nature portfolio

Peer Review File

Glycaemic variability underlies myocyte dysfunction and myocardial injury risk in diabetes

Corresponding Author: Professor Nathan Palpant

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study sought to find risk factors for cardiac dysfunction and myocardial injury in diabetic patients, and they concluded that glycaemic variability was an important factor. The study was carried out mainly through cohort studies, ex vivo and in vivo experiments and clinical samples. The design of the study was relatively comprehensive, but there were some problems with the presentation of the findings and logic, as described below:
1.Major concerns
1.1. Figure S1B shows 4 of the 10 sets of clinical data showing differences in post-MI cardiac function between DM and non-DM populations, which is inconsistent with the authors' conclusions on line 82 in the manuscript. Also, the values of change in post-MI cardiac functon(LVEF) between DM and non-DM populations in different data sets are very high (~30-60%), what were the authors' criteria for selecting these data? In addition, both Figure S1A and Figure S1B results are based on diabetic and non-diabetic MI populations, yet the authors selected data from different sources, what were the considerations? How do these data from different sources correspond to or represent the findings and conclusions of subsequent studies?
1.2. In general, the mortality rate after MI in normal normal animals is about 10-30%, and in the context of diabetes, it is further elevated, and it is hard to believe that the authors showed a mortality rate of only 9.1% after MI in diabetic cG mice in Figure 2C. To confirm the validity of the MI procedure, the authors should have shown the cardiac function parameters and cardiac pathology characteristics of the mice before and after MI. Otherwise, it cannot be concluded that the risk of acute mortality after MI in diabetic patients is mainly determined by hyperglycemic variability.
1.3. Numerous studies have shown that glucose oxidation is inhibited and fatty acid oxidation is further upregulated in diabetic, especially type 2 diabetic, cardiomyocytes. Curiously, the authors show in Figure 3D that fatty acid oxidation is decreased and glucose oxidation is generally elevated in the cG and vG groups compared to the CTRL group. the same is true for Figure 3I. The authors are requested to verify the original RNA-seq data and provide an explanation. In addition, the analytical screening conditions for the RNA-seq data should be described to provide replication.
1.4. The results of in vitro Figures 3G-3H and in vivo Figure 2B are not entirely consistent. Specifically, the in vivo experiments did not observe a statistical difference in EF (even though FS was not shown), however there was a change in cardiac output, which I guess is strongly related to the decrease in heart rate, in other words, the authors should have provided more detailed information about the measured parameters of echocardiography, or even included ECG. And in the in vitro experiments, the amplitude or contractility of hiPSC-CM in different groups had significant differences, which contradicted the in vivo EF data. In addition, in Figure 3H, the Control group showed abnormal raw contraction trace, which should be carefully confirmed.
1.5. The use of patient blood samples and the conclusions drawn in Figure 4 are highly problematic, specifically in two main ways. One, the aforementioned in vivo mouse and in vitro cellular models are biased towards type 2 diabetes modeling (high fat/high glucose, not treated with STZ), whereas in vitro samples were collected from 12 patients with type 1 diabetes, which is confusing in the use of the model, as insulin resistance is different in the two types, and the authors' conclusions are therefore informed by unknown factors. Second, the details of the blood samples used for the processing of hiPSC-CM were inconsistent in the methodology of the accessory material. According to the description, plasma samples were used for cell processing, while serum samples were used for experimental replication. This also limits the accuracy of the authors' study as the two components are different.
2.Other minor issues
2.1. In summary, hyperglycemic biomarkers do not account for risk of post-infarction mortality in diabetics. It should not be a surprise that there are many risks of post heart attack death in diabetics, please change this sentence.
2.2. “Using in vivo and in vitro models, we demonstrate that glycaemic variability rather than hyperglycaemia alone is a

dominant risk factor for heart muscle dysfunction and myocardial injury sensitivity in diabetes." Please use specialized terms.
2.3. line65-"Emerging evidence suggests glycaemic variability is a major risk factor for cardiovascular disease 14".The conclusion needs to be supported by more and more definitive evidence.
2.4. In Figure 3B, the contrast of the Oil Red O staining is quite different, so please adjust it on the same background.
2.5. The heat map of differential genes in Figure 3C is too small and does not show enough valid information, especially in the vertical axis.

Reviewer #2

(Remarks to the Author)
Using biobank-scale human population data, the authors demonstrated that glucose regulation-related factors cannot effectively predict post-myocardial infarction mortality risk. Building on this finding, they further evaluated the relationship between glycemic stress and acute cardiovascular injury/death risk in diabetes, while exploring underlying mechanisms to establish glycemic variability as a cardiovascular risk factor. Through integrated epidemiological, experimental, and clinical evidence, the authors substantiated its pathophysiological role in cardiac dysfunction. My comments are as follows:

Despite the inclusion of a large sample size for analysis, it remains imperative to investigate additional variables including lifestyle factors and medication adherence. Furthermore, given that T2 constitutes the predominant diabetic population, accounting for over 90% of diabetes-related cardiovascular complications, and considering the potential differences in glycemic variability patterns and lipid metabolism profiles between T1 and T2 patients and other diabetic subgroups, future studies should stratify analyses accordingly.

While the causal relationship between glycemic variability and cardiomyocyte injury has been validated, it remains imperative to develop targeted therapeutic interventions addressing glucose fluctuations and substantiate their cardioprotective efficacy in preclinical models.

The investigation was limited to observing acute (short-term) contractile function alterations, without assessing the potential recovery of cardiomyocyte function post-serum withdrawal. Implementing time-course experiments with graded exposure durations could delineate the reversibility of glycemic fluctuation-induced injury, thereby establishing temporal parameters for optimal therapeutic intervention windows.

Serum collection was not strictly matched to patients' cardiovascular events (e.g., MI history), making it difficult to determine whether glycemic variability is a cause or a consequence (e.g., heart failure may contribute to metabolic disorders). Future prospective cohort studies should track the temporal relationship between serum markers and cardiovascular outcomes to clarify causality.

Reviewer #3

(Remarks to the Author)
In the clinical part of this study, Cao et al analyzed the relation of conventional cardiometabolic factors with the incidence of MI and post-MI survival in diabetic and non-diabetic individuals in the UK Biobank Cohort. The authors showed that both DM and non-DM individuals with higher HbA1c levels were more likely to have an MI than those with lower HbA1c levels. However, no difference in post-MI survival was observed between DM patients with lower and higher HbA1c levels. So the authors postulated that other cardiometabolic factors, such as glycemic variability, rather than hyperglycemia per se underlie myocardial dysfunction and injury in patients with diabetes.
However, there are some issues that should be clarified.
1. A major problem of this part is that the authors confused the concepts of MI death and all-cause death after MI. In the manuscript, it seems that the authors analyzed survival from all-cause mortality after MI. However, in the figure legends of Figure 1 and Figure S1, the authors stated the 'death from MI'. The post-MI event should be explicitly defined. If the MI death or cardiovascular death was adopted, non-cardiovascular death should be treated as a competing risk in the analysis.
2. The authors compared the incidence of MI and post-MI mortality between patients in the up and bottom 30% quantile of cardiometabolic factors including HbA1c and LDL-C. Why did you choose 30%? Especially for glycemic levels, hypoglycemia and hyperglycemia are both risk factors for MACE. This might be the reason that post-MI survival was not different between these 2 quantiles. Cardiovascular events in full quantiles of these cardiometabolic factors should be analyzed to confirm the relationship.
3. Another issue is that high glycemic variability usually comes with high glycemic level. In other words, if the cardiovascular events are not related to HbA1c level, it might be unrelated to glycemic variability as well. So I do not think these data are a good start of this study. This point should at least be discussed.
Minor:
1. 'HbA1c' rather than 'Hba1c' should be used.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
1、While the authors have made textual revisions to acknowledge the inconsistency in the clinical data, the core methodological and interpretive issues raised in the initial query have not been fully addressed.
First, the authors mention "predefined inclusion criteria" for data selection, but they have not clearly detailed what these criteria are in terms of the range of acceptable values for sample size, or how they defined "sufficient sample size". Second, regarding the high values of change in post - MI cardiac functon(LVEF) between DM and non - DM populations (~30 - 60%), the authors have not addressed whether these extreme values are outliers in the field and how they might impact the overall analysis and conclusions. Third, although the authors aim to provide a reference point for interpreting animal modelling studies, they have not explicitly discussed how the inconsistencies in the clinical data relate to the potential limitations or generalizability of their animal model results. These aspects need to be further elaborated to strengthen the study's validity and credibility.
2、The authors' inclusion of echocardiographic and histologic data is a positive step. However, key concerns persist. While the 40-minute ischemia model may explain lower mortality, its clinical relevance to the high-risk diabetic MI population you aim to model is unclear. Could you provide comparative mortality data from your lab or literature for permanent ligation in this model to better contextualize your findings?
Furthermore, the new functional data show a decline in EF, but the absolute post-MI values appear relatively high. Please provide the actual infarct size percentages quantified from your histology. It is critical to demonstrate that the infarct size is substantial and equivalent between groups to firmly conclude that the difference in mortality is solely attributable to glycemic variability and not to a milder, non-lethal injury.
3、The cited Wende et al. 2020 study seems to have results different from the general understanding but not fully in line with your data either. Given that numerous studies show increased FAO and decreased glucose oxidation in diabetic cardiomyocytes, how do the authors explain their findings of decreased FAO and increased glucose oxidation in cG and vG groups? Could the acute nature of glycaemic variability be the key factor here, and if so, how does it override the chronic metabolic changes typically seen in diabetes?
4、The authors' response regarding systemic compensation in vivo is plausible but remains speculative without direct experimental support. While the new in vitro beat rate data provides some alignment, the core contradiction in contractility amplitude between systems persists. Specifically, if glycemic variability significantly impairs intrinsic cardiomyocyte contraction as shown in vitro, what specific compensatory mechanisms (e.g., sympathetic activation, preload changes) maintain near-normal EF in vivo? Please provide the full echocardiography parameters (including FS, heart rate, and ventricular dimensions) as supplementary data to allow proper assessment of loading conditions.

Additionally, regarding the corrected contraction trace: could you clarify how many replicates were re-examined and confirm the statistical robustness of this correction? The concern about the trace's abnormality now extends to its representativeness across the dataset.
5、Even with expanded patient samples, the fundamental issue of model mismatch between T1D patient samples and T2D-like preclinical models remains. How can you convincingly argue that GV's effect is diabetes-type independent without direct T2D patient sample validation?
Could you provide experimental data or cite literature demonstrating that the specific molecular mechanisms of glycemic variability-induced cardiotoxicity you propose (e.g., transcriptional changes in Fig. 3) are indeed conserved between T1D and T2D? Furthermore, for the 32 patients, were key covariates like diabetes duration, insulin therapy regimen, and presence of autoantibodies matched between high and low GV groups? These factors could confound the observed biomarker and contractility differences more than GV itself.
6、While the authors have addressed most technical points, the response to comment 2.3 remains inadequate. Merely stating that "revisions are provided throughout" does not allow for a proper assessment. Could you specifically indicate which new data figures or analyses most definitively support the claim that glycaemic variability is a "major risk factor"? Please highlight the key experimental evidence that distinguishes GV's effect from other comorbid risk factors.
Additionally, for the revised heatmap (Fig. 3C/Suppl. Fig. 3), please confirm in the figure legend that all displayed gene clusters passed statistical significance thresholds after multiple testing correction, and indicate the specific clustering method used.

Reviewer #2

(Remarks to the Author)
The authors responded to the raised questions. I have no further questions.

Reviewer #3

(Remarks to the Author)
The authors have addressed my previous concerns satisfactorily.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study aimed to identify novel predictors of adverse cardiovascular events (primarily myocardial infarction) in diabetic patients. Analysis of UK Biobank cohort data revealed limitations in the traditional HbA1c metric for predicting mortality following myocardial infarction in diabetics, thereby highlighting the potential of glycaemic variability (GV) as an alternative predictive indicator. Subsequent investigators conducted animal studies, cellular experiments, and patient plasma assays to further elucidate the predictive potential of glycaemic variability (GV). Overall, the article exhibits clear logic and holds considerable clinical significance. The authors have addressed to a certain extent the issues raised in our previous two rounds of peer review, significantly enhancing the paper's overall quality. However, the following major issues remain:
Comment 1: The researchers have still not addressed Comment 3(Given that numerous studies show increased FAO and decreased glucose oxidation in diabetic cardiomyocytes, how do the authors explain their findings of decreased FAO and increased glucose oxidation in cG and vG groups? Would the acute nature of glycaemic variability be the key factor here, and if so, how does it override the chronic metabolic changes typically seen in diabetes?) raised previously. The metabolomic data presented herein do not align with the metabolic alterations observed in diabetes. However, it is perplexing that the researchers merely relocated the data to the supplementary figures without elucidating the underlying cause.
Comment 2: Regarding the selection of animal models, although the authors propose this model as a mouse model of glycaemic variability, its modelling approach differs from established diabetic animal models. Consequently, the discussion section ought to include a more explicit description of the model's limitations and how it diverges from recognised and commonly used diabetic models. This would assist researchers in the field to comprehend and select appropriate models.
Comment 3: Regarding the in vitro cell culture section, as per the previous review comments, Figure 3b (Oil Red O staining) requires image replacement. The current image quality is not convincing.
Comment 4: In the cellular experiments, what was the rationale for selecting a concentration of 10 nM Hi1a? The paper lacks supporting references or experimental data regarding the concentration selection.

Reviewer #1 comments:

This study sought to find risk factors for cardiac dysfunction and myocardial injury in diabetic patients, and they concluded that glycaemic variability was an important factor. The study was carried out mainly through cohort studies, ex vivo and in vivo experiments and clinical samples. The design of the study was relatively comprehensive, but there were some problems with the presentation of the findings and logic, as described below:

1.Major concerns

1.1. Figure S1B shows 4 of the 10 sets of clinical data showing differences in post-MI cardiac function between DM and non-DM populations, which is inconsistent with the authors' conclusions on line 82 in the manuscript. Also, the values of change in post-MI cardiac function (LVEF) between DM and non-DM populations in different data sets are very high (~30-60%), what were the authors' criteria for selecting these data? In addition, both Figure S1A and Figure S1B results are based on diabetic and non-diabetic MI populations, yet the authors selected data from different sources, what were the considerations? How do these data from different sources correspond to or represent the findings and conclusions of subsequent studies?

Response to Comment 1.1: We appreciate the reviewer's comments regarding data sources and their relevance to the study conclusions. Supplementary Fig. 1a and Supplementary Fig. 1b both address differences between diabetic and non-diabetic populations following MI, but they were designed to highlight different clinical aspects: acute mortality due to MI and post-MI cardiac function, specifically LVEF. Due to the limited availability of studies that report both mortality outcomes and cardiac function measures stratified by diabetes status within the same cohort, we sourced data from separate but clinically relevant studies. Selection was based on predefined inclusion criteria, including stratification by DM status, sufficient sample size, and clear reporting of either mortality outcomes or LVEF.

Our purpose in providing these meta-analyses of clinical cohorts was to provide a reference point for readers to help interpret clinical relevance of our animal modelling studies specifically regarding post-MI mortality and heart function. We acknowledge that these clinical studies show inconsistent differences in post-MI left LVEF between DM and non-DM populations. To address this, we have revised the statement on **lines 97-98** to accurately reflect the data:

"Among patients that do survive, post-MI heart function is not consistently significantly different between DM and non-DM populations (Supplementary Fig. 1b)."

We have also revised statements throughout the results and discussion to ensure they accurately reflect the clinical findings in the context of results presented in this study.

1.2. In general, the mortality rate after MI in normal animals is about 10-30%, and in the context of diabetes, it is further elevated, and it is hard to believe that the authors showed a mortality rate of only 9.1% after MI in diabetic cG mice in Figure 2C. To confirm the validity of the MI procedure, the authors should have shown the cardiac function parameters and cardiac pathology characteristics of the mice before and after MI. Otherwise, it cannot be concluded that the risk of acute mortality after MI in diabetic patients is mainly determined by hyperglycemic variability.

Response to Comment 1.2: We thank the reviewer for raising this point. We acknowledge that reported mortality rates following myocardial infarction (MI) can vary widely depending on several experimental variables, particularly the duration of ischaemia, the surgical model used (permanent ligation vs. ischaemia-reperfusion), mouse strain, age, glycaemic burden, and perioperative care protocols.

In our study, we utilised a moderate-duration ischaemia-reperfusion injury model (40 minutes of LAD ligation followed by reperfusion), which is known to induce reproducible infarction with relatively lower acute mortality rates compared to models with longer ischaemic durations or permanent occlusion. This protocol was selected to model clinically relevant, survivable MI, particularly in the context of studying post-MI survival under controlled metabolic perturbations such as glycaemic variability. To reduce variability in infarct size and other procedural factors, MI surgeries were conducted by a single experienced surgeon blinded to group assignments. This approach reduces the likelihood that differences between experimental groups were due to variation in MI induction. Thus, the lower acute mortality observed in the cG group (9.1%) likely reflects the milder injury burden imposed by this model, rather than technical inconsistency or a lack of MI induction.

To confirm the validity and consistency of our MI procedure, we have now both included pre- and post-MI echocardiographic measurements, which demonstrate a significant decline in ejection fraction (EF) across all groups, including cG and vG, consistent with successful MI induction and reduced systolic function. These data are now provided in **Fig. 2d** and detailed in the revised Results section (**lines 145–147**). In addition, we have included histological analyses using Masson's trichrome staining (**Methods lines 217–243**), which show equivalent infarct size and fibrotic remodelling between glycaemic variability groups post-MI in the revised **Fig. 2e-f** and the revised Results section (**lines 147–149**). Together, these data confirm consistent and reproducible infarct injury across groups.

In summary, we believe our revised data provide strong evidence for the validity of the MI model, and support our conclusion that glycaemic variability, rather than total hyperglycaemic burden, is a critical determinant of early post-MI survival.

1.3. Numerous studies have shown that glucose oxidation is inhibited and fatty acid oxidation is further upregulated in diabetic, especially type 2 diabetic, cardiomyocytes. Curiously, the authors show in Figure 3D that fatty acid oxidation is decreased and glucose oxidation is generally elevated in the cG and vG groups compared to the CTRL group. the same is true for Figure 3I. The authors are requested to verify the original RNA-seq data and provide an explanation. In addition, the analytical screening conditions for the RNA-seq data should be described to provide replication.

Response to Comment 1.3: We agree with the reviewer that studies have demonstrated a metabolic shift in cardiomyocytes toward increased fatty acid oxidation (FAO) and decreased glucose oxidation in the context of chronic type 2 diabetes (T2D). This shift is typically attributed to *chronic* metabolic stress associated with sustained insulin resistance, mitochondrial dysfunction, and broader systemic stressors.

However, our study focusses on acute glycaemic variability (GV) rather than sustained hyperglycaemia or long-term insulin resistance. GV introduces intermittent glucose surges that exert unique metabolic and transcriptional effects distinct from chronic diabetic states.

In support of our findings, we draw attention to recent studies (e.g. see Wende et al. 2020, *Diabetes*[1]), demonstrating that enhancing glucose uptake in diabetic mouse hearts leads to a reduction in both FAO and glucose oxidation. Importantly, increasing myocardial glucose (which would occur under conditions of high glucose variability even in the presence of chronic hyperglycaemia) exacerbates mitochondrial dysfunction. This finding supports our data (**Supplementary Fig. 3b** and **Fig. 3f-g** and **Supplementary Fig. 4**) showing repression of FAO genes and activation of glucose metabolism pathways in response to glycaemic fluctuations.

Our study supports prior studies showing a distinct glucotoxic transcriptional signature, likely driven by repeated glucose surges rather than prolonged hyperinsulinaemia or lipotoxicity. This response may reflect an early maladaptive response, whereby glycaemic oscillations modulate gene networks governing substrate utilisation and mitochondrial function. We have revised our description of these results (see **lines 170-174**) to clarify this concern and ensure our findings are supported by evidence showing consistency with the broader literature in the discussion (see **lines 221-226**).

1.4. The results of *in vitro* Figures 3G-3H and in vivo Figure 2B are not entirely consistent. Specifically, the in vivo experiments did not observe a statistical difference in EF (even though FS was not shown), however there was a change in cardiac output, which I guess is strongly related to the decrease in heart rate, in other words, the authors should have provided more detailed information about the measured parameters of echocardiography, or even included ECG. And in the in vitro experiments, the amplitude or contractility of hiPSC-CM in different groups had significant differences, which contradicted the in vivo EF data. In addition, in Figure 3H, the Control group showed abnormal raw contraction trace, which should be carefully confirmed.

Response to Comment 1.4: We acknowledge the reviewers comment highlighting the challenges of interpreting cardiac functional assessment across biological systems. First, we note that in vivo contractility measurements such as ejection fraction (EF) and fractional shortening (FS) are influenced not only by intrinsic myocardial contractility but also by loading conditions, ventricular geometry, vascular tone, and heart rate, among other systemic factors. These systemic variables are known to compensate for intrinsic contractility defects which could only be demonstrated by isolating intrinsic heart contractility away from system loading.

The following changes have been made to address this concern and help align outcomes from *in vivo* and *in vitro* studies more clearly. We agree with the reviewer that the reduction in cardiac output is likely driven primarily by differences in heart rate and now provide supplementary data showing reduction in beat rate for *in vitro* studies that align with the *in vivo* data (See new added **Supplementary Fig. 5**). Second, we highlight excellent alignment of isovolumic relaxation time (IVRT) *in vivo* and changes in velocity of contraction *in vitro* that both align in demonstrating impacts of glycaemic variability on contractile kinetics. While we observe a small but insignificant difference in EF and FS, we have now discussed the contribution of loading effects to *in vivo* pump performance that may account for this difference.

Furthermore, as requested, we have re-examined the raw contraction traces and identified that the original trace provided for the Control group in Fig. 3H was not fully representative of the typical contraction profile observed in that group, and this has been corrected in the revised **Fig. 3ei**.

In summary, while discrepancies exist between *in vitro* and *in vivo* functional parameters, these are expected and underscore the distinct advantages and limitations of each system. The in vivo model captures integrative cardiovascular physiology, including baroreflexes, autonomic tone, and vascular

coupling, which can obscure or compensate for subtle cellular deficits. In contrast, in vitro hiPSC-CM assays offer high-resolution insight into the primary effects of metabolic stress on cardiomyocyte contractility, without systemic buffering. Taken together, the findings from both models provide complementary perspectives on the impact of glycaemic variability on cardiac function.

1.5. The use of patient blood samples and the conclusions drawn in Figure 4 are highly problematic, specifically in two main ways. One, the aforementioned in vivo mouse and in vitro cellular models are biased towards type 2 diabetes modeling (high fat/high glucose, not treated with STZ), whereas in vitro samples were collected from 12 patients with type 1 diabetes, which is confusing in the use of the model, as insulin resistance is different in the two types, and the authors' conclusions are therefore informed by unknown factors. Second, the details of the blood samples used for the processing of hiPSC-CM were inconsistent in the methodology of the accessory material. According to the description, plasma samples were used for cell processing, while serum samples were used for experimental replication. This also limits the accuracy of the authors' study as the two components are different.

Response to Comment 1.5: We agree with the reviewer regarding the challenge in aligning preclinical modelling studies with studies using patient-derived blood samples.

As the reviewer notes, our *in vivo* and *in vitro* models indeed reflect type 2 diabetes (T2D)-like metabolic stress, whereas the patient plasma samples used for hiPSC-CM treatment were derived from individuals with type 1 diabetes (T1D). As stated in the Limitations of the Study section, our access to patient samples was constrained by current Australian federal policy under the National Diabetes Services Scheme (NDSS), which provides continuous glucose monitoring (CGM) funding only for patients with T1D. Consequently, only T1D plasma samples with available CGM data were accessible under our existing ethics approvals. Thus, our work with patient plasma represents a proof-of-concept study aiming to explore whether GV, irrespective of diabetes type, could induce functional changes in hiPSC-CMs.

To strengthen these findings, we have significantly expanded our patient-focussed study. In the revised manuscript, we expanded the blood sample analysis from 12 to 32 patients (**Table 1 and Supplementary Table 1**). The overall findings remain the same as our original findings, showing that cardiomyocyte contractility is significantly reduced in patients with high glycaemic variability. This effect is lost when patients are ranked by their hyperglycaemic state (% time very high).

Further, to investigate whether these plasma samples are associated with evidence of subclinical cardiac injury, we performed quantification of cardiac biomarkers using high-sensitivity immunoassays. Importantly, none of the patients had a documented history of cardiovascular disease, yet patients with high GV displayed elevated levels of BNP, ANP, CK-MB, and cTnI. These findings indicate a potential biochemical signature of subclinical cardiac stress or injury in individuals with high GV, reinforcing the pathophysiological relevance of GV and aligning with our preclinical data. All these results are now presented in the revised **Fig. 4**, with corresponding updates to the Results (**lines 184-204**) and Methods (**lines 244–285**) sections.

Regarding the issue of blood sample used in our studies, we have now revised the manuscript and figures to consistently state that plasma samples were used throughout all hiPSC-CM experiments. No serum samples were used. The methods section on **lines 244-258** has been updated accordingly to avoid ambiguity.

In summary, while we recognize the limitations of model alignment between T1D patient samples and T2D-like preclinical models, we believe our expanded data set and biomarker analysis substantially strengthen the translational value of our findings. Together, they support the conclusion that glycaemic variability, regardless of diabetes type, may contribute to cardiac dysfunction and early myocardial stress, underscoring the clinical importance of GV monitoring in diabetes care.

2. Other minor issues

2.1. In summary, hyperglycemic biomarkers do not account for risk of post-infarction mortality in diabetics. It should not be a surprise that there are many risks of post heart attack death in diabetics, please change this sentence.

Response to Comment 2.1: In response, we have revised the manuscript text for improved clarity and accuracy. The updated sentences now read:

Revised sentence on **lines 34–36**:

"We use human population genetic data to demonstrate that classical cardiovascular disease risk biomarkers, including common measures of hyperglycaemia, do not fully account for the increased risk of post-MI mortality in patients with diabetes."

Revised sentence on **lines 122–124:**

"Together, these findings show that classical cardiovascular disease risk biomarkers, including common measures of hyperglycaemia, do not fully capture the elevated post-MI mortality risk in diabetes, highlighting a significant area of need for identifying new clinical biomarkers."

These revisions more accurately reflect our findings from the UK Biobank cohort, where commonly used biomarkers, including HbA1c, were associated with MI incidence but did not stratify risk of post-MI mortality in diabetic individuals.

2.2. "Using *in vivo* and *in vitro* models, we demonstrate that glycaemic variability rather than hyperglycaemia alone is a dominant risk factor for heart muscle dysfunction and myocardial injury sensitivity in diabetes." Please use specialized terms.

Response to Comment 2.2: We thank the reviewer for the suggestion. In response, we have revised the sentence on **lines 37-39** to incorporate more specialised terminology. The updated sentence now reads: "Using *in vivo* and *in vitro* models, we demonstrate that glycaemic variability, rather than sustained hyperglycaemia alone, is a key risk factor for cardiomyocyte dysfunction and increased susceptibility to myocardial injury in diabetes."

2.3. line65-"Emerging evidence suggests glycaemic variability is a major risk factor for cardiovascular disease 14". The conclusion needs to be supported by more and more definitive evidence.

Response to Comment 2.3: We have revised every aspect of the current manuscript to provide further evidence supporting this conclusion. These include new analysis of human genetic data, additional data supporting *in vivo* MI model, new studies supporting *in vitro* modelling work, and significant new studies expanding our analysis of human blood samples from diabetes patients. The detailed revisions are provided in response to reviewer comments throughout the revised study. In addition to findings outlined in our study, we have also added several additional references to reflect the broader literature

that supports a link between glycaemic variability as a risk factor for cardiovascular disease[1,2] (see **lines 78–84**).

2.4. In Figure 3B, the contrast of the Oil Red O staining is quite different, so please adjust it on the same background.

Response to Comment 2.4: To address this concern, we have reprocessed the images in Figure 3 using identical background levels and contrast settings to ensure consistent visual presentation across all groups. The updated version of **Fig. 3b** (**page 9**) has been included in the revised manuscript.

2.5. The heat map of differential genes in Figure 3C is too small and does not show enough valid information, especially in the vertical axis.

Response to Comment 2.5: We appreciate the reviewer's suggestion. To clarify, we performed hierarchical clustering (Euclidean distance, complete linkage) on differentially expressed (DE) genes from all comparisons, identifying two major gene clusters: one comprising 1,482 genes upregulated in cG and vG samples relative to controls, and another with 1,266 genes upregulated in controls relative to diabetic groups.

In response to this comment, we have improved the readability of the original Figure 3C by expanding the figure panel to display gene clusters on both horizontal and vertical axes. Additionally, we have included this heatmap as a new **Supplementary Fig. 3**, which also highlights dysregulation of gene programs controlling insulin resistance, fatty acid (FA) oxidation, and glucose oxidation. The updated figure and corresponding descriptions are included in the revised manuscript.

Reviewer #2 comments:

Using biobank-scale human population data, the authors demonstrated that glucose regulation-related factors cannot effectively predict post-myocardial infarction mortality risk. Building on this finding, they further evaluated the relationship between glycemic stress and acute cardiovascular injury/death risk in diabetes, while exploring underlying mechanisms to establish glycemic variability as a cardiovascular risk factor. Through integrated epidemiological, experimental, and clinical evidence, the authors substantiated its pathophysiological role in cardiac dysfunction. My comments are as follows:

Comment 2.1. **Despite the inclusion of a large sample size for analysis, it remains imperative to investigate additional variables including lifestyle factors and medication adherence. Furthermore, given that T2 constitutes the predominant diabetic population, accounting for over 90% of diabetes-related cardiovascular complications, and considering the potential differences in glycemic variability patterns and lipid metabolism profiles between T1 and T2 patients and other diabetic subgroups, future studies should stratify analyses accordingly.**

Response to this comment: Acknowledging the differences in disease profiles between T1 and T2 DM patients, the analysis in the UK Biobank only focuses on T2 DM patients. There are insufficient T1 DM patients in the UK Biobank to have the statistical power to detect the impact of blood glucose measures. We further acknowledge that there are additional differences in lifestyle characteristics between DM and non-DM individuals. To account for these, we identified significant differences in exercise volume, smoking status and alcohol intake frequency between DM and non-DM individuals, and included them as covariates in the Cox proportional hazards models. These results have been added to **Supplementary Fig. 1d-f** and **Supplementary Fig. 2a-b** in the revised manuscript. Unfortunately, medication adherence is infeasible to be accurately estimated in the UK Biobank.

Comment 2.2. While the causal relationship between glycemic variability and cardiomyocyte injury has been validated, it remains imperative to develop targeted therapeutic interventions addressing glucose fluctuations and substantiate their cardioprotective efficacy in preclinical models.

Response to this comment: We appreciate the reviewer's insightful comment highlighting the importance of translating the causal relationship into therapeutic strategies. Our current study primarily focuses on establishing glycaemic variability (GV) as a major risk factor for cardiomyocyte injury and exploring potential cellular and molecular pathways involved. While the development of new therapeutic interventions managing GV is beyond the scope of this manuscript, we highlight numerous studies that have demonstrated that intensive glycaemic control treatment in patients improves outcomes post-MI by reducing oxidative stress and inflammation[3-5], which is added into the Discussion (see **lines 241–242**).

However, recent consensus statements by global peak bodies have highlighted the significant failure of cardioprotective therapeutics at least part attributable to poor preclinical models that do not accurately reflect patients most likely to be treated by these drugs in the clinic studies[6,7]. This highlights a critical need to test novel cardioprotective drugs in co-morbid models, like those developed in the current study. We have recently investigated the peptide Hi1a, a selective inhibitor of the acid-sensing ion channel 1a (ASIC1a) as a cardioprotective therapy that improves the viability of hearts subjected to ischaemia-reperfusion injury[8,9]. A variation of this drug has been developed and successfully completed IND-enabling studies to support progression to a Phase 1 clinical trial. Therefore, in the revised study, we

evaluated therapeutic efficacy of Hi1a in our GV *in vitro* model and observed that Hi1a significantly decreased ischaemia-induced cardiomyocyte death compared to control conditions. This supports the role for ASIC1a inhibitors as therapeutically effective even in complex co-morbid conditions like diabetes. This new data has been added to the revised manuscript (**page 9**, **Fig. 3i**) and accordingly been updated in the Results (lines **177–183**).

Comment 2.3. The investigation was limited to observing acute (short-term) contractile function alterations, without assessing the potential recovery of cardiomyocyte function post-serum withdrawal. Implementing time-course experiments with graded exposure durations could delineate the reversibility of glycemic fluctuation-induced injury, thereby establishing temporal parameters for optimal therapeutic intervention windows.

Response to this comment: We agree that time-course studies assessing recovery following serum withdrawal or restoration of normoglycaemic conditions would provide valuable information about the persistence and reversibility of glycaemic variability (GV)-induced dysfunction. We note that the importance of sustained glycaemic control in reducing cardiovascular risk is already well established in the clinical literature. Landmark studies such as the DCCT/EDIC (Type 1 diabetes) and UKPDS (Type 2 diabetes) have demonstrated that maintaining stable glycaemic profiles lowers the long-term risk of heart failure and myocardial infarction. These findings underscore that glycaemic instability is itself a driver of cardiovascular risk, highlighting the value of reversing effects caused by glucose fluctuations. Based on this literature, we did not carry out additional wet lab studies directly addressing this concern and instead focused our efforts on developing stronger claims around our current data package. In particular, we have added new analyses or studies across every aspect of the project (statistical genetics (**Fig. 1d-e** and **Supplementary Fig. 1d-f** and **Supplementary Fig. 2**), *in vivo* and *in vitro* modelling (**Fig. 2d-f** and **Fig. 3i** and **Supplementary Fig. 5a**), and patient sample analyses (**Fig. 4** and **Supplementary Fig. 5b**) to help strengthen the link between GV and acute cardiac injury responses. To directly address this concern, we have added discussion into the revised manuscript highlighting the need for future studies incorporating time-course analyses, reversibility assays, and interventions targeting glycaemic variability (see **lines 238–240**).

Comment 2.4. Serum collection was not strictly matched to patients' cardiovascular events (e.g., MI history), making it difficult to determine whether glycemic variability is a cause or a consequence (e.g., heart failure may contribute to metabolic disorders). Future prospective cohort studies should track the temporal relationship between serum markers and cardiovascular outcomes to clarify causality.

Response to this comment: Thank you for bringing this concern. To clarify, none of the patients included in our plasma analysis had a documented history of myocardial infarction (MI) or other overt cardiovascular disease at the time of plasma collection (**Supplementary Table 1**). Selection was based only on the availability of continuous glucose monitoring (CGM) data, independent of cardiovascular status.

Thus, our work with patient plasma represents a proof-of-concept study aiming to explore whether GV, irrespective of diabetes type, could induce functional changes in hiPSC-CMs. To strengthen these findings, we have significantly expanded our patient-focused study. In the revised manuscript, we expanded the blood sample analysis from 12 to 32 patients (see **Table 1** and **Supplementary Table 1**). The overall findings remain the same as our original findings, showing that cardiomyocyte contractility

is significantly reduced in patients with high GV. This effect is lost when patients are stratified by their hyperglycaemic state (% time very high), data which is represented in **Fig. 4a-c**.

Further, to investigate whether these plasma samples are associated with evidence of subclinical cardiac injury, we performed quantification of cardiac biomarkers using high-sensitivity immunoassays. Importantly, as mentioned above, none of the patients had a documented history of cardiovascular disease (**Supplementary Table 1**), yet patients with high GV displayed elevated levels of injury biomarkers, including BNP, ANP, CK-MB, and cTnI. These findings indicate a potential biochemical signature of subclinical cardiac stress or injury in individuals with high GV, reinforcing the pathophysiological relevance of GV and aligning with our preclinical data. These results are now presented in revised **Fig. 4d-e**, with corresponding updates to the Results (**lines 184-204**) and Methods (**lines 244–258**) sections. We acknowledge that although our patient history data is limited, preventing definitive causal claims, the collective evidence from our *in vivo* mouse model, *in vitro* stem cell studies, and patient plasma assays strongly indicates that GV is a primary contributor to cardiomyocyte dysfunction in diabetes. All experimental models consistently demonstrated significant reductions in cardiac beat rate and contraction kinetics under conditions of high GV (**Fig. 2b**, **Fig. 3e**, **Fig. 4b** and **Supplementary Fig. 5a-b**).

We note that Reviewer 1 also raised issues with interpreting study conclusions between type 1 and type 2 diabetes. Our *in vivo* and *in vitro* models indeed reflect type 2 diabetes (T2D)-like metabolic stress, whereas the patient plasma samples used for hiPSC-CM treatment were derived from individuals with type 1 diabetes (T1D). As stated in the Limitations of the Study section, our access to patient samples was constrained by current Australian federal policy under the National Diabetes Services Scheme (NDSS), which provides CGM funding only for patients with T1D. Consequently, only T1D plasma samples with available CGM data were accessible under our existing ethics approvals.

In summary, while we recognise the limitations of model alignment between T1D patient samples and T2D-like preclinical models, we believe our expanded analyses substantially strengthen the translational value of our findings. The absence of known cardiovascular disease in our patient cohort, combined with the biochemical evidence of subclinical cardiac stress and injury and concordant functional data, supports the conclusion that GV, regardless of diabetes type, may contribute to cardiac dysfunction and early myocardial stress, underscoring the clinical importance of GV monitoring in diabetes care.

Reviewer #3 comments:

In the clinical part of this study, Cao et al analyzed the relation of conventional cardiometabolic factors with the incidence of MI and post-MI survival in diabetic and non-diabetic individuals in the UK Biobank Cohort. The authors showed that both DM and non-DM individuals with higher HbA1c levels were more likely to have an MI than those with lower HbA1c levels. However, no difference in post-MI survival was observed between DM patients with lower and higher HbA1c levels. So the authors postulated that other cardiometabolic factors, such as glycemic variability, rather than hyperglycemia per se underlie myocardial dysfunction and injury in patients with diabetes. However, there are some issues that should be clarified.

Comment 3.1. A major problem of this part is that the authors confused the concepts of MI death and all-cause death after MI. In the manuscript, it seems that the authors analyzed survival from all-cause mortality after MI. However, in the figure legends of Figure 1 and Figure S1, the authors stated the 'death from MI'. The post-MI event should be explicitly defined. If the MI death or cardiovascular death was adopted, non-cardiovascular death should be treated as a competing risk in the analysis.

Response to comment: In this manuscript, only death attributed to cardiovascular causes was used to evaluate mortality from MI. Individuals who died from other causes were excluded from the analysis. We have clarified this definition on **lines 101-102** "We confirmed the increased incidence of both MI (Fig. 1a) and post-MI mortality due to MI (Fig. 1b) in DM individuals compared…". Given that the majority of death occurred within days of the MI, we do not believe that excluding individuals with non-cardiovascular death confounds the analysis.

Comment 3.2. The authors compared the incidence of MI and post-MI mortality between patients in the up and bottom 30% quantile of cardiometabolic factors including HbA1c and LDL-C. Why did you choose 30%? Especially for glycemic levels, hypoglycemia and hyperglycemia are both risk factors for MACE. This might be the reason that post-MI survival was not different between these 2 quantiles. Cardiovascular events in full quantiles of these cardiometabolic factors should be analyzed to confirm the relationship.

Response to comment: We selected 30% to provide separation between the quantiles while maximising statistical power to detect differences. To address whether the differences between quantiles were driven by hypoglycaemia, we added a middle quantile to all analyses, capturing individuals between 30-70% of the biomarker. Furthermore, each biomarker also has a continuous estimate of its contribution, not divided into quantiles. These changes have been reflected in **page 7 Fig. 1** and **Supplementary Fig. 2**. Finally, we also visualise the distribution of HbA1c and random blood glucose and indicate the quantiles. The results show that the bottom 30% of individuals is not hypoglycaemic, but normal/still slightly elevated glucose, particularly in diabetic individuals. This has been updated in **Supplementary Fig. 2**.

Comment 3.3. Another issue is that high glycemic variability usually comes with high glycemic level. In other words, if the cardiovascular events are not related to HbA1c level, it might be unrelated to glycemic variability as well. So I do not think these data are a good start of this study. This point should at least be discussed.

Response to comment: We note that a major finding of this data is that HbA1c cannot predict post-MI mortality, to emphasise an area of need to identify new clinical markers. To determine if HbA1c fully captures any potential glycaemic variability in the UK Biobank cohort, we added a new analysis showing that there is a significant proportion of variation in random glucose that is not captured by HbA1c and fasting time. Furthermore, we show that random glucose non-significantly stratifies post-MI mortality in DM individuals. These results have been added in **Supplementary Fig. 2f-i**.

To further investigate if this data suggests a role for glycaemic variability in determining post-MI mortality in DM individuals, we regressed the effect of HbA1c and fasting time on random glucose to obtain a measure of variation in random glucose not explained by these factors. In DM individuals, this measure stratifies individuals more than both HbA1c and random glucose, suggesting a potential role for glycaemic variability. These results have been updated in **Supplementary Fig. 2j-k**.

Minor:

Comment 3.4. 'HbA1c' rather than 'Hba1c' should be used.

Response to comment: Thank you for pointing this out. We have corrected all instances of "Hba1c" to the standard format "HbA1c" throughout the manuscript.

REFERENCES

1 Wende, A. R. *et al.* Maintaining myocardial glucose utilization in diabetic cardiomyopathy accelerates mitochondrial dysfunction. *Diabetes* **69**, 2094-2111 (2020).

2 Gerbaud, E. *et al.* Glycemic variability is a powerful independent predictive factor of midterm major adverse cardiac events in patients with diabetes with acute coronary syndrome. *Diabetes Care* **42**, 674-681 (2019).

3 Marfella, R. *et al.* Tight glycemic control reduces heart inflammation and remodeling during acute myocardial infarction in hyperglycemic patients. *Journal of the American College of Cardiology* **53**, 1425-1436 (2009).

4 Hemmingsen, B. *et al.* Intensive glycaemic control for patients with type 2 diabetes: systematic review with meta-analysis and trial sequential analysis of randomised clinical trials. *Bmj* **343** (2011).

5 Wong, V. W., McLean, M., Boyages, S. C. & Cheung, N. W. C-reactive protein levels following acute myocardial infarction: effect of insulin infusion and tight glycemic control. *Diabetes Care* **27**, 2971-2973 (2004).

6 Lecour, S. *et al.* IMproving Preclinical Assessment of Cardioprotective Therapies (IMPACT) criteria: guidelines of the EU-CARDIOPROTECTION COST Action. *Basic Res Cardiol* **116**, 52, doi:10.1007/s00395-021-00893-5 (2021).

7 Cao, Y. *et al.* New Drug Targets and Preclinical Modelling Recommendations for Treating Acute Myocardial Infarction. *Heart, Lung and Circulation* **32**, 852-869, doi:10.1016/j.hlc.2022.12.015 (2023).

8 Redd, M. A. *et al.* Therapeutic inhibition of acid-sensing ion channel 1a recovers heart function after ischemia–reperfusion injury. *Circulation* **144**, 947-960 (2021).

9 Redd, M. A. *et al.* Acid-sensing ion channel 1a blockade reduces myocardial injury in rodent models of myocardial infarction. *European Heart Journal*, ehad793 (2023).

Reviewer #1 comments:

Comment 1. While the authors have made textual revisions to acknowledge the inconsistency in the clinical data, the core methodological and interpretive issues raised in the initial query have not been fully addressed.

First, the authors mention "predefined inclusion criteria" for data selection, but they have not clearly detailed what these criteria are in terms of the range of acceptable values for sample size, or how they defined "sufficient sample size".

Response: We have now detailed these criteria in the revised METHODS section (page 9, lines 285–298) and added new Supplementary Tables 1 and 2, which specify the predefined inclusion criteria and sample size ranges used for data selection.

Second, regarding the high values of change in post - MI cardiac function (LVEF) between DM and non - DM populations (~30 - 60%), the authors have not addressed whether these extreme values are outliers in the field and how they might impact the overall analysis and conclusions.

Response: We have reviewed all studies reported in Supplementary Table 2 to confirm the accuracy of the values. Notably, studies reporting larger LVEF differences often included higher-risk patients, such as those with delayed reperfusion or greater comorbidity burden, which can amplify post-MI systolic dysfunction (as discussed in previous reviews such as Zeng et al., 2024[1]). In addition to adding new citations and discussion (page 5, lines 222–229), new Supplementary Table 2 now annotates the clinical characteristics of each cohort to help readers interpret the range of observed LVEF changes.

Third, although the authors aim to provide a reference point for interpreting animal modelling studies, they have not explicitly discussed how the inconsistencies in the clinical data relate to the potential limitations or generalizability of their animal model results. These aspects need to be further elaborated to strengthen the study's validity and credibility.

Response: The clinical literature on diabetes and MI is heterogeneous because of variation in endpoint measures (early post-MI mortality vs. LVEF among survivors are rarely reported in the same cohort) and biomarker choice (HbA1c vs. fasting/random glucose vs. CGM-derived glycaemic variability). We have revised the manuscript to more clearly connect these sources of heterogeneity and contextualise the contribution of our animal and cell modelling:

First, across clinical cohorts, diabetes associates with higher early post-MI mortality, whereas LVEF among survivors is not consistently different. Our variable-glycaemia (vG) model reproduces this specific pattern: greater 48-h mortality in vG vs. constant glycaemia (cG) (25% vs. 9.1%; Fig. 2c) without a between-group difference in day-15 EF or fibrotic area (Fig. 2d–f). These findings support the validity of our models in terms of evaluating the *early mortality* phenotype but does not show EF differences among survivors, which mirrors the mixed LVEF findings in patients.

Second, UK Biobank, HbA1c predicts MI incidence but not post-MI mortality in DM (Fig. 1c–e), whereas in new analyses performed for this revision, we now show that proxies of glycaemic variability (GV) show stronger alignment with mortality trends (new Supplementary Fig. 2f–k). Our *in vivo* paradigm matches total glucose load between cG and vG and varies only the temporal variance (Fig. 2a), thereby isolating GV as the mechanistic variable the clinical biomarkers fail to capture. Furthermore, in studies we have completed for this revision, we show that, independent of neurohormal stressors, vG impairs contractility and increases ischaemic cell death compared to cG *in vitro* (new Fig. 3h–j), and patient plasma stratified by CGM-derived GV show elevated levels of cardiac-injury biomarkers and also reduces hiPSC-CM contractility (Fig. 4b–e). Together, these data help identify GV as a culprit cause of cardiac disease risk in diabetes that is not explained by measures of average glycaemia (HbA1c).

The new analyses used to justify the above can be found in new Supplementary Figure 2, new Figure 3h–j, expanded Discussion, and additional comments in Study Limitations (page 6, lines 296–300).

Comment 2. The authors' inclusion of echocardiographic and histologic data is a positive step. However, key concerns persist. While the 40-minute ischemia model may explain lower mortality, its clinical relevance to the high-risk diabetic MI population you aim to model is unclear. Could you provide comparative mortality data from your lab or literature for permanent ligation in this model to better contextualize your findings?

Response: We assume the reviewer is referring to mortality data on our ischaemia-reperfusion injury (IRI) model as opposed to permanent ligation model as indicated in the comment. Since reperfusion therapy is standard of care for patients with an MI, IRI is more clinically relevant and allows for better interpretability of outcomes relative to clinical outcome findings that we reported in Supplemental Figure 1a-b and Supplementary Tables 1-2. To address this concern we cite two of our previous studies (Redd et al., Circulation, 2021[2]; Redd et al., EHJ, 2021[3]) in which we have performed the same IRI modelling and in which we found no mortality in vehicle or treated animals. Notably, the mild injuries were explicitly acknowledged as a limitation for assessing outcomes in our EHJ paper (Redd et al, EHJ, 2023[3]). Consistent with the increased mortality in patients with diabetes, the mortality outcomes in this study are more severe compared to studies involving healthy wildtype mice. In alignment with recent international guidelines[4,5], this study underscores the importance of incorporating co-morbid metabolic stress into preclinical models to better capture clinical risk and therapeutic performance of new drugs. We have added these citations and provided additional commentary on model comparison in the DISCUSSION (page 5, lines 254–259).

Furthermore, the new functional data show a decline in EF, but the absolute post-MI values appear relatively high. Please provide the actual infarct size percentages quantified from your histology. It is critical to demonstrate that the infarct size is substantial and equivalent between groups to firmly conclude that the difference in mortality is solely attributable to glycemic variability and not to a milder, non-lethal injury.

Response: The post-MI injuries in our current study show an ~20% drop in ejection fraction at 1 week post-MI compared to baseline performance. This level of functional decline is equivalent to the most severe forms of heart attack, where an EF of <40% is associated with ST-Segment Elevation Myocardial Infarction (STEMI). We added a reference (Wohlfahrt et al., 2022[6]) to support the injury severity as clinically relevant. Furthermore, in our previous studies (Redd et al, EHJ, 2023[3]), we reported about a 10-15% drop in EF at 1 week post-MI, indicating that the outcomes from these diabetic models are more severely affected compared to our standard MI mouse model in healthy wildtype animals. Regarding infarct size area, this is most typically measured using evans blue/TTC staining at 24 hours post-MI. We did not perform this analysis and instead evaluated fibrotic area as a surrogate indicator of infarct severity, which is our typical approach to measuring myocardial injury by histology[7,8]. We have added citations (Fishbein et al., 1981[9]; Voelkl et al., 2011[10]) showing that infarct size by MRI is strongly associated with fibrotic area to justify the method. We also ensured the article language is accurate in reflecting conclusions about fibrotic area as outcome and avoid over-stating conclusions regarding infarct size, which we did not directly measure. These changes are now included in the DISCUSSION (page 5, lines 222–229) and changes to the wording in the RESULTS (page 3, lines 148–152).

Comment 3. The cited Wende et al. 2020 study seems to have results different from the general understanding but not fully in line with your data either. Given that numerous studies show increased FAO and decreased glucose oxidation in diabetic cardiomyocytes, how do the authors explain their findings of decreased FAO and increased glucose oxidation in cG and vG groups? Could the acute

nature of glycaemic variability be the key factor here, and if so, how does it override the chronic metabolic changes typically seen in diabetes?

Response: The reviewer raises an important point. We agree that the findings are likely influenced by the acute and short-term glycaemic stress imposed in our model, which differs from the chronic metabolic reprogramming typically observed in diabetes. Importantly, our experiments were performed in an *in vitro* system with simplified media conditions, which cannot fully replicate the complex and chronic metabolic environment of the diabetic heart. To acknowledge the limitations of the metabolic data, we moved all metabolic and metabolomic data (previously Figure 3 panels f-g) to the supplemental material (new Supplementary Figure 4), as these results are less central to the study. We have also softened our conclusions regarding these findings and explicitly acknowledge the limitations of our model relative to the well-established metabolic changes in diabetes (page 4, lines 173–179).

Comment 4. The authors' response regarding systemic compensation *in vivo* is plausible but remains speculative without direct experimental support. While the new *in vitro* beat rate data provides some alignment, the core contradiction in contractility amplitude between systems persists. Specifically, if glycemic variability significantly impairs intrinsic cardiomyocyte contraction as shown *in vitro*, what specific compensatory mechanisms (e.g., sympathetic activation, preload changes) maintain near-normal EF *in vivo*? Please provide the full echocardiography parameters (including FS, heart rate, and ventricular dimensions) as supplementary data to allow proper assessment of loading conditions.

Response: To address this, we now include citations to clinical studies demonstrating that patients with diabetes exhibit elevated sympathetic tone and altered autonomic regulation, which can transiently augment contractility and maintain near-normal ejection fraction (EF) despite underlying subclinical myocardial impairment (López-Cano et al., 2019[11]; El Tantawyet al., 2022[12]). This phenomenon can mask early contractile deficits observable only under stress or at the cellular level. We have cited these in our Discussion (page 5, lines 227–229).

We have also now included additional supplementary material with baseline and post-MI echocardiographic parameters as requested in the comment (new Supplementary Figure 7). These new data focus on ventricular dimensions at baseline and post-MI because heart rate and pump function (EF) are already provided in the main Figure 2. These new data support our overall findings by showing post-MI myocardial dilation of the ventricle (increased end systolic and end diastolic volumes) consistent with adverse remodelling. No differences were found between cG and vG groups which is also consistent with no difference in post-MI pump function (Figure 2d).

Additionally, regarding the corrected contraction trace: could you clarify how many replicates were re-examined and confirm the statistical robustness of this correction? The concern about the trace's abnormality now extends to its representativeness across the dataset.

Response: We appreciate the reviewer's attention to data reproducibility. The representative contraction trace shown in Figure 3 was derived from one biological replicate for each condition, selected to illustrate the characteristic waveform observed under each treatment. Quantitative analyses (bar plots, right to the trace) in Figure 3e were performed using data from three independent biological replicated hiPSC-CMs, each with three technical replicates per condition. We have ensured all replicate numbers and statistics for this analysis are provided in the manuscript legend and/or methods. To further demonstrate reproducibility, we have provided two additional biological replicated individual contraction traces as an appendix for the editor and reviewers to review.

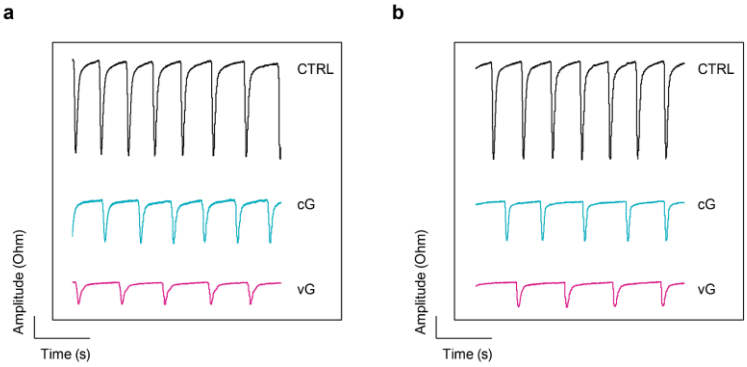

Figure | Impedance-based contractility assessment using the CardioExcyte 96 platform. Representative raw contractility traces from two additional biological replicates are shown for CTRL, constant glucose (cG), and variable glucose (vG) conditions (**a-b**). These traces illustrate the reproducibility of contractile dynamics across independent hiPSC-CM preparations.

Comment 5. Even with expanded patient samples, the fundamental issue of model mismatch between T1D patient samples and T2D-like preclinical models remains. How can you convincingly argue that GV's effect is diabetes-type independent without direct T2D patient sample validation?

Response: We appreciate the reviewer's comment and have undertaken additional experiments to address this concern and further strengthen the connection between our clinical and preclinical data.

First, we sought to include plasma samples from individuals with T2D to align directly with our T1D plasma analyses. However, only eight T2D samples were available, and just one had continuous glucose monitoring (CGM) data. We therefore evaluated surrogate biomarkers of glycaemic stress (1,5-anhydroglucitol and HbA1c) to assess their relationships with cardiomyocyte contractile function *in vitro*. As expected, the low number of samples and the use of static biomarkers did not reveal statistically meaningful differences. Given the limited value of these results, we did not add them into the revised manuscript.

Our second approach to address this issue focused on developing results showing a diabetes-independent mechanism governing heart injury via glycaemic variability. To do this, we performed new *in vitro* experiments exposing hiPSC-derived cardiomyocytes to vG and cG glucose conditions without the addition of T2D-associated stimuli (cortisol and endothelin-1). These new data (now shown in revised Figure 3h–j) demonstrate that variable glycaemic stress alone, independent of hormonal context, significantly reduces contractility and increases susceptibility to ischaemia compared with both control and constant glucose groups. This supports the conclusion that GV exerts deleterious effects on cardiomyocyte function through a core, diabetes-type-independent mechanism. These findings are provided in Figure 3h–j, and discussed on pages 5, lines 234–237.

Importantly, our study provides clinically significant findings by providing evidence of cardiac injury in young individuals with T1D which goes well beyond simply reproducing T2D cardiovascular disease risk in an experimental setting. The ability to measure early cardiac injury biomarkers (BNP, ANP, CK-MB, cTnI) in plasma from T1D patients (none of whom reported prior cardiovascular disease) highlights the translational impact of our findings. Demonstrating subclinical cardiac injury in this population underscores the immediate relevance of GV as a modifiable but previously unrecognised risk factor for early cardiovascular intervention in diabetes management. This point is discussed in the revised manuscript to further emphasise the significance of this study in the context of its clinical impact (page 6, lines 272–277).

We appreciate the reviewer's persistence on requesting further work for this study to help align the different modelling studies and markedly strengthened the impact of the study. We believe

the new experiments and integrated datasets now provide a cohesive and generalisable framework linking glycaemic variability to cardiomyocyte dysfunction and ischaemic vulnerability across diabetes types. They also establish a transition dataset that justifies our experimental workflow and supports GV as a mechanistic driver of cardiovascular risk, independent of whether the underlying diabetes is type 1 or type 2.

Could you provide experimental data or cite literature demonstrating that the specific molecular mechanisms of glycemic variability-induced cardiotoxicity you propose (e.g., transcriptional changes in Fig. 3) are indeed conserved between T1D and T2D?

Response: Our new experimental data now demonstrate that glycaemic variability (GV) alone (independent of additional T2D-associated neurohormonal stimuli) is sufficient to induce contractile dysfunction and ischaemic sensitivity in cardiomyocytes (new Fig. 3h–j). These findings localise the injury mechanism to the glycaemic stress itself, rather than to disease-specific factors such as insulin deficiency, hyperlipidaemia, or hormonal milieu. In addition to these new data, we have added discussion regarding prior studies showing that oscillating glucose elicits shared cellular stress pathways including ER stress, mitochondrial dysfunction, and NF-κB–mediated inflammation are shared across both diabetes types (Wu et al. (2022)[13], Mordel et al. (2023)[14], Zhang et al. (2022)[15], and Zhou (2020, review)[16]) (page 5–6, lines 261–269).

Furthermore, for the 32 patients, were key covariates like diabetes duration, insulin therapy regimen, and presence of autoantibodies matched between high and low GV groups? These factors could confound the observed biomarker and contractility differences more than GV itself.

Response: We have included all available patient demographics in new Supplementary Table 3, detailing variables including age, sex, diabetes duration, HbA1c, insulin treatment type, available CGM parameters, and available autoantibody data. In addition, although none of the covariates have a strong association with GV, we have provided a new Supplementary Table 4 summarising the covariates for the 32 T1D patients, comparing high versus low GV conditions. This supports our previous findings that classical cardiovascular disease risk biomarkers, including common measures of hyperglycaemia, do not fully capture the elevated risk in diabetes, highlighting a significant area of need for identifying new clinical indicators of heart disease risk. Finally, we have revised the LIMITATIONS section (page 6, lines 296–300) to explicitly acknowledge that, while GV appears to be a key determinant, residual clinical covariates such as diabetes duration or treatment regimen cannot be fully excluded given the modest sample size.

Comment 6. While the authors have addressed most technical points, the response to comment 2.3 remains inadequate. Merely stating that "revisions are provided throughout" does not allow for a proper assessment. Could you specifically indicate which new data figures or analyses most definitively support the claim that glycaemic variability is a "major risk factor"? Please highlight the key experimental evidence that distinguishes GV's effect from other comorbid risk factors.

Response: Our conclusion that glycaemic variability is a major risk factor is supported by evidence across epidemiology, *in vivo, in vitro*, and human plasma, with designs that isolate GV from other disease confounders:

1. **In our UK Biobank analysis, we show that HbA1c predicts MI incidence but not post-MI mortality in diabetes (Fig. 1c–e). In contrast, proxies of GV (random glucose deviation after accounting for fasting time and HbA1c) better stratify mortality among people with diabetes (OR = 1.83, p = 0.08; trend) (Supplementary Fig. 2f–k), and therefore highlight risk not captured by average glycaemia.**
2. **Our mouse model matches total glucose load between constant (cG) and variable glycaemia (vG) and alters only temporal variance (see protocol schematic, Fig. 2a). Under identical HFD background and MI surgery conditions we show that vG elevates 48-h post-MI mortality (25% vs 9.1%; Fig. 2c) and impairs relaxation/outputs pre-MI (IVRT↑, CO↓,**

HR↓; Fig. 2b). Importantly, post-MI EF decline (Fig. 2d) and fibrotic area (Fig. 2e–f) are similar to sever STEMI injuries but are comparable between glycaemic stress models, indicating GV specifically heightens fatal vulnerability to MI.

3. **Using *in vitro* studies, we show that vG reduces contractility more than cG (Fig. 3e) and increases IR-induced cell death (Fig. 3f). Crucially, new experiments without T2D neurohormonal stimuli (no cortisol/ET-1) show the same vG-specific defects (reduced contractility and heightened IR sensitivity; new Fig. 3h–j) that further reinforce the role of GV to heart disease risk.**

4. **Lastly, we use human plasma samples to show that GV predicts cardiotoxicity while hyperglycaemic burden does not. hiPSC-CMs exposed to plasma from individuals stratified by CGM show reduced contractility in high-GV samples (CV > 36%) (Fig. 4b), whereas percent time very high glucose fails to stratify contractility (Fig. 4c). In the same donors, young people with T1D without reported CVD, show significantly elevated cardiac injury biomarkers (BNP, ANP, CK-MB, cTnI) in the high-GV group (Fig. 4d–e) that underscore clinical impact beyond simply validating heart disease risk in T2D risk.**

Together, these additions support our central claim that glycaemic variability, rather than chronic hyperglycaemia, is a major, mechanistically distinct risk factor for heart disease risk in diabetes.

Additionally, for the revised heatmap (Fig. 3C/Suppl. Fig. 3), please confirm in the figure legend that all displayed gene clusters passed statistical significance thresholds after multiple testing correction, and indicate the specific clustering method used.

Response: We thank the reviewer for this important point. We confirm that all genes displayed in the current Supplementary Fig. 3 passed the statistical significance threshold (FDR < 0.05) after multiple-testing correction in the RNA-seq differential expression analysis. As described in the METHODS (page 5, lines 103–107), hierarchical clustering was performed using Euclidean distance and the complete-linkage method to group genes by expression dynamics between diabetic and control conditions. We have clarified these details in the corresponding figure legends (Supplementary Figure 3).

REFERENCES

1 Zeng, M., Sun, E., Zhu, L. & Deng, L. Influence of prediabetes on the prognosis of patients with myocardial infarction: a meta-analysis. *Diabetology & Metabolic Syndrome* **16**, 160 (2024).

2 Redd, M. A. *et al.* Therapeutic inhibition of acid-sensing ion channel 1a recovers heart function after ischemia–reperfusion injury. *Circulation* **144**, 947-960 (2021).

3 Redd, M. A. *et al.* Acid-sensing ion channel 1a blockade reduces myocardial injury in rodent models of myocardial infarction. *European Heart Journal*, ehad793 (2023).

4 Cao, Y. *et al.* New Drug Targets and Preclinical Modelling Recommendations for Treating Acute Myocardial Infarction. *Heart, Lung and Circulation* **32**, 852-869 (2023). https://doi.org/10.1016/j.hlc.2022.12.015

5 Lecour, S. *et al.* IMproving Preclinical Assessment of Cardioprotective Therapies (IMPACT) criteria: guidelines of the EU-CARDIOPROTECTION COST Action. *Basic Res Cardiol* **116**, 52 (2021). https://doi.org/10.1007/s00395-021-00893-5

6 Wohlfahrt, P. *et al.* Trajectories and determinants of left ventricular ejection fraction after the first myocardial infarction in the current era of primary coronary interventions. *Frontiers in Cardiovascular Medicine* **9**, 1051995 (2022).

7 Redd, M. A. *et al.* Acid-sensing ion channel 1a blockade reduces myocardial injury in rodent models of myocardial infarction. *European Heart Journal* (2023). https://doi.org/10.1093/eurheartj/ehad793

8 Redd, M. A. *et al.* Therapeutic Inhibition of Acid-Sensing Ion Channel 1a Recovers Heart Function After Ischemia-Reperfusion Injury. *Circulation* **144**, 947-960 (2021). https://doi.org/10.1161/CIRCULATIONAHA.121.054360

9 Fishbein, M. C. *et al.* Early phase acute myocardial infarct size quantification: validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. *American heart journal* **101**, 593-600 (1981).

10 Voelkl, J. G. *et al.* Cardiac imaging using clinical 1.5 t MRI scanners in a murine ischemia/reperfusion model. *BioMed Research International* **2011**, 185683 (2011).

11 López-Cano, C. *et al.* Sympathetic hyperactivity and sleep disorders in individuals with type 2 diabetes. *Frontiers in endocrinology* **10**, 752 (2019).

12 El Tantawy, A. *et al.* Cardiac autonomic neuropathy linked to left ventricular dysfunction in type 1 diabetic patients. *Cardiovascular Endocrinology & Metabolism* **11**, e0272 (2022).

13 Wu, L.-D. *et al.* Glucose fluctuation promotes cardiomyocyte apoptosis by triggering endoplasmic reticulum (ER) stress signaling pathway in vivo and in vitro. *Bioengineered* **13**, 13739-13751 (2022).

14 Mordel, P., Fontaine, F., Dupas, Q., Joubert, M. & Allouche, S. Glucose fluctuation promotes mitochondrial dysfunctions in the cardiomyocyte cell line HL-1. *Plos one* **18**, e0289475 (2023).

15 Zhang, Z.-Y. *et al.* Glucose fluctuations aggravate myocardial fibrosis via the nuclear factor-κB-mediated nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome activation. *Frontiers in Cardiovascular Medicine* **9**, 748183 (2022).

16 Zhou, Z., Sun, B., Huang, S., Zhu, C. & Bian, M. Glycemic variability: adverse clinical outcomes and how to improve it? *Cardiovascular diabetology* **19**, 102 (2020).

Reviewer #1 comments:

This study aimed to identify novel predictors of adverse cardiovascular events (primarily myocardial infarction) in diabetic patients. Analysis of UK Biobank cohort data revealed limitations in the traditional HbA1c metric for predicting mortality following myocardial infarction in diabetics, thereby highlighting the potential of glycaemic variability (GV) as an alternative predictive indicator. Subsequent investigators conducted animal studies, cellular experiments, and patient plasma assays to further elucidate the predictive potential of glycaemic variability (GV). Overall, the article exhibits clear logic and holds considerable clinical significance. The authors have addressed to a certain extent the issues raised in our previous two rounds of peer review, significantly enhancing the paper's overall quality. However, the following major issues remain:

Comment 1: The researchers have still not addressed Comment 3 (Given that numerous studies show increased FAO and decreased glucose oxidation in diabetic cardiomyocytes, how do the authors explain their findings of decreased FAO and increased glucose oxidation in cG and vG groups? Would the acute nature of glycaemic variability be the key factor here, and if so, how does it override the chronic metabolic changes typically seen in diabetes?) raised previously. The metabolomic data presented herein do not align with the metabolic alterations observed in diabetes. However, it is perplexing that the researchers merely relocated the data to the supplementary figures without elucidating the underlying cause.

Response to Comment 1: We apologise that it was not clear where we had addressed this in the revised manuscript. In response to the reviewer's original comments on this point in reviews 1 and 2, we have added explicit text to explain why the metabolic profile observed in our cG and vG conditions differs from the canonical pattern described in long-standing diabetes, and to emphasise the acute nature of the glycaemic perturbation in our models.

***Results* section – In the *Results* section (page 4), we state: "Complementary metabolic assays showed increased glucose consumption and lactate production, with a trend toward reduced ATP generation in vG-treated cells (Supplementary Fig. 6e). These patterns are consistent with acute metabolic adaptations to short-term glycaemic fluctuations rather than the chronic metabolic remodelling typically observed in diabetes" and support this by reference to Wende et al., Diabetes, 2020[1].**

This sentence was added in response to the reviewer's comment from review #1 to clarify that our metabolomic and functional data reflect short-term glycaemic fluctuations, not the fully established chronic metabolic reprogramming characteristic of long-duration diabetes.

***Discussion* section (page 5) – We also expanded the *Discussion* to link our findings to prior work and to explain how acute increases in glucose availability can alter FAO and glucose metabolism.**

"At a metabolic level, recent studies (Wende et al., Diabetes, 2020[1]) have shown that enhancing glucose uptake in diabetic mouse hearts suppresses both FA oxidation and glucose oxidation. Notably, increased myocardial glucose availability, such as that observed under conditions of high GV, even alongside chronic hyperglycaemia, has been linked to exacerbated mitochondrial dysfunction. These findings are consistent with our metabolic data, which demonstrate repression of FA oxidation-associated genes and upregulation of glucose metabolic pathways in response to glycaemic fluctuations."

Based on a study that has just been published in *Nature*[2] that directly supports the outcomes of this study, we have also added the following to the discussion (page 6):

"We also note that our interpretation of GV as a biologically distinct and clinically relevant stressor, separable from chronic hyperglycaemia, is supported by a very recent large-scale human study published during the revision of this manuscript (Lutsker et al., Nature, 2026). This study trained a self-supervised foundation model on >10 million CGM measurements and demonstrated that dynamic glucose patterns, rather than static HbA1c or mean glucose levels, more effectively predicted long-term cardiometabolic outcomes, including cardiovascular mortality. Importantly,

the study showed that representations derived from short-term CGM captured prognostic information that was not explained by baseline HbA1c, reinforcing the concept that transient and fluctuating glucose dynamics exert independent biological effects. These results support the findings of this study and reinforce the need for preclinical models involving GV for more accurate modelling of diabetic stress."

This text explains that:

- **Our cG and vG paradigms represent acute or subacute glycaemic stress, not long-standing, fully remodelled diabetic cardiomyopathy.**
- **Experimental models with increased glucose availability can show suppressed FAO and altered glucose pathway utilisation, consistent with our observations.**
- **The apparent discrepancy with the "classic" diabetic pattern (increased FAO, reduced glucose oxidation) is therefore interpreted as a short-term, maladaptive response to glycaemic variability, rather than a contradiction of the chronic diabetes literature.**
- **The recent Nature study findings[2] are highly concordant with our experimental observations. Specifically, they support the notion that acute or subacute glycaemic instability can induce maladaptive metabolic responses that differ from the canonical metabolic remodelling observed in long-standing diabetes. Thus, the apparent divergence of our metabolomic data from chronic diabetic profiles does not represent a contradiction of established literature, but rather reflects a distinct and clinically relevant metabolic state driven by glucose variability itself.**

We confirm that the above sentences in the *Results* and *Discussion* were introduced in direct response to the reviewer's initial comments to clarify the metabolic interpretation and the role of acute GV. The reviewer has not offered any more specific suggestions for how to better address this concern despite raising it several times. We hope the reviewer and editor can see that the underlying concern about the apparent mismatch with chronic diabetic metabolic profiles has already been explicitly addressed in the current text and figure revisions.

Comment 2: Regarding the selection of animal models, although the authors propose this model as a mouse model of glycaemic variability, its modelling approach differs from established diabetic animal models. Consequently, the discussion section ought to include a more explicit description of the model's limitations and how it diverges from recognised and commonly used diabetic models. This would assist researchers in the field to comprehend and select appropriate models.

Response to Comment 2: To address this concern we have added the following text (page 5) into the *Discussion*:

"It is important to note that the glycaemic-variability (GV) model used in this study differs from classical diabetic mouse models such as long-term high-fat diet feeding, STZ-induced insulin deficiency, or genetic models (e.g., db/db mice), which recapitulate chronic hyperglycaemia, insulin resistance, and multi-organ metabolic remodelling. In contrast, our approach was intentionally designed to isolate the specific contribution of *temporal glucose fluctuations* by matching total glucose exposure between groups while varying its delivery pattern. As such, this model does not reproduce the full chronic diabetic phenotype but instead serves as an acute/subacute co-morbidity model that captures the cardiovascular vulnerability associated with glycaemic instability. This distinction is important for selecting appropriate experimental systems: while standard models are suited to studying long-term diabetic cardiomyopathy, the GV paradigm provides a targeted tool for examining glycaemia-driven stress responses that better reflect the clinical context of fluctuating glucose patterns in diabetes."

The clinical relevance of isolating glucose variability from cumulative glycaemic exposure is further underscored by the recent *Nature* study by Lutsker et al. (2026)[2], where short-duration

CGM recordings (independent of long-term glycaemic burden) were sufficient to stratify individuals for future diabetes progression and cardiovascular mortality more effectively than HbA1c. These findings reinforce our rationale for employing an experimental model that specifically decouples temporal glucose fluctuations from total glucose exposure, rather than attempting to recapitulate all features of chronic diabetes.

Accordingly, our GV model should be viewed not as an alternative to established diabetic models, but as a complementary system designed to study the cardiovascular consequences of glycaemic instability which is increasingly recognised in clinical populations but is not adequately captured by conventional models.

Comment 3: Regarding the *in vitro* cell culture section, as per the previous review comments, Figure 3b (Oil Red O staining) requires image replacement. The current image quality is not convincing.

Response to Comment 3: We have replaced the original images in Figure 3b with higher-resolution Oil Red O staining images to address the reviewer's concerns about image quality. In addition, we added arrows to highlight regions of lipid accumulation indicative of lipid toxicity. The updated images and revised figure legend are now included in Figure 3b.

Comment 4: In the cellular experiments, what was the rationale for selecting a concentration of 10 nM Hi1a? The paper lacks supporting references or experimental data regarding the concentration selection.

Response to Comment 4: The rationale for using 10 nM Hi1a is based on previously published studies demonstrating that this concentration provides robust and reproducible cardioprotection in both rodent hearts and hiPSC-cardiomyocytes subjected to ischaemia–reperfusion injury. We have revised the text (page 4) to make our justification for this concentration more directly linked to our previous studies (Redd et al., 2021[3]; Redd et al., 2023[4]).

References

1 Wende, A. R. *et al.* Maintaining myocardial glucose utilization in diabetic cardiomyopathy accelerates mitochondrial dysfunction. *Diabetes* **69**, 2094-2111 (2020).

2 Lutsker, G. *et al.* A foundation model for continuous glucose monitoring data. *Nature*, 1-9 (2026).

3 Redd, M. A. *et al.* Therapeutic inhibition of acid-sensing ion channel 1a recovers heart function after ischemia–reperfusion injury. *Circulation* **144**, 947-960 (2021).

4 Redd, M. A. *et al.* Acid-sensing ion channel 1a blockade reduces myocardial injury in rodent models of myocardial infarction. *European Heart Journal*, ehad793 (2023).