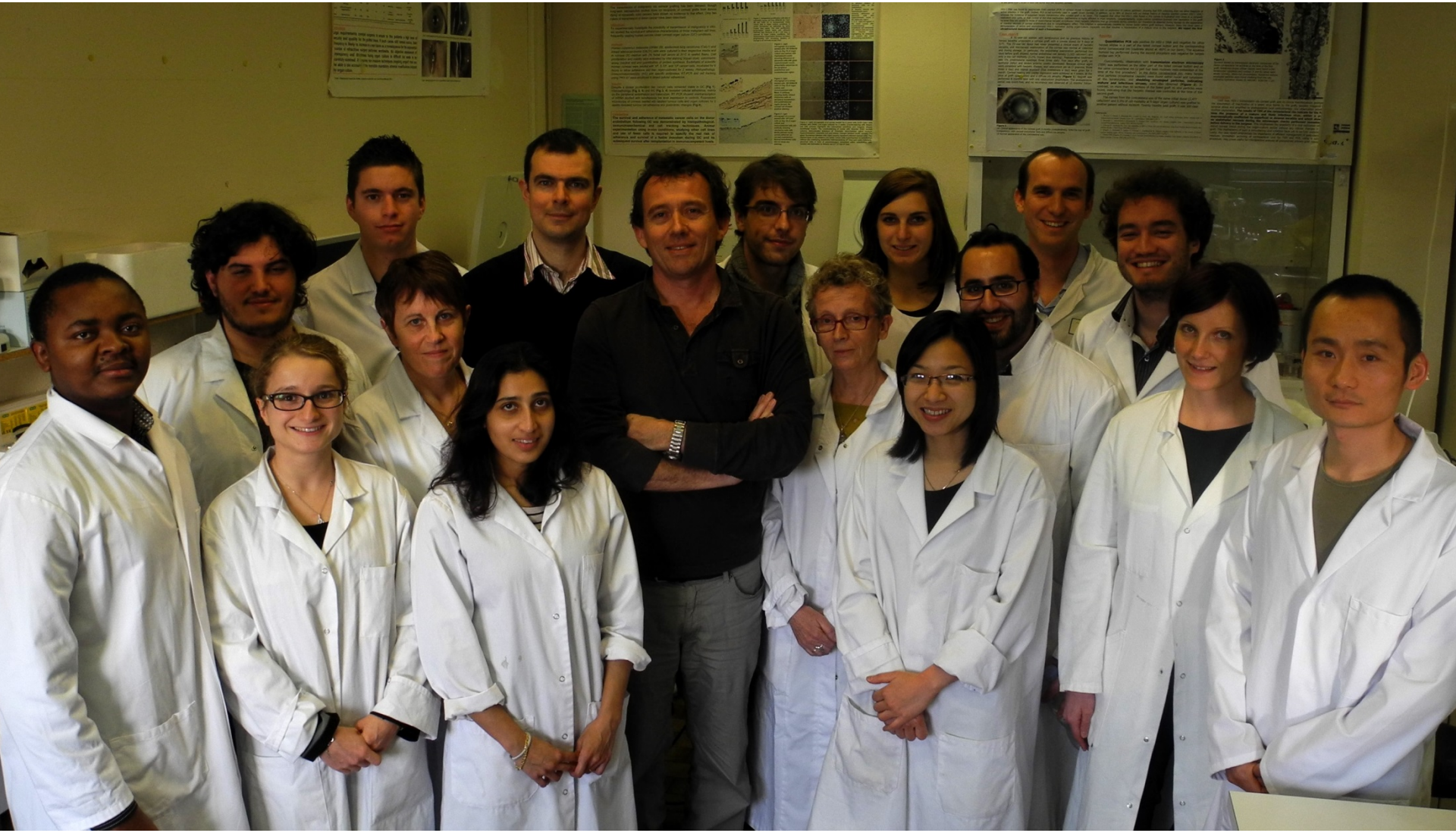

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ARISTON

Comparison of the Endothelial Cell Density of Organ Cultured Corneas With the Cornea Donor Study

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Background

- Determination of the endothelial cell density (ECD) by eye banks is paramount in donor cornea qualification.
- Internal calibration of the counting method is essential.
- 12 years ago, we developed and validated an image analyser specifically for corneal endothelial images (Gain BJO 2002;86:801, Deb-Joardar IOVS 2006;47:4373, Thuret BJO 2007;91:265, Deb-Joardar IOVS 2007;48:2062, Deb-Joardar IOVS 2007;48:3077, Acquart IOVS 2010;51:1356)

- But we thought that an external validation would add an extra stage in the assessment reliability.
- Data published by the multicenter Cornea Donor Study (CDS) in 2005 **are a reference** [Sugar A et al.; Cornea Donor Study Group. *Baseline donor characteristics in the Cornea Donor Study. Cornea*. 2005 May;24(4):389-96].

AIM

To compare ECD determined within a single eye bank, which uses calibrated image analysis software designed for transmitted light microscopy (TLM) images of organ cultured corneas, with the CDS data determined on specular microscopy (SM) images of corneas stored at 4°C.

MATERIALS AND METHODS (1/3)

- Data were prospectively registered in a **single eye bank** (Auvergne-Loire French Blood Center, Saint-Etienne, France) **between January 2005 and July 2013**.
- Cell-counting materials and process remained unchanged.
- Counts were performed by 3 skilled technicians.
- Corneas were retrieved by ophthalmology residents using in situ corneoscleral excision only.
- Corneas were immersed in glass vials containing OC medium (CorneaMax; Eurobio, les Ulis, France) and transferred to a 31°C dry incubator.
- No upper age limit for corneal donation in France

MATERIALS AND METHODS (2/3)

Cell-Counting Method and Calibration

- Endothelial cells are made visible for the transmitted light microscopy (TLM) through trypan blue and sodium chloride incubations.
- Corneal endothelium was observed under a direct TLM (Leica, Leitz laborlux, Wetzlar, Germany) equipped with a digital camera.
- Ten microscopic fields of 768 x 576 µm were acquired in the 8-mm central area.
- ECs were counted using the variable-frame method with Sambacornea software (TribVn, Chatillon, France).
- The entire analysis chain was calibrated in X and Y with a certified Leitz micrometer.

MATERIALS AND METHODS (3/3)

Statistics

- Data were expressed as mean (SD) and median (25th an 75th percentiles).
- Means were compared using analysis of variance.
- $P < 0.05$ considered as significant.

RESULTS (1/6)

Donor Characteristics

Age groups replicated those of the CDS **excepts for the last and largest group (over 75 years).**

TABLE 1. Donor Characteristics

Donor Characteristics	This Study, n (%)	CDS, n (%)
Age, yr		
<41.0	46 (2.9)	128 (12)
41.0 to <51.0	83 (5.2)	132 (12)
51.0 to <61.0	230 (14.5)	281 (26)
61.0 to <66.0	153 (9.6)	172 (16)
66.0 to <71.0	158 (9.9)	220 (20)
71.0 to <75	149 (9.4)	168 (15)
>75	772 (48.5)	—
Median (25th, 75th percentiles)	74 (62, 83)	61 (52, 69)
Gender		
Female	622 (39)	376 (34)
Retrieval type		
Non–heart-beating donors	1448 (91)	NC

This study recruited 1591 donors, whereas the CDS recruited 1101 donor corneas. The CDS data are reproduced for easier comparison.

RESULTS (2/6)

The ECD did not differ significantly between the years (P = 0.062, analysis of variance)

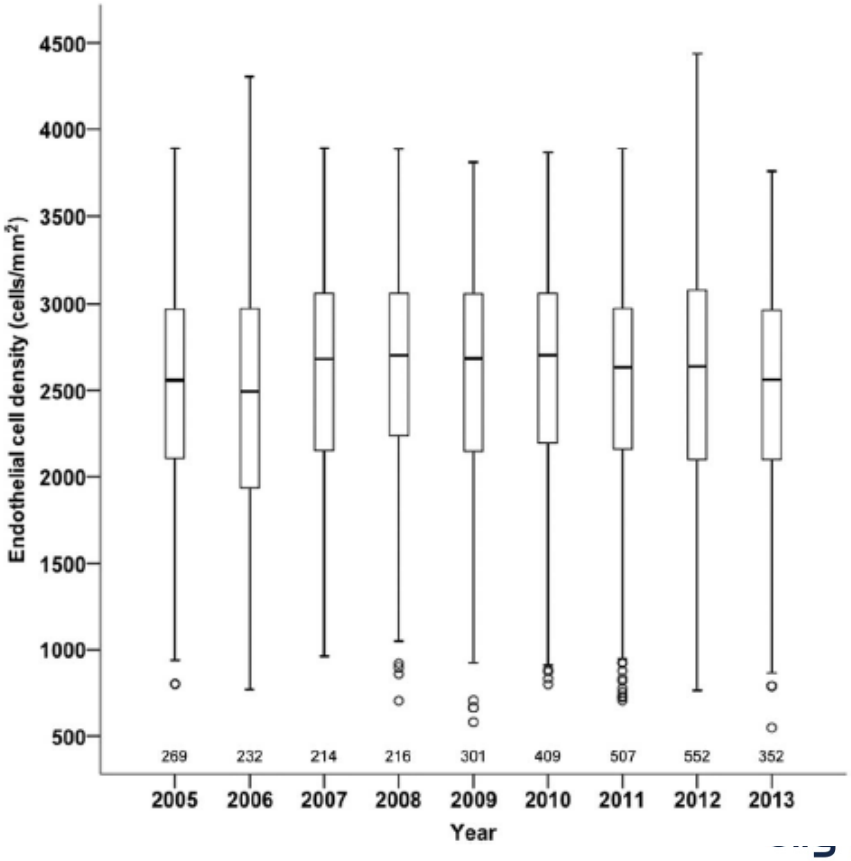


FIGURE 1. Box plots of eye bank ECD by year of analysis. No drift was observed. The thick horizontal lines show the distribution median; boxes, the interquartile range (IQR); and individual circles, outliers. Whiskers show the highest and lowest nonoutlying values. A circle is between 1.5 and 3 times the IQR. Numbers of corneas are indicated above the horizontal axis.

RESULTS (3/6)

Endothelial Cell Density of the Donor Corneas

- Cell counts were performed on average of 5 days after retrieval.
- The relationship between eye bank ECD and donor age was nonlinear.
- The ECD decreased conventionally with increasing age.

TABLE 2. Eye Bank Endothelial Cell Density According to Donor Age of the Whole Series, Presented in the Same Way as the CDS for Easier Comparison

	Donor Age, yr							
Cell Density (cells/mm²)	Total (N = 3052)	<41.0 (N = 88)	41.0 to <51.0 (N = 159)	51.0 to <61.0 (N = 431)	61.0 to <66.0 (N = 297)	66.0 to <71.0 (N = 310)	71.0 to 75.0 (N = 288)	≥75.0 (N = 1479)
<2300	32%	6%	11%	17%	22%	23%	32%	44%
2300–2499	10%	0%	5%	11%	9%	12%	9%	11%
2500–2699	12%	9%	15%	15%	15%	11%	14%	10%
2700–2899	13%	10%	23%	16%	12%	15%	14%	12%
2900–3099	12%	15%	21%	16%	16%	11%	13%	10%
3100–3300	10%	13%	13%	15%	14%	13%	8%	7%
>3300	11%	48%	13%	11%	13%	15%	9%	7%
Mean (SD)	2552 (643)	3225 (514)	2848 (442)	2760 (470)	2723 (511)	2686 (590)	2559 (592)	2355 (680)
Median	2633	3240	2869	2812	2782	2778	2636	2413
(25th, 75th percentile)	(2127, 3026)	(2903, 3549)	(2594, 3105)	(2437, 3113)	(2393, 3124)	(2338, 3159)	(2179, 2972)	(1866, 2872)

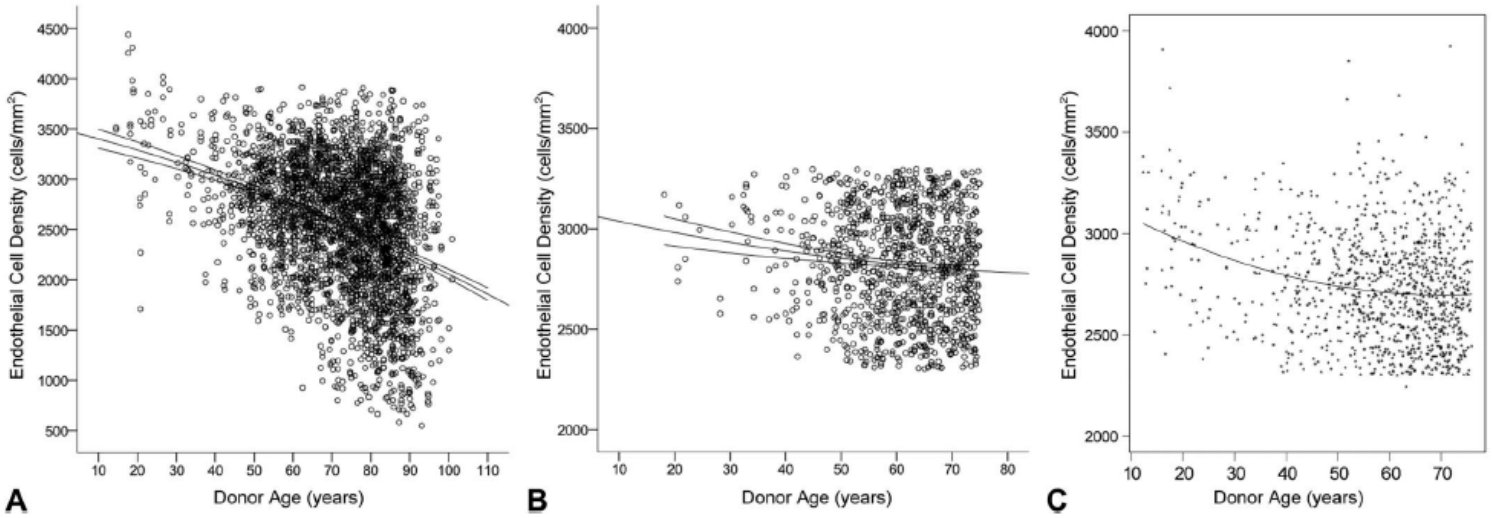


FIGURE 2. Endothelial cell density according to donor age. A, Whole series of 3052 corneas. The line and its 95% confidence interval were produced by quadratic polynomial regression, $Y = 3500 - 9.606 \times x - 0.048 \times x^2$ ($R^2 = 0.129$, $P < 0.001$). B, Scatter plots after data censoring as per the CDS ($2300 < ECD < 3300$ and age <75 years) and displayed with the same vertical and horizontal axis for easier comparison. The line and its 95% confidence interval were produced by quadratic polynomial regression, $Y = 3099 - 6.538 \times x + 0.032 \times x^2$ ($R^2 = 0.012$, $P = 0.002$). C, For comparison, the corresponding figure in the CDS is reproduced with permission from Sugar et al. Baseline donor characteristics in the Cornea Donor Study. *Cornea*. 2005;24:389–396. $Y = 3215 - 14.7 \times x + 0.1 \times x^2$.

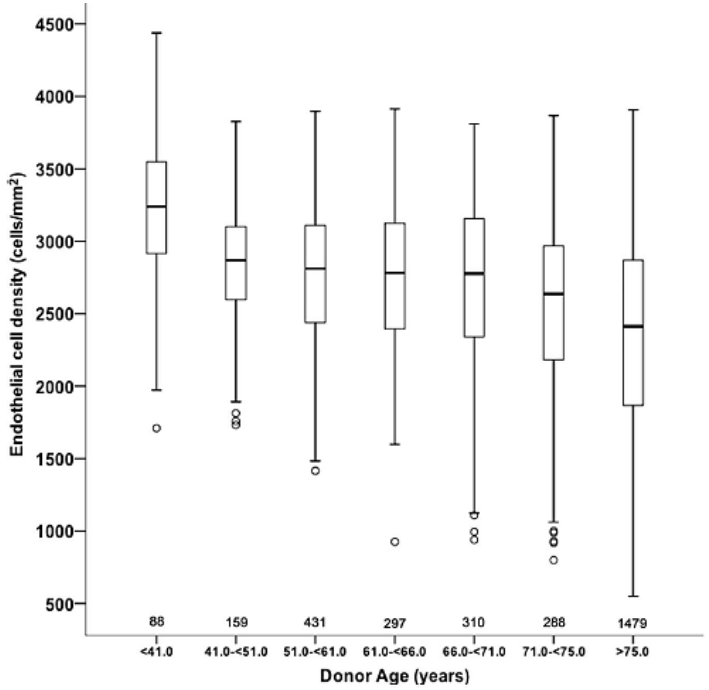


FIGURE 3. Box plots of ECD according to the CDS donor age groups, with an extra group of donors >75 years. Thick horizontal lines show the distribution median; boxes, the interquartile range (IQR); and individual circles, the outliers. Whiskers mark the highest and lowest nonoutlying values. A circle is between 1.5 and 3 times the IQR. Numbers of corneas are indicated above the horizontal axis.

RESULTS (5/6)

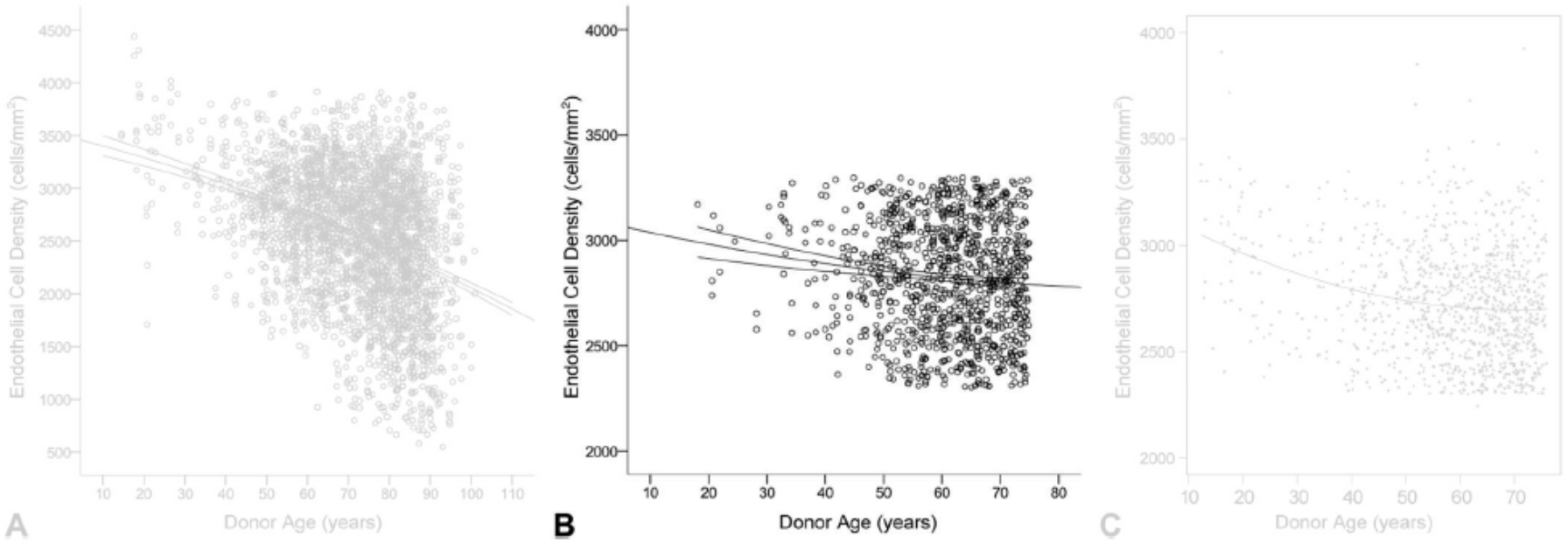
Comparison With the Cornea Donor Study

For each age group: light microscopy eye bank ECD was 100 (±25) cells/mm² above specular eye bank ECD (P<0.001) = overestimation of 3.7 (1.0)%

TABLE 3. Eye Bank Endothelial Cell Density According to Donor Age of the Data, Censored for Age >75 Years and 2300 < ECD < 3300 to Strictly Correspond to the Donor Population of the CDS (Shown in Bold)

	Donor Age, yr						
Cell Density (cells/mm²)	Total N = 1030; N = 1101	<41.0 N = 41; N = 128	41.0 to <51.0 N = 121; N = 132	51.0 to <61.0 N = 311; N = 281	61.0 to <66.0 N = 195; N = 172	66.0 to <71.0 N = 192; N = 220	71.0 to 75.0 N = 170; N = 168
2300–2499	18%	0%	7%	15%	14%	19%	16%
	21%	**13%**	**16%**	**21%**	**27%**	**23%**	**26%**
2500–2699	20%	20%	19%	20%	23%	18%	24%
	26%	**20%**	**24%**	**27%**	**24%**	**27%**	**29%**
2700–2899	23%	22%	31%	22%	18%	25%	24%
	28%	**20%**	**33%**	**29%**	**30%**	**27%**	**26%**
2900–3099	22%	32%	27%	23%	25%	18%	22%
	15%	**22%**	**21%**	**14%**	**11%**	**16%**	**12%**
3100–3300	17%	27%	17%	21%	21%	21%	14%
	8%	**20%**	**6%**	**7%**	**6%**	**6%**	**7%**
>3300	0	0	0	0	0	0	0
	2%	**5%**	**0**	**3%**	**1%**	**0.5%**	**1%**
Mean (SD)	2800 (271)	2941 (227)	2854 (234)	2824 (274)	2829 (274)	2810 (279)	2788 (257)
	2733 (271)	**2872 (309)**	**2742 (230)**	**2742 (275)**	**2687 (259)**	**2703 (246)**	**2692 (273)**
Median	2805	2994	2864	2834	2839	2809	2789
	2715	**2867**	**2731**	**2717**	**2679**	**2694**	**2664**
(25th, 75th percentile)	**(2525, 2900)**	**(2632, 3095)**	**(2563, 2904)**	**(2552, 2893)**	**(2481–2849)**	**(2515, 2874)**	**(2487, 2843)**

RESULTS (6/6)



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DISCUSSION (1/5)

- Is ECD overestimed by TLM or underestimed by SM ?

Previous study = SM ECD was underestimated by 6% (95%CI, (1%-11%)) [Thuret G et al. *Assessment of the human corneal endothelium: in vivo Topcon SP2000P specular microscope versus ex vivo sambacornea eye bank analyser*. Br J Ophthalmol. 2007 Feb;91(2):265-6]

- Calibrations errors can be ruled out in our case (certified internal calibration and external validation)

- We developed and certified slides comprising mosaics (Keratotest) with known predetermined ECD that can also be visualized by SM.

[Flury M, He Z, Campolmi N, Gain P, Kress B, Thuret G. *Fabrication of optical mosaics mimicking human corneal endothelium for the training and assessment of eye bank technicians.* Opt Lett. 2012 Jan 1;37(1):22-4]

DISCUSSION (2/5)

- The intrisic variability that may be partly due to inaccurate calibration of certain SMs, but can probably not explain the systematic difference.

- **The influence of different storage methods must be considered.**

- 2 mutually nonexclusive explanations for this systematic difference:

  1) Differences in the way cell boundaries are taken into acount in the variable-frame counting method.

  For several SM software program, the ECD is the ratio of the number of Ecs pointed out on the total drawn.

  Our software draws the contours or each EC.

Individual EC area was the sum of the inner pixels plus half of the area of the pixel on the boundaries.

Total area was the sum of all individual EC areas.

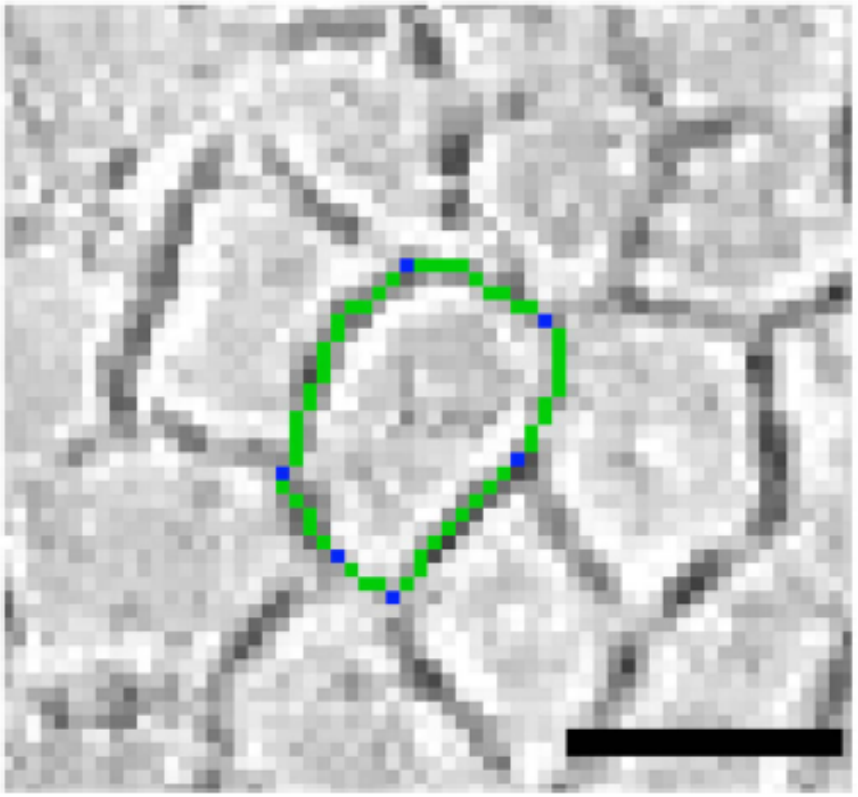


FIGURE 4. Principle of determining individual cell area in the software used in this study to measure ECD on transmitted light microcopy images. Cell boundaries were reduced to only 1 pixel width. Because boundaries are by definition shared by 2 neighboring cells, the area was the sum of the inner pixels (273 in this example), half of the green pixels of the boundaries shared by 2 cells (50/2), and one third of the blue pixels shared by 3 cells (6/3). Scale bar 20 μm.

DISCUSSION (4/5)

2) True differences due to relatively higher corneal shrinkage in OC compared with corneas stored at 4°C.

Optisol-GS contains macromolecules that limit stromal swelling.

- ECD of very old donors (>75 years, can be suitable for graft and even of excellent quality (7% had 3300 cells/mm^2)

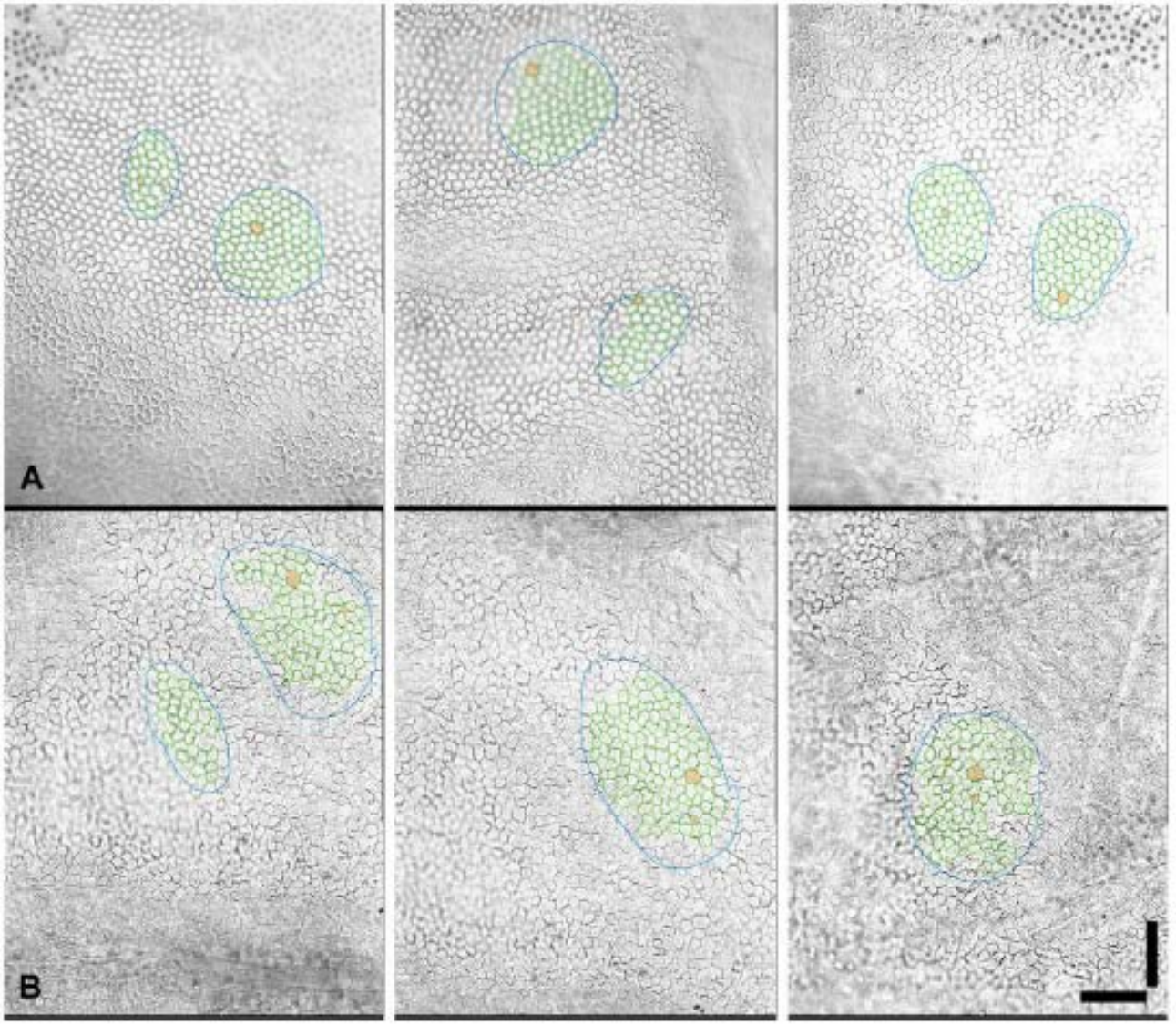


FIGURE 5. Representative example of endothelial images with cell contours of outliers with the highest ECD in both extreme age groups. A, A 17-year-old donor with ECD of 4438 cells per square millimeter. B, An 85-year-old donor with ECD of 3887 cells per square millimeter. The 3 images used for cell-counting are shown. The blue lines roughly outlined the regions of interest chosen at the beginning of the analysis process, and were not the external border of the variable frame, which was constituted by the external borders of the most peripheral delineated cells. Scale bar 100 μm.

CONCLUSIONS

- ECD determined by a computer-aided method from TLM images compares favorably with the American CDS reference series.

- The slight systematic difference on either side of Atlantic Ocean could be due to:

1) differences in counting principles and/or

2) higher shrinkage of the cornea caused by stromal edema in organ culture.

But…

What does the Eye Bank ECD indicates?

Notion of viable ECD (vECD)

- Simple concept: only living ECs are useful for the recipient
- vECD <eye bank ECD (even for perfect count)
  - Black box of the deswelling step (OC)
  - Black box of the storage time (4°C)
  - Dying cells not taken into account
  - Denuded area (folds) not counted
  - Too small sampling size (established from specular microscopy in living patients not adapted for eyebanking)
- Even the best cell count overestimate the number of ECs by 12% (range 3-26%) on exp series of 5 corneas
- Surgeons graft fewer cells than they think !

Corneal modifications during storage

- folds: in organ culture (deep, numerous) as well as 4° C (moderate)
- at best revealed and quantifyed by triple Hoecht/Ethidium/Calceine-AM staining + image analysis (CorneaJ for ImageJ)

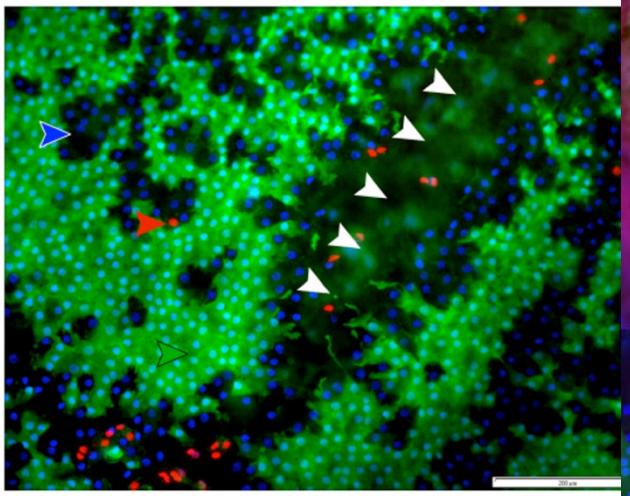
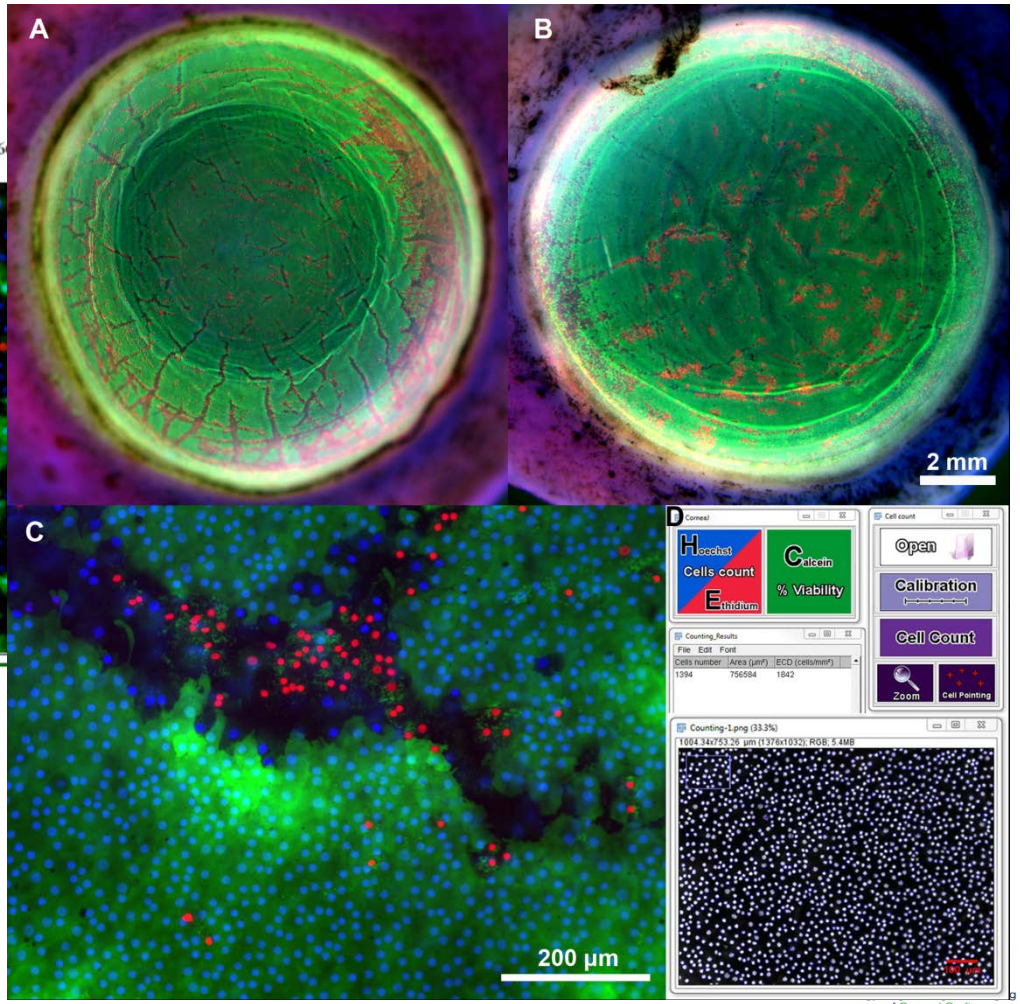


FIGURE 1. Triple endothelial labeling with Hoechst 33342 (H), ethidium homodimere (E), and calcein-AM (C). ×10 objective. *Green arrowhead*: living cell C positive. *Red arrowhead*: dead cell E positive. *Blue arrowhead*: dying cell without metabolic activity (C negative) but still adherent, either isolated or at fold edges. *White arrowheads*: fold deprived of cell. Scale bar, 200 μm.

Pipparelli, IOVS 2011;52:6021
Bernard, Cornea 2014;33:604



Grazie

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