# Supplementary Appendix

**Frohnert et al.: Refining the Definition of Stage 1 Type 1 Diabetes: An Ontology-Driven Analysis of the Heterogeneity of Multiple Islet Autoimmunity**

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## Measurement of islet autoantibodies by study site

### BABYDIAB/BABYDIET:

Measurements of IAA, GADA, IA-2A, and ZnT8A antibodies in venous blood samples were performed using radiobinding assays (RBAs) at the Institute of Diabetes Research, Helmholtz Center Munich. Positivity thresholds were based on the upper 99th centile and Q-Q plots of results from control children as described elsewhere (1–3). IAA levels were measured using a competitive RBA (4). Assays were evaluated by the Diabetes Antibody Standardization Program (DASP) and the Islet Autoantibody Standardization Program (IASP). (5–8)

### DAISY:

All antibodies were measured, in duplicate, from frozen serum samples. Autoantibody detection assays have been updated over the course of the 24 years study history as described below:

*Glutamic acid decarboxylase antibodies (GADA) and Islet antigen-2 antibodies (IA-2A):*

- From June 8, 1994 to Feb. 16, 2010, autoantibodies to GAD65 were measured utilizing a modified radioassay as described previously (9,10). Results were expressed as an index: (sample cpm-negative control cpm)/(positive control cpm-negative control cpm).
- From Nov. 17, 1995 to Feb. 16, 2010, autoantibodies to islet cell antigen 512 (IA-2) were measured utilizing a modified radioassay with in vitro transcribed and translated human ICA512bdc protein construct labeled with [$^{35}$S]methionine. (10,11) The GADA and IA-2A autoantibodies were measured in a combined assay.
- From Feb. 22, 2010 to the present, the GADA and IA-2A assays were standardized to be brought into alignment with Bristol, UK and Munich, Germany labs. (3)

Samples which tested positive were confirmed using a separately aliquoted sample from the same clinic visit blood draw.

Threshold for GADA was defined as the 97$^{th}$ percentile and IA-2A were defined as the 99$^{th}$ percentile of antibody level for healthy population controls .(3)

*Insulin autoantibodies (IAA):*

- From March 15, 1994 to Dec. 18, 1998, insulin autoantibodies (IAA) were measured using a quantitative radioimmunoassay, competitive insulin assay (cIAA) as described previously (12). Due to the large serum sample required, testing was not routinely repeated.
- From Jan. 27, 1999 to present, IAA is measured using a micro-IAA assay (mIAA), which utilizes ELISA techniques and Protein A-Sepharose (13). Positive samples were repeated for confirmation.

Threshold for IAA was defined as the 99$^{th}$ percentile of antibody level for healthy population controls (13)

**DEW-IT:**

Radiolabeled whole Glutamate Decarboxyase (GAD65), the intracellular portion of insulinoma antigen-2 (IA2), and human insulin were incubated separately in triplicate wells with 2 to 5 uL of patient serum, then captured by Protein A, washed extensively to remove unbound antigen, and counted. Results were expressed using an index relative to specific positive control and negative control sera as described, with each cutoff defined as the 99$^{th}$ percentile of >200 individual healthy population controls. (14) Samples close to the cutoff, and those where triplicate well CV's were >30%, were reflexively repeated. The laboratory participates in a DASP/IASP proficiency testing, with DEW-IT assays exceeding mean performance of all participating laboratories for all 4 antigens over each of 12 consecutive workshops. The four autoantibodies assays are each CLIA certified (CLIA# 50D0982418).

**DiPiS:**

*Autoantibodies to GAD65 and IA-2: Analysis in dried blood spots, serum or plasma*

GAD65 autoantibodies (GADA) and IA-2 autoantibodies (IA2A) were analyzed from dried blood spots (DBS) with a radioligand binding assay (RBA) as previously described (9). GADA and IA2A levels were expressed as units per mL (U/mL) derived from the WHO standard 97/550. The samples were considered to be positive if the IA2A levels were above 5 U/mL or the GADA levels were above 34 U/mL, which represent the 99$^{th}$ percentile for healthy controls (15).

*Autoantibodies to IAA*

Analysis for IAA was first performed using a noncompetitive method with radiolabeled insulin and serum samples. Positive samples for IAA were further analyzed in duplicate using a competitive method and radiolabeled insulin. IAA levels were calculated as relative units and were related to positive controls. Positivity for IAA was set to 1.9 relative units, which represents the 99$^{th}$ percentile for healthy controls (15). The competitive method was used to verify false-positive binding in the noncompetitive assay. In subsequent analysis, the competitive assay results were used.

**DIPP:**

Islet autoantibodies reported in this analysis were analyzed in the Research Laboratory, Department of Pediatrics, University of Oulu, Oulu, Finland. The biochemical autoantibodies IAA, GADA, and IA-2A were analyzed with specific radiobinding assays. ICA was used as the primary screening tool for the beta-cell autoimmunity in children born in 1994-2002. In all children with ICA positivity, IAA, IA-2A and GADA were then measured from all samples. For children born 2003 or later , ICA, IAA, IA-2A and GADA were measured at every visit. The reference values for the IAA, GADA, and IA-2A assays were based on the 99$^{th}$ percentile of more than 370 non-diabetic Finnish children and adolescents.

*Islet cell antibodies (ICA)*

ICA were quantified by standard immunofluorescence on sections of frozen human pancreas, blood group O donor. Fluorescein-conjugated antihuman IgG (Sigma-Aldrich Corp., St. Louis, MO) was used for detection. The end-point dilution titers of the ICA-positive samples

were recorded, and the results were expressed in Juvenile Diabetes Foundation units (JDF-U). The detection limit was 2.5 JDF-U.

*Insulin Autoantibodies (IAA)*

Serum levels of IAA were quantified with a microassay modified from the method described by Williams et al. (16,17) The IAA titers representing specific binding were expressed in relative units (RU) based on a standard curve run on each plate. A subject was considered positive for IAA when the specific binding exceeded 99th percentile in 371 nondiabetic Finnish subjects.

*Autoantibodies to GAD65 (GADA)*

GADA were measured by a radiobinding assay as previously described (18). The results were expressed in relative units on the basis of a standard curve. The cut-off limit for positivity was at the 99th percentile for 373 nondiabetic Finnish children and adolescents.

*Autoantibodies to islet antigen 2 (IA-2A)*

IA-2A were quantified by a radiobinding assay as previously described by (19). Antibody titers were expressed in relative units based on a standard curve. The cut-off limit for positivity was defined by the 99th percentile in 374 healthy Finnish children and adolescents.

**<u>Supplementary Table 1</u>: Representation of persistence or reversion of islet autoantibodies (IAb) at two time points ($t_0$, $t_1$) for those who begin with multiple islet mIA/Persistent/2 positive status. NA: not available**

| Number of IAb @ $t_0$ | Number of IAb @ $t_1$ | mIA/Persistent/2 category at $t_1$ | N at $t_1 = 2 \pm 0.5$ years (total N=521) |
|---|---|---|---|
| 3 | 3 | M-Sustained | 251 |
| 2 | 2 | M-Sustained | |
| 2 | 3 | M-Sustained | 117 |
| 3 | 2 | M3-M2 | 42 |
| 2 or 3 | 1 | M-Single | 87 |
| 2 or 3 | 0 | excluded | 2 |
| 2 or 3 | NA | excluded | 22 |

**Supplementary Table 2: P-values for pairwise comparisons of mIA definitions (Figure 1B).** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | mIA/SameVisit | mIA/Persistent/1 | mIA/Persistent/2 |
|---|---|---|---|
| ***mIA/Any*** | 0.036 | 0.0014 | 2.8 e-8 |
| ***mIA/SameVisit*** | | 0.15 | 4.9 e-7 |
| ***mIA/Persistent/1*** | | | 5.7 e-8 |

**Supplementary Table 3: P-values for pairwise comparisons of Progression to mIA/Persistent/2 by Quartiles at sIA/Persistent (Figure 2A).** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | Q2 (2.0, 3.5] | Q3 (3.5, 7.1] | Q4 (7.1, 18.7] |
|---|---|---|---|
| ***Q1*** *(0.0, 2.0]* | 3.1 e-5 | 7.8 e-17 | 6.2 e-22 |
| ***Q2*** *(2.0, 3.5]* | | 5.8 e-6 | 3.0 e-9 |
| ***Q3*** *(3.5, 7.1]* | | | 0.14 |

**Supplementary Table 4: P-values for pairwise comparisons of Progression to stage 3 Type 1 diabetes by Quartiles at mIA/Persistent/2 status (Figure 2B).** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | Q2 (2.0, 3.5] | Q3 (3.5, 7.1] | Q4 (7.1, 18.7] |
|---|---|---|---|
| ***Q1*** *(0.0, 2.0]* | 0.0093 | 0.0016 | 3.5 e-5 |
| ***Q2*** *(2.0, 3.5]* | | 0.677 | 0.14 |
| ***Q3*** *(3.5, 7.1]* | | | 0.29 |

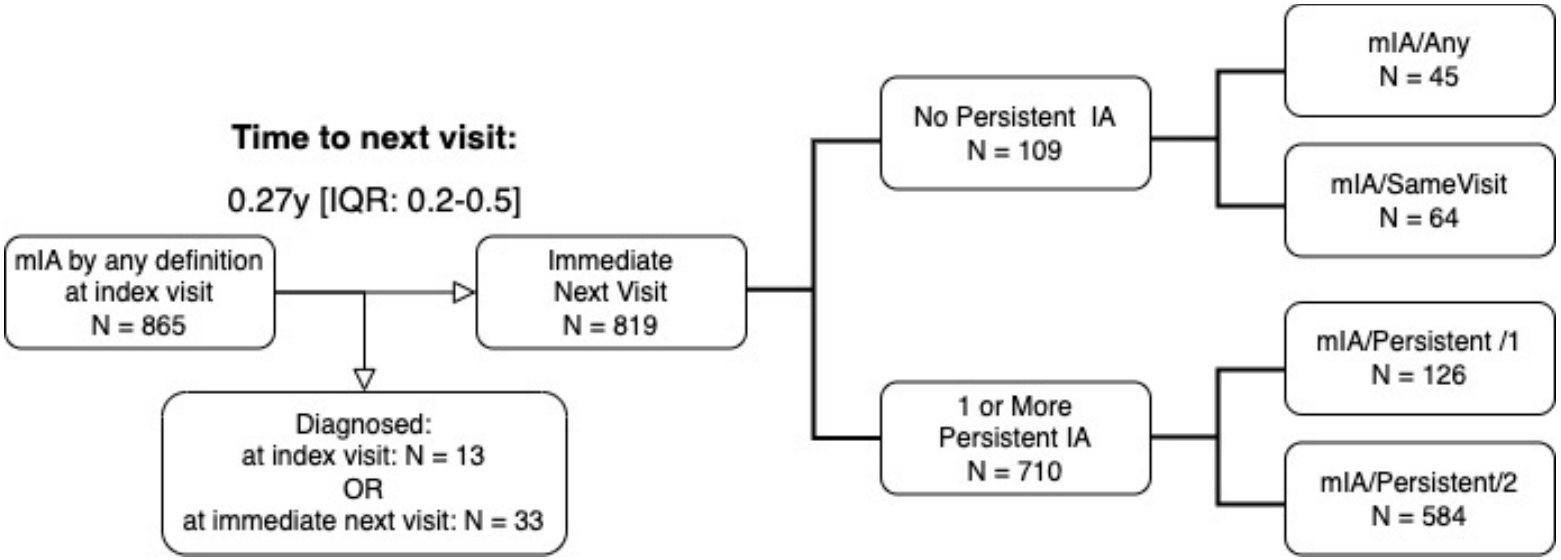
**Supplementary Table 5: P-values for pairwise comparisons of mIA definitions at visit 2 ± 0.5 years after baseline (Figure 3A).** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | mIA/SameVisit | mIA/Persistent/1 | mIA/Persistent/2 |
|---|---|---|---|
| ***mIA/Any*** | 0.84 | 0.18 | 3.1 e-05 |
| ***mIA/SameVisit*** | | 0.16 | 2.4 e-06 |
| ***mIA/Persistent/1*** | | | 9.0 e-06 |

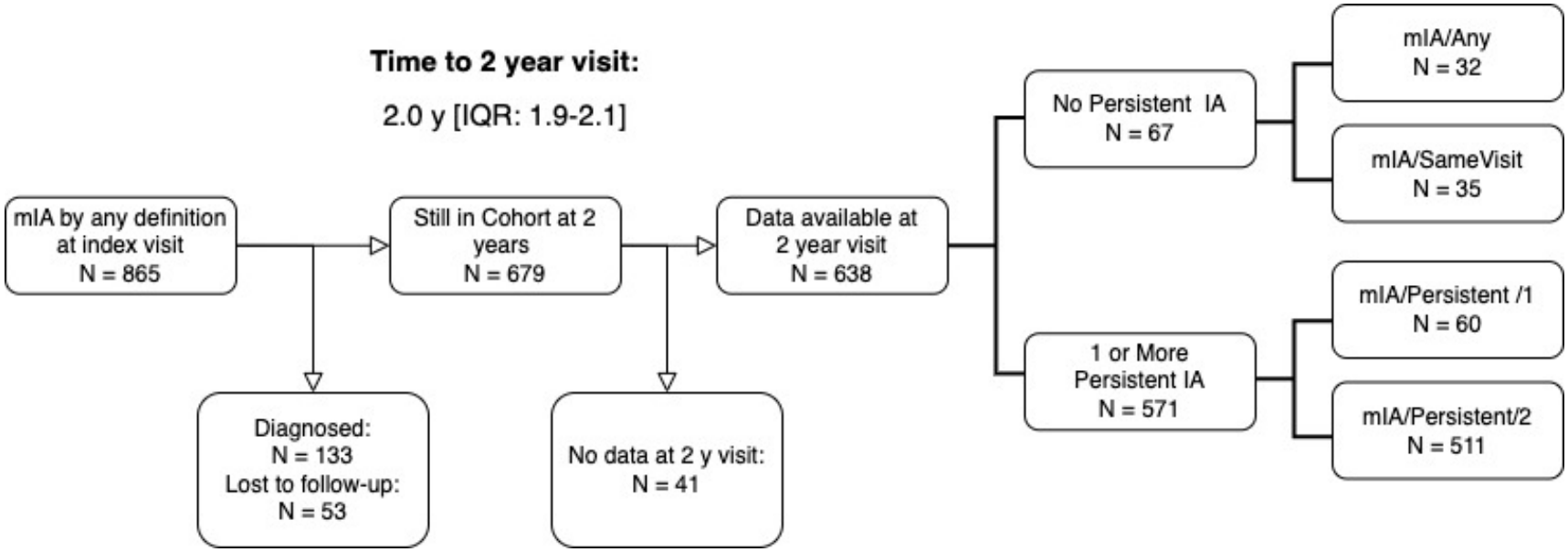
**Supplementary Table 6: P-values for pairwise comparisons of persistence category (Figure 3B).** P-values significant with Bonferroni correction for multiple comparisons (p<0.017) indicated with shading.

| | M-Sustained | M-Single |
|---|---|---|
| ***M3-M2*** | 0.002 | 0.0013 |
| ***M-Sustained*** | | 0.30 |

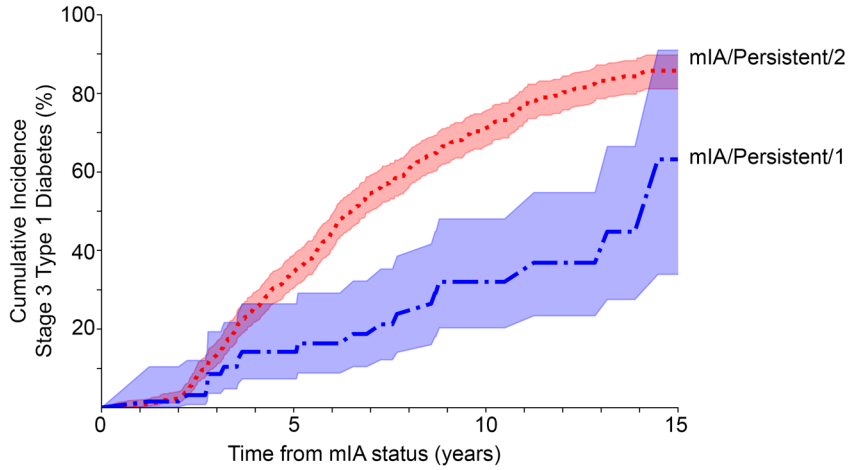
**<u>Supplementary Figure 1:</u> Highest stringency and persistence at immediate next visit after mIA positivity.**



**<u>Supplementary Figure 2</u>: Persistence Status for Figure 3A. Cumulative incidence of type 1 diabetes from mIA positive status in the infant-toddler cohort by highest stringency in 2 ± 0.5 years later visit.** Median age at index visit was 3.8 [IQR: 2.0-7.3] years and was 5.8 years (IQR 4.0-9.0) at the 2-year visit.

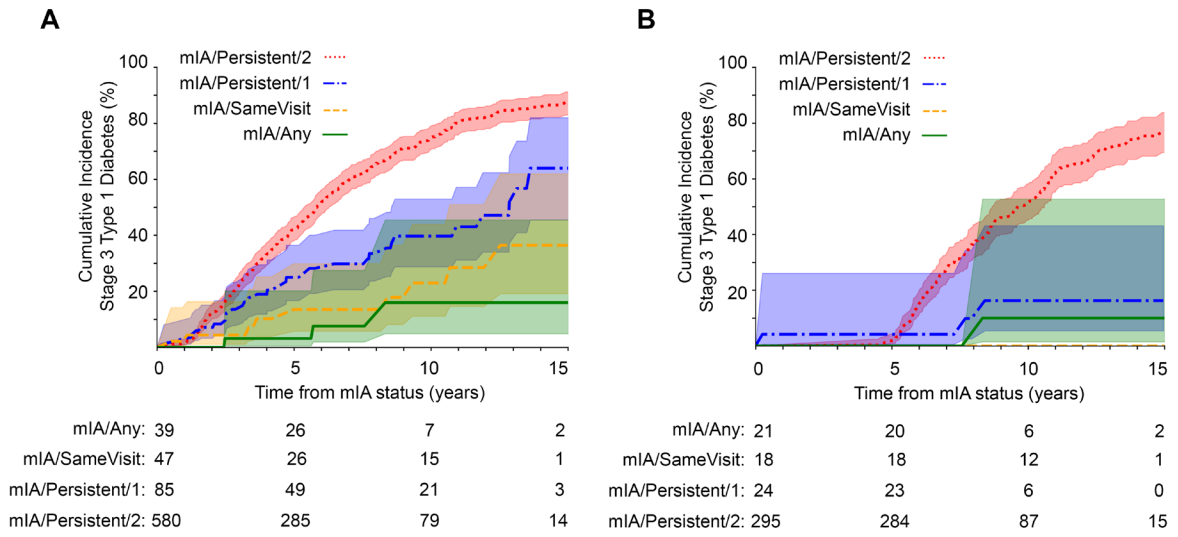


**<u>Supplementary Figure 3</u>: Cumulative incidence of type 1 diabetes in 15 years in the infant-toddler cohort for those who were mIA/Persistent/1 or mIA/Persistent/2 at baseline (N=591), stratified by persistence category at 2-year follow-up (P = 2.031e-06).**




| | | | | |
|---|---|---|---|---|
| mIA/Persistent/1 | 65 | 42 | 16 | 1 |
| mIA/Persistent/2 | 526 | 302 | 83 | 15 |

**<u>Supplementary Figure 4</u>: Cumulative incidence of type 1 diabetes in mIA individuals stratified at by highest stringency of mIA definition achieved at (A) 1 ± 0.5 and (B) 5 ± 0.5 years follow-up. (C) and (D) tables of pair-wise comparisons.**



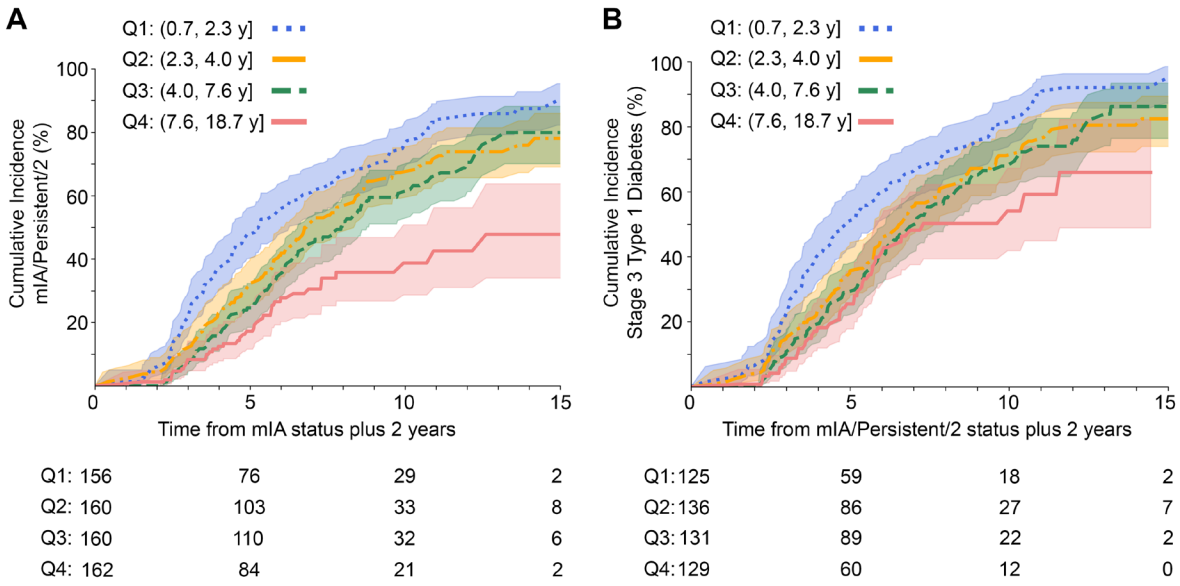

**(C) P-values for pairwise comparisons of mIA definitions at visit 1 ± 0.5 years after baseline (panel A above).** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | **mIA/SameVisit** | **mIA/Persistent/1** | **mIA/Persistent/2** |
|---|---|---|---|
| ***mIA/Any*** | 0.23 | 0.0059 | 3.1 e-07 |
| ***mIA/SameVisit*** | | 0.056 | 3.4 e-07 |
| ***mIA/Persistent/1*** | | | 1.6 e-06 |

**(D) P-values for pairwise comparisons of mIA definitions at visit 5 ± 0.5 years after baseline (panel B above).** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | **mIA/SameVisit** | **mIA/Persistent/1** | **mIA/Persistent/2** |
|---|---|---|---|
| ***mIA/Any*** | 0.25 | 0.51 | 0.0018 |
| ***mIA/SameVisit*** | | 0.11 | 4.3 e-05 |
| ***mIA/Persistent/1*** | | | 0.0029 |

**<u>Supplementary Figure 5</u>: Cumulative incidence of type 1 diabetes by age quartile based on (A) any definition of mIA and more stringent (B) mIA/Persistent/2 at 2 ± 0.5 years of follow up. (C) and (D) tables of pair-wise comparisons.**




**(C) P-values for pair-wise comparisons for panel A.** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | **Q2 (2.3, 4.0]** | **Q3 (4.0, 7.6]** | **Q4 (7.6, 18.7]** |
|---|---|---|---|
| ***Q1*** *(0.7, 2.3]* | 0.0113 | 0.0004 | 3.205e-10 |
| ***Q2*** *(2.3, 4.0]* | | 0.228 | 6.898e-05 |
| ***Q3*** *(4.0, 7.6]* | | | 0.0022 |

**(D) P-values for pair-wise comparisons for panel B.** P-values significant with Bonferroni correction for multiple comparisons (p<0.0083) indicated with shading.

| | **Q2 (2.3, 4.0]** | **Q3 (4.0, 7.6]** | **Q4 (7.6, 18.7]** |
|---|---|---|---|
| ***Q1*** *(0.7, 2.3]* | 0.006 | 0.0012 | 1.43 e -05 |
| ***Q2*** *(2.3, 4.0]* | | 0.583 | 0.062 |
| ***Q3*** *(4.0, 7.6]* | | | 0.205 |

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