

Mapping histologic and functional maturation of human endocrine pancreas across early postnatal periods

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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)

Saunders et al present an impressive study of the temporal and spatial changes in the endocrine pancreas using a large cohort of young human pancreas. They set up a pipeline for analyzing many of the characteristics of the endocrine pancreas during this postnatal time (neonatal : birth to 3 months, infancy 3m to 2 y, childhood 2 y-10y) providing data on composition, architecture, density, proliferation, vascularization, innervation and importantly insulin and glucagon secretion. There are several important findings. First, identification in the infants of both PTF1A+Nkx6.1+ GP2+ cells showing what is thought to be multipotential progenitor cells (MPC) and Pax6+ hormone negative protoendocrine cells up to the age of 20 months; both suggesting continued differentiation of new endocrine cells (neogenesis) in the human pancreas. Second, up to 2 years islets are mainly core-mantle structure, after which they become more intermingled and indistinguishable from adult islets. Thirdly, beta and alpha cell proliferation were unchanged (about 1%) across this age span whereas delta cell proliferation decreased. Fourth, that while there were no real differences in insulin secretory responses across the age span even to adult, the maturation of glucagon secretion was later and came only in childhood. Fifth, innervation of the islets was essentially absent in infancy and only developed in childhood even though the vascularization was unchanged. The authors acknowledge that more studies are needed to understand the mechanisms responsible for the observed changes.

While some of the metrics used are unusual and difficult to relate to, the study presents a framework of human postnatal development that has been lacking and should be very useful for the field especially since the images are in the Pancreasatlas. There are some issues that need further details:

1. The finding of MPC and protoendocrine cells is important and exciting but there was little information concerning this given. It is stated that these cells were found up to 20 m age but the images shown do not indicate the age of the donor and quantification is lacking.
2. Data are presented compared to pancreatic weight, and not age with the rationale that there were variable growth rates. However, this does not seem consistent with their speculation that the pancreatic size is established early (intrauterine) and with a strong genetic influence. Additionally presenting data with age as the x-axis would be easier for the reader to relate to other studies.
3. The measurement of location 20um from periphery for assessing the islet architecture is odd and would seem somewhat dependent on the islet size.
4. While 115 pancreases from donors of neonatal (birth -3months age) to 10 years were included, the numbers for various assessments are far less: functional assessment (perifusion) used islets from 2 neonate, 8 infants 18 children and 14 adults; islet architecture used 5 neonatal (1d,1d,1d, 4d,3m) and 5 childhood (7m 20 m, 4y,5y, 10 y); endocrine density and composition used 24 neonatal, 21 infant (3m-24m), 33 childhood (2y-10 y); 27 are only used for pancreatic weight. The text should reflect these smaller samples rather than the whole collection, particularly in the Discussion.
- 5.While it is impressive to have perfused islets from 8 infants and 20 children, the authors should acknowledge (and cite) the studies Henquin did on islets from 5 infants/toddlers (11-36 months) (PLos1, 2016). He showed clearly that by 1 year the islets responded as adult islets but they secreted a smaller fraction of their content and even so their stimulation index was the same as adults because they had a lower basal secretion.

6. There should be a caveat re the Ki67 staining for proliferation. Ki67 protein decreases with ischemia (warm and cold) and is reported to have a half-life of less than 1 hr. It may be that the larger organs have longer warm ischemia.

Minor issues:

- Data for Ki67 are referred to as rates, but rate is a value over a time denominator, thus these are no rates, just frequency of Ki67+ cells.
- Suppl Fig2 only provides additional images, again without indication of age of donor.

Reviewer #2

(Remarks to the Author)

The manuscript 'Mapping histologic and functional maturation of human endocrine pancreas across early postnatal periods' by Saunders and colleagues examines the differentiation and morphogenesis of human endocrine cells from birth to ten years old. Analyzing 115 pediatric pancreata, the study categorizes samples into three age groups: Neonatal (birth to <3 months), Infancy (3 to 24 months), and Childhood (2 to 10 years). Using fluorescence imaging techniques like confocal microscopy, the research maps morphological changes with 15 antibodies and highlights differences in insulin and glucagon secretion maturation and cellular markers across age groups.

The manuscript is well-written and structured, including material, antibodies, assays, and images. While primarily descriptive, the sample collection's scope and diversity stand out, providing valuable insights into Type 1 Diabetes risk and genetic data related to islet biology.

Concerns:

This study uses image-based microscopy, so it should include essential documentation on image processing procedures, software, workflows, and classification. The online portal and figures seem to show processed images without the originals?

Most analysis depends on fluorescence labelling of cells by antibodies, requiring segmentation. Only Ki-67 and CD31 methods for immunohistochemistry quantification are mentioned, suggesting manual processing. Parameters/configurations for commercial systems are not provided.

Considering the image-based approach and the expected impact of visual access through an online portal, why have the authors not included appropriate schemes for red-green color blindness, which affects 8% of men?

The study categorizes the pancreas by head, body, and tail but does not analyze endocrine islet development across age groups.

The anencephaly versus other causes of death for the neonatal group is analyzed, but other confounders may affect different age groups (e.g., procurement, origin, ischemia). Particularly, the infancy group's secretion assay should be tested for these factors (Fig. 4F and I).

The observation that endocrine cell numbers per tissue have increased from the neonatal stage to infancy (Fig. 3, D) is based on segmentation, as well as the size and volume of islets in 3D, which is not accounted for by area percentage. Could you normalize for this?

The differences in cell compositions between the neonatal and infancy groups could be due to image processing (Fig. 3E)?

The online images and Fig. 3 (A, B, C) illustrate the presence of intermingled endocrine cells only in childhood islets. In contrast, Fig. 4 (C, D) shows that these endocrine cells are primarily located at the periphery of islets, comprising about 20%, rather than intermingling. Is there any possibility that the selection of the measured 30 islets could be biased?

The rationale for selecting markers/antibodies is not mentioned.

The proposed model and curves may be speculative given the aforementioned points.

Minor:

Defining the gene GP2 as a marker for multipotent cells is challenging, as iPSCs from cell cultures show high GP2 expression, which may differ in tissues and proteins.

The findings on the vascularization and innervation of the neonatal pancreas are noteworthy and warrant discussion in the context of neurons in the neonatal group (neuronal marker).

Comments:

This research improves the understanding of pediatric pancreatic islet histology, though it may not be fully analyzed. Multiplex Immunostaining (e.g., PTF1A+/NKX6-1+ cells with PAX6) with machine learning image processing would also be beneficial.

This data resource with limited findings may not suit the Nature communication readership, expecting more in depth documented analysis from this valuable data.

Reviewer #3

(Remarks to the Author)

Pancreatic islets form the endocrine compartment of the pancreas responsible for maintaining blood glucose homeostasis. Alterations in the development and function of islet β -cells contribute to the development of diabetes. While the structure and function of human adult islets have been well characterized, postnatal pancreatic islet development remains poorly understood, primarily due to the limited availability of human pediatric pancreatic tissue. Leveraging a cohort of 115 pediatric pancreata spanning birth to 10 years of age, this study provides a comprehensive characterization of the structural and functional changes of human pancreatic islets, such as the dynamic changes in islet cell composition, spatial organization, proliferative capacity, vascularization, innervation, and the functional maturation of α - and β -cells. The dataset is unique and even though information on some aspects of the work was already available there are many new insights and no study of this breadth has been published to date on this topic. These insights significantly enhance our understanding of postnatal pancreatic islet development and have important implications for diabetes research and regenerative medicine. Notably, the long co-expression of NKX6.1 and PTF1A, as well the presence of pre-endocrine cells (PAX6+) could signify a perdurance of multipotent pancreas progenitors postnatally, which may be used as a period for intervention.

This work fills a significant gap in our understanding of islet development and maturation after birth, while also providing useful guidance for improving the structure and function of stem cell-derived islets (SC-islets). Overall, the paper has strong scientific merit and will form an important resource and reference. It is also supported by solid data and clear arguments. Particularly, there is an excellent analysis of functional features of beta cells and alpha cells on a subset of the samples (about 1/3rd). Additionally, the risk of data being skewed by specific pathologies (anencephaly) is well analyzed.

Many other things would have been worth analyzing in this cohort such as acinar maturation, delta cell functional maturation and immune cells, as well as single-cell data to see cell maturation trajectories. However, considering the amount of work to date this would have to wait for a subsequent manuscript.

The reviewer recommends to clarify a few points, requiring a few extra experiments, revise some claims and improve one aspect of the narrative. The specific points are highlighted below:

Line 101: the number of months seems to be missing. From figures I would infer 3 months.

In Figure 1E, there appears to be a difference in the weight-index between the age of 2-6 and 6-10. It would be useful to include a corresponding comparison in Figure S1D.

Figure S1: It would be valuable to represent the relation of pancreas weight to body weight in neonates. They cannot be appreciated in the combined graph and it would help to know if there is a clear correlation at birth. In Supplemental Figure 1E, the p-value is 0.011, which appears to contradict the statement in lines 121-122.

Line 110 mentions the variation in pancreas weight at birth. It would be helpful to include a supplementary chart illustrating the fold difference in islet weight changes across different postnatal age groups.

Lines 127-128: If expressed in acinar cells GP2 would not be expected to strictly be a multipotent progenitor marker. The sentence is misleading.

Lines 140-141: the sentence is unclear. Why would the authors stress the repression of PTF1A by NKX6.1 while they show they remain co-expressed for a substantial amount of time.

The overlap between PTF1a and NKX6.1 is important and contrasts with mice. Though it indeed raises the possibility that endocrine cells continue to be produced after birth this remains unknown. The statement on lines 146-147 is too strong a claim. When does this stop? Is this resolved in the adult? The presence of partially differentiated cells expressing PAX6 but not hormones is also interesting and also suggestive of an ongoing process. Are they also NEUROD1 and INSM1-positive, which would be expected from observations in mice? Are NEUROG3+ cells still present at any of these stages?

In Figure 2 the overlap between PTF1A and NKX6.1 is not so clear and may affect only a subset of the cells. Can the authors document this more clearly with single-channels and ideally quantify the proportion of co-expressing cells among the PTF1a-positives?

In the paragraph on endocrine cell ratios could the authors provide absolute hormonal cell numbers or endocrine mass? Were polihormonal cells observed and at which frequency? In This could be added to Figure 3E and be compared at different ages.

Lines 177-178: Ki67 does not mark proliferation rates but rather non-quiescent cells (and even this is a relative notion: <https://doi.org/10.1016/j.celrep.2018.06.110>)

The paragraph starting on line 194 validates previous reports which should be cited.

The paragraph starting line 262 belongs to a discussion rather than results.

Line 265: the statement may be too strong. Is there any argument against some endocrine cells continuously forming in the trunk? If anything it should be NEUROG3 expression patterns which are not analyzed in this study.

Line 327: is this really senescence?

In the discussion or into it would be worth citing <https://doi.org/10.1038/s41467-024-47686-> and discussing any spatial heterogeneities seen during the early postnatal period.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Saunders et al present an impressive study of the temporal and spatial changes in the endocrine pancreas using a large cohort of young human pancreas. They set up a pipeline for analyzing many of the characteristics of the endocrine pancreas during this postnatal time (neonatal : birth to 3 months, infancy 3m to 2 y, childhood 2 y-10y) providing data on composition, architecture, density, proliferation, vascularization, innervation and importantly insulin and glucagon secretion. As stated in previous review this study presents some interesting findings and a framework of human postnatal development that has been lacking and should be very useful for the field, especially since the images are in the Pancreasatlas.

The revision is impressive for adding CODEX data for a separate cohort of donors. Additionally the improved Fig1 and Table S1 give clearer understanding of which donors were used for what. However, the expanded Table S1 raises the issue of overstating that their "unprecedented cohort of 123 donor organs" from birth to 10 years age. While 123 donors may be listed, 6 neonates, 7 infants and 5 childhood are used ONLY for pancreatic weights. At least 5 neonatal donors are preemies being less than 36 weeks gestational age. It is a plus to have all the studies done but the implication in new Fig 1A is that all this cohort was extensively studied functionally and phenotypically, which is a bit of a stretch. Several of the CODEX samples are only CODEX; only 2 of the CODEX also had perfusion, and only 2 infant and 6 children had both traditional IHC and perfusion.

It is not adequate to add that information re time on ventilator for dealing with Ki67+ data. The issue is that time with warm and cold ischemia reduces the amount of Ki67 protein(PMC3218923;PMC4407864), in fact the half life of Ki67 is less than 1 hour (PMID10653597). Yet there are several cases that have over 1 hour ischemia that are included in the Ki67 data, perhaps this is why the Ki67+ % are lower than other reports. The legend is also confusing ; what is meant by "italicized letters denote comparison among cell types within each age group"?

Normalizing secretion of either insulin or glucagon to IEQ is problematic since the proportion of beta/alpha cells is reported to differ at the different ages (Fig4b, Fig S6A).

In many of the figures pancreatic polypeptide cells are ignored, but they are a major cell type of the islet.

It would be helpful to have y axis labeled in Fig 1 G,H, J.

What is meant by the purple boxes in Fig2J?

For the perfusion studies, Fig5D gives data for 8 infants ,20 children and 12 adults but the Table S1 lists 1 neonate, 7 infants, 20 children and 14 adults were used for perfusion studies. The numbers should be consistent.

Reviewer #2

(Remarks to the Author)

I am pleased to report that the authors have thoroughly addressed the comments and suggestions, and as a result, the revisions have led to a significant enhancement of both the content and the conclusions presented in the manuscript.

Reviewer #3

(Remarks to the Author)

In the revised version of their article, Saunders et al. addressed most comments of the reviewers. Only few suggestions for improvements were not addressed experimentally such as Ki67 or INSM1/NEUROG3 stainings investigating more directly proliferation and endocrine differentiation. The ratios of polyhormonal cells were also not addressed though many readers would be curious about this. The authors argue that a new pipeline of analysis would be needed. NEUROD1 imaging was partially addressed due to technical difficulties.

On the other hand, the authors have added new data (not asked by reviewers) such as CODEX multiplexed stainings, which also added information on macrophages and immune cells. The images are of outstanding quality. Moreover, the data analysis is much improved (notably on islet organization) and the new table is rich in source information for specialists. A few more samples were also included. Taken together, this is a really impressive piece of work, the fruit of many years of patience, clearly a future reference paper for the field. The links with the Pancreasatlas will also contribute to making it a useful resource. It is also well written, not claiming more than it should.

There are a few minor points the authors may want to look into to polish the article:

1. In figure 1J, it is surprising to see so many neonates with a body weight of less than 2kg but it may be linked to the cause of death.

2. In Figure S1G, one neonate younger than 8 days appears to have a body weight exceeding 8 kg. Please check whether this value is accurate or if it might represent a data entry error, all the more that in figure 1J and S1G, the body weight of neonates are different.

3. In line 104, the total number of neonates is reported as 36. However, the number of data points shown in the figures appears to be smaller (Figure 1J: 29; Figure S1F: 32; Figure S1G: 31; Figure S1H: 29). Was some data excluded? Why?

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Response to Reviewer Comments

Introduction

We appreciate the reviewers' thoughtful comments and suggestions, which have guided us in making substantial improvements to strengthen our revised manuscript. These revisions include the addition of new data and figures summarized below, as well as detailed responses to specific reviewer suggestions provided in the accompanying point-by-point responses.

Since the original submission, we have continued our analyses of this unique collection of human pancreata by performing CO-Detection by indEXing (CODEX) multiplex antibody-based imaging on a subset of samples. This technology enabled quantitative analyses of proto-endocrine cells, transcription factor expression, immune cell populations, vascular cells, and additional cell types. To our knowledge, this is the first time such technology has been applied to the endocrine pancreas from young human donors. All this data is included in the revised manuscript.

For these reasons, we provide not only a response to each reviewer comment but also a summary of the new data, which we believe offers additional novel insights into events in postnatal pancreas development. Below is a summary of the modifications in the manuscript since the original submission:

- Six primary figures and five supplementary figures from the original manuscript have been revised
 - Islet size and cell arrangement data from the original cohort have been added to Figures 3, 5, and S9
 - CODEX data has been added to the originally presented, traditional IHC data in Figures 2- 4, 6, S7, and S11
- The manuscript now includes one additional primary figure (Figure 7) and six additional supplementary figures (Figures S3-4, 8, and 12-14) featuring primarily CODEX data

In our response, we use the Figure numbers in the revised manuscript. Here is a summary of the figures in the original and revised manuscripts:

- Original Figure 1 remains Figure 1 (accompanied by Figure S1), with minor panel additions to depict cohort demographics
- Original Figure 2 remains Figure 2 (accompanied by Figures S2-4), strengthened by CODEX data describing the proto-endocrine cell population
- Original Figure 3 was expanded to Figures 3-4 (accompanied by Figures S5-8)
- Original Figure 4 is now Figure 5 (accompanied by Figures S9-10)
- Original Figure 5 is now Figure 6 (accompanied by Figure S11)
- Figure 7, a new figure in the revised manuscript (accompanied by Figures S12-14), shows that immune cells, including macrophages and T lymphocytes, are present throughout postnatal pancreas development
- Original Figure 6 is now Figure 8

Reviewer 1 Response

Reviewer 1 remarks: Saunders et al present an impressive study of the temporal and spatial changes in the endocrine pancreas using a large cohort of young human pancreas. They set up a pipeline for analyzing many of the characteristics of the endocrine pancreas during this postnatal time (neonatal: birth to 3 months, infancy 3m to 2 y, childhood 2 y-10y) providing data on composition, architecture, density, proliferation, vascularization, innervation and importantly insulin and glucagon secretion. There are several important findings. First, identification in the infants of both PTF1A+Nkx6.1+ GP2+ cells showing what is thought to be multipotential progenitor cells (MPC) and Pax6+ hormone negative proto-endocrine cells up to the age of 20 months; both suggesting continued differentiation of new endocrine cells (neogenesis) in the human pancreas. Second, up to 2 years islets are mainly core-mantle structure, after which they become more intermingled and indistinguishable from adult islets. Thirdly, beta and alpha cell proliferation were unchanged (about 1%) across this age span whereas delta cell proliferation decreased. Fourth, that while there were no real differences in insulin secretory responses across the age span even to adult, the maturation of glucagon secretion was later and came only in childhood. Fifth, innervation of the islets was essentially absent in infancy and only developed in childhood even though the vascularization was unchanged. The authors acknowledge that more studies are needed to understand the mechanisms responsible for the observed changes.

While some of the metrics used are unusual and difficult to relate to, the study presents a framework of human postnatal development that has been lacking and should be very useful for the field especially since the images are in the Pancreasatlas. There are some issues that need further details:

Our response: We thank the reviewer for highlighting the important findings of our study. In response, we have added substantial analyses that further strengthen, refine, and clarify these results, making them more accessible and more interpretable.

1. **Reviewer 1:** The finding of MPC and protoendocrine cells is important and exciting but there was little information concerning this given. It is stated that these cells were found up to 20 m age but the images shown do not indicate the age of the donor and quantification is lacking.

Our response: We appreciate the reviewer's interest in obtaining additional information on the multipotent progenitor cells and proto-endocrine cells identified in our pediatric cohort.

Using the expanded CODEX imaging data and updated image analysis, we have added the requested quantification data for MPC (GP2+) cells (see **Figure S2C-D**) and proto-endocrine cells (see **Figure 2** and **Figure S4**). These new data also include analyses of transcription factor co-expression within these cell populations, as well as quantification of the spatial relationships between proto-endocrine cells and ductal structures.

To address concerns about the clarity of donor information in representative images, we have added specific donor ID references to all figure legends that include images. This will allow readers to readily link each representative image to the corresponding detailed donor information provided in **Table S1**.

2. **Reviewer 1:** Data are presented compared to pancreatic weight, and not age with the rationale that there were variable growth rates. However, this does not seem consistent with

their speculation that the pancreatic size is established early (intrauterine) and with a strong genetic influence. Additionally presenting data with age as the x-axis would be easier for the reader to relate to other studies.

Our response: We believe the reviewer is referring to the presentation of endocrine cell subtypes by pancreas weight (in the original **Figure S4**). We have added these data to revised **Figure S6D** and **S6F**, along with the analysis of estimated endocrine cell subtype mass by age (**Figure S6H**).

3. **Reviewer 1:** The measurement of location 20um from periphery for assessing the islet architecture is odd and would seem somewhat dependent on the islet size.

Our response: We appreciate the reviewer's feedback and, in response, have adopted a revised approach analyzing islet architecture, as described in the text and shown in **Figure 5A**. This new approach incorporates a normalization algorithm to account for differences in islet size and shape, enabling a more granular analysis of islet architecture than was previously possible. New data generated using this method are presented in **Figure 5B-C** and **Figure S9**. While the overall results are consistent with our prior analysis, we are grateful to the reviewer for suggesting this refinement to better account for islet size variability.

4. **Reviewer 1:** While 115 pancreases from donors of neonatal (birth -3 months age) to 10 years were included, the numbers for various assessments are far less: functional assessment (perifusion) used islets from 2 neonate, 8 infants 18 children and 14 adults; islet architecture used 5 neonatal (1d,1d,1d, 4d,3m) and 5 childhood (7m 20 m, 4y,5y, 10 y); endocrine density and composition used 24 neonatal, 21 infant (3m-24m), 33 childhood (2y-10 y); 27 are only used for pancreatic weight. The text should reflect these smaller samples rather than the whole collection, particularly in the Discussion.

Our response: We appreciate the reviewer's suggestion to clarify that experimental and cost limitations prevented us from performing all analyses on every pancreas in our cohort. In response, we have added clarification at multiple points in the revised manuscript. Specifically, in the first section of the Results, where the cohort is introduced, we now explicitly state that not all donors were used for every analysis. Readers are directed to **Table S1**, which provides detailed donor information, including which donors were included for each analysis. We have also added clarifying information in the text and figure legends regarding the *n* numbers used for each corresponding analysis. Finally, additional statements highlighting the limitations associated with smaller cohorts have been included in the revised text.

5. **Reviewer 1:** While it is impressive to have perfused islets from 8 infants and 20 children, the authors should acknowledge (and cite) the studies Henquin did on islets from 5 infants/toddlers (11-36 months) (PLoS1, 2016). He showed clearly that by 1 year the islets responded as adult islets but they secreted a smaller fraction of their content and even so their stimulation index was the same as adults because they had a lower basal secretion.

Our response: We thank the reviewer for the suggestion to incorporate this reference. We have added the citation and included an acknowledgement and discussion of this study in the context of prior studies and our findings.

6. **Reviewer 1:** There should be a caveat re the Ki67 staining for proliferation. Ki67 protein decreases with ischemia (warm and cold) and is reported to have a half-life of less than 1 hr. It may be that the larger organs have longer warm ischemia.

Our response: We thank the reviewer for pointing out the need for this additional caveat. In addition to discussing this potential concern regarding these proliferation metrics in the text, we do have data analyzing whether on-ventilator time (where available) affects our Ki67+ numbers (see **Figure S7A-B**). Unfortunately, we do not have enough donors with ischemic time data available to analyze the effect on Ki67+ numbers; however, this limitation was noted in the text.

Minor concerns:

7. **Reviewer 1:** Data for Ki67 are referred to as rates, but rate is a value over a time denominator, thus these are no rates, just frequency of Ki67+ cells.

Our response: We appreciate the reviewer noting this. References in the text and figure legends to Ki67 proliferation data have been revised to remove language implying proliferation “rates.”

8. **Reviewer 1:** Suppl Fig2 only provides additional images, again without indication of age of donor.

Our response: As noted above in response to a similar concern, we have added specific donor ID references in all figure legends, including **Figure S2**. This allows readers to link each representative image to the detailed donor information found in **Table S1**.

Reviewer 2 Response

Reviewer 2 remarks: The manuscript 'Mapping histologic and functional maturation of human endocrine pancreas across early postnatal periods' by Saunders and colleagues examines the differentiation and morphogenesis of human endocrine cells from birth to ten years old. Analyzing 115 pediatric pancreata, the study categorizes samples into three age groups: Neonatal (birth to <3 months), Infancy (3 to 24 months), and Childhood (2 to 10 years). Using fluorescence imaging techniques like confocal microscopy, the research maps morphological changes with 15 antibodies and highlights differences in insulin and glucagon secretion maturation and cellular markers across age groups.

The manuscript is well-written and structured, including material, antibodies, assays, and images. While primarily descriptive, the sample collection's scope and diversity stand out, providing valuable insights into Type 1 Diabetes risk and genetic data related to islet biology.

Our response: We are grateful to the reviewer for the feedback on the valuable insights our study provides and appreciate the suggestions for improvement, which we have addressed below.

1. **Reviewer 2:** This study uses image-based microscopy, so it should include essential documentation on image processing procedures, software, workflows, and classification. The online portal and figures seem to show processed images without the originals?

Our response: We appreciate the reviewer's concern regarding image availability and processing. The Methods section has been updated to clarify that images were uploaded directly to the Pancreatlas™ portal following acquisition, with only reversible brightness and/or contrast adjustments and pseudocoloring applied to improve signal visualization.

2. **Reviewer 2:** Most analysis depends on fluorescence labelling of cells by antibodies, requiring segmentation. Only Ki-67 and CD31 methods for immunohistochemistry quantification are mentioned, suggesting manual processing. Parameters/configurations for commercial systems are not provided.

Our response: We acknowledge the reviewer's request for additional clarification of the image analysis methods. In response, we have added a more detailed description of the image analysis pipeline used for the majority of the IHC and CODEX images, including a link that directs readers to the Python script used for these analyses. A small subset of IHC image analyses was performed using alternative tools and methods; in those cases, the specific software and algorithms employed are listed, and the corresponding figures containing these data are appropriately referenced.

3. **Reviewer 2:** Considering the image-based approach and the expected impact of visual access through an online portal, why have the authors not included appropriate schemes for red-green color blindness, which affects 8% of men?

Our response: We appreciate the reviewer's concern and regret that manuscript figures provide limited space for single-channel images (unfortunately, there are no ideal, accessible color schemes for images with 4+ unique channels that highlight colocalization between channels). As noted, we have uploaded all images included in this study to the Pancreatlas online portal to ensure maximum accessibility. This platform allows individual

markers within each image to be independently pseudocolored, enabling visualization in any preferred color scheme, including options for those with red-green color blindness.

4. **Reviewer 2:** The study categorizes the pancreas by head, body, and tail but does not analyze endocrine islet development across age groups.

Our response: We are grateful for the opportunity to clarify this point. Pancreatic tissue sections from the head, body, and/or tail were analyzed for each donor. Individual donor totals for analysis were compiled by combining data from all available sections for each donor. This has been clarified in the text. In addition, the specific pancreas region corresponding to each image is indicated on Pancreatlas, and the pancreatic regions analyzed by CODEX imaging are detailed in **Table S2B**.

5. **Reviewer 2:** The anencephaly versus other causes of death for the neonatal group is analyzed, but other confounders may affect different age groups (e.g., procurement, origin, ischemia). Particularly, the infancy group's secretion assay should be tested for these factors (Fig. 4F and I).

Our response: We agree with the reviewer that confounding factors could differ among age groups and potentially influence our findings. Unfortunately, due to limited information available for many of these samples, we do not have sufficient data to investigate potential confounding variables (e.g., ischemic time) and perform a multivariate analysis to address this issue.

For the secretion assays, specifically, all islets from the infancy group were isolated and analyzed at the same site, eliminating processing location as a potential confounder. However, we are unable to evaluate additional confounding variables for the reasons outlined above. To ensure transparency, we have included detailed information on included and excluded donors for the secretion assays, together with reasons for exclusion (see **Table S3A**).

6. **Reviewer 2:** The observation that endocrine cell numbers per tissue have increased from the neonatal stage to infancy (Fig. 3, D) is based on segmentation, as well as the size and volume of islets in 3D, which is not accounted for by area percentage. Could you normalized for this?

Our response: We appreciate the reviewer bringing this concern to our attention. In the original **Figure 3D**, the y-axis was labeled “% Endocrine cells per pancreas”; however, this metric was derived from counts of endocrine cells in 2D tissue sections rather than based on 3D islet volume measurements. To clarify this distinction, the figure has been revised (now **Figure 4A**), and the y-axis has been relabeled as “% Endocrine cells per total cells” to more accurately reflect the analysis performed.

Data collection from 2D images, as noted by the reviewer, is an important limitation of this study and is discussed in the revised Discussion (see **Limitations** section). We have also added estimated islet and endocrine cell mass (see **Figure S6G-H**).

7. **Reviewer 2:** The differences in cell compositions between the neonatal and infancy groups could be due to image processing (Fig. 3E)?

Our response: The reviewer raises an important point, as differential image processing across groups could confound downstream analyses. However, in our study, all images were acquired and processed identically across developmental stages, thereby minimizing the possibility of image-processing differences as a confounder. Cell segmentation was performed using a convolutional neural network-based approach trained to identify cell boundaries using local image features (e.g., intensity, gradients, texture, and spatial context), rather than size-based thresholds. As a result, segmentation performance does not rely on assumptions about cell size or morphology, which can vary across developmental stages. All downstream cell-type classification models were trained on manually annotated cells pooled across neonatal, infancy, and childhood donors. This strategy explicitly avoids age-specific tuning and ensures that classifiers learn marker-expression patterns shared across developmental stages. These image annotations are also publicly available on Pancreatlas.

8. **Reviewer 2:** The online images and Fig. 3 (A, B, C) illustrate the presence of intermingled endocrine cells only in childhood islets. In contrast, Fig. 4 (C, D) shows that these endocrine cells are primarily located at the periphery of islets, comprising about 20%, rather than intermingling. Is there any possibility that the selection of the measured 30 islets could be biased?

Our response: We appreciate the reviewer's feedback and, in response, adopted a revised approach to the analysis of islet architecture, as described in the text and illustrated in **Figure 5A**. This new approach applies a normalization algorithm to account for differences in islet size and shape and increases the number of islets analyzed per group. In total, 63,238 islets and clusters were identified by CODEX analysis of 17 donors, and 167,388 by IHC of 43 donors. For CODEX, sample sizes were as follows: neonatal (n=6 donors; 34,330 clusters; 2,995 islets), infancy (n=5 donors; 11,978 clusters; 2,036 islets), and childhood (n=6 donors; 10,461 clusters; 1,428 islets). For IHC, sample sizes were as follows: neonatal (n=9 donors; 65,333 clusters; 7,464 islets), infancy (n=11 donors; 37,395 clusters; 5,508 islets), and childhood (n=23 donors; 44,646 clusters; 7,042 islets). This expanded dataset enabled a more granular analysis of islet architecture than was previously possible and reduces potential bias inherent in our previous methodology. New data from this approach are presented in **Figure 5B-C** and **Figure S9**. While the overall results are consistent with those from our prior analysis, we are grateful to the reviewer for suggesting refinements to our analytical approach and expansion of the dataset.

9. **Reviewer 2:** The rationale for selecting markers/antibodies is not mentioned.

Our response: We used antibodies in both traditional IHC staining and the new CODEX image analysis to target signature makers of pancreatic cell types, transcription factors, and other pancreatic features, enabling visualization and characterization of these cells and their structures. Specific antibodies were selected to optimize visualization based on screening of tissue sections and are listed for traditional IHC and CODEX imaging in **Table S4A** and **Table S2A**, respectively. We have added a 'Rationale' column to clarify the use of each marker.

Reviewer 2: The proposed model and curves may be speculative given the aforementioned points.

Our response: We agree that the model (now shown in **Figure 8**) is speculative based on the data presented in our studies, which we have substantially strengthened with revised

and additional analyses. However, we have clarified in the text and figure legends that this is a proposed model based on these data.

Minor concerns:

10. **Reviewer 2:** Defining the gene GP2 as a marker for multipotent cells is challenging, as iPSCs from cell cultures show high GP2 expression, which may differ in tissues and proteins.

Our response: We agree with the reviewer that GP2 has been described in iPSCs as a pancreatic progenitor cell marker, and in IHC data from native pancreas; we have cited these studies in the text. As such, the current study is not the first to link GP2 expression in pancreatic progenitor iPSCs to co-expression with PTF1A and NKX6-1 in developing human pancreata. The text now explicitly situates our findings in the context of prior studies, highlighting that our results provide new evidence for the importance of this marker in human pancreatic development. We have further strengthened these findings by adding new image analysis quantifying and characterizing transcription factor co-expression in GP2+ cells (see **Figure S2**).

11. **Reviewer 2:** The findings on the vascularization and innervation of the neonatal pancreas are noteworthy and warrant discussion in the context of neurons in the neonatal group (neuronal marker).

Our response: We believe this comment refers to the comparatively lower percentage of islets with interior nerve fibers in the neonatal and infancy stages compared to childhood (shown in revised **Figures 6E-H** and **S11B**). We appreciate the reviewer's interest in the vascular and neuronal findings, which we have strengthened through additional analyses of vascular cells (see **Figures 6L-M** and **S11E-I**) and visualization of intrapancreatic ganglia in CODEX images (see **Figure 6I-J**). The Discussion underscores the need for further studies to characterize the timeline of innervation in the developing human pancreas.

Comments:

12. **Reviewer 2:** This research improves the understanding of pediatric pancreatic islet histology, though it may not be fully analyzed. Multiplex Immunostaining (e.g., PTF1A+/NKX6-1+ cells with PAX6) with machine learning image processing would also be beneficial. This data resource with limited findings may not suit the Nature communication readership, expecting more in-depth documented analysis from this valuable data.

Our response: We appreciate this valuable feedback from the reviewer and, in response, have added substantial additional data generated through multiplexed antibody-based imaging (CODEX) on a subset of donor tissues, as suggested. These analyses enabled improved quantification and characterization of MPC and proto-endocrine cells, including assessment of transcription factor co-expression in both precursor populations and differentiated endocrine cells. We have also incorporated extensive additional quantification and analysis of immune, vascular, and other cell types. Collectively, these new data account for a large portion of the two additional primary figures (**Figure 4** and **Figure 7**) and 13 supplementary figures included in the revised manuscript. Furthermore, making the data and images available on Pancreatlas will allow other scientists to make additional assessments.

Reviewer 3 Response

Reviewer 3 remarks: Pancreatic islets form the endocrine compartment of the pancreas responsible for maintaining blood glucose homeostasis. Alterations in the development and function of islet β -cells contribute to the development of diabetes. While the structure and function of human adult islets have been well characterized, postnatal pancreatic islet development remains poorly understood, primarily due to the limited availability of human pediatric pancreatic tissue. Leveraging a cohort of 115 pediatric pancreata spanning birth to 10 years of age, this study provides a comprehensive characterization of the structural and functional changes of human pancreatic islets, such as the dynamic changes in islet cell composition, spatial organization, proliferative capacity, vascularization, innervation, and the functional maturation of α - and β -cells. The dataset is unique and even though information on some aspects of the work was already available there are many new insights and no study of this breadth has been published to date on this topic. These insights significantly enhance our understanding of postnatal pancreatic islet development and have important implications for diabetes research and regenerative medicine. Notably, the long co-expression of NKX6.1 and PTF1A, as well the presence of pre-endocrine cells (PAX6+) could signify a perdurance of multipotent pancreas progenitors postnatally, which may be used as a period for intervention.

This work fills a significant gap in our understanding of islet development and maturation after birth, while also providing useful guidance for improving the structure and function of stem cell-derived islets (SC-islets). Overall, the paper has strong scientific merit and will form an important resource and reference. It is also supported by solid data and clear arguments. Particularly, there is an excellent analysis of functional features of beta cells and alpha cells on a subset of the samples (about 1/3rd). Additionally, the risk of data being skewed by specific pathologies (anencephaly) is well analyzed.

Many other things would have been worth analyzing in this cohort such as acinar maturation, delta cell functional maturation and immune cells, as well as single-cell data to see cell maturation trajectories. However, considering the amount of work to date this would have to wait for a subsequent manuscript.

The reviewer recommends to clarify a few points, requiring a few extra experiments, revise some claims and improve one aspect of the narrative.

Our response: We appreciate the reviewer's comments regarding the importance of our study and agree that additional analysis would be valuable. In response, as described in the introduction, we have now included substantial new analyses from CODEX imaging that further strengthen the impact of our findings. As the reviewer notes, many additional analyses could be conducted on this cohort; however, these are beyond the scope of this current manuscript. To support future investigations, we are providing all source data of original images, including those from the newly added CODEX images (available on Pancreatlas), with annotations. We hope these efforts will provide a valuable resource for the broader scientific community and facilitate further discovery.

1. **Reviewer 3:** Line 101: the number of months seems to be missing. From figures I would infer 3 months.

Our response: We appreciate the reviewer catching this typo; it has been corrected in the revised text.

2. **Reviewer 3:** In Figure 1E, there appears to be a difference in the weight-index between the age of 2-6 and 6-10. It would be useful to include a corresponding comparison in Figure S1D.

Our response: We appreciate the reviewer's suggestion. This analysis was performed and is now included in **Figure S1E**.

3. **Reviewer 3:** Figure S1: It would be valuable to represent the relation of pancreas weight to body weight in neonates. They cannot be appreciated in the combined graph and it would help to know if there is a clear correlation at birth.

Our response: An inset of the XY plot comparing pancreas weight to body weight has been added (revised **Figure 1J**). We provide further analysis of this age group in revised **Figure S1F-H**, which is described in the response below.

4. **Reviewer 3:** In Supplemental Figure 1E, the p-value is 0.011, which appears to contradict the statement in lines 121-122.

Our response: We appreciate the reviewer noting this error in the discussion of now **Figure S1H**. The text has been updated to clarify that the observed difference in the weight index is likely due to variations in body weight resulting from missing brain tissue (which accounts for 10-12% of body weight at birth) in anencephalic donors, rather than differences in pancreas mass. To support this clarification, we have added additional data on body and pancreas weight in this cohort (see **Figure S1F-G**). Of note, the p-value, though still significant, has been updated after we consulted a biostatistician, who recommended switching to a nonparametric test (updated to Mann-Whitney from t-test).

5. **Reviewer 3:** Line 110 mentions the variation in pancreas weight at birth. It would be helpful to include a supplementary chart illustrating the fold difference in islet weight changes across different postnatal age groups.

Our response: We appreciate the reviewer's suggestion and have added a figure showing the estimated mass of each endocrine cell type by age (see **Figure S6E**). Recognizing that alternative visualization approaches and data representations may also be valuable, we are providing the source data to enable readers to perform additional analyses as desired.

6. **Reviewer 3:** Lines 127-128: If expressed in acinar cells GP2 would not be expected to strictly be a multipotent progenitor marker. The sentence is misleading.

Our response: We appreciate the reviewer's suggestion to clarify our discussion of GP2. The revised text now specifies that GP2 marks pancreatic multipotent progenitor cells and acinar progenitors, based on co-expression with transcription factors PTF1A and NKX6.1 (beginning on line 132). Analyses of newly added data further quantify and characterize transcription factor co-expression in these GP2+ cells (see **Figure S2**).

7. **Reviewer 3:** Lines 140-141: the sentence is unclear.

- Why would the authors stress the repression of PTF1A by NKX6.1 while they show they remain co-expressed for a substantial amount of time.
- The overlap between PTF1a and NKX6.1 is important and contrasts with mice. Though it indeed raises the possibility that endocrine cells continue to be produced after birth this remains unknown.

Our response: We appreciate the reviewer's feedback and have clarified our discussion of the interactions between PTF1A and NKX6.1 as co-repressive determinants of endocrine and acinar lineage specification in multipotent progenitor cells. We have also strengthened the supporting data by adding quantification of GP2+ cells and analysis of PTF1A and NKX6.1 co-expression, as mentioned above (see **Figure S2**). The reviewer is correct that this is observational data based solely on protein colocalization, and we have emphasized the need for follow-up studies in the revised manuscript text.

8. **Reviewer 3:** The statement on lines 146-147 is too strong a claim. When does this stop? Is this resolved in the adult? The presence of partially differentiated cells expressing PAX6 but not hormones is also interesting and also suggestive of an ongoing process. Are they also NEUROD1 and INSM1-positive, which would be expected from observations in mice? Are NEUROG3+ cells still present at any of these stages? In Figure 2 the overlap between PTF1A and NKX6.1 is not so clear and may affect only a subset of the cells. Can the authors document this more clearly with single-channels and ideally quantify the proportion of co-expressing cells among the PTF1a-positives?

Our response: We thank the reviewer for their feedback and have added substantial new data from CODEX image analysis characterizing both multipotent progenitor cells (see **Figure S2**) and proto-endocrine cells (see **Figure 2** and **Figure S4**) as suggested. These additions include quantification of co-expressed transcription factors and analyses supporting a ductal niche for the PAX6+ proto-endocrine cells. We have also revised the sentence the reviewer indicated (now lines 159-160). All images showing PTF1A and NKX6.1 staining are available for interactive viewing in Pancreatlas, where individual channels can be isolated and pseudocolored as desired.

We also appreciate the reviewer's interest in analyzing additional markers (e.g., NEUROD1, ISM1, NEUROG3). Unfortunately, some transcription factors in our CODEX panel did not have enough donor samples passing QC for robust analysis (see **Table S2B**), while others have not been optimized for CODEX and are beyond the scope of this manuscript. Images that passed QC are available for visualization on Pancreatlas, and further analysis of these markers will be an important focus for future studies.

9. **Reviewer 3:** In the paragraph on endocrine cell ratios, could the authors provide absolute hormonal cell numbers or endocrine mass? Were polyhormonal cells observed and at which frequency? In This could be added to Figure 3E and be compared at different ages.

Our response: We appreciate the reviewer's suggestion and have added additional figures showing the estimated mass of each endocrine cell type by age and developmental stage (see **Figure S6G-H**). In optimizing our cell annotation process, to minimize false positives and ensure we were not missing true positives, the computational pipeline "forced" each cell that was annotated into a single hormonal label. This does not preclude this data from being analyzed but it was not reasonable with our pipeline to manually review each one to quantify this metric for this manuscript. However, recognizing that alternative visualization and quantification approaches may be valuable, we will provide readers with the source data and the original images (on Pancreatlas) to allow for additional assessment, such as that suggested by the reviewer.

10. **Reviewer 3:** Lines 177-178: Ki67 does not mark proliferation rates but rather non-quiescent cells (and even this is a relative notion: <https://doi.org/10.1016/j.celrep.2018.06.110>)

Our response: We appreciate the reviewer noting this semantic error; the text and figure legend references to Ki67 data have been adjusted to remove language referencing proliferation “rates.”

11. **Reviewer 3:** The paragraph starting on line 194 validates previous reports which should be cited.

Our response: We appreciate the reviewer noting this omission. Appropriate additional references have been added.

12. **Reviewer 3:** The paragraph starting line 262 belongs to a discussion rather than results.

Our response: We agree with the reviewer’s suggestion and have appropriately moved our discussion of the proposed model from Results to Discussion.

13. **Reviewer 3:** Line 265: the statement may be too strong. Is there any argument against some endocrine cells continuously forming in the trunk? If anything it should be NEUROG3 expression patterns which are not analyzed in this study.

Our response: We appreciate the reviewer’s feedback and have expanded the discussion to provide a more nuanced consideration of additional studies needed to clarify postnatal endocrine cell differentiation (see **Postnatal processes in human endocrine cell neogenesis**). We have also added a caveat that the model we propose is based on how our findings build upon prior knowledge, while acknowledging that many questions remain to be answered.

14. **Reviewer 3:** Line 327: is this really senescence?

Our response: We appreciate the reviewer’s comment and have clarified in the text that we are drawing a parallel to low proliferation observed in adult β cells. Whether this reflects cellular quiescence versus senescence is beyond the scope of our study and does not materially affect the observations reported here.

15. **Reviewer 3:** In the discussion or into it would be worth citing <https://doi.org/10.1038/s41467-024-47686-> and discussing any spatial heterogeneities seen during the early postnatal period.

Our response: The link provided by the reviewer was unfortunately incomplete; we presume they were referring to Lehrstrand et al. (*Nature Communications* 2024). This reference has been cited in the revised Discussion, which has also been strengthened by our inclusion of new data demonstrating heterogeneity of cluster and islet size and composition (see **Figure 3**).

Response to Reviewer Comments

Introduction

We appreciate the reviewers' additional thoughtful comments and suggestions, which have guided us in clarifying the results to strengthen our revised manuscript. These revisions are detailed in the accompanying point-by-point responses to specific reviewer remarks and suggestions.

Reviewer #1 Response

Reviewer #1 Remarks: Saunders et al present an impressive study of the temporal and spatial changes in the endocrine pancreas using a large cohort of young human pancreas. They set up a pipeline for analyzing many of the characteristics of the endocrine pancreas during this postnatal time (neonatal : birth to 3 months, infancy 3m to 2 y, childhood 2 y-10y) providing data on composition, architecture, density, proliferation, vascularization, innervation and importantly insulin and glucagon secretion. As stated in previous review this study presents some interesting findings and a framework of human postnatal development that has been lacking and should be very useful for the field, especially since the images are in the Pancreasatlas.

Our response: We thank the reviewer for highlighting the important findings of our study and Pancreatlas resource. We have addressed the remaining remarks below by further refining and clarifying the text as detailed in our responses.

1. **Reviewer #1:** The revision is impressive for adding CODEX data for a separate cohort of donors. Additionally the improved Fig1 and Table S1 give clearer understanding of which donors were used for what. However, the expanded Table S1 raises the issue of overstating that their “unprecedented cohort of 123 donor organs” from birth to 10 years age. While 123 donors may be listed, 6 neonates, 7 infants and 5 childhood are used ONLY for pancreatic weights. At least 5 neonatal donors are preemies being less than 36 weeks gestational age. It is a plus to have all the studies done but the implication in new Fig 1A is that all this cohort was extensively studied functionally and phenotypically, which is a bit of a stretch. Several of the CODEX samples are only CODEX; only 2 of the CODEX also had perfusion, and only 2 infant and 6 children had both traditional IHC and perfusion.

Our response: We appreciate the reviewer's suggestion to provide additional clarification on the analysis of our pediatric cohort. Though the cohort remains unprecedented, we have edited the introduction (line 73) to include a caveat from the beginning that analyses were performed on subsets of the cohort, and to reference **Table S1**. This is in addition to the more detailed statement in the results (lines 113-114).

2. **Reviewer #1:** It is not adequate to add that information re time on ventilator for dealing with Ki67+ data. The issue is that time with warm and cold ischemia reduces the amount of Ki67 protein (PMC3218923; PMC4407864), in fact the half life of Ki67 is less than 1 hour (PMID10653597). Yet there are several cases that have over 1 hour ischemia that are included in the Ki67 data, perhaps this is why the Ki67+ % are lower than other reports. The legend is also confusing ; what is meant by “italicized letters denote comparison among cell types within each age group”?

Our response: We agree with the reviewer that it would be ideal to have donors with shorter ischemia times and more complete clinical data for all donors analyzed. Unfortunately, due to the limited opportunities we have to obtain these rare pediatric samples, we do not have enough donors with ischemic time data available to analyze the effect on Ki67+ numbers. However, this limitation regarding ischemia time was expanded upon in the text of our revision to include possible effects on Ki67 with additional reference added (lines 243-247).

Regarding statistical analysis denoted by italicized letters, these are the comparisons among cell types (e.g., α , β , δ) within rather than between each age group (neonatal, infancy, childhood); columns with the same letters in each age group are not statistically different, and different letters within the specific age group are $p < 0.05$. This has been updated for clarification in the figure legend.

3. **Reviewer #1:** Normalizing secretion of either insulin or glucagon to IEQ is problematic since the proportion of beta/alpha cells is reported to differ at the different ages (Fig4b, Fig S6A).

Our response: We thank the reviewer for this observation. This is why we have also provided secretion normalized to insulin or glucagon content (**Fig S10A-H**), detailed statistical analyses for the perfusion data (**Table S3B**), and the source data.

4. **Reviewer #1:** In many of the figures pancreatic polypeptide cells are ignored, but they are a major cell type of the islet.

Our response: We agree with the reviewer that pancreatic polypeptide cells (also known as γ cells, as labeled in our figures) are a major endocrine cell type. Due to limitations of the traditional immunohistochemistry markers, analysis of these additional endocrine cell types was primarily performed on donors using CODEX (see **Table S1**). Despite these limitations, we have included pancreatic polypeptide cells (γ cells) in a substantial number of our analyses, including specific γ cell subtype analysis and images in **Figures 4B, D-E, G; S6B-G; S7F; S8A-F**, and included in total endocrine cell analyses and images in **Figures 2F-F", G-G"; 4F; 6K, L-M; 7J-J'; S3C-D; S11C; and S12A-B**.

5. **Reviewer #1:** It would be helpful to have y axis labeled in Fig 1 G,H, J.

Our response: These axis labels have been added as the reviewer suggested.

6. **Reviewer #1:** What is meant by the purple boxes in Fig2J?

Our response: We thank the reviewer for this question. The purple boxes around PAX6, ISL1, and NKX2-2 highlight the point made in the text that the vast majority (73.2%) of PAX6+ hormone-negative cells were also producing transcription factors ISL1 and NKX2-2, with most of those cells (84.8%) positive for either PDX1 or ARX (not highlighted), consistent with the expected developing lineage-specific TF profiles for β and α cells, respectively. A brief explanation of this point was added to the figure legend to help clarify this for the readers.

7. **Reviewer #1:** For the perfusion studies, Fig5D gives data for 8 infants ,20 children and 12 adults but the Table S1 lists 1 neonate, 7 infants, 20 children and 14 adults were used for perfusion studies. The numbers should be consistent.

Our response: We appreciate the reviewer noting the donors included in perfusion studies. Unfortunately, due to the nature of these samples and assays, not all donors included in perfusion studies had detectable extract for all measured metrics, which explains the differences between the numbers in **Figure 5D** and **Table S1**. This is why we detail these issues in the methods and provide a reference to **Table S3A**, which provides detailed information on which assays were included for each donor and parameters used for inclusion versus exclusion.

Reviewer #2 Response

Reviewer #2 Remarks: I am pleased to report that the authors have thoroughly addressed the comments and suggestions, and as a result, the revisions have led to a significant enhancement of both the content and the conclusions presented in the manuscript.

Our response: We thank the reviewer for their invaluable comments and suggestions that guided our original revisions.

Reviewer #3 Response

Reviewer #3 Remarks: In the revised version of their article, Saunders et al. addressed most comments of the reviewers. Only few suggestions for improvements were not addressed experimentally such as KI67 or INSM1/NEUROG3 stainings investigating more directly proliferation and endocrine differentiation. The ratios of polyhormonal cells were also not addressed, though many readers would be curious about this. The authors argue that a new pipeline of analysis would be needed. NEUROD1 imaging was partially addressed due to technical difficulties. On the other hand, the authors have added new data (not asked by reviewers) such as CODEX multiplexed stainings, which also added information on macrophages and immune cells. The images are of outstanding quality. Moreover, the data analysis is much improved (notably on islet organization) and the new table is rich in source information for specialists. A few more samples were also included. Taken together, this is a really impressive piece of work, the fruit of many years of patience, clearly a future reference paper for the field. The links with the Pancreaatlas will also contribute to making it a useful resource. It is also well written, not claiming more than it should. There are a few minor points the authors may want to look into to polish the article:

Our response: We thank the reviewer for highlighting the important findings of our study

8. **Reviewer #3:** In figure 1J, it is surprising to see so many neonates with a body weight of less than 2kg but it may be linked to the cause of death

Our response: We agree with the reviewer that we suspect that the cause of death is linked to body weight, particularly given that anencephalic neonates on average tend to be <10th percentile for weight corrected for gestational age; this population makes up a significant part of our neonatal cohort, and the body weight difference in these infants is noted in **Figure S1G**.

9. **Reviewer #3:** In Figure S1G, one neonate younger than 8 days appears to have a body weight exceeding 8 kg. Please check whether this value is accurate or if it might represent a data entry error, all the more that in figure 1J and S1G, the body weight of neonates are different.

Our response: We thank the reviewer for pointing out this atypical value. We went back to the patient chart and discovered that it was a transcription data-entry error in our Laboratory Inventory Management System. The information for this donor (DON18) has been updated in **Table S1** and the source material, resulting in updates to **Figure 1** and **S1**.

10. **Reviewer #3:** In line 104, the total number of neonates is reported as 36. However, the number of data points shown in the figures appears to be smaller (Figure 1J: 29; Figure S1F: 32; Figure S1G: 31; Figure S1H: 29). Was some data excluded? Why?

Our response: We appreciate the reviewer's question. Due to the nature of these donations, we do not always get body weight and/or pancreas weight data for all donors. **Table S1** provides information on which of the 36 neonatal donors are included in the pancreas weight analyses (32 neonatal donors). Of these donors, 3 do not have body weight data available but do have pancreas weight. These differences between donors that have body weight only, pancreas weight only, or both explain the variable numbers included in each graph; specific donor information included in the graphs is available in the source data.