

# ELECTRONIC SUPPLEMENTARY MATERIAL

## DETAILED AUTOANTIBODY MEASUREMENT PROCEDURES

### *RECOMBINANT LUCIFERASE-TAGGED ANTIGENS PRODUCTION*

The coding sequences of major T1D and CD autoantigens were obtained from human pancreatic islet cDNA following reverse transcription and cDNA amplification or as synthetic genes (Eurofins Genomics GmbH, Munich, Germany) as previously described. Briefly, modified cDNA sequences of GAD65, insulin, IA-2, ZnT8 and TGM2 were sub-cloned into modified pCMVTNT™ vectors (Promega, Madison WI, USA) in frame with a NanoLuc™ (NLuc) luciferase reporter (Promega). To express luciferase-tagged insulins, PC1/PC2 convertase recognition sites were replaced with furin convertase sites, allowing for the cleavage of C-peptide. Constructs were modified by site-directed mutagenesis using the GeneArt® Site-Directed Mutagenesis PLUS Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) to insert point mutations. Recombinant antigens with deletions were produced by sub-cloning synthetic genes with deleted regions into pCMVTNT™ vectors.

Antigens were expressed by transfection into Expi293F™ cells (Expi293™ Expression System, Thermo Fisher Scientific Life Technologies). After 48 h, cells were centrifugated and the antigens were harvested either from the culture medium (i.e., insulin) or from the cells, lysed using the Passive Lysis 5X Buffer (Promega) (i.e., tGAD65, IA-2, ZnT8, TGM2). The recovered luciferase activity was quantified by seeding the wells of a 96-well OptiPlate™ (PerkinElmer, Waltham, MA, USA) with serial ten-fold dilutions of the antigen, adding the Nano-Glo® substrate (Promega), and measuring the light units (LU) for 2 s/well in a Berthold Centro XS3 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The antigens were then stored frozen at –80°C as single-use aliquots.

### *SCLIPS AND LIPS IMMUNOASSAYS*

LIPS and scLIPS assays of cytoplasmic antigens share similarities with prior radiobinding assays that captured liquid phase immune complexes using either protein A coated beads or plates. Each assay is characterized using the corresponding recombinant nanoluciferase-tagged antigen(s). The overall procedure consists of the following steps:

- 1) Coincubation of serum and recombinant nanoluciferase antigen(s) for 2 hr at room temperature in a 96-deep well polystyrene plate (Sarstedt, Nümbrecht, Germany) for LIPS or polypropylene 96-well plate for scLIPS (Sarstedt). For competitive antibody binding assays (IAA screening and confirmatory tGADA tests), replicate serum samples were incubated either with or without unlabelled competitor (Actrapid®, NovoNordisk, Denmark for IAA and recombinant tGAD65 expressed in Expi293F for GADA, respectively)

- 2) Capture of immune complexes by addition of rProtein A Sepharose 4 fast flow resin (GE Healthcare Life Sciences, Amersham, UK) for LIPS or transfer to a 96-well plate (NUNC™ MaxiSORP, Thermo-Fisher) pre-coated with recombinant Protein A (Sino Biological, China) for scLIPS, followed by incubation for 1hr with orbital shaking (~700 rpm) at 4°C. For LIPS, PAS pellets are then transferred into the corresponding well of a 96-well OptiPlates™ (Perkin-Elmer) using a multichannel liquid handler
- 3) Wash of the immune-complexes with Tris-buffered saline plus Tween to remove unbound antigens using a 96 well plate BioTek Elx405 automated plate washer (Agilent Technologies, Santa Clara, CA, USA)
- 4) Addition of Nano-Glo® Luciferase Assay Reagent luciferase substrate followed by read-out (2 seconds/well) of recovered luciferase activity in a Centro X960 luminometer
- 5) Acquired LU are then converted to arbitrary units (AU) using a standard curve constructed from doubling dilutions in normal human serum of antibody-positive sera and linear regression model based on log transformed LU. The distribution of autoantibody measurements was then used to establish assay thresholds.

#### *OTHER ASSAYS*

Additional antibody tests on follow-up venous serum samples were conducted according to the manufacturer's instructions using the following immunoassays:

Bridge-ELISA (RSR Ltd, Llanishen, Cardiff, UK) for GADA, IA-2A, and ZnT8A measurements

Direct ELISA for TGA-IgA and TGA-IgG (Eurospital S.p.A., Trieste, Italy)

CLIA for TGA-IgA and TGA-IgG on a BIO-FLASH platform (Inova Diagnostics, San Diego, USA)

Anti-endomysial antibodies by indirect immunofluorescence using a NOVA Lite® Monkey Esophagus kit (Werfen, Barcelona, Spain)

#### CHARACTERISTICS OF AUTOANTIBODY DISTRIBUTIONS AND THRESHOLD SELECTION

All autoantibody measurements displayed markedly non-normal distributions, as confirmed by Shapiro-Wilk tests (all  $p < 0.001$ ) and skewness analyses. These distributions were characterized by right-skewness (ranging from 7.80 for TGA-IgA to 37.98 for IAA) and high kurtosis, with a majority of negative samples and a smaller subset of samples with elevated antibody levels. For all assays, a substantial proportion of values exceeded their respective upper statistical fences ( $Q3 + 1.5 \cdot IQR$ ), ranging from 5.8% for the 3-screen scLIPS to 11.7% for TGA-IgA LIPS.

To establish optimal thresholds for autoantibody positivity, we performed quantile-quantile (QQ) plot analysis. Specifically, we utilized detrended QQ plots to visually identify inflection points or "transition zones" within the distributions of antibody levels (Supplementary Figure 2). These transition zones represent shifts from background noise to potentially meaningful signals. Thresholds were then strategically defined based on the expected prevalence of autoimmunity in the general population: for low-prevalence markers (e.g., type 1 diabetes-associated autoantibodies), thresholds were placed at the lower boundary of the transition region to enhance sensitivity; for markers with higher expected prevalence (e.g., celiac disease autoantibodies), thresholds were set at the upper boundary to optimize specificity.

For confirmatory testing of insulin autoantibodies (IAA) in individuals who had already tested positive using the 3-screen scLIPS assay, we adopted a Bayesian-informed approach. Specifically, we applied a lower threshold for IAA positivity, acknowledging that the prior probability of true autoimmunity in this subgroup was elevated, thereby improving the balance between sensitivity and specificity in follow-up screening.

## STATISTICAL ANALYSIS SUPPLEMENTARY INFORMATION

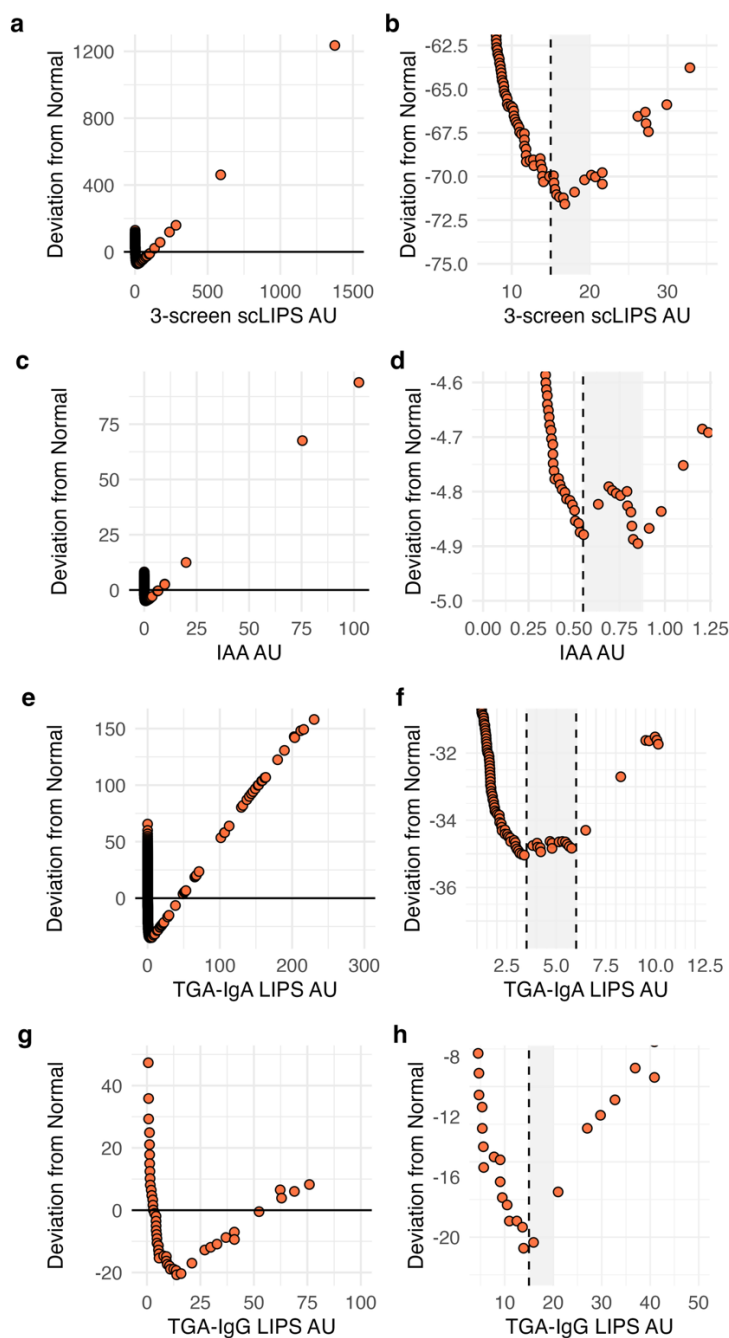
All statistical analyses were performed using R version 4.5.1 via RStudio (2024.12.0, Build 467). Data were initially processed in Microsoft Excel 365. The following R packages were used for analysis and visualization: tidyverse (version 2.0.0) for data manipulation and transformation; ggplot2 (version 3.4.4) for data visualization; rstatix (version 0.7.2) for statistical testing; flextable (version 0.9.8.007) for generating summary tables.

For age-stratified analyses, subjects were categorized into the following groups: children/early adolescents:  $\leq 15$  years; adolescent/young adults: 16-30 years; adults: 31-45 years; middle-aged adults: 46-60; seniors: 61-75 years; elderly:  $>75$  years.

For comparisons of autoantibody concentrations between age groups, due to the non-normal distribution of values, non-parametric tests (Mann-Whitney U test for two-group comparisons and Kruskal-Wallis test for multi-group comparisons) were employed. Post-hoc pairwise comparisons with Bonferroni correction were performed when the overall Kruskal-Wallis test was significant.

Concordance between different assay formats was calculated as the percentage of samples with matching results (positive or negative) in both tests. For analyses of concordance by antibody level, samples were divided into terciles based on the distribution of values in the initial screening test.

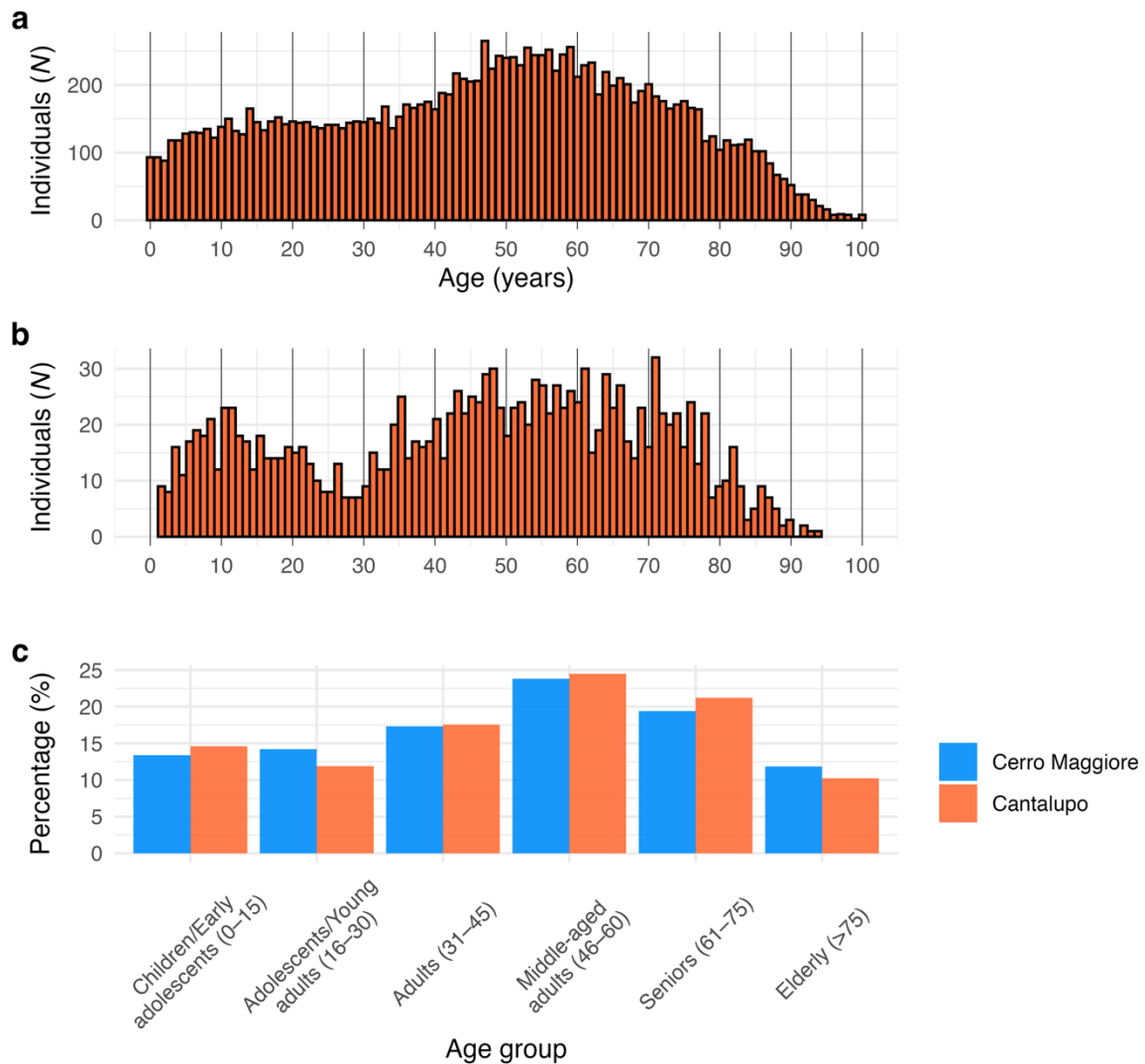
ESM FIG. 1



**ESM Fig. 1 Detrended normal Q-Q plots for LIPS assay results.** Panels a-b: 3-screen sCLIPS assay (a) Full-scale plot showing deviations from normal distribution. (b) Magnified threshold region (7-35 AU). Highlighted area (15-20 AU): transition zone. Dashed line at 15 AU: assay threshold for positivity. Panels c-d: IAA LIPS assay (c), full-scale plot (d). Magnified threshold region (0-1.2 AU). Highlighted area (0.55-0.875 AU): transition zone. Dashed line at 0.55 AU: assay threshold for positivity. Panels e-f: TGA-IgA LIPS assay (e), full-scale plot (f). Magnified threshold region (1-12 AU). Highlighted area (3.5-6 AU): borderline zone. Dashed line at 6 AU: assay threshold for positivity. Panels g-h: TGA-IgG LIPS assay (g), full-scale plot (h). Magnified threshold region (5-50 AU). Highlighted area (15-20 AU): transition zone. Dashed line at 15 AU: assay threshold for positivity. Points in all panels show individual measurements' deviation from expected normal distribution ( $y=0$  line), demonstrating non-normal distribution patterns of autoantibody measurements in the population.

ESM FIG. 2

Age distribution comparison  
Cantalupo population vs Cerro Maggiore population



**ESM Fig. 2. Age distribution comparison between Cantalupo (UNISCREEN study population) and Cerro Maggiore (general population).** Panel a: Histogram showing the age distribution of the general population in Cerro Maggiore municipality (n = 15021), displayed as number of subjects per age (years). Panel b: Histogram showing the age distribution of participants enrolled in the UNISCREEN study from Cantalupo (n = 1532), displayed as number of subjects per age (years). Panel c: Comparative bar chart showing the percentage distribution by age groups in both populations. The Cantalupo study population (orange) is compared to the Cerro Maggiore general population (blue).

**ESM Table 1. Distribution and parameters of autoantibody screening assays AU**

<b>Distribution parameters of autoantibody screening assays</b>								
<b>Assay</b>	<b><i>N</i></b>	<b>Mean</b>	<b>Median</b>	<b>SD</b>	<b>Skew</b>	<b>Kurt</b>	<b>p-value</b>	<b>Tail %</b>
3-screen scLIPS	1532	5.5	2.3	40.1	28.5	920.5	<0.001	5.8
IAA LIPS	1532	0.1	0.0	1.9	37.6	1447.1	<0.001	14.0
TGA-IgA LIPS	1532	3.4	0.2	20.6	7.8	66.7	<0.001	11.7

Note: Tail % = values > Q3 + 1.5\*IQR. p-value from Shapiro-Wilk test (all p<0.001, indicating non-normal distributions).

**ESM Table 2: Islet autoantibody screening results stratified by diabetes status**

Test	Status	Diabetes status			Overall
		Type 1 diabetes	Type 2 diabetes	No diabetes	
3-screen scLIPS	Positive	1 (20.0) [0.5–71.6]	4 (5.5) [1.5–13.4]	31 (2.1) [1.5–3.0]	36 (2.3) [1.7–3.2]
3-screen scLIPS	Negative	4 (80.0) [28.4–99.5]	69 (94.5) [86.6–98.5]	1423 (97.9) [97.0–98.5]	1496 (97.7) [96.8–98.3]
IAA LIPS	Positive	4 (80.0) [28.4–99.5]	3 (4.1) [0.9–11.5]	2 (0.1) [0.0–0.5]	9 (0.6) [0.3–1.1]
IAA LIPS	Negative	1 (20.0) [0.5–71.6]	70 (95.9) [88.5–99.1]	1452 (99.9) [99.5–100.0]	1523 (99.4) [98.9–99.7]
Combined (scLIPS or IAA)	Positive	4 (80.0) [28.4–99.5]	7 (9.6) [3.9–18.8]	33 (2.3) [1.6–3.2]	44 (2.9) [2.1–3.8]
Combined (scLIPS or IAA)	Negative	1 (20.0) [0.5–71.6]	66 (90.4) [81.2–96.1]	1421 (97.7) [96.8–98.4]	1488 (97.1) [96.2–97.9]

Values are presented as *n* (%), with [95% CI] on the line below when applicable

**ESM Table 3: Concordance of single autoantibody LIPS results in capillary vs venous samples**

<b>Antibody</b>	<b>Global</b>	<b>Low tercile</b>	<b>Medium tercile</b>	<b>High tercile</b>
		<b>(15.3-21.6 AU)</b>	<b>(21.6-62.8 AU)</b>	<b>(71.8-1375.1 AU)</b>
tGADA	90.0 (18/20)	66.7 (2/3)	85.7 (6/7)	100.0 (10/10)
tGADA + competition	85.0 (17/20)	100.0 (3/3)	71.4 (5/7)	90.0 (9/10)
IA-2A	85.0 (17/20)	66.7 (2/3)	85.7 (6/7)	90.0 (9/10)
ZnT8A	90.0 (18/20)	100.0 (3/3)	85.7 (6/7)	90.0 (9/10)
IAA	95.0 (19/20)	100.0 (3/3)	100.0 (7/7)	90.0 (9/10)

Values shown as percentage (concordant/total). Terciles based on 3-screen sLIPS antibody levels at screening. AU = arbitrary units.

**ESM Table 4: Concordance of single autoantibody LIPS vs bridge-ELISA results in venous samples**

<b>Antibody</b>	<b>Global</b>	<b>Low tercile</b>	<b>Medium tercile</b>	<b>High tercile</b>
		<b>(15.3-21.6 AU)</b>	<b>(21.6-62.8 AU)</b>	<b>(71.8-1375.1 AU)</b>
tGADA	50.0 (10/20)	33.3 (1/3)	28.6 (2/7)	70.0 (7/10)
tGADA + competition	75.0 (15/20)	66.7 (2/3)	57.1 (4/7)	90.0 (9/10)
IA-2A	90.0 (18/20)	100.0 (3/3)	85.7 (6/7)	90.0 (9/10)
ZnT8A	85.0 (17/20)	100.0 (3/3)	85.7 (6/7)	80.0 (8/10)

Values shown as percentage (concordant/total). Terciles based on 3screen sLIPS antibody levels at screening. AU = arbitrary units.

**ESM Table 5. Antibody positivity stratified by age group**

<b>Antibody Test</b>	<b>Overall N = 1532</b>	<b>0-15 years n = 224</b>	<b>16-30 years n = 182</b>	<b>31-45 years n = 269</b>	<b>46-60 years n = 375</b>	<b>61-75 years n = 325</b>	<b>76+ years n = 157</b>	<b>p-value</b>
<b>3-screen sLIPS</b>								0.049
Negative	1496 (97.7)	217 (96.9) [93.4-98.6]	179 (98.4) [94.9-99.6]	265 (98.5) [96.0-99.5]	372 (99.2) [97.5-99.8]	312 (96.0) [93.1-97.8]	151 (96.2) [91.5-98.4]	
Positive	36 (2.3)	7 (3.1) [1.4-6.6]	3 (1.6) [0.4-5.1]	4 (1.5) [0.5-4.0]	3 (0.8) [0.2-2.5]	13 (4.0) [2.2-6.9]	6 (3.8) [1.6-8.5]	
<b>IAA LIPS</b>								0.10
Negative	1523 (99.4)	224 (100.0) [97.9-100.0]	180 (98.9) [95.7-99.8]	267 (99.3) [97.0-99.9]	375 (100.0) [98.7-100.0]	323 (99.4) [97.6-99.9]	154 (98.1) [94.1-99.5]	
Positive	9 (0.6)	0 (0.0) [0.0-2.1]	2 (1.1) [0.2-4.3]	2 (0.7) [0.1-3.0]	0 (0.0) [0.0-1.3]	2 (0.6) [0.1-2.4]	3 (1.9) [0.5-5.9]	
<b>TGA-IgA LIPS</b>								<0.001
Negative	1464 (96.4)	200 (90.9) [86.1-94.2]	173 (96.1) [91.8-98.3]	254 (95.5) [92.0-97.5]	361 (97.0) [94.6-98.4]	320 (99.1) [97.1-99.8]	156 (99.4) [96.0-100.0]	
Positive	54 (3.6)	20 (9.1) [5.8-14]	7 (3.9) [1.7-8.2]	12 (4.5) [2.5-8.0]	11 (3.0) [1.6-5.4]	3 (0.9) [0.2-2.9]	1 (0.6) [0.0-4.0]	

Data are presented as n (%), with [95% CI] on the line below when applicable. Abbreviation: CI = Confidence Interval. p-values from Pearson's Chi-squared test comparing positivity rates across age groups are shown.

**ESM Table 6. Characteristics of T2D patients stratified by islet autoantibody status**

Characteristic	N	3-screen+ and/or IAA+ (no insulin therapy) <i>n</i> = 5 <sup>1</sup>	Other <sup>2</sup> <i>n</i> = 68	p-value <sup>3</sup>
Age (years)	73	72 (66, 83)	71 (53, 92)	0.6
BMI (kg/m <sup>2</sup> )	73	25.8 (21.5, 31.3)	26.8 (17.6, 38.1)	0.7
HbA1c (mmol/mol)	73	46 (43, 71)	48 (34, 91)	0.6
Diabetes therapy	73			>0.9
no		1 (20.0)	10 (14.7)	
insulin		0 (0.0)	5 (7.4)	
other		4 (80.0)	49 (72.1)	
insulin plus others		0 (0.0)	4 (5.9)	

<sup>1</sup>Median (Min, Max); *n* (%)

<sup>2</sup>Autoantibody-negative or IAA+ receiving insulin therapy

<sup>3</sup>Wilcoxon rank sum test; Fisher's exact test

**ESM Table 7. Coeliac disease autoantibody screening results stratified by coeliac disease status**

TGA-IgA LIPS	Coeliac Disease Status		Overall
	CD	No CD	
positive	6 (37.5) [15.2–64.6]	48 (3.2) [2.3–4.2]	54 (3.5) [2.7–4.6]
borderline	0 (0.0%)	14 (0.9%) [0.5–1.5]	14 (0.9%) [0.5–1.5]
negative	10 (62.5%) [35.4–84.8]	1454 (95.9%) [94.8–96.9]	1464 (95.6%) [94.4–96.5]

Values are presented as *n* (%), with [95% CI] on the line below when applicable