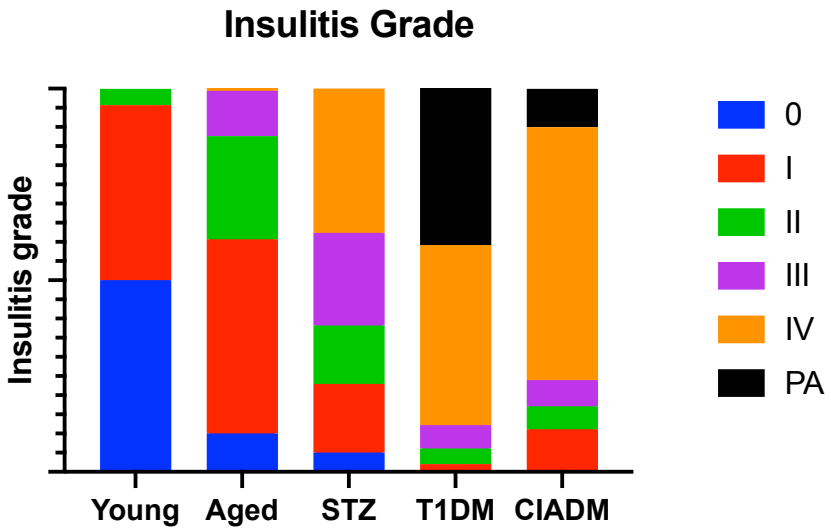


5.4.4. Insulitis

Figure 5.7 depicts insulitis grades across each group studied. Young 6 week NOD female mice had the highest proportions of normal insulin staining islets, with relatively minimal insulitis (45% grade I, 4% grade II, 51% of islets normal). In comparison aged non-diabetic NOD female mice had higher proportions of active insulitis with grade I (51%), II (27%) and III (12%) insulitis seen. CIADM and type 1 diabetes cohorts both had very high prevalence of grade IV insulitis (66% and 47% respectively), however the CIADM cohort had lower proportion of pseudoatrophic (PA) islets (10% versus 41% in NOD mice with diabetes). The STZ treated cohort had moderate levels of insulitis across all grades with more grade IV insulitis than aged controls (37%) but not as severe as that which was seen in CIADM or type 1 diabetes. All differences between groups were highly significant ($p<0.0001$, Chi square test).

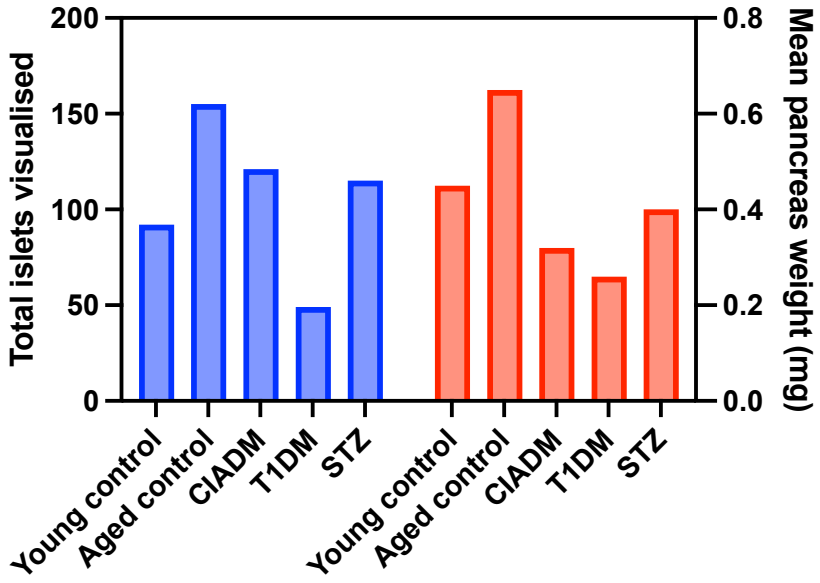
Figure 5.7. Insulitis grading for each cohort.

Islets were scored as follows: 0 for islets with normal appearance and no inflammatory infiltrate, 1 for mild mononuclear infiltrate (<25%) of islet at periphery (peri-insulitis), 2 for moderate infiltration (25-50%), 3 for severe infiltration (50-75%) and 4 for destructive (>75%). Pseudoatrophic (PA) islets are devoid of inflammatory infiltrate and also no longer have any insulin positive staining. All differences between groups were highly significant ($p<0.0001$, Chi square test).



Aged control NOD mice had higher numbers of islets visualised than younger controls (155 and 92 respectively) and had relatively higher pancreatic weights (Figure 5.8, 0.65mg and 0.45mg respectively). STZ mice had fewer islets visualised than aged controls (115 total) and had lower pancreatic weights (0.4mg). CIADM and T1DM cohorts had the lowest pancreatic weights of all cohorts (0.32mg and 0.26mg) respectively, although more islets were visualised in the CIADM than T1DM cohort (121 and 49 islets respectively). The weights and number of islets visualised differed significantly between groups (one way ANOVA, $p=0.002$ and $p=0.003$ respectively).

Figure 5.8. Total number of islets visualised for insulitis grading (left) and mean pancreas weight (right), 2 pancreata per group.

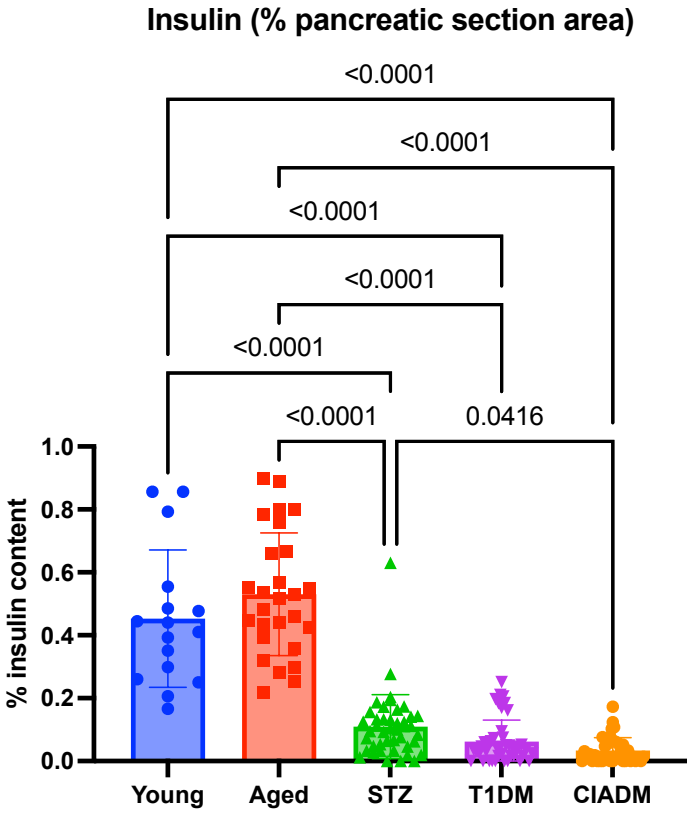


5.4.5. Insulin IHC quantification

Figures 5.9 shows the quantitative analysis of % insulin positive staining cells from immunohistochemistry staining for each cohort.

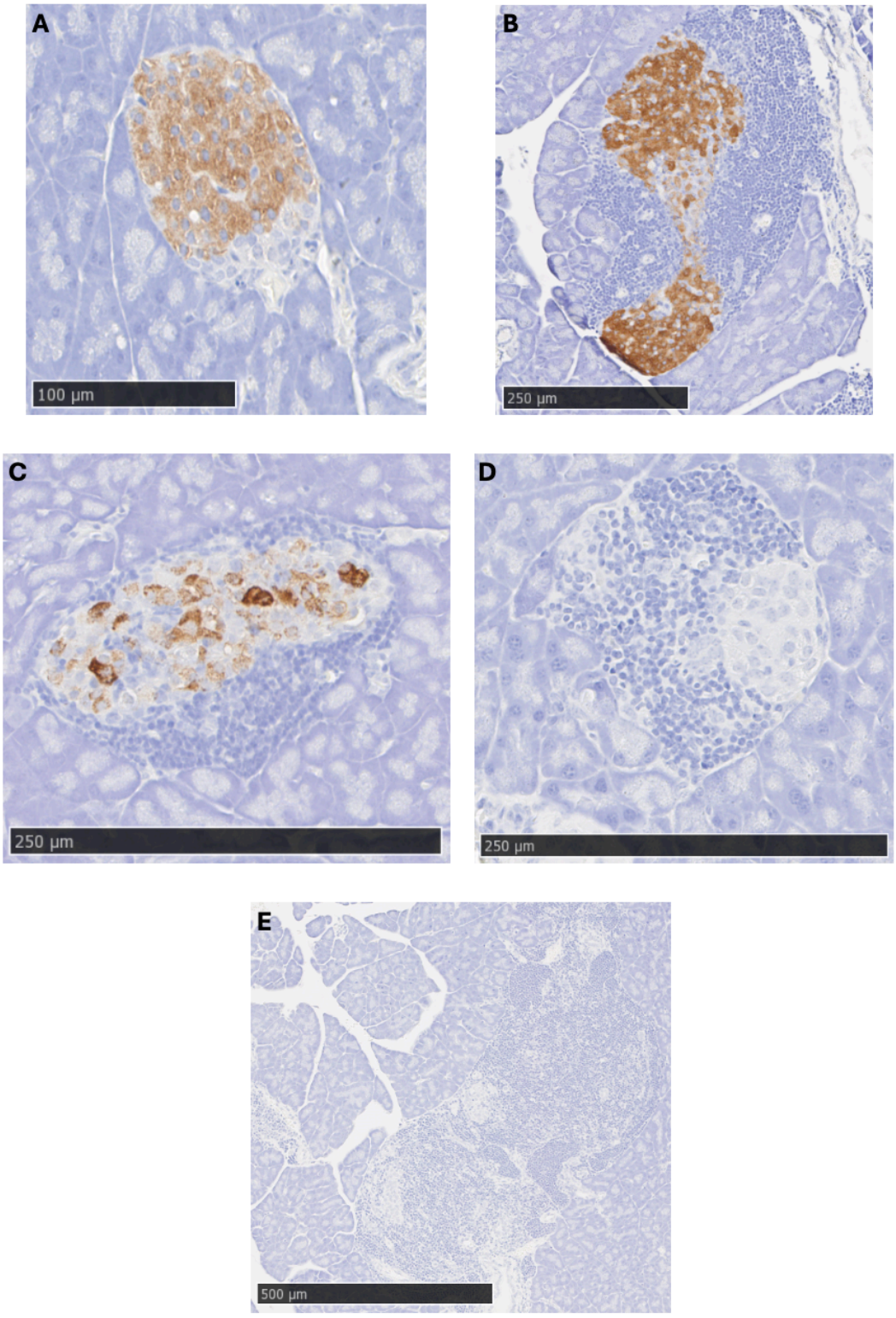
The non-diabetic cohorts (6 week NOD and aged NOD) had significantly higher insulin content than diabetic groups, despite the increased insulitis in the aged controls demonstrated in Figure 5.7 above. The STZ cohort had higher insulin content than CIADM or T1DM groups but lower than non-diabetic groups. The CIADM group had the lowest insulin content of all cohorts.

Figure 5.9. Insulin % content per section by mouse cohort (insulin DAB positive/pancreatic section total area*100). One way ANOVA testing showed significant differences between each group ($p<0.0001$). All significant ($p<0.05$) pairwise comparisons p values are depicted. P values adjusted for multiple comparisons (Tukey's). Error bars represent mean ± SD.



Representative images of insulin staining in islets for each cohort are shown in Figure 5.10. Young (6 week old) NOD mice had strong insulin staining and small islets with limited insulitis. In contrast, aged NOD mice had larger islets with retained insulin staining but had more obvious insulitis. STZ mice had weaker insulin staining suggesting beta cell stress and evidence of insulitis. CIADM mice had florid insulitis with virtually no insulin staining. T1DM mice had few islets and which had little to no insulin staining, with variable insulitis.

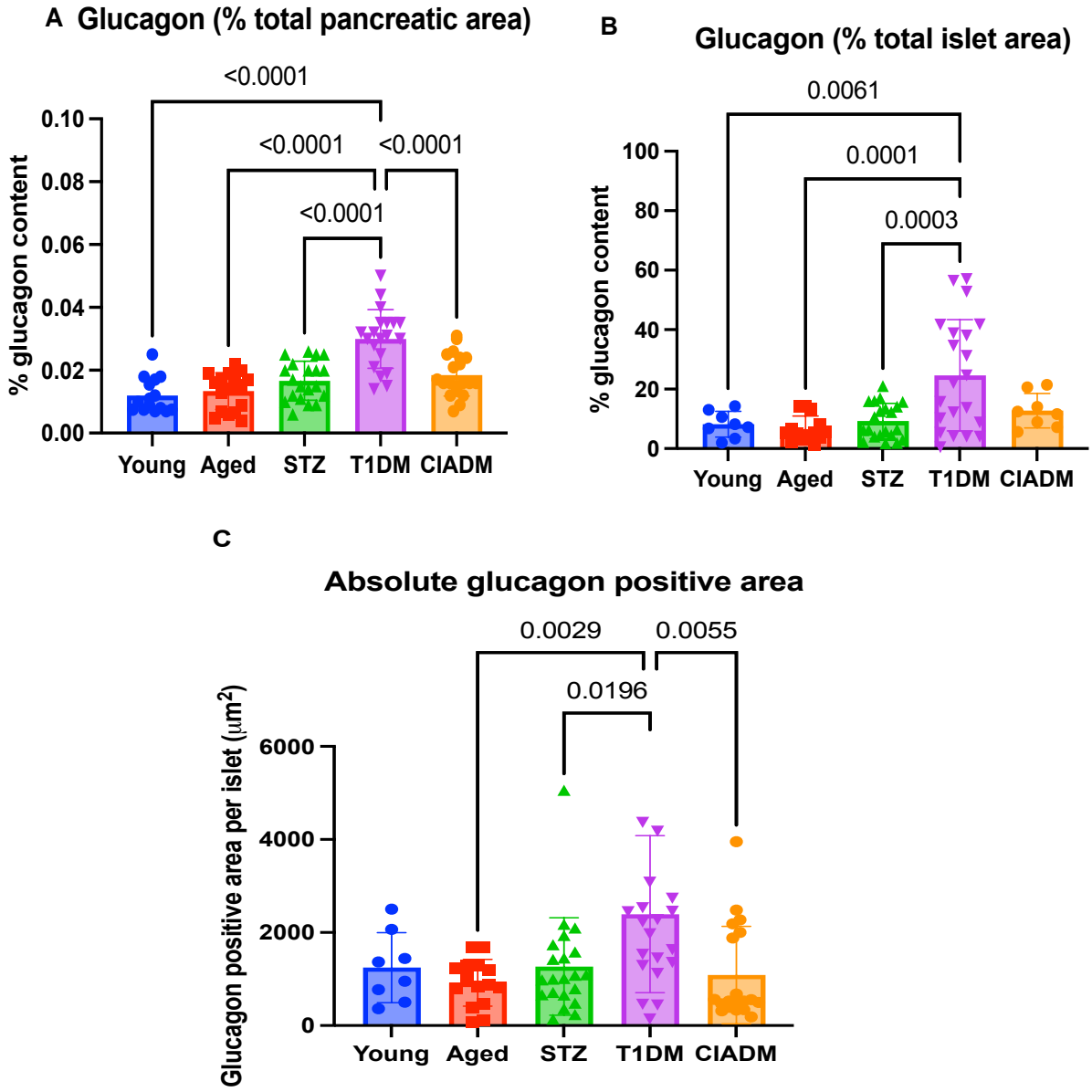
Figure 5.10. Representative images of insulin immunohistochemistry by mouse cohort. A= 6 week NOD mice, B = aged NOD mice, C = STZ induced diabetes NOD mice, D = type 1 diabetes NOD mice, E = CIADM NOD mice.



5.4.6. Glucagon IHC quantification

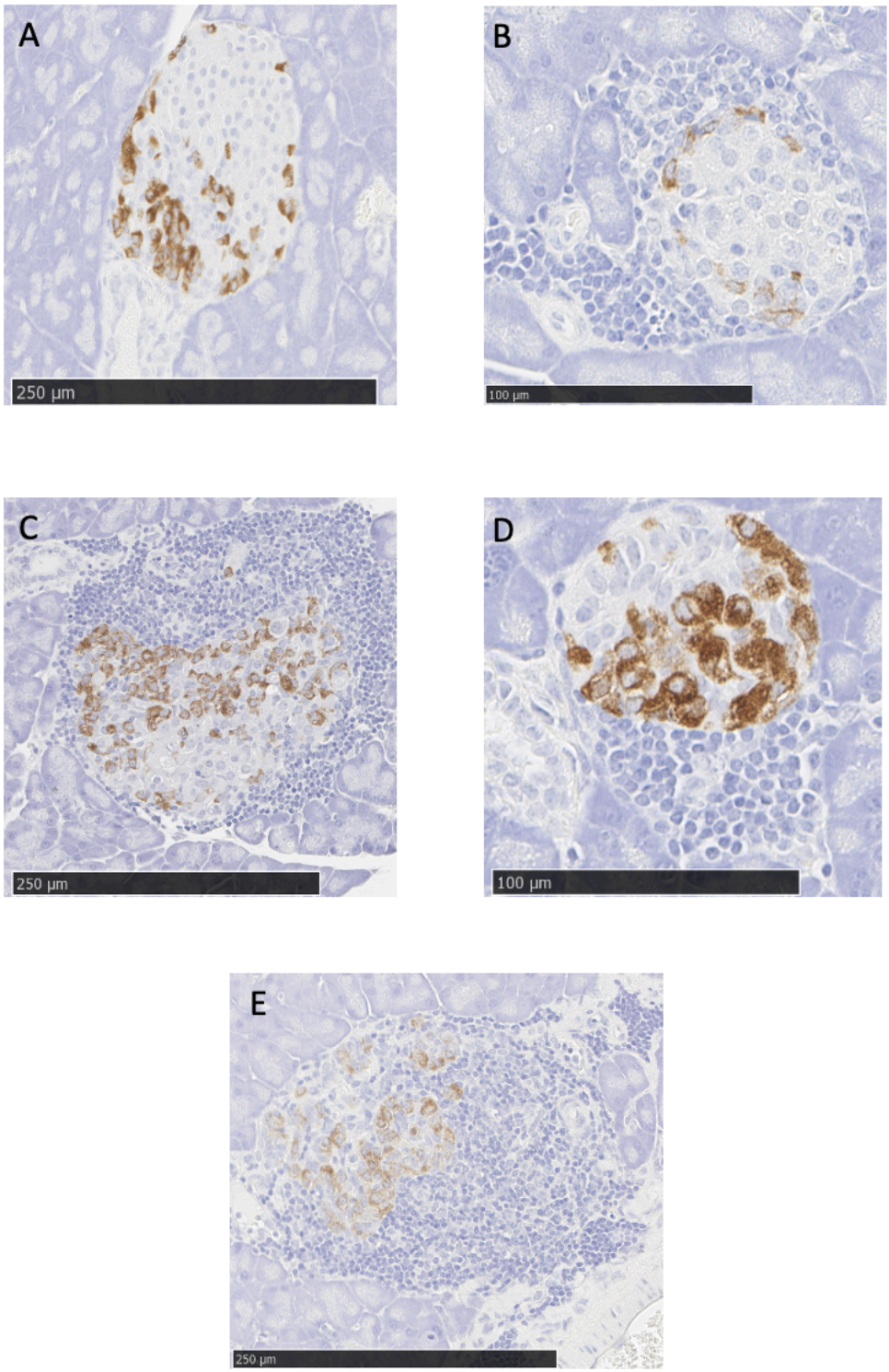
As shown in Figure 5.11, glucagon content as % of pancreatic area was significantly higher in the T1DM group compared to others ($p<0.0001$). As the measured glucagon % was low (range 0-0.05% of pancreatic area) we repeated measurements as a % of total islet area measuring individual islet areas to improve sensitivity. Glucagon content as % of total islet area varied also significantly between groups ($p<0.0001$) and was highest in the T1DM group. Finally we calculated absolute glucagon content per islet which again significantly higher in the T1DM group compared with others ($p=0.001$).

Figure 5.11. A. Glucagon content as % of pancreas area (glucagon DAB positive/total pancreatic area *100). B. Glucagon content as % of total islet area from 50 measured islets per pancreas (glucagon DAB positive/total islet area*100). C. Absolute glucagon positive area per islet. Islet glucagon content was significantly different between groups both using pancreatic area and using total islet area in calculations (One way ANOVA, p<0.0001 and p<0.0001 respectively). Significant pairwise comparisons are as depicted. P values are adjusted for multiple comparisons (Tukey's). Error bars represent mean ± SD.



In Figure 5.12, representative images of glucagon IHC from each group are shown. Young NOD mice (6 weeks old) retained normal glucagon staining and islet alpha cell arrangement with intense DAB staining in peripheral islet cells. This is relatively preserved also in the aged NOD control group. STZ mice and CIADM mice cohort had higher glucagon content however it should be noted that the total islet area for these diabetic mice was lower. T1DM mice had highest % glucagon content, and these islets appeared shrunken consistent with depleted beta cell mass reducing total islet area. Absolute area of glucagon positive staining per islet was therefore calculated to determine if glucagon % is only elevated due to absence of beta cells. Absolute glucagon area was also significantly higher in T1DM mice than other groups.

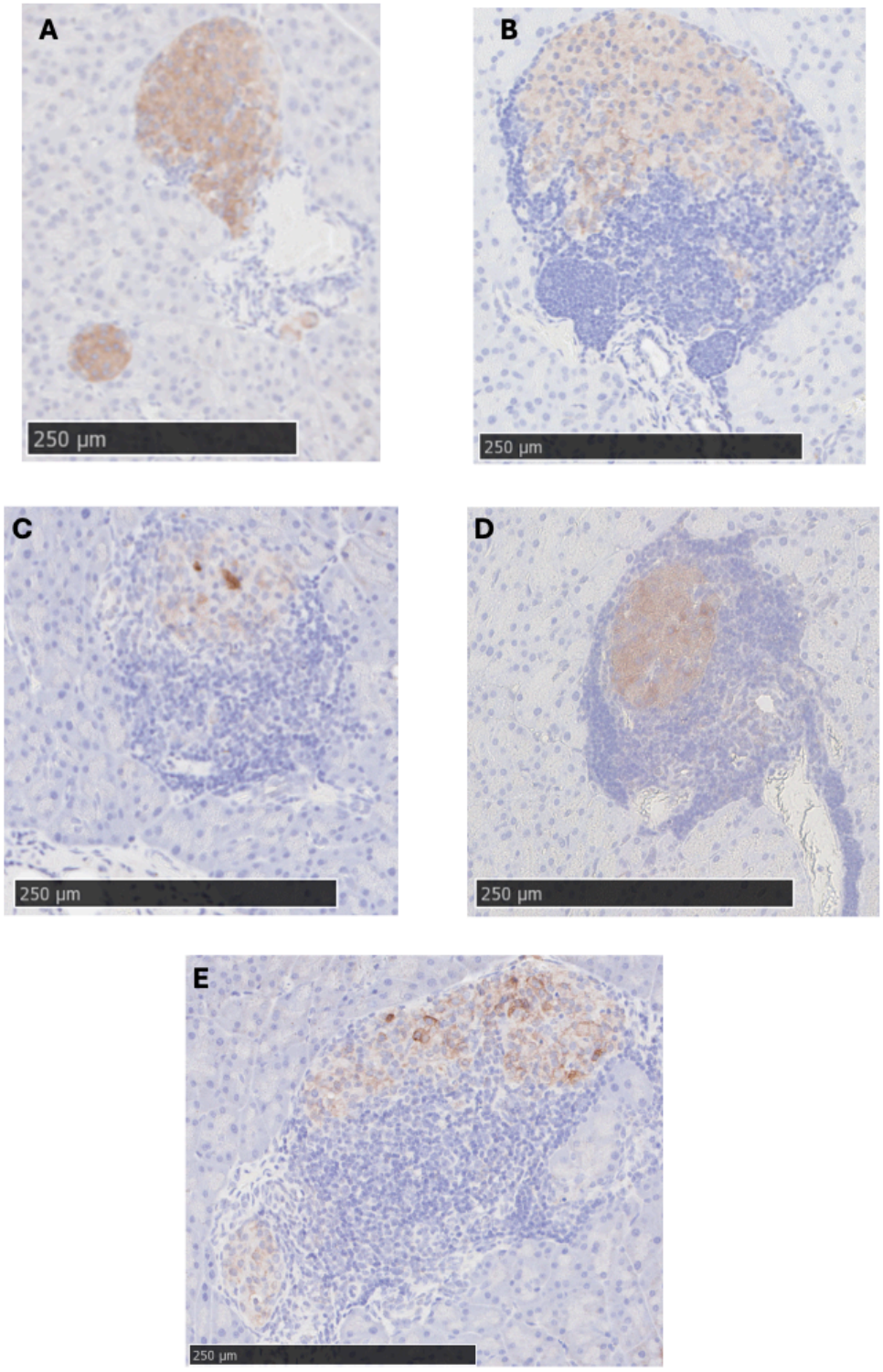
Figure 5.12. Representative images of glucagon IHC. A= 6 week NOD mice, B = aged NOD mice, C = STZ induced diabetes NOD mice, D= type 1 diabetes NOD mice, E= CIADM NOD mice.



5.4.7. PD-L1 IHC

Islet images after staining for PD-L1 are shown in Figure 5.13 for each group. All islets had some PD-L1 positive areas, with strong PD-L1 expression even from 6 weeks of age that was retained with age. CIADM mice and T1DM had fewer islets but those that were visualised had high PD-L1 expression in remaining islet cells. Exocrine tissue did not express PD-L1 in any cohort.

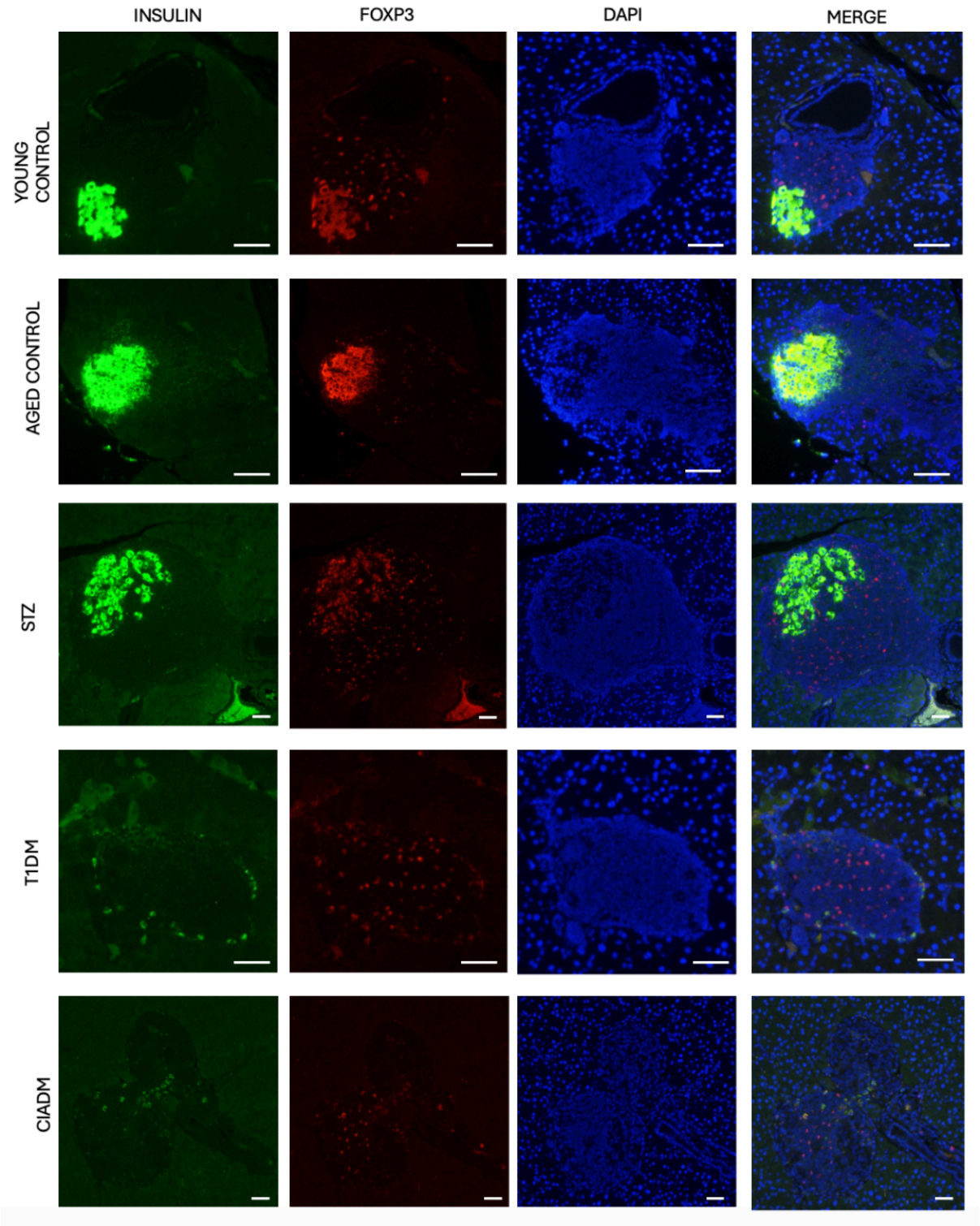
Figure 5.13. Representative images of PD-L1 IHC. A= 6 week NOD mice, B = aged NOD mice, C = STZ induced diabetes NOD mice, D = type 1 diabetes NOD mice, E = CIADM NOD mice,



5.4.8. Foxp3 immunofluorescence

Figure 5.14 shows Foxp3 with insulin and DAPI staining via immunofluorescence (IF) for each mouse cohort. Foxp3 positive cells were evident in the inflammatory infiltrate in all islets affected by insulitis across each cohort. Insulin staining via IF was consistent with previous IHC findings.

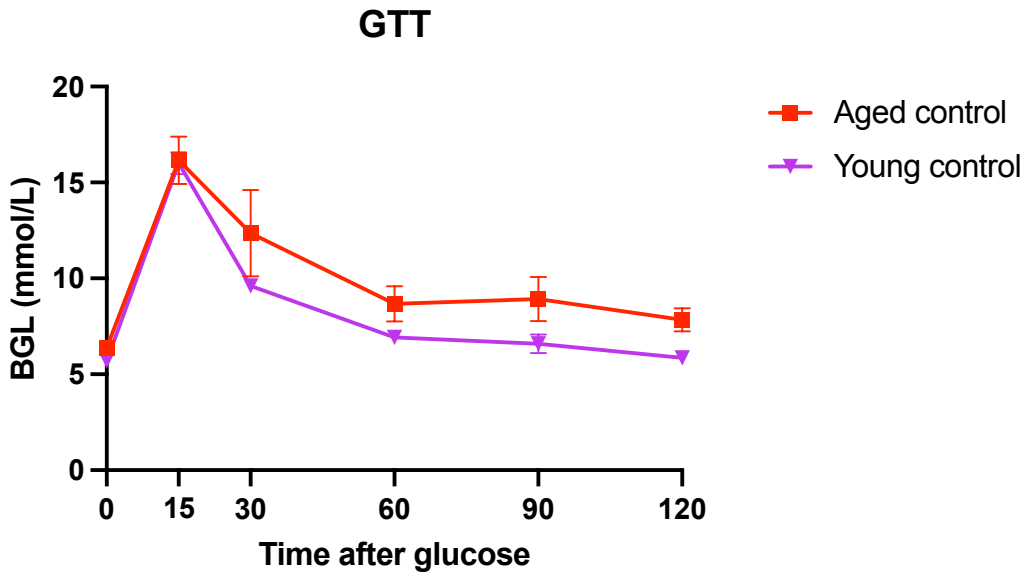
Figure 5.14. Representative images of immunofluorescent staining for insulin (green), Foxp3 (red), DAPI (blue) and merged channels across each mice cohort. Scale bars 50 µm.



5.4.9. Glucose and insulin quantification

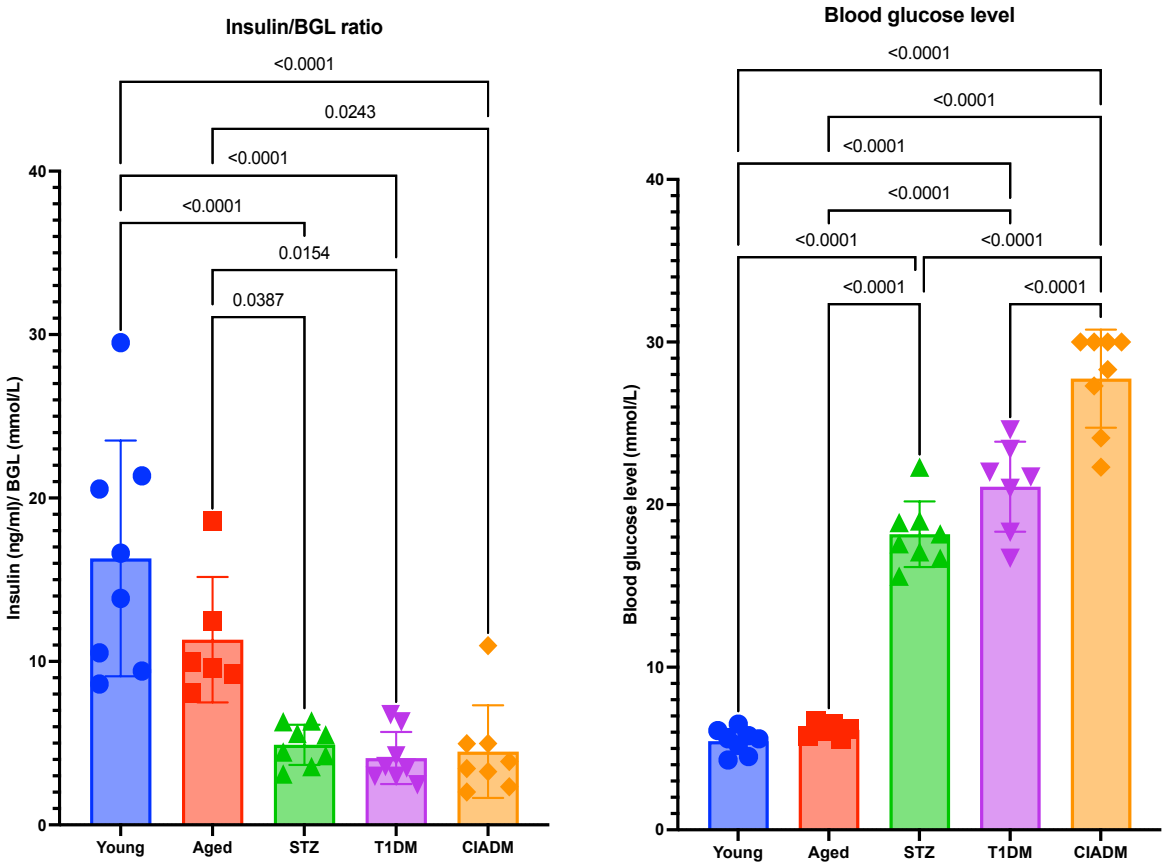
Glucose tolerance tests were performed in mice that did not display overt diabetes prior to cull as shown in Figure 5.15. Aged control mice exhibited slightly impaired glucose tolerance when compared to 6 week control mice with a higher area under the curve however this difference was not significant (p=0.47).

Figure 5.15. Glucose tolerance tests performed prior to cull for non-diabetic mice. N=6 for aged control NOD mice and n=8 for 6 week control NOD mice. Two way repeated measures ANOVA analysis with Bonferroni's test for multiple comparisons was performed. Area under the curve was 1214 in the aged control and 992.3 in the 6 week NOD mice group which was not significantly different between groups (p=0.47). Error bars represent mean ± SEM.



Peak blood glucose level prior to cull was recorded during the GTT. CIADM mice had significantly higher peak glucose levels than the other models, including those with diabetes ($p<0.0001$). Insulin was determined by serum insulin ELISA and expressed as a ratio to serum blood glucose level. Diabetic mice (CIADM, T1DM and STZ groups) had significantly lower insulin to BGL ratios compared to non-diabetic mice, indicating inadequate insulin production to control serum glucose. There were no significant differences in insulin/BGL ratio between different diabetic groups.

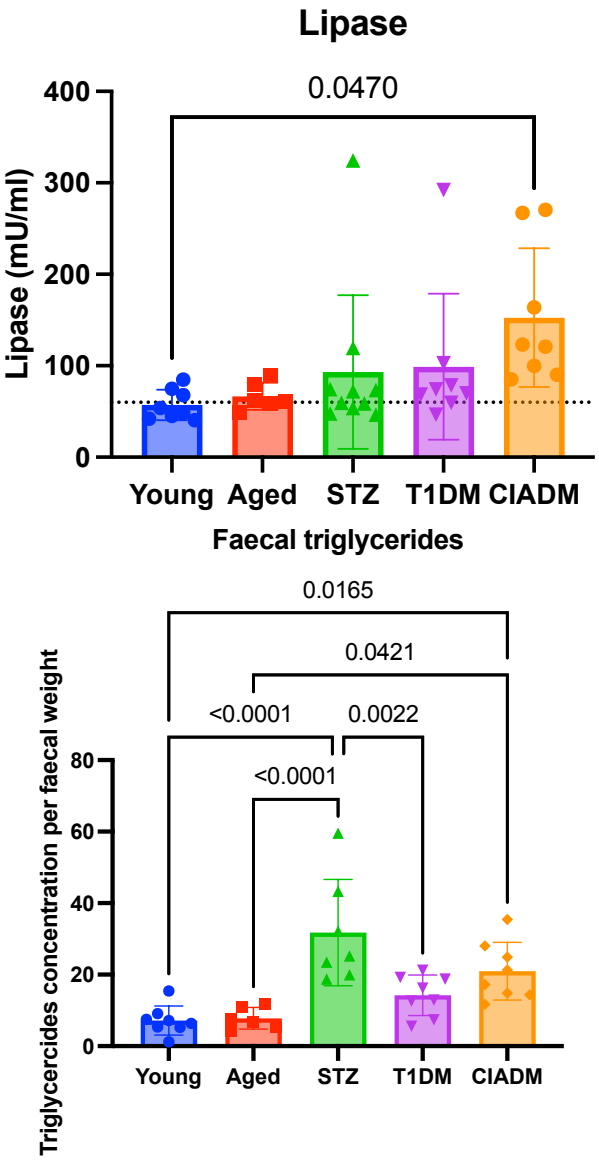
Figure 5.16. Left: Peak blood glucose level recorded by mice cohort. Right: Insulin/blood glucose level (BGL) ratio by mice cohort. One way ANOVA with multiple comparisons (Tukey's test adjusted) analysis performed with all significant p values as depicted. N=8 for all groups except aged control mice (n=6). Column indicates mean and error bars represent SD.



5.4.10. Exocrine function

Assessment of exocrine function was performed using serum lipase to assess for active exocrine pancreatic inflammation and faecal triglyceride levels as a marker of impaired fat absorption. Results are depicted in Figure 5.17. Lipase was significantly higher in the CIADM group compared to young controls, and no other significant differences were noted. Each of the diabetic cohorts had outliers with high levels of lipase despite passing tests for normality (Shapiro-Wilk). Faecal triglycerides were significantly higher in CIADM mice and STZ mice when compared to controls. T1DM mice in comparison did not exhibit significantly different faecal triglycerides to controls.

Figure 5.17. Lipase and faecal triglyceride levels. Dotted line at 60mU/ml for lipase represents cut off for normal serum lipase in mice[87]. There was no statistically significant difference in proportion of mice with elevated lipase between groups (Chi squared test, p=0.07). Faecal triglycerides are corrected for weight of each faecal pellet (µg/µg*100). N=8 for all groups except aged control mice, where n=6. One way ANOVA with correction for multiple comparisons (Tukey's test adjusted) analysis performed with all significant p values as depicted. Error bars represent mean ± SD.



5.4.11. Flow cytometry

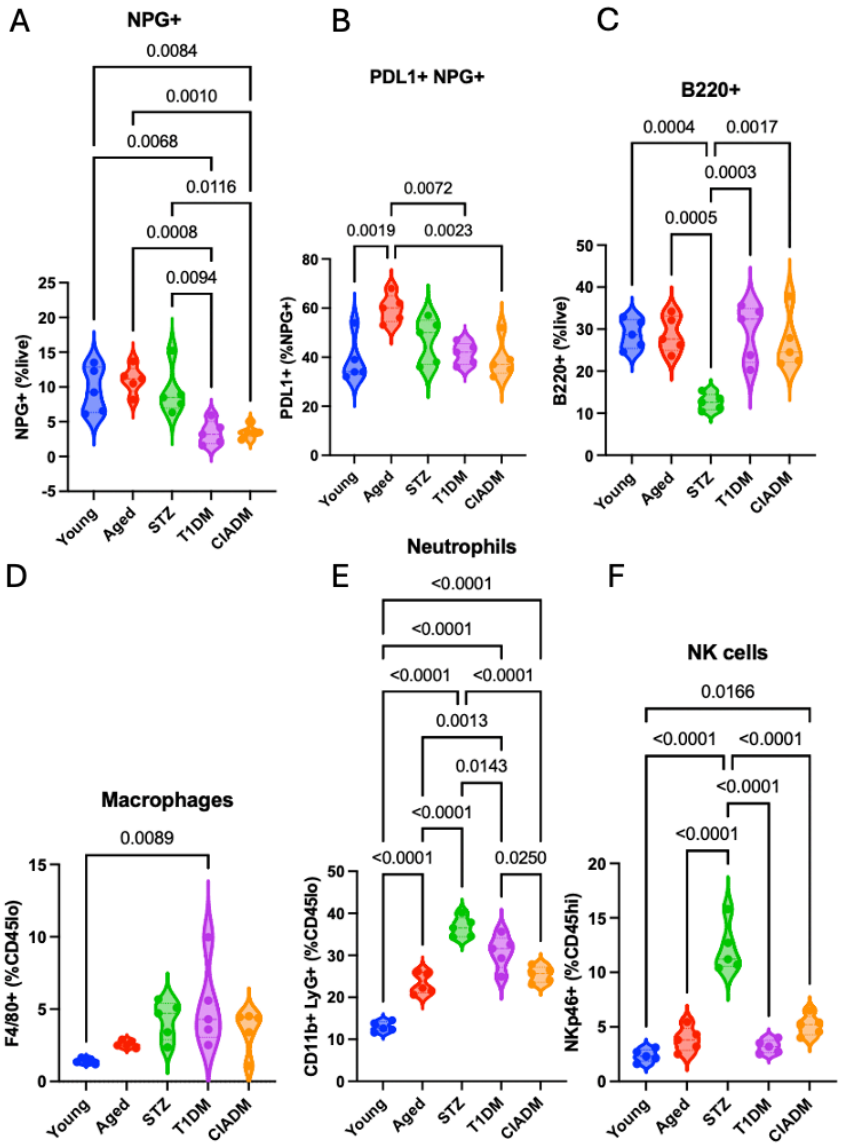
Results from flow cytometry analysis of islet single cell suspensions are shown in Figure 5.18. Newport green positive cells (beta cells) were lower in prevalence in CIADM and T1DM than the NOD control groups, shown in Figure 5.18A. This is also consistent with the beta-cell mass data from Figures 5.9 and 5.10. Surprisingly, there was not a significant difference between the STZ-diabetes mice and the NOD controls.

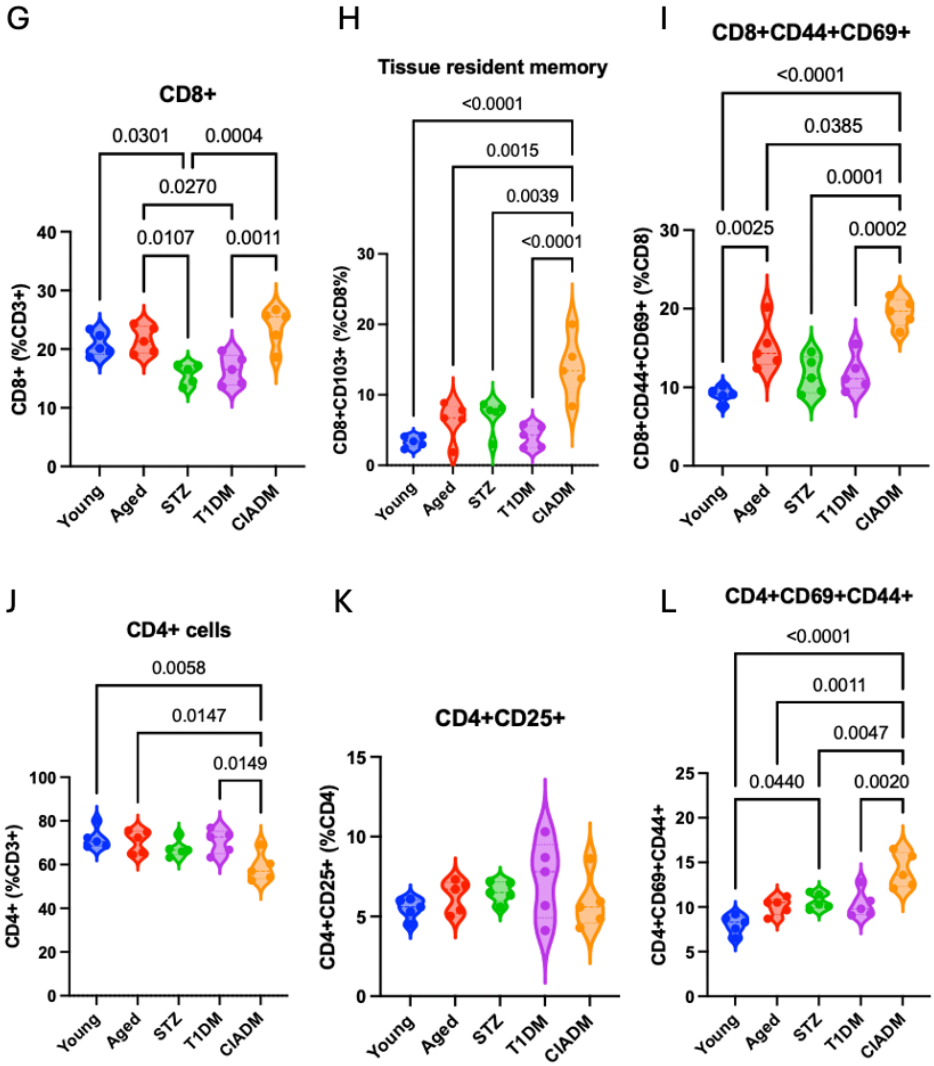
PD-L1 expression in beta cells was significantly more common in the aged NOD controls compared to each of the other groups, except for the STZ mice (Figure 5.18B).

B220+ B cells did not differ between young and aged controls, T1DM and CIADM mice (Figure 5.18C). Unexpectedly, they were significantly less frequent in STZ diabetic mice than each of the other groups.

Figure 5.18G shows CD8+ T cells, which were more prevalent in CIADM mice than T1DM mice ($p=0.001$). Among the CD8+ cells, CIADM mice had significantly higher prevalence of tissue resident memory cells (Figure 5.18H) and activated CD8+CD69+CD44+ cells (Figure 5.18I) than all other groups. CIADM mice also had significantly higher prevalence of activated CD4+CD69+CD44+ cells than other groups (Figure 5.18L).

Figure 5.18. Box violin plots depicting flow cytometry results across each group. N=5 pancreata analysed per group. Row 1 from left to right: A. Newport Green positive beta cells (NPG+), B. PDL1+ beta cells (NPG+), C. B220+ B cells. Row 2 from left to right: D. F4/80+ macrophages, E. CD11b+ Ly6G+ neutrophils, F. NKp46+ NK cells. Row 3 from left to right: G. CD8+ T cells, H. CD8+CD69+CD103+ tissue resident memory cells, I. CD8+CD44+CD69+ activated cells. Row 4 from left to right: J. CD4+ T cells, K. CD4+CD25+ T regulatory cells, L. CD4+CD44+CD69+ activated cells. One way ANOVA with Tukey's test for multiple comparisons performed and all significant p values ($p<0.05$) are depicted.




G
CD8+
0.0301
0.0004
0.0270
0.0107
0.0011
CD8+ (%CD3+)
Young
Aged
STZ
T1DM
CIADM
H
Tissue resident memory
<0.0001
0.0015
0.0039
<0.0001
CD8+CD103+ (%CD8%)
I
CD8+CD44+CD69+
<0.0001
0.0385
0.0001
0.0025
0.0002
CD8+CD44+CD69+ (%CD8)
J
CD4+ cells
0.0058
0.0147
0.0149
CD4+ (%CD3+)
K
CD4+CD25+
CD4+CD25+ (%CD4)
L
CD4+CD69+CD44+
<0.0001
0.0011
0.0047
0.0440
0.0020
CD4+CD69+CD44+

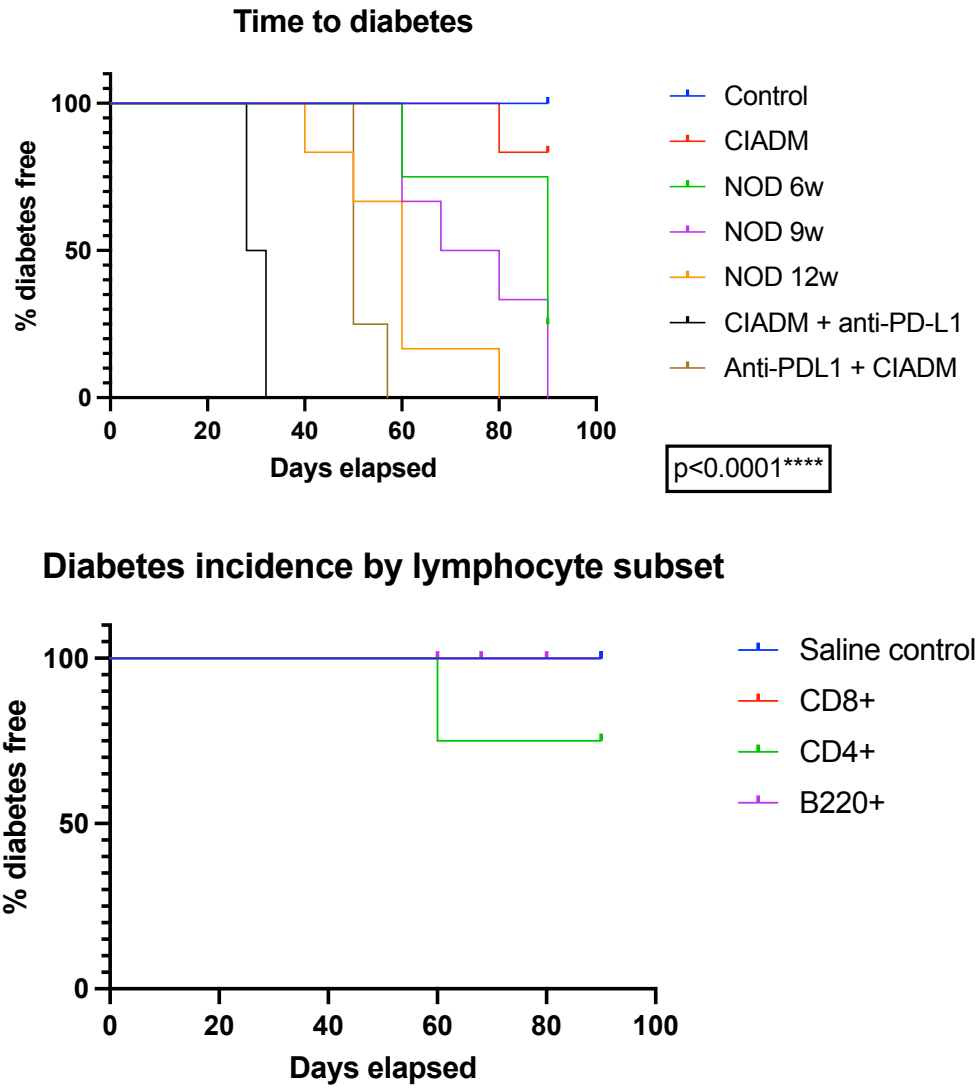
5.4.12. Adoptive transfers

Results from adoptive transfer of splenocytes from different cohorts of NOD mice are shown in Figure 5.19. Older NOD splenocytes were more diabetogenic than young. NOD-scid mice which received splenocytes from 12 week old NOD mice (n=6) had a median survival of 60 days to develop diabetes and 100% diabetes incidence, whilst NOD-scid mice receiving splenocytes from 9 week old NOD mice (n=6) had a significantly shorter median survival of 74 days to diabetes and 100% diabetes incidence ($p<0.0001$). Those transferred 6 week NOD splenocytes (n=4) had a median survival of 90 days to diabetes and ¾ developed diabetes.

Of the 6 NOD-scid mice that received CIADM splenocytes only 1 developed diabetes at 80 days, whilst those that received CIADM splenocytes along with anti-PDL1 had a 100% incidence of diabetes by 1 month. Administration of anti-PDL1 prior to adoptive transfer of 6 week old NOD splenocytes led to more rapid development of diabetes ($p<0.0001$) than administration of 6-week NOD splenocytes alone, but diabetes onset was slower than administration of anti-PDL1 after adoptive transfer. Overall, CIADM splenocytes were less diabetogenic than young NOD splenocytes however anti-PDL1 administration either pre or post adoptive transfer both led to diabetes development (Log-rank test, $p<0.0001$).

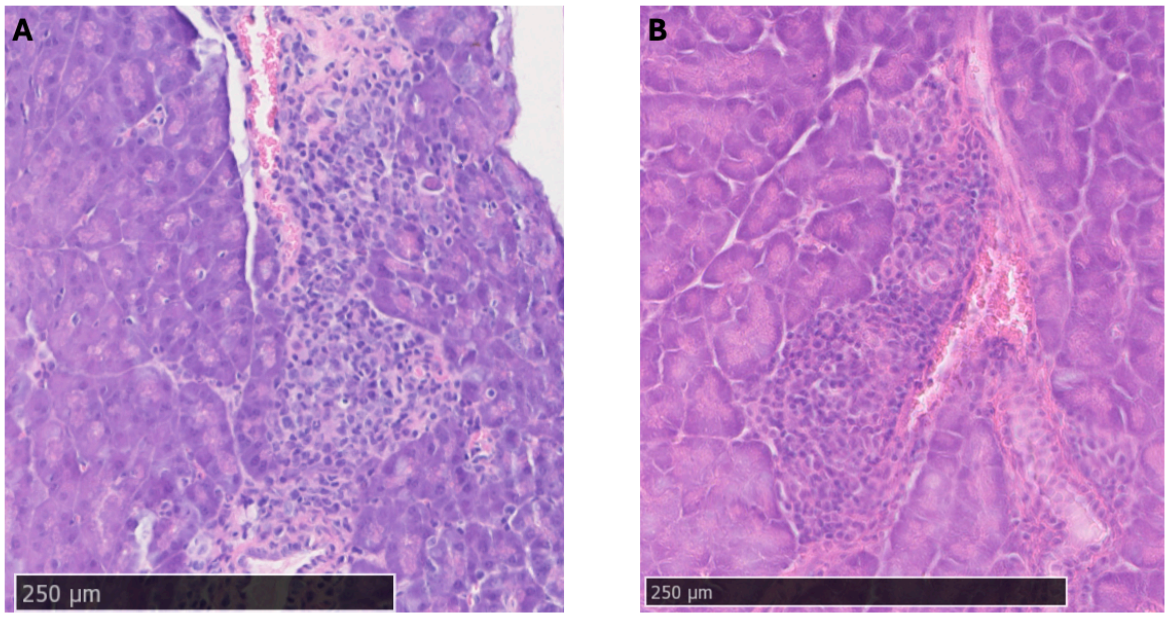
NOD-scid mice were transferred saline, B220+, CD4+ or CD8+ NOD lymphocyte subsets as shown in Figure 5.19. Only one NOD-scid mouse transferred CD4+ lymphocytes developed diabetes, whereas B220+ cells and CD8+ cells in isolation were not pathogenic.

Figure 5.19. Top: Survival curves depicting time to onset of diabetes in NOD-scid mice from adoptive transfer. Saline controls (n=4), CIADM NOD splenocytes (n=6), 6 week NOD splenocytes (n=4), 9 week NOD splenocytes (n=6), 12 week NOD splenocytes (n=6), CIADM NOD splenocytes followed by anti-PDL1 treatment (n=4) and anti-PDL1 prior to adoptive transfer of CIADM splenocytes (n=4). Bottom: Survival curves after adoptive transfer of NOD lymphocyte subsets. CD8+ (n=5), CD4+ (n=4), or B220+ (n=5) subsets were transferred in comparison to saline controls (n=3). Only 1 mouse developed diabetes, which was after CD4+ transfer.



Histology performed on NOD-scid pancreas (Figure 5.20) showed evidence of prominent perivascular inflammatory infiltrate and insulitis was seen in direct vicinity to these blood vessels.

Figure 5.20. Representative sections with haematoxylin and eosin staining of NOD-scid pancreas post adoptive transfer of 12 week NOD splenocytes leading to diabetes (left) and CIADM splenocytes leading to diabetes (right).



5.5 Discussion

5.5.1 CIADM has a distinct endocrine and exocrine profile to other models of autoimmune diabetes

Table 5.7 summarises the endocrine and exocrine profile of each model of diabetes. All diabetic mouse groups had reduced islet numbers, pancreatic mass, impaired insulin secretion and elevated glucose levels. CIADM mice groups had a more severe diabetes phenotype with significantly higher glucose levels than type 1 diabetes and STZ diabetes. STZ diabetes appeared milder with an incomplete insulin secretion defect and moderately elevated glucose levels.

Beta cell mass (as measured by % insulin staining positive) correlated with diabetes status. CIADM mice had significantly lower beta cell mass than control mice of both ages ($p<0.0001$) and STZ mice ($p=0.0416$) but similar to that seen in T1DM. This supports that in a mice model of CIADM, metabolic parameters of diabetes and glucose tolerance are similar to what is noted in humans, which is that of rapid and profound insulin deficiency[88].

Whilst in general increase in insulitis correlated with reduced beta cell mass, we found that despite the significant increase in insulitis seen, aged controls had a non-significant trend towards higher beta cell mass than young controls. It has been shown previously that chronic inflammation from insulitis leads to increased beta cell proliferation [89,90] and leads to development of a low insulin expressing subset of beta cells that appear more resistant to immune killing with high PD-L1 expression[91].

We found a significantly higher alpha cell mass in the T1DM mouse group than other groups. Previous NOD mouse studies have found relative preservation of alpha cell mass [92], increasing alpha cell mass with age [93] and one study found higher levels of plasma glucagon in diabetic NOD mice than controls with no difference in alpha cell mass [94]. One potential contributor to elevated alpha cell mass in addition to increased age, is the chronic lack of suppression by insulin, which has been demonstrated to suppress glucagon release in both mice and humans [95,96].

Reductions in islet and pancreatic mass were seen in all diabetic mouse groups and were most profound in CIADM and type 1 diabetes groups. This is similar to what has been documented for CIADM and type 1 diabetes in humans previously [38,97]. Histology demonstrated a reduction in beta cell mass that was not adequate to fully account for these changes with evidence of concurrent exocrine tissue atrophy. This supports the hypothesis that insulin itself has a trophic effect on local exocrine tissue. Previous mouse studies have found that regeneration of exocrine tissue via stem cell transplant could ameliorate insulitis, further supporting the concept of a symbiotic relationship between the endocrine and exocrine compartments of the pancreas [98].

Interestingly although STZ and CIADM mice had increased faecal triglycerides, T1DM mice had relatively normal faecal triglycerides. To our knowledge exocrine function of T1DM NOD mice models has not previously been reported, although STZ treatment has previously been demonstrated to lead to exocrine insufficiency [99]. Preservation of faecal triglyceride levels in T1DM mice may be due slower onset T1DM allowing exocrine pancreatic regeneration, and/or less exocrine cell damage in comparison to the STZ and CIADM groups. In humans with type 1 diabetes, clinical exocrine insufficiency is

uncommon, however studies have detected subclinical levels of exocrine insufficiency previously [100,101]. Our human data reported that some patients with CIADM and evidence of exocrine insufficiency did not display normal exocrine function on follow-up [97].

Table 5.7. Summary table of endocrine and exocrine profile by mice model of diabetes.

	Islet number	**Pancreatic mass**	**Glucose**	**Insulin**	**Glucagon**	**Exocrine function**
Young NOD	Normal	Normal	Normal	Normal	Normal	Normal
Aged NOD	Normal	Normal	Normal	↓	Normal	Normal
STZ	↓	↓	↑	↓ ↓	Normal	↓
T1DM	↓ ↓	↓ ↓ ↓	↑ ↑	↓ ↓	Normal	Normal
CIADM	↓ ↓	↓ ↓	↑ ↑ ↑	↓ ↓	↑	↓

5.5.2 CIADM is associated with fulminant diabetes and increased activated autoimmune infiltrate

Key differences in the immune profile of each model of diabetes are summarised in Table 5.8. Insulitis grade increased with age and diabetes status. CIADM mice had the highest proportion of severe insulitis (Grade IV), whereas T1DM mice had a greater proportion of pseudoatrophic islets, indicative of past autoimmune destructive insulitis. These findings are in keeping with the metabolic profile of severe, rapid hyperglycaemia in CIADM mice we noted earlier and with human post mortem studies of CIADM [102,103] and T1DM patients [84].

Islet PD-L1 expression was seen in all mice groups on immunohistochemistry, and when % PD-L1 positive Newport Green positive cells were quantified, aged NOD mice had significantly higher PD-L1 positive beta cells. This suggests that this increased PD-L1 expression was protective in tolerising the insulitis in comparison to the lower PD-L1 expression seen in T1DM and CIADM groups. These findings mirror those previously reported, with an age related increase in islet PD-L1 expression and with increased PD-L1 expression in beta cells that resist immune destruction [91,104]. The CIADM group had no significant difference in PD-L1 expression to other groups. This supports that anti-PDL1 leads to diabetes by functional inhibition of the receptor and does not appear lead to any compensatory changes to pancreatic PD-L1 expression.

Whilst all groups had evidence of inflammatory islet infiltrate, the immune profile of each cohort differed. CIADM mice displayed a highly activated lymphocyte population with high prevalence of CD8+CD69+CD44+ and CD4+CD69+CD44+ cells. CD44+ is a marker of effector/memory status and is also constitutively expressed in mouse tissue

resident memory cells[105]. CD44+ expression in particular has been previously associated with insulitis[106] and anti-CD44 therapy induces resistance to diabetes[105], CD44 initiated adhesion pathways have been shown to mediate T cell extravasation to inflamed sites and enhance suppressive capacity of T regulatory cells [107]. CD69+ is a marker of early T cell activation[108] and also is constitutively expressed in tissue resident memory cells[109].

CIADM mice had high numbers of CD8+ tissue resident memory (TRMs; CD8+CD69+CD103+) T cells in their pancreatic islets in comparison to other groups.CD8+ TRMs are a subset of memory T cells that persist in peripheral tissue long term to provide protection against reinfection[110]. In CD8+ TRMs, CD69+ assists in maintaining TRM retention and prevents egress into circulation [111] and CD103+ allows for adherence of TRMs and persistence in the tissue[112]. CD8+ TRM T cells have been shown to be a hallmark cell type of a number of autoimmune diseases[113,114], immune related adverse events [115,116] and responsible for Th1/Th17 pathway activation [117]. CD8+ TRMs have been found to be an important cell type in pancreatic insulitis in recent onset T1DM in humans and have an important role in local immune responses [118].

In our study, we found that CD8+ TRM T cells are more prevalent CIADM in comparison to de novo T1DM. Sasson et al previously demonstrated that in a similar manner humans with checkpoint inhibitor related colitis had increased activated CD8+ TRM in comparison to idiopathic ulcerative colitis[116]. RNA-sequencing data from this study also suggests that these CD8+ TRM have significant upregulation of activation and cytotoxic markers in comparison to the more homeostatic signature seen in healthy controls[116]. Luoma et al's study of patients with ICI related colitis identified that a significant proportion of colitis associated CD8+ cells originated from CD8+ TRMs[119] Similarly in irAE dermatitis, spatial

transcriptomics studies identified a key role of CD4+ and CD8+ TRMs[115]. We hypothesise that checkpoint inhibitors primarily unleash TRMs and change their profile from homeostasis to cytotoxic, and thus patients who have previously tolerised islet autoimmune responses can develop rapid onset diabetes.

Compared to young NOD mice, aged NOD mice had increased PD-L1 expression, increased activated lymphocyte subsets and increased neutrophil infiltration. This is in keeping with the increased insulitis seen on histology. Increase in PD-L1 expression and activated lymphocytes with age in NOD mice is consistent with previous reports [91,92] and supports that PD-L1 expression is induced by ongoing autoimmunity and suggests this expression may be either directly protective or evidence of protection against development of destructive insulitis.

Our T1DM cohort had similar infiltrate characteristics to aged controls, albeit with lower PDL1 expression in beta cells and higher macrophage and neutrophil counts. The lack of increase in activated lymphocytes is consistent with the profile of pseudoatrophic burnt out insulitis visualised. Macrophage and neutrophil infiltration is likely explained by the need for phagocytosis of dead beta cells post insulitis[120]. Macrophages[121] have previously also demonstrated an important role in type 1 diabetes and aid in T cell differentiation.

In contrast, STZ mice had high numbers of neutrophils, macrophages and NK cells, with relatively lower prevalence of B cells and CD8+ cells. There was a stepwise increase in neutrophils from young to old non-diabetic mice, then CIADM, NOD T1DM and finally STZ diabetic mice had the highest prevalence of neutrophils (Figure 5.18E). NK cells, shown in Figure 5.18F were markedly elevated in the STZ group compared to all other

groups. Whilst increase in macrophages[122] and neutrophils[123] have been noted previous in STZ induced diabetes, NK cells have not previously been quantified.

No discernible differences in Foxp3 immunofluorescence or CD4+CD25+ prevalence were detected between groups, which was surprising given the significant differences seen in degree of inflammatory activity. This may be due to inadequate profiling of T regulatory cells as due an already extended protocol we did not perform intracellular Foxp3 staining. However, other NOD mouse studies have previously also demonstrated that T regulatory cells can be impaired in function rather than quantity in autoimmune diabetes [96,124].

Table 5.8. Summary table of immune changes by mice model of diabetes.

	Predominant insulitis grade	PD-L1 expression	Predominant islet infiltrate compared to young controls	Activation
Young NOD	0-I	+	Reference group	-
Aged NOD	II-III	++	Increased neutrophils and activated lymphocytes	+
STZ	III-IV	+	Neutrophils and NK cells predominant, low B cell and unchanged CD8+ prevalence compared to young controls	+
T1DM	IV-PA	+	B220+ predominant, moderate CD8+, high macrophage and neutrophil prevalence	+
CIADM	IV	+	CD8+ predominant, high tissue resident memory CD8+ cells, high B cell, moderate neutrophil and NK cell infiltrate	+++

5.5.3 Exposure of anti-PDL1 to splenocytes in isolation is not adequate to induce CIADM

Our adoptive transfer experiments demonstrated firstly that NOD splenocytes have diabetogenic potential even in the absence of diabetes in the NOD mouse, and this pathogenicity increases with increasing age of the NOD mouse. This confirms what has been shown in previous studies the direct pathogenic role of lymphocytes in T1DM on adoptive transfer [125].

Figure 5.20 demonstrates a slightly different pattern of insulitis to that seen in NOD mice. In particular there was a predominance in both NOD splenocyte and CIADM splenocyte transferred NOD-scids of perivascular inflammatory infiltrate. This is keeping with a model of adoptively transferred diabetes rather than a pre-existing local infiltrate. Although pancreatic histology in NOD-scids transferred NOD splenocytes has been reported[126,127] specific location in relation to vasculature was not noted.

CIADM splenocytes unexpectedly demonstrated lower pathogenicity than normal NOD mice with only 1/6 mice developing diabetes within 90 days of adoptive transfer. It is possible that counterregulatory tolerance mechanisms had been upregulated in CIADM splenocytes that led these cells to have reduced pathogenicity compared to untreated NOD splenocytes. We hypothesised that this was due to lack of exposure of the local immune system, pre-existing tissue resident memory cells and pancreatic tissue in recipient mice to anti-PDL1. The absence of the ‘unleashing step’ of checkpoint inhibitor administration directly to CD8+ TRMs as seen in CIADM may account for why the adoptive transfer model had low incidence of diabetes.

Therefore, we tested groups of recipients that were treated with anti-PDL1 prior to and after adoptive transfer of CIADM lymphocytes. Exposure to systemic anti-PDL1 both prior to and after adoptive transfer led to increased incidence of diabetes, which was most accelerated in the group that had anti-PDL1 exposure immediately post after adoptive transfer. This again supports our hypothesis that the crucial pathogenic step of CIADM is the effect of the checkpoint inhibitor on the local tissue resident memory cell.

We did not identify any reports testing whether CIADM can be adoptively transferred in the literature. However, CD4+ cells are known to be most pathogenic on adoptive transfer from NOD mice [128] whereas CD8+ cells in isolation do not usually transfer T1DM [128,129]. We wondered whether the lower incidence of diabetes seen in CIADM transfer could be due to the relatively higher proportion of CD8+ cells and lower proportion of CD4+ seen in this group. Importantly, adoptive transfer of ICI treated peripheral lymphocytes alone may not be adequate to trigger CIADM due to the absence of pathogenic TRM cells. On transferring lymphocyte subsets, it was difficult to draw strong conclusions as to which lymphocyte subset is most pathogenic in CIADM as CIADM splenocytes themselves had relatively lower rates of diabetes induction. We did however observe one case of adoptive transfer of diabetes in the group transferred CIADM CD4+ splenocytes which was suggestive that in CIADM this is a key pathogenic cell type.

5.5.4. Limitations and future directions

This study would be strengthened through the identification and quantification specifically of islet antigen specific T cells and further RNA analysis of the infiltrate to characterise expression of inflammatory markers. Furthermore, we are limited in our statistical power to detect smaller differences by the relatively small cohort numbers in each group.

In the future, improved characterisation of insulitis in relation to local structures through the use of spatial transcriptomics would be useful in understanding how the local architecture of the pancreas can influence CIADM related insulitis and the impacts on the exocrine tissue which was not able to be analysed on flow cytometry. Consideration of use of immunotherapies that target CD8+ TRMs such as an anti-CD103+ antibody, could be used to further determine the role of this cell type in pathogenesis of CIADM.

Chapter 6: Clinical Guidance for Identification and Management of Immune Checkpoint Inhibitor related Hyperglycaemia

Manuscript title:

Hyperglycaemia in patients treated with immune checkpoint inhibitors: key clinical challenges and multi-disciplinary consensus recommendations

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This guideline has been endorsed by the Australian Diabetes Society and the Endocrine Society of Australia through working group peer review.

Abbreviations

BGL = blood glucose level

CIADM = checkpoint inhibitor associated diabetes mellitus

DKA = diabetic ketoacidosis

GAD = glutamic acid decarboxylase

IA2= islet antigen 2

ICI = immune checkpoint inhibitors

IRAE = immune-related adverse events

RCT = randomised control trial

T1DM = type 1 diabetes mellitus

T2DM = type 2 diabetes mellitus

ZnT8 = zinc transporter 8

Executive summary

Background

Immune checkpoint inhibitors (ICIs) have an expanding role in the management of numerous cancers. Hyperglycaemia is commonly seen in patients treated with ICIs. However, the differential diagnosis for hyperglycaemia is broad, and incorrect diagnosis can have serious consequences.

Objectives

To review the available literature on causes of hyperglycaemia in ICI treated patients and expert guidelines on management and provide an updated synthesis of expert multidisciplinary recommendations.

Key Recommendations

- Intensity of screening for hyperglycaemia should be based on a patient's risk level, including assessment of factors such as corticosteroid use, pre-existing diabetes, baseline HbA1c and fasting blood glucose levels (BGL).
- People with new onset hyperglycaemia should undergo initial assessment to determine severity and aetiology, including bedside capillary BGL, and formal bloods including lipase, C-peptide with matching glucose, electrolytes and renal function and in some cases type 1 diabetes autoantibodies.
- People with BGL >15mmol/L (or those receiving SGLT2 inhibitors with BGL >10mmol/L) should additionally have ketones measured.
- Patients with a high risk of diabetic ketoacidosis (BGL>15 mmol/L, ketones >2 mmol/L) and/or risk of hyperosmolar hyperglycaemic state (BGL persistently >20 mmol/L or reading 'HI') should be referred directly to hospital for emergency assessment and management.
- Further management of hyperglycaemia should be tailored to the underlying cause(s), as discussed in the Tables below and in Figure 1.

6.1 Introduction

Hyperglycaemia is a common clinical complication of immune checkpoint inhibitor (ICI) therapy. It can pose both diagnostic and management challenges, with a broad range of presentations ranging from mild asymptomatic steroid induced hyperglycaemia to life-threatening diabetic ketoacidosis (DKA) from checkpoint inhibitor associated autoimmune diabetes mellitus (CIADM). Corticosteroid induced hyperglycaemia is common as up to 35% of ICI treated patients receive corticosteroids as treatment for immune related adverse events (IRAE) (1).

Retrospective reviews report an incidence of hyperglycaemia ranging from 13-27% of all ICI treated patients (2,3). In patients with known diabetes, cohort analyses report hyperglycaemia in 42-72% of patients treated with ICI (2,3). A large meta-analysis of 52 ICI related randomised controlled trials (RCTs) quotes an incidence of not related to pre-existing diabetes or corticosteroids at 2.26% (95% CI 1.28-3.48) and serious grade hyperglycaemia at 0.28% (95% CI 0.16-0.42) (4). Drug-attributed hyperglycaemia was more common in those treated with combination of 2 or more ICIs (3.37% all grade, 0.47% serious hyperglycaemia) and there was no evidence of increased risk with higher doses of ICIs (4).

Patients with cancer have an increased risk of developing hyperglycaemia and new onset diabetes irrespective of treatment factors (5,6). Several studies have shown an association between hyperglycaemia and lower overall survival and increased risk of cancer recurrence (7–9).

6.2 Key differentials and diagnostic challenges

The potential aetiologies underlying hyperglycaemia in patients receiving ICI therapy are broad. Of patients who developed new onset hyperglycaemia, 68-76% were associated with administration of corticosteroids (2,3). CIADM was reported in 0.4-1.9% (10–15). Pancreatitis or autoimmune lipodystrophy related hyperglycaemia are largely limited to case reports (16–18).

Table 1 summarises the reported incidence of the different known aetiologies of ICI-related hyperglycaemia and key supportive features to guide diagnosis.

Table 1. Causes of hyperglycaemia in patients on ICI and supportive clinical findings

Cause for ICI-associated hyperglycaemia	**Proportion of patients**	**Supportive features**
Corticosteroids	68-76% (2,3)	Corticosteroid treatment
Type 2 diabetes	42-72% (2,3)	Past history of T2DM. Diabetes without low C-peptide, steroid treatment, T1DM autoantibodies or lipase elevation
CIADM	0.4-1.9% (10–15)	Exposure to anti-PD1 or anti-PDL1 therapy. Hyperglycaemia with low C-peptide (<0.4nmol/L). May have elevated ketones, positive T1DM auto-antibodies (anti-GAD, -IA2, -insulin, -ZnT8) and/ or elevated lipase
Pancreatitis	Case series only(18) 17.6% with new lipase elevation had diabetes	Elevated lipase, symptoms and/or CT changes of pancreatitis. ICI exocrine pancreatitis can lead to partial or complete insulin deficiency (unlike CIADM which targets β-cells)
Autoimmune lipodystrophy	Case reports only (16,17)	Hyperlipidaemia, clinical, radiologic and/or histologic lipodystrophy

T1DM= type 1 diabetes, T2DM = type 2 diabetes, GAD = glutamic acid decarboxylase, IA2= islet antigen 2, ZnT8 = zinc transporter 8

6.3 Current clinical guidance

Several national and international oncology societies have provided guidelines and recommendations for investigation and management of hyperglycaemia during ICI therapy. These are summarised in Table 2. The terminology for CIADM varies between guidelines and is, in some cases, labelled ‘type 1 diabetes’.

Table 2. Review of recommendations in oncological guidelines for ICI related hyperglycaemia

Group	Screening and investigation	Management
American Society of Clinical Oncology (ASCO), 2021(38)	· Monitor for symptoms · Measure baseline glucose. Repeat each treatment cycle for ≥6months · Measure ketones, anion gap, C-peptide and autoantibodies if considering CIADM	Grade 1 (asymptomatic, fasting glucose >8.9mmol/L) · Continue ICI, refer to general practitioner (GP) for oral therapy, close follow up Grade 2 toxicity (symptomatic, fasting glucose 8.9-13.9mmol/L) · Consider withholding ICI. If CIADM suspected, endocrine consult and insulin commencement · Consider hospital admission if symptomatic. Admit for diabetic ketoacidosis (DKA). Grade 3-4 toxicity (Severe symptoms, glucose >13.8mmol/L, ketosis or other metabolic abnormality) · Withhold ICI. Endocrine consultation and insulin commencement. Consider hospital admission if symptomatic. Admit for DKA.
Society for Immunotherapy of Cancer (SITC), 2021 (39)	· Patients with new-onset T1DM; test HbA1c, C-peptide, and autoantibodies · Evaluate hyperglycaemic patients for DKA	· If patients have severe hyperglycaemia or DKA, they should be hospitalised · Withhold ICIs until DKA resolved · Patients with T1DM need counselling on diabetes management, diet and lifestyle changes, glucose monitoring, and insulin treatment.

European Society of Medical Oncology (ESMO), 2022 (40)	· Monitor HbA1c and glucose 4-6 weekly while on ICI. Repeat HbA1c if suspected T1DM · If new hyperglycaemia requiring insulin, test glucose, ketones, anion gap, C-peptide, lipase and autoantibodies	· Mild asymptomatic hyperglycaemia without ketosis: continue ICI, optimise oral agents if pre-existing T2DM, insulin replacement, liaise with endocrinology · Moderate hyperglycaemia without ketosis: as above but withhold ICI until stable, insulin management, hydration · Severe or life-threatening symptoms, ketosis: withhold ICI until stable, hospitalisation, DKA management, insulin management

Whilst these guidelines contain valuable information for oncologists for broader management of irAE, there is limited recognition of the complexity of the differential diagnosis of hyperglycaemia and how management may differ in each instance. In particular, there is a need to incorporate risk stratification to guide appropriate use of resources and current guidelines to not provide risk stratification or follow up guidelines to identify and more closely monitor those at highest risk of deterioration. Without appropriate screening, it is difficult to promptly identify and intervene on the patients at high risk of CIADM.

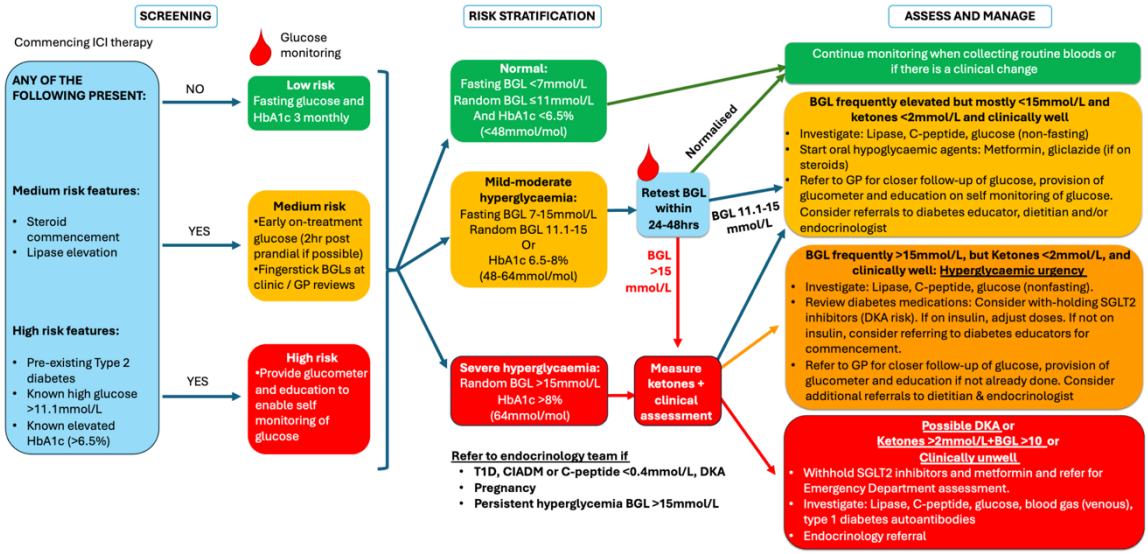
6.4 Multidisciplinary Recommendations

In view of the rapidly developing body of knowledge of ICI related hyperglycaemia and increasing use of ICIs, a more detailed discussion and review of evidence is required. In the following sections we provide further updated expert recommendations based on recent research.

Screening

The frequency and modality of screening for hyperglycaemia and diabetes in people receiving ICIs should be dynamic and tailored to the patient's risk of significant hyperglycaemia (Figure 1).

Figure 1. Approach to initial investigations and management of ICI-related hyperglycaemia



Screening recommendations for each risk category are:

- LOW (e.g. newly commencing ICI): serum glucose (fasting preferred) included with bloods for treatment cycles and 3 monthly HbA1c.
- MODERATE (e.g. commencing corticosteroids, raised lipase): Serum glucose (2hr postprandial preferable) and/or fingerpick BGL at clinic/GP review. Fasting BGL will not always capture severity of hyperglycaemia. We recommend monitoring within 2 weeks of steroid commencement and with dose increases, and at 4 weekly intervals thereafter, at minimum, until cessation or resolution of hyperglycaemia after dose-reduction. Patients deemed intermediate risk should receive education on warning symptoms of severe hyperglycaemia to prompt earlier medical review.
- HIGH (e.g. newly detected hyperglycaemia >11mmol/L/ raised HbA1c >6.5%, pre-existing type 2 diabetes): provision of glucose meter for daily patient self-monitoring of glucose. All patients deemed high risk on screening should receive education on self-monitoring of glucose levels, warning symptoms of severe hyperglycaemia and when to seek medical attention. Frequency of monitoring should thereafter be tailored to the degree of hyperglycaemia but for patients who have glucose levels above >10mmol/L we recommend ongoing daily monitoring at a minimum.

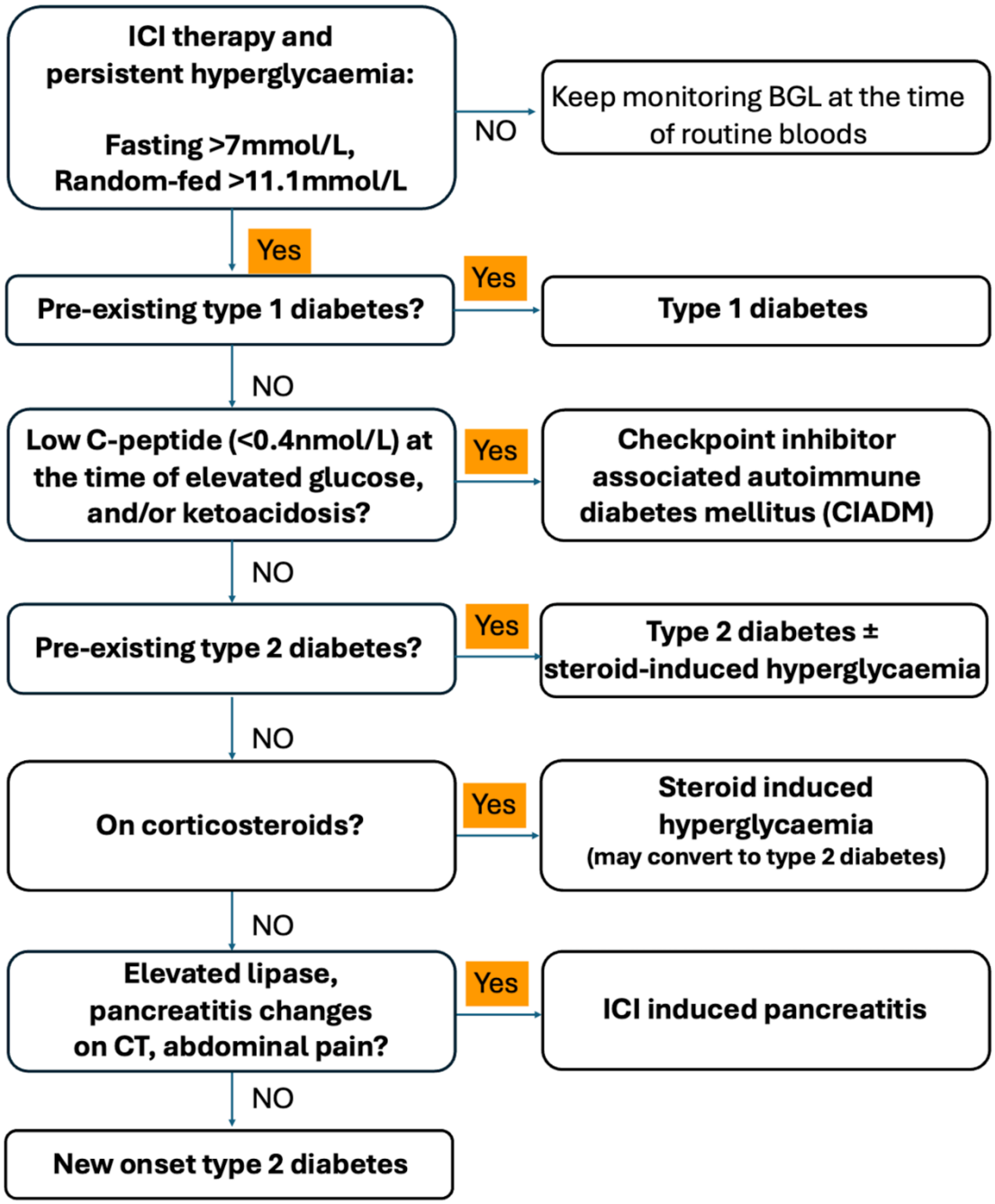
The duration of screening for CIADM is debated; the median onset time of CIADM is 12 weeks after initiation of ICI with a small number of cases reported years after initiation. Therefore, we recommend monitoring while on treatment as per risk stratification and for 12 months post completion followed by normal long-term monitoring of HbA1c with the patient's usual doctor as per local guidelines for T2DM screening.

6.5 Management

Whilst oncologists inevitably must play an invaluable role in the detection and early assessment of ICI related hyperglycaemia, ongoing management should be shared with the patient's primary care physician and, where appropriate, an endocrinologist. We recommend early communication and shared care with a primary care physician for management for all patients with hyperglycaemia, with referral to endocrinology where insulin is expected to be required. In high-risk patients, and those who are already on an SGLT2 inhibitor, re-education about the risk of DKA, and particularly, euglycaemic DKA should be provided. Patients should have capability to measure ketones as well as self-monitoring blood glucose. If this is not feasible, consider whether SGLT2 inhibitors could be ceased.

After initial screening and investigations are conducted as per Figure 1, once hyperglycaemia is identified we recommend utilising the algorithm in Figure 2 to determine underlying aetiology and referring to the relevant section below for further management guidance.

Figure 2. Flowchart for differential diagnosis of hyperglycaemia on ICI therapy.



6.5.1 Corticosteroid induced hyperglycaemia

Steroids are a common cause of hyperglycaemia in the general oncology setting and in particular with ICI therapy, steroids are often commenced to manage immune related adverse events. Steroids lead to hyperglycaemia via a multitude of mechanisms including impaired beta cell insulin secretion, increased total body insulin resistance and increased hepatic gluconeogenesis (19). The incidence of steroid use was 35% in those who received ICI therapy (2). A retrospective study of ICI related hyperglycaemia found hyperglycaemia in 12.4% and 39.5% of those on ICIs with hyperglycaemia were due to steroids (3).

The expected profile of steroid-induced hyperglycaemia differs depending on the pharmacokinetics of the steroid prescribed, with prednisone and dexamethasone being the most commonly used. Prednisone prescribed daily in the morning typically leads to peak hyperglycaemia and insulin resistance around 8 hours after the dose. This corresponds to elevations in BGL in the late morning and afternoon (20). Dexamethasone which has been well studied in the setting of COVID-19, led to a peak in hyperglycaemia at 7-9 hours and intravenous dosing triggered greater degrees of hyperglycaemia (21). The degree of hyperglycaemia correlates with the dose of steroid. Adjustments to steroid doses frequently necessitate adjustment of the associated diabetes treatment regimen.

Risk factors for developing steroid induced hyperglycaemia and diabetes include steroid dose and duration, previous hyperglycaemia on steroid therapy, previous impaired glucose tolerance or impaired fasting glucose, concurrent diabetogenic medications as well as traditional diabetes risk factors (obesity, older age, previous gestational diabetes, polycystic ovarian syndrome, ethnicity, positive family history) (19).

Diagnosis

Persistent hyperglycaemia with 2 abnormal tests (random BGL ≥11.1mmol/L on different occasions and/or newly elevated HbA1c ≥6.5%) and use of corticosteroids is adequate to make a diagnosis of steroid induced diabetes.

Management

All patients with steroid induced hyperglycaemia should receive education regarding glucose monitoring, symptoms of severe hyperglycaemia, and safety thresholds for presentation to hospital. Those receiving glucose lowering therapy which may induce low BGLs should also be educated on hypoglycaemia management. It should be emphasised that as steroids are the primary driver for hyperglycaemia, adjustments to steroids should necessitate a review of the associated diabetes treatment regimen. Failure to down-titrate therapy with significant steroid dose reduction is a frequent cause of hypoglycaemia.

We recommend treatment to a general glucose target of 5-10 mmol/L. Different targets may be desirable depending on age and comorbidities, for example, in the palliative setting 6-15 mmol/L is a reasonable compromise to balance avoidance of symptomatic hyperglycaemia and hypoglycaemia. Efforts should be made in this setting to rationalise medications and avoid medications such as insulin and sulfonylureas that can lead to hypoglycaemia where possible. We recommend monitoring glucose 4 times daily in the short term; including fasting and 2hr post meals to capture the lowest and highest predicted values on therapy. For patients who have stable glucose values within target range 5-10mmol/L with no changes to steroid regimen, frequency of monitoring can be reduced to once daily. Increased monitoring should be undertaken at the time of steroid dose changes,

or intercurrent illness. For those who are able to afford it, continuous glucose monitoring may be very helpful, and reduce testing burden.

In milder cases of corticosteroid-induced hyperglycaemia (capillary BGLs <15 mmol/L), in line with other steroid related hyperglycaemia guidelines we recommend commencing oral agents (22–24). Our first line agent of choice in this situation is modified release gliclazide 60 mg daily to be taken with the morning steroid dose and increased up to 120 mg daily if required. Gliclazide should be avoided in severe hepatic and renal impairment and patients should be warned of the risk of hypoglycaemia on these agents. There is no comparative evidence for immediate versus delayed release sulfonylureas in this situation, but these could also be considered for isolated daytime hyperglycaemia. Metformin can be added as an additional agent in those with preserved renal and hepatic function, with some evidence that metformin can alleviate some of the metabolic effects of steroids (25). There is little data for use of SGLT2 inhibitors in this space, but if a person has significant hyperglycaemia, the risk of euglycaemic DKA may warrant consideration of substituting with DPP4i. We would not usually recommend commencing a new GLP1RA due to the known risk of pancreatitis with ICI therapy. People already taking a GLP1RA should be counselled about the symptoms of pancreatitis and offered the option to change therapies.

In more severe cases of steroid-induced hyperglycaemia (capillary BGLs >15 mmol/L and/or not reaching glucose targets on the above oral agents) we recommend insulin therapy with guidance of the local endocrinology team. The preferred insulin therapies are insulin isophane (e.g. Humulin NPH or Protaphane) due to the matching duration of action with most intermediate acting steroids with demonstrated efficacy (26,27). Standard long acting basal insulin glargine (Optisulin) was noninferior to intermediate insulin in clinical

trials and would be especially useful if patients have more prolonged elevation of their glucose (28,29).

Current guidelines recommend basal bolus insulin with once daily Optisulin and Novorapid with each meal would be commenced at 0.3-0.5units/kg, split 50/50 between Optisulin and Novorapid for more severe cases of hyperglycaemia where the addition of rapid acting insulin to control the prandial rise is desirable (30). Alternatively for those that may struggle to manage 4 injections a day, mixed insulin including intermediate-acting insulins such as Novomix 30 (30% rapid acting insulin aspart/70% intermediate protamine insulin aspart) given at time of steroid dosing at breakfast can be used. Consideration of higher starting doses may be warranted in certain cases, as weight, baseline HbA1c, pre-existing diabetes and diabetes therapy have been shown to independently influence insulin requirements in steroid induced hyperglycaemia(31). Initial doses may be lower in the elderly and those with renal impairment. Doses need to be down-titrated as steroids are reduced.

In very severe cases of steroid-induced hyperglycaemia, hyperosmolar hyperglycaemic state (HHS) can develop. This is a life-threatening condition driven by persistent hyperglycaemia leading to profound dehydration and severe hyperosmolality. Patients should be warned that in settings where capillary BGLs are persistently above 20 mmol/L despite treatment, or the glucose meter is reading ‘HI’ they should present to hospital to receive further assessment and therapy.

6.5.2 Type 2 diabetes (T2DM)

Pre-existing and new onset T2DM are both common in patients on immune checkpoint inhibitor therapy. As outlined earlier (Figure 1) patients with pre-existing T2DM are at high risk for hyperglycaemia and should have a blood glucose meter for regular self-monitoring for glucose levels. We recommend initial monitoring 4 times daily (fasting and 2hrs post meals) and continuing at this frequency if readings are out of range, diabetogenic medications such as corticosteroids are introduced, or when significant changes to the patient's renal function or oral intake have occurred. Glucose levels persistently outside of the target range of 5-10mmol/L warrant medical review with their primary care physician for consideration of medication adjustment and ongoing close monitoring.

In people with T2DM we recommend seeking further advice from their local endocrinology service if any of the following are present: blood glucose persistently ≥15 mmol/L, HbA1c ≥9% (unless palliative/end of life). People with T2DM in these scenarios will often require insulin commencement.

Detailed discussion regarding general T2DM management is outside of the scope of this article and we would refer any interested readers to the American Diabetes Association (32) and Australian Diabetes Society guidelines for glycaemic management of T2DM (33) and Australian Evidence-Based Clinical Guidelines for Diabetes.

6.5.3 Checkpoint inhibitor related autoimmune diabetes mellitus (CIADM)

CIADM is a rare complication of ICI therapy occurring in 0.4-1.9% of patients (10–15). It is nearly exclusively associated with anti-PD1/anti-PDL1 therapy and risk is increased in those receiving combination anti-CTLA4 therapy in combination with anti-PD1/anti-PDL1. Median time from commencing ICI to onset of CIADM is 12 weeks; however, some case reports describe CIADM occurring years after ICI therapy, indicating an ongoing risk (34). Diabetic ketoacidosis (DKA) is often present at diagnosis of CIADM (45-67% of presentations) (34).

Diagnostic criteria

In a systematic review of all published cases of CIADM we identified key features to support a diagnosis of CIADM (34):

1) New onset hyperglycaemia (≥11.1 mmol/L and/or HbA1c ≥6.5%)
2) Low C-peptide (<0.4 nmol/L) within one month of presentation

These criteria were sufficient to pick up all confirmed cases of CIADM. Type 1 diabetes autoantibodies were only positive in 40%; therefore, these are a supportive criterion but not required for diagnosis of CIADM (34). Lipase was elevated in 69% however this may be an underestimate as testing was limited (34).

Management

As DKA in patients with CIADM is common and life-threatening, we recommend all patients suspected to have CIADM be tested for capillary ketones and if elevated, or if serum bicarbonate is <16 mmol/L without alternative cause, they should be referred to hospital for work up of potential DKA. DKA management often requires intensive care admission for close metabolic monitoring, fluid and electrolyte replacement and insulin infusion.

Patients with CIADM have absolute and fulminant beta cell failure as indicated by persistently low C-peptide on follow up, and therefore should remain on lifelong insulin therapy. The lack of residual beta cell function in case series has led to higher levels of glycaemic variability and more difficult glycaemic control in a population often already burdened with comorbidities (10). We recommend all patients with CIADM be under the care of an endocrinology service to provide initial education and stabilisation. We usually prescribe once daily long acting insulin (e.g. insulin glargine) starting at 0.2-0.3 units/kg/day (usually higher doses with higher starting body weight) plus fast-acting insulin to be given three times daily with meals (e.g. insulin aspart) at 0.05-0.1 units/kg/meal.

Patients with CIADM should be considered eligible for supportive technologies available to those with type 1 diabetes including insulin pump therapy and continuous glucose monitoring (CGM). All patients with CIADM should also receive sick day management education including hypoglycaemia management, stress dosing and ketone monitoring and diabetic ketoacidosis education. Patients with T1D may benefit from diabetes education around sick day management and DKA if this has not occurred in the preceding 5 years. Glucose targets should be tailored to the individual patient. In general, a glucose target of 5-10 mmol/L is reasonable; however, more flexible targets are appropriate if the patient is elderly, palliative or experiencing frequent unpredictable hypoglycaemia.

Case series suggest an under-recognised proportion of patients with CIADM have concurrent pancreatic exocrine insufficiency (35). Faecal elastase can be tested in CIADM patients, in particular in those with symptoms of gastrointestinal malabsorption and if low

replacement with pancreatic enzymes (e.g. Creon 25,000 units with main meals) may be appropriate to improve these symptoms.

6.5.4 Type 3c diabetes

Type 3c diabetes (also termed pancreatogenic diabetes) can occur in the setting of acute or chronic pancreatitis secondary to ICI therapy. This is a rare complication and literature is limited to cases series only (18).

Diagnostic criteria

Diagnosis of type 3c diabetes requires the following criteria to be met

1) New onset hyperglycaemia (≥11.1 mmol/L) and/or HbA1c ≥6.5%
2) Evidence of pancreatitis (lipase elevation >2x ULN and supportive symptoms and CT changes within the pancreas)
3) Evidence of pancreatic exocrine insufficiency is typically present, and can be assessed using faecal elastase in conjunction with malabsorptive symptoms

The delineation between CIADM and type 3c diabetes is a topic of ongoing debate. It should be noted that CIADM as previously discussed can feature elevated lipase and pancreatic atrophy. Whilst all patients with CIADM by definition will have low C-peptide (<0.4nmol/L) indicating of absolute insulin deficiency, in Type 3c diabetes insulin is not always required as insulin deficiency only emerges at late stages of disease (36). In the event of a low C-peptide (<0.4nmol/L) all patients should be managed with insulin as discussed below.

Management

Type 3c diabetes management varies depending on the extent of pancreatic damage and residual beta cell function. For this reason we recommend that persons with type 3c diabetes are managed by an endocrinology team.

In mild cases of type 3c diabetes with robust C-peptide levels and mild dysglycaemia, oral agents (metformin, DPP4 inhibitors, sulfonylurea, GLP1RAs, SGLT2i) can be used and management is similar to type 2 diabetes. It should be noted that DPP4 inhibitors and GLP1Ras have been rarely reported to be associated with pancreatitis and therefore should be used with caution in this population. SGLT2i are associated with increased risk of DKA, so those that are prescribed this drug should ideally have capability to assess ketones at home.

In more severe cases, in particular with evidence of inappropriately normal/low C-peptide levels, insulin therapy is required and management is similar to people with type 1 diabetes/CIADM. In those with low faecal elastase, we recommend dietitian review and commencement of pancreatic enzyme replacement therapy. Enzyme replacement improves nutritional outcomes and may also assist in stabilising glycaemia in those with type 3c diabetes (37). A full replacement regimen would typically consist of Creon 25,000 IU with meals and 10,000IU with snacks.

6.6 Conclusions

The differential diagnosis for a patient with new onset hyperglycaemia whilst on immune checkpoint inhibitor is broad and risk profiles are diverse. This article provides a synthesis of current evidence and expert opinion as summarised in Table 3 to support clinicians managing such patients. Further targeted research in this emerging field is required to strengthen these clinical recommendations.

Table 3. Summary of recommendations (Level of Evidence)

SCREENING

• Intensity of screening for hyperglycaemia should be based on a person's risk level (Figure 1). **(Level V – Expert Opinion).** Options include:
  • HIGH RISK: E.g. newly detected hyperglycaemia / raised HbA1c, pre-existing type 2 diabetes; provision of glucometer for daily patient self-monitoring of glucose and education. Capability to self-test ketones is desirable.
  • MODERATE RISK: E.g. commencing corticosteroids, raised lipase; Serum glucose (2hr postprandial or 8 hours after prednisolone dosing preferable) and capillary BGL at clinic/GP review. Fasting BGL will not always capture severity of hyperglycaemia.
  • LOW RISK: Commencing ICI but not fitting into the above categories; Serum glucose on bloods with treatment cycles and 3 monthly HbA1c.
• Duration of follow up: As CIADM has a median onset of 12 weeks after initiation of ICI with cases reported even years after initiation, we recommend initial follow-up based on risk for 12 months followed by long term monitoring as per standard type 2 diabetes screening with GP. **(Level V– Expert Opinion).**

ASSESSMENT
• Persons who have new onset hyperglycaemia should undergo initial assessment to determine severity and aetiology. • Initial investigations should include bedside capillary blood glucose and ketones, and formal bloods including lipase, C-peptide with matching glucose, electrolytes and renal function and in some cases type 1 diabetes autoantibodies.

- Patients with a high risk of diabetic ketoacidosis (BGL>15 mmol/L, ketones >2 mmol/L) and/or hyperosmolar hyperglycaemic state (BGL persistently >20 mmol/L or reading 'HI') should be referred directly to hospital for emergency assessment and management

MANAGEMENT

Steroid induced hyperglycaemia (Level II-III – Controlled studies and RCT).

- Diagnosis of steroid induced hyperglycaemia is based on repeated glucose measurements ≥11.1 mmol/L in the setting of steroid use, without previous history of diabetes. In those that also have a new elevation in HbA1c ≥6.5% this is considered steroid induced diabetes.
- Management of mild steroid induced hyperglycaemia consists of oral agents such as gliclazide and metformin. DPP4/ GLP1RA are appropriate to consider as 2nd line therapy if pancreatitis and elevated lipase are absent. Moderate to severe cases will require insulin therapy matched to the steroid dose and profile, such as intermediate acting insulin isophane or mixed insulin given in the morning.

Type 2 diabetes.

- Clinicians can refer to established type 2 diabetes guidelines such as American Diabetes Association guidelines for guidance
- Clinicians should seek further advice from the local endocrinology team for high risk patients with type 2 diabetes: blood glucose >15 mmol/L persistently, HbA1c >9% (unless palliative/end of life) (**Level V – Expert opinion**).

Checkpoint inhibitor associated diabetes mellitus (CIADM) (Level IV – Case series).

- Diagnosis of CIADM is based on new onset hyperglycaemia (≥11.1 mmol/L and/or HbA1c ≥6.5%) and low C-peptide (<0.4 nmol/L) within one month of presentation. Type 1 diabetes autoantibodies are specific but not sensitive.
- Management of CIADM is similar to type 1 diabetes and requires lifelong insulin therapy. Adjunctive technologies including continuous glucose monitoring and insulin pump therapy to assist in management should considered

Type 3c diabetes (Level V – Expert Opinion).

- A diagnosis of type 3c diabetes is based on new onset hyperglycaemia (≥11.1 mmol/L and/or HbA1c ≥6.5%)with evidence of pancreatitis (lipase elevation >2x ULN and supportive symptoms and CT changes within the pancreas). Evidence of pancreatic exocrine insufficiency is typically present.
- Management of type 3c diabetes is dependent on assessment of residual beta cell function (C-peptide levels). Mild cases with robust C-peptide can be managed as per type 2 diabetes with some oral agents. As mentioned in the text, due to the risk of pancreatitis with ICI therapy, we would not usually recommend DPP4 inhibitors or GLP1RA in people with current or recent ICIs (<3m since cessation).
- Patients with 3c diabetes and inappropriately low/normal C-peptide will require insulin therapy.
- All patients should be assessed for exocrine insufficiency and receive pancreatic enzyme replacement if present

Disclosures

LW, WC, RCB, VT, MC , TT , YH, MO and JEG have no relevant disclosures.

AMM has served on advisory boards for BMS, MSD, Novartis, Roche, Pierre-Fabre, and QBiotics.

GVL is consultant advisor for Agenus, Amgen, Array Biopharma, AstraZeneca, Bayer, BioNTech, Boehringer Ingelheim, Bristol Myers Squibb, Evaxion, GI Innovation, Hexal AG (Sandoz Company), Highlight Therapeutics S.L., Immunocore, Innovent Biologics USA, IOBiotech, Iovance Biotherapeutics, MSD, Novartis, PHMR Ltd, Pierre Fabre, Regeneron, Scancell and SkylineDX B.V.

AMM is supported by a NHMRC Investigator Grant, Nicholas and Helen Moore and Melanoma Institute Australia. JEG received support from NHMRC Program Grant Complexity in Nutrition AppID 1149976 and an NHMRC Investigator Grant AppID 2033227. GVL is supported by a NHMRC Investigator Grant and the University of Sydney Medical Foundation.

Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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Chapter 7: **Discussion**

7.1 Introduction

Following the advent of immune checkpoint inhibitor (ICI) use for cancer therapy in 2014, the first case of checkpoint inhibitor associated autoimmune diabetes mellitus (CIADM) was subsequently reported in 2015. There are now over 11 ICIs approved across 43 indications, now extending into neoadjuvant and adjuvant settings[130]. Subcutaneous ICI forms have recently also been approved [131], further increasing the accessibility and broadening use of ICIs. With up to 43% of cancer patients in USA now estimated to be eligible for ICI treatment[132], CIADM prevalence is predicted to correspondingly increase.

This research project aimed to increase understanding of the novel form of diabetes CIADM from both the bench and the bedside. **Chapter 1** outlines the parallels between type 1 diabetes and what is known of the newly emerging entity termed 'checkpoint inhibitor associated autoimmune diabetes'. **Chapter 2** includes a multicentre case series profiling in details the clinical, biochemical and radiologic characteristics of patients with CIADM. Findings are then examined alongside the broader literature in a systematic review in (**Chapter 3**), which provided evidence to develop a standardised set of diagnostic criteria for CIADM.

Chapter 4 moves beyond diagnosis and treatment of CIADM to potential ways to identify risk of CIADM prior to disease onset. Baseline low grade type 1 diabetes autoantibody titres, lower pancreatic volume and PBMC phenotyping characteristics formed a predictive model for CIADM with a predicted sensitivity of 90% and specificity of 89.2%. **Chapter 5** reports NOD mice model data of CIADM in comparison to traditional mouse models of Type 1 diabetes to improve the understanding of the pathogenesis of CIADM. Using these models, differences in immune islet infiltrate, endocrine and exocrine function were

observed between CIADM, type 1 diabetes models and control mice. Finally, **Chapter 6** translates above findings to recommendations to improve clinical practice and discuss key challenges in recognition for patients who may have CIADM.

7.2. Clinical phenotype of checkpoint inhibitor associated diabetes mellitus

Chapter 2 contains a multicentre case series of patients with CIADM which enabled confirmation of several key findings. CIADM was established to be exclusively associated with anti-PD1/anti-PDL1 therapy with a median onset of 15 weeks. Similar to previous case series [133–135], CIADM had a lower incidence of autoantibody prevalence than seen in type 1 diabetes (43%), and a concerningly high incidence of presenting with diabetic ketoacidosis (69%). C-peptide was universally low (<0.4nmol/l) by one month after diagnosis, indicating rapid loss of insulin secretory capacity. Finally, a high incidence of lipase elevation (48%) was identified, peaking prior to CIADM onset and for the first time lower baseline and on-treatment pancreatic volumes in CIADM patients compared to controls was identified.

Given autoantibodies are unreliable in the diagnosis of CIADM, the initial aim was to investigate what alternative diagnostic criteria could be used. A key characteristic from the CIADM case series was that by one month, all cases universally had low levels of serum C-peptide. It was hypothesised that serum C-peptide was therefore an ideal candidate to use as a potential diagnostic criterion. A systematic review was performed in **Chapter 3** of all published cases and case series of CIADM and identified across 146 papers 192 patients with CIADM. This paper characterised on the largest possible scale the clinical phenotype of CIADM. Patients with positive type 1 diabetes autoantibodies were found to have earlier onset diabetes, higher lipase levels and higher incidence of DKA than those

without. 91.6% of cases had a low C-peptide at presentation and those that did not have a low C-peptide on presentation progressed rapidly to low C-peptide on follow up. On this basis recommendations for diagnostic criteria for CIADM were established that incorporates a low C-peptide as a more sensitive criterion over T1DM autoantibodies. To our knowledge this remains the only paper to provide evidence to support a diagnostic criteria for CIADM.

Importantly, the case series demonstrated that 40% of patients who had glucose tested in the month prior to their diagnosis of CIADM had already met criteria for diabetes. This statistic confirmed anecdotal reports from clinical practice, that CIADM was under-recognised. With the goal to optimise the window of opportunity to diagnose CIADM prior to onset of DKA and thus avoid the associated morbidity, a multidisciplinary consensus statement was written on collaboration with medical oncologists guiding the initial diagnosis and management for patients who develop hyperglycaemia after ICI therapy (**Chapter 6**). One of the key challenges identified in the multidiscliplinary discussion process was that differentials for ICI related hyperglycaemia are broad and CIADM although serious is uncommon. Therefore, a balance in terms of appropriate use of resources and risk stratification to improve the yield of our investigations and burden of testing was required. In this paper, the available literature from international oncology societies is synthesised, evidence from clinical trials and multicentre expert opinion and further gained endorsement from the Endocrine Society of Australia and Australasian Diabetes Society. This is the first paper to our knowledge to provide an in-depth clinical approach to hyperglycaemia in patients on ICIs.

One remaining aspect of the clinical phenotype of CIADM that there was inadequate long term data to fully characterise was the exocrine function. Lipase elevation occurred in 48% at diagnosis and 90% of patients tested met criteria for pancreatic exocrine insufficiency in our case series, yet few reported symptoms of overt steatorrhoea. Of the two patients with follow up testing after 5 years, one patient had returned to normal faecal elastase levels. The mouse data presented in **Chapter 5** demonstrated elevated faecal triglycerides (young p=0.01, aged p=0.04) and serum lipase (young p=0.047; aged p=NS) in CIADM mice compared to controls.

Exocrine function in CIADM patients has not been extensively characterised in the literature to date. In type 1 diabetes, meta-analysis has confirmed that patients with type 1 diabetes have lower pancreatic volume, with evidence of exocrine insufficiency (faecal elastase <200µg/g) in 38.6% [136]. Furthermore, in type 1 diabetes it was noted that low C-peptide levels correlated with greater incidence of pancreatic exocrine insufficiency [101]. Impairment of the exocrine pancreas may be due to the loss residual insulin secretion, as it has been shown that endogenous insulin can exert local effects on acinar cells, and that endogenous concentrations are higher in the pancreas than that of exogenous subcutaneous insulin [137]. Finally, glucotoxicity has been shown to impair exocrine function. Based on the above it is likely that exocrine function in CIADM is impaired at least initially in the setting of glucotoxicity and lack of insulin secretion, although the degree to which this may lead to symptoms or change over time remains to be determined [138].

7.3. Risk factors and predictors of checkpoint inhibitor associated diabetes mellitus

As the indications for immune checkpoint inhibitors expand, it becomes increasingly desirable to develop biomarkers to predict onset of irAEs such as CIADM to aid in the

decision-making process and open a window for preventative strategies. In **Chapter 4** the largest reported cohort of CIADM patient sample data, incorporating serological, immune and radiologic parameters was utilised to determine potential predictors of CIADM in comparison to matched controls. This paper highlighted that CIADM patients have higher pre-treatment anti-GAD and lower pancreatic volume than matched controls. Furthermore, CIADM patients at baseline had higher Th17 T cell prevalence, higher central memory CD4+ T cell and lower naïve CD4+ T cell prevalence than controls and greater dynamic rise in activated CD4+CD38+HLA-DR+ T cell subsets on treatment compared to controls. Similar changes in Th17 T cell prevalence and dynamic rises in activated CD4+ T cell populations have been noted in prior comparable studies [63,64,66].

We identified significant differences in cytokine levels at baseline and on treatment in presence of CIADM and all irAE in comparison to controls. CIADM patients had significantly increased levels of TNF-α, IFN-gamma and IL-2 at time of diagnosis of CIADM compared to baseline not seen in controls on ICI, suggestive of Th1 predominant response. Interestingly IL-4, a Th2 pathway cytokine also just reached significance. IL-17A which we would have expected to be raised given higher baseline Th17 subsets seen in CIADM patients, was more elevated in CIADM patients but this did not reach significance.

It was interesting to find that although T1DM autoantibodies are not as commonly found in CIADM compared to T1DM, the titre of anti-GAD was significantly higher at baseline in CIADM patients than controls. Anti-GAD is the T1DM antibody associated with latent autoimmune diabetes of adulthood, a form of autoimmune diabetes associated with a less aggressive disease [139]. Several studies have shown that anti-GAD positivity is present in a

small proportion of the general population [140], with the Islet Autoantibody Accreditation Program reporting a median specificity of 98.9% [141] and a large Norwegian study finding an anti-GAD prevalence of 1.7% in the non-diabetic Norwegian adult population [142]. Anti-GAD seropositivity in this study was associated with thyroid autoimmunity and prediabetes, which suggests that anti-GAD positivity may relate to true islet autoimmunity. Furthermore, a study in prediction of irAE has similarly noted that low levels of autoantibodies (ANA, RF, anti-CCP) at baseline were associated with organ specific irAE[67].

Although several studies had identified reduction in pancreatic volumes with onset of CIADM [103,134,143], this is the first study to identify a lower baseline volume than controls. In line with evidence that early stages of T1DM also exhibit lower pancreatic volumes prior to overt stage 3 T1DM [39,144], this may also reflect prior pancreatic autoimmunity.

When combined this anti-GAD positivity with the findings of lower baseline pancreatic volume and more experiences immune cell subsets, these biomarkers support a hypothesis of pre-existing islet autoimmunity, with islet tolerance broken as a result of anti-PD1/anti-PDL1 exposure. This may explain the relatively low incidence of CIADM amongst ICI treated patients as anti-GAD (as evidence of previous or current autoimmunity) at baseline is only positive in a low proportion of the general population. Whilst flow cytometry based immunophenotyping may fail to discriminate between patients with irAEs generally versus CIADM specifically, the addition of baseline pancreatic volume and anti-GAD provide a risk prediction model with a predicted sensitivity of 92.6% and specificity of 90.1%. These parameters will require prospective testing with larger validation cohorts in order to further determine their value on a broader scale.

7.4. Pathogenesis of checkpoint inhibitor associated diabetes mellitus

A NOD mouse model of CIADM was used in **Chapter 5** to further elucidate endocrine and local immune changes in the pancreas that were difficult to access in humans. Combining the key observations of Chapter 4 and Chapter 5 this builds upon the preliminary hypothesis on the pathogenesis of CIADM.

Similar to CIADM in humans, in mice fulminant insulitis was identified, rapid loss of beta cells mass and more severe diabetes after anti-PDL1 administration than spontaneous diabetes or administration of streptozotocin. A relative preservation of alpha cell mass was noted, and this is in keeping with the normal glucagon levels in limited number of CIADM patients who have had this tested[133]. Similar to the case series, key findings included lower pancreatic volume, elevated lipase and higher faecal triglycerides indicative of exocrine pancreatic involvement.

In humans, one post-mortem study of a patient with CIADM found persistent PD-L1 expression in beta cells [103], whilst another found no PD-L1 expression [102]. We found that PD-L1 expression is maintained in the remaining beta cells of CIADM mice. PD-L1 expression increased with age and was highest in aged non-diabetic NOD mice, supporting that islet PD-L1 expression is a protective factor against development of diabetes. The ongoing presence of PD-L1 expression in CIADM and T1DM beta cells at similar levels however suggests that the mechanism of CIADM pathogenesis is independent of changes to beta cell PD-L1 expression.

Tissue resident memory cells have been identified as a key pathogenic cell type in gut and mucosal irAEs [115,116,119] and T1DM [118,145], not previously studied in CIADM. For the first

time, the increased prevalence of tissue resident memory cells in CIADM is identified, in comparison to aged controls, T1DM controls and STZ control cohorts. The adoptive transfer study results also indicate that exposure of anti-PDL1 to splenocytes/systemic immune cells alone is not adequate to induce diabetes (i.e. transfer of splenocytes from a mouse with CIADM), whereas exposure to anti-PDL1 again after these CIADM splenocytes have had a chance to engraft locally in the host mouse had a high incidence of diabetes. This data also supports the role of activation of local pancreatic tissue resident memory cells in CIADM pathogenesis.

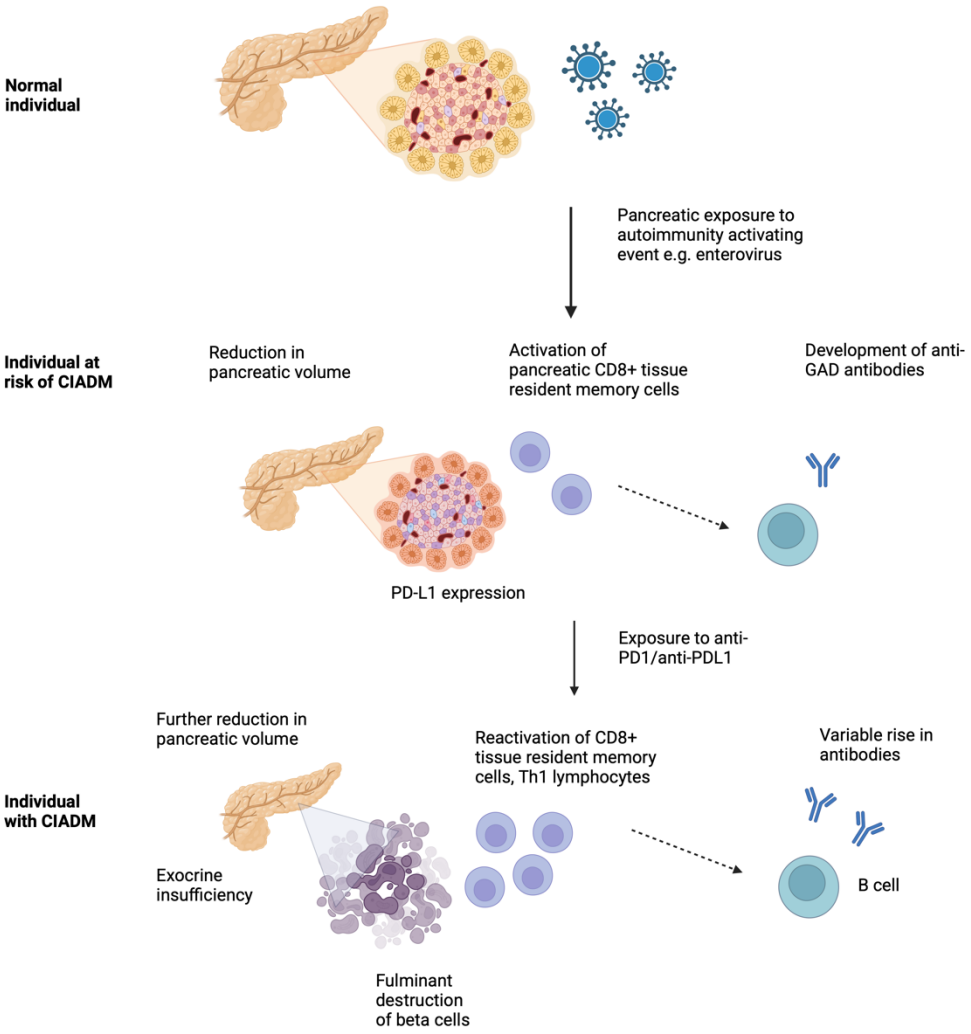

Figure 7.1. Proposed pathogenesis of checkpoint inhibitor associated diabetes mellitus. A normal individual has an environmental exposure such as enterovirus, leading to priming of the local pancreatic immune system. This initial immune response is associated with insulitis with development of pancreatic CD8+ tissue resident memory T cells, anti-GAD antibodies, pancreatic PD-L1 expression and mild reduction in pancreatic volume. If this is tolerised, the individual does not develop Type 1 diabetes. When the person is later exposed to anti-PD1 or anti-PDL1 reactivation of tissue resident memory T cells occurs, then rise in Th1 lymphocytes and antibodies, fulminant islet destruction, and further reduction in pancreatic volume ± exocrine insufficiency.

7.5 Limitations

One of the biggest challenges to the study of CIADM in humans has been the low incidence of disease which has limited the ability to power studies and detect small differences. The biomarker study in humans had a case to control ratio of 1:2 as a pilot study, however a case to control ratio of 1:99 would be required to more closely emulate the real world scenario in prospective validation studies. The limited sample size elevates the positive and negative predictive values of the proposed biomarkers and thus highlight the need for further validation.

The study of CIADM in humans is limited by the difficulty in accessing pancreatic tissue for analysis of local immune changes. We sought to overcome this by utilising a mouse model of CIADM, however it must be acknowledged that murine data is limited by the same differences between mouse and human models that have made T1DM studies in mice difficult to translate to humans. We had a 100% incidence of CIADM after ICI exposure, indicating key differences in predisposition to diabetes between species. Analysis of predictors of CIADM development is therefore also not possible in our mouse study. Again, as an exploratory study, the cohort sizes were smaller (n=4-8) and would require validation in larger cohort studies.

Finally, islet-specific lymphocyte subsets in humans or mice were not tested but may have been useful in determining the specificity of immune changes seen. In humans this is challenging due to the need for HLA subtyping in the case of tetramer assays which further limits sample size, and prospective collection in a disease of such low incidence would require extended research timelines beyond the scope of this thesis. In mice, we aimed to

overcome this barrier by isolating immune cells from islets directly, which are likely to be specific based on their location.

7.6. Future directions

Prospective validation of our proposed biomarkers in a larger prospective cohort would be of great value. This would ideally be a prospective study in collaboration with established biobanks including CT volumetry, serum antibody analysis and flow cytometric analysis. More detailed cytometric analysis using technologies such as single-cell RNA-Seq and mass cytometry would be desirable in the CIADM group. With effective tools for predicting risk of irAE, clinicians can better inform patients of the relative risk and benefit of ICIs prior to initiation. This is particularly relevant in neoadjuvant and adjuvant settings where the benefits in overall survival may be less definitive and alternative therapeutic options of comparable efficacy are available.

Prevention and early diagnosis of CIADM remains a high priority in this field as treatment of CIADM is challenging, given the majority of beta cells have been destroyed by time of onset of diabetes. Steroids have been shown to be ineffective in treating CIADM[146–148]. Whilst there has been one case report of effective use of infliximab to reverse CIADM[149], this patient never demonstrated a low C-peptide consistent with insulin deficiency, and additionally had steroid exposure, making steroid-induced diabetes a more likely differential.

In addition to validation of results, the prominent role of tissue resident memory T cells we identified in our study, lends value to the therapeutic potential of targeting this cell type. JAK/STAT pathway inhibitors have been shown to inhibit development of type 1 diabetes

and CIADM in mice [103] and delay onset in humans recently [150], making them a promising therapeutic agent for use in CIADM. Case reports of effective use of JAK inhibitors already approved for use in autoimmune diseases such as baricitinib and tofacitinib in patients with severe irAE have emerged [151,152]. In ICI related colitis in particular numerous case reports have found tofacitinib an effective agent for treatment of refractory colitis[116,153,154].

One significant concern with use of JAK/STAT inhibitors and inhibition of tissue resident memory cells however is the evidence that these pathways are also crucial for anti-tumour efficacy of ICIs [155–157]. Whilst this has not been specifically investigated for JAK inhibitors, in a broader registry study of second line immunosuppressive therapies for treatment of irAEs after steroids, 217 patients received a second line nonsteroidal immunosuppressive therapy [158]. No significant difference in tumour response was seen in comparison to steroid treated controls with irAEs. More studies of the comparative effects of anti-autoimmune and anti-tumour effects of such agents in mice is required before determining their potential use in humans.

7.7. Conclusion

Checkpoint inhibitor associated autoimmune diabetes is an uncommon but morbid immune related adverse event. It is characterised by rapid loss of C-peptide and requires distinct diagnostic criteria to its de novo equivalent type 1 diabetes due to its relatively low incidence of islet autoantibodies. Baseline lower pancreatic volume, higher anti-GAD titres and a more experienced Th17 predominant immunophenotype prior to checkpoint inhibitor therapy in our preliminary study are predictors of CIADM development. Mouse islet analysis demonstrates that exposure of CD8+ tissue resident memory T cells to checkpoint inhibitors are a critical pathogenic step in CIADM development and potential therapeutic target. Further research is required to validate these findings for potential clinical applications.

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(Note: Includes references for non-published portion of Chapter 1, Chapter 5 and Chapter 7; does not include references listed in published manuscripts)

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8.0 Supplemental material

PDF versions of published papers

8.1 PDF version of published manuscript from Chapter 1: Unravelling Checkpoint Inhibitor Associated Autoimmune Diabetes: from Bench to Bedside

8.2 PDF version of published manuscript from Chapter 2: Checkpoint Inhibitor Associated Autoimmune Diabetes Mellitus is Characterised by Rapid C-peptide Loss and Pancreatic Atrophy

8.3 PDF version of Letter to the Editor from Hao and Xue for "Checkpoint Inhibitor-associated Autoimmune Diabetes Mellitus Is Characterized by C-Peptide Loss and Pancreatic Atrophy" (Chapter 2)

8.4 PDF version of Response to Letter to the Editor from Hao and Xue: "Checkpoint Inhibitor-associated Autoimmune Diabetes Mellitus Is Characterized by C-Peptide Loss and Pancreatic Atrophy" (Chapter 2)

8.5 PDF version of published manuscript from Chapter 3: Risk Factors and Characteristics of Checkpoint Inhibitor–Associated Autoimmune Diabetes Mellitus (CIADM): A Systematic Review and Delineation From Type 1 Diabetes