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# In elderly Caucasian women, younger facial perceived age correlates with better forearm skin microcirculation reactivity

Christelle Guéré<sup>1</sup> | Armelle Bigouret<sup>1</sup> | Alex Nkengne<sup>1</sup> | Katell Vié<sup>1</sup> | Anthony Gélis<sup>2</sup> | Joshua Dulong<sup>2</sup> | Jérôme Lamartine<sup>2</sup> | Bérengère Fromy<sup>2</sup>

<sup>1</sup>Laboratoires Clarins, Pontoise, France

<sup>2</sup>Laboratoire de Biologie Tissulaire et d'Ingénierie Thérapeutique (LBTI), UMR 5305 CNRS/UCBL1, 7 passage du Cedex 07, France

## Correspondence

Bérengère Fromy, Laboratoire de Biologie tissulaire et Ingénierie thérapeutique (LBTI), UMR 5305 CNRS/UCBL1, 7 passage du Vercors, 69367 LYON Cedex 07.  
Email: berengere.fromy@univ-lyon1.fr

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## Abstract

**Background:** Visual and molecular changes occurring upon aging are rather well characterized. Still, aging signs show great significant inter-individual variations, and little is known concerning the link between perceived age and cutaneous microcirculation.

**Materials and Methods:** To investigate this point, we recruited Caucasian women in their mid-50's to mid-70's and subsampled women looking older or younger than their age. We studied their facial skin color, as well as their microvascular reactivity to local heating assessed in the forearm skin. We also used skin biopsies from some of these women for gene expression or immunohistochemical analysis.

**Results:** Clinical and instrumental analysis of skin color revealed that subjects who look 5 years younger differ only by a higher glowing complexion. Our most striking result is that subjects looking 5 years younger than their age present a higher microcirculation reactivity in forearm skin. Transcriptome comparison of skin samples from women looking older or younger than their age revealed 123 annotated transcripts differentially expressed, among which MYL9 relates to microcirculation. MYL9 is downregulated in the group of women looking younger than their real age. Microscopy shows that the labeling of MYL9 and CD31 are altered and heterogeneous with age, as is the morphology of microvessels.

**Conclusion:** Therefore, assessing generalized vascular reactivity in non-photo-exposed skin to focus on the intrinsic aging allows subtle discrimination of perceived age within elderly healthy subjects.

## KEYWORDS

aging, microcirculation, MYL9, perceived age, skin, transcriptome

## 1 | INTRODUCTION

As our societies grow older, skin aging has become an area of increasing research interest. Indeed, skin aging is the most obvious sign of aging. In this context, perceived age, the age someone is

visually estimated to have, is socially essential to many individuals, as exemplified by the success of the cosmetic industry, and surgical solutions. It also has implications that go beyond mere aesthetics as it is a robust predictor of mortality and age-related diseases.<sup>1-3</sup>

Jérôme Lamartine and Bérengère Fromy co-last authors.

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As a consequence, many studies have focused on the changes occurring in aging skin.<sup>4-7</sup> These changes result from two distinct biological processes. The first one is intrinsic aging whatever the skin area, which is genetically determined and occurs with time. The intrinsic aging signs are linked to many structural and molecular changes appearing in all parts of the skin. In particular, the cutaneous microcirculation is altered with age.<sup>8</sup> The thickness of the basement membranes is decreased, as is the number of perivascular cells, two changes that result in decreased perfusion, and increased capillary fragility.<sup>9</sup> Several functional studies commonly reported endothelial dysfunction with advanced aging in human and rodent skin.<sup>10-14</sup>

Skin aging can be aggravated by environmental insults (eg, sun exposure,<sup>15,16</sup> tobacco,<sup>17,18</sup> pollution<sup>19,20</sup>...), a process known as extrinsic aging that mainly affects the exposed areas of the body: the face and the neck. If each process leads to characteristic signs, both combine to lead to the progressive decay of the skin and visible changes in topographic and color cues: wrinkles, sagging, and uneven skin tone.<sup>4,15</sup>

These aging changes influence one's appearance and show significant interindividual variations that are partially genetically determined.<sup>21,22</sup> They also have different relative importance on perceived age.<sup>22-26</sup>

All these works pinpoint that inter-individual variation occurs with biological aging. The clinical signs revealing this heterogeneity, and influencing perceived age, are well described. Nevertheless, little is known about the biological markers that underly this heterogeneity and explain the perceived age. If biological age have an influence on skin microcirculation properties, the link between perceived age and skin microcirculation properties have not been previously explored. We hypothesized that women looking younger have better skin microcirculation properties than women looking older. For this purpose, we recruited 55- to 75-year-old subjects who were looking younger or older than their real age. We evaluated and measured skin color parameters that could relate to microcirculation on the subjects of these two groups. We also studied the reactivity of the cutaneous microcirculation in response to local heating in non-photo-exposed skin (forearm) to highlight possible differences on intrinsic aging and performed a microarray analysis to identify molecular markers that would be differentially expressed depending on perceived age.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics

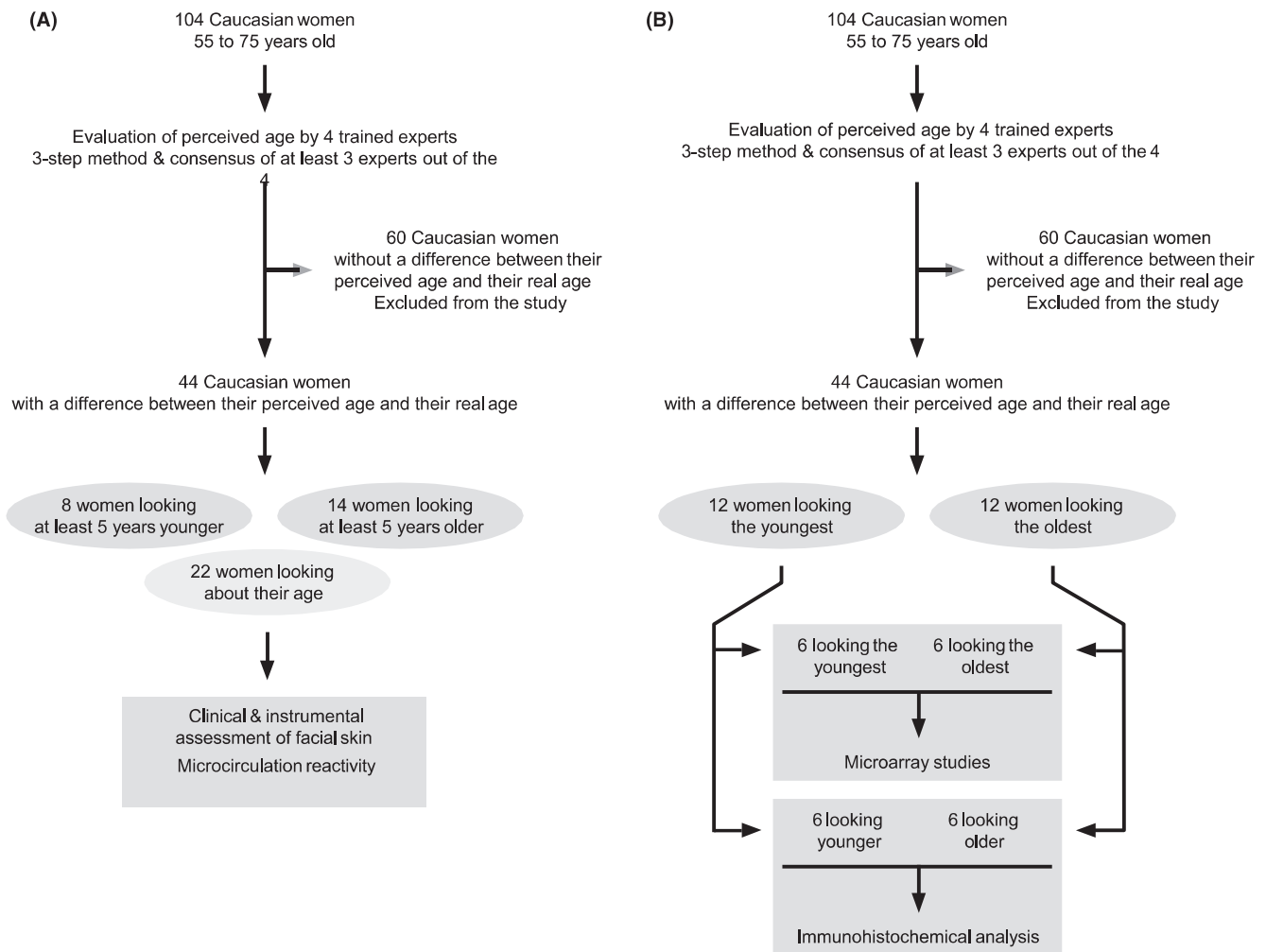
This study was conducted according to Good Clinical Practices and followed the principles of the Declaration of Helsinki. It was approved by an Independent Ethics Committee, the "Comité de Protection des Personnes" of the "Centre Léon Bérard - Sud-Est IV" on May 11th, 2016 and authorized by the "Agence Nationale de Sécurité du Médicament et des Produits de Santé," the competent authority, on June 10th, 2016. Prior to enrolling in the study, written informed consent was obtained from every subject.

### 2.2 | Subjects, perceived age groups, treatments, and biopsies sampling

An initial panel of 104 Caucasian women was recruited. All of them were healthy subjects with age ranging between 55 and 75 years old, a body mass index between 20 and 28, a skin belonging to phototype II or III on the Fitzpatrick scale. They were non-smokers or have stopped smoking for at least 5 years and without any dermatological pathology affecting the face. The volunteers were asked to avoid sun exposition during the full month before the test in order to prevent self-tanning, which could affect their natural skin complexion and glow. Their living habit toward smoking and sun exposure was also collected and analyzed to ensure similarities among volunteers (Supplementary Information S1). The volunteers did not apply any cosmetic product or makeup on the day of the visit. Four trained experts estimated the perceived age of each subject. One of these experts is a dermatologist, the other 3 are clinical evaluation specialists with more than 12 years of experience, and who had undertaken several training sessions (on average every 2 or 3 weeks) to evaluate the skin parameters clinically. They assessed the perceived age on 4000 × 6000px large standardized pictures (Nikon D7100 equipped with a 60 mm lens, F/22, 1/125s, ISO-250) of the full face taken under normal diffuse light, with open eyes and a neutral expression (no smile). The estimation of perceived age was performed in two steps. In a first step, experts estimated the perceived age of all subjects based on the sole pictures, without any reference, and focusing on the full skin facial attributes. In a second step, they were told the real age and, upon this knowledge, decided if the subject was looking younger than her age, looking at her age, or looking older than her age. Subjects were further considered in the study if their perceived age given by three of the four experts were consistently younger or older than their real age. Subjects who were looking younger than their age and those looking older than their age were split into different groups, as depicted in Figure 1.

Clinical assessments and instrumental measurements of skin's parameters and microcirculation studies (Figure 1A), were performed on 44 subjects whose average perceived age show at least a 1-year difference from their real age. These volunteers were divided into three groups: one group of 8 women looking at least 5 years younger than their real age, a second group of 14 women looking at least 5 years older than their real age, and the third group containing the 22 last volunteers having a perceived age close to their real age (less than a 5-year difference with their real age). The results of this third group are presented in supplementary information (S1 and S2).

Four millimeter punch skin biopsies were sampled by a clinician in an area of the neck, close to the ear. The subjects selected for these biopsies were the 24 subjects showing the most substantial difference between their perceived age and their real age (Figure 1B). Twelve of them were looking younger than their real age, and 12 more were looking older. In each group, the 6 biopsies from subjects showing the largest difference between perceived and real age were used for microarray studies, and the 6 remaining biopsies were used for immunohistochemical analysis.



**FIGURE 1** Scheme of subjects' selection and of their inclusion for: A, The clinical and instrumental assessment of facial skin as well as for the study of the microcirculation reactivity, B, The microarray studies, and the immunohistochemical analysis

### 2.3 | Clinical assessments and instrumental measurements of facial skin color

The skin color of each subject was clinically graded by three trained experts using standardized full-face pictures. The parameters studied were the pink complexion, the beige complexion, the uniformity of the complexion, and the glowing complexion, this last parameter corresponding to the global luminosity/reflection that emanates from the entire face. These parameters were all evaluated on a 0 (none/not at all) to 15 (very) unstructured scale. Skin color ( $L^*a^*b^*$ ) was also instrumentally measured using a CM700-d spectrophotometer (Konica Minolta) on an 8 mm diameter zone of the cheek.

### 2.4 | Assessment of the reactivity of cutaneous microcirculation (forearm skin)

Cutaneous microcirculation was studied by analyzing reactivity of the skin microcirculation in response to local heating in the forearm skin, which is of great interest in providing information on the

underlying mechanisms of non-photo-exposed skin to focus on internal aging.<sup>27</sup> The experiment started after a 30-minute acclimation in a temperature- and humidity-controlled environment ( $22 \pm 2^\circ\text{C}$  and 35 to 55% hygrometry). During these 30 minutes, the subjects were in a semi-recumbent position, and the temperature of the skin was stabilized between 33.5 and 35°C. Skin blood flow was recorded on the volar surface of the forearm using Laser Doppler probe (457-PI, Perimed) in response to local heating of 42°C thanks to a Temp Unit (PF 5020, Perimed) as previously reported.<sup>14</sup> Data collection included a one-minute baseline period before the onset of local heating. Local heating and recording were continued for 20 minutes. The recording included measuring blood flow at the site of heating, blood flow outside of the heated zone (3 to 5 cm away), and measurement of cutaneous temperature (440 sensors; Perimed) to check the stability of the skin temperature.

To ensure that results are indeed due to skin microcirculation's sole reactivity, we performed several controls during the acclimation period. The first one is a cutaneous perception threshold test on the internal face of each forearm of all subjects using Von Frey monofilaments to detect possible impaired touch perception, previously

1 reported with advancing age.<sup>28</sup> We also monitored non-invasively  
2 the systolic arterial blood pressure, the diastolic arterial blood pres-  
3 sure, and the heart rate, comparing results before and after the mi-  
4 crocirculation recordings.

## 2.5 | Gene expression profiling (neck skin)

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9 After temporary storage of skin biopsies in RNeasy<sup>®</sup> (Thermo  
10 Fischer Scientific) at 4°C, RNAs were rapidly extracted using mir-  
11 Vana<sup>®</sup> isolation kit (Thermo Fischer Scientific) according to the man-  
12 ufacturer's instructions with only one modification: biopsies were  
13 crushed in the lysis buffer using a plastic RNase free pellet pestle<sup>®</sup>  
14 (Kimble<sup>®</sup>). Microarray processing and data analysis were performed  
15 at the ProfileXpert core facility (Lyon, France) using a high-density  
16 oligonucleotide array (Human Transcriptome Array GeneChip HG-  
17 ST 2.0, Affymetrix). RNA (100 ng) was reverse-transcribed, pre-  
18 amplified, and labeled with the GeneChip WT PLUS reagent kit  
19 (Affymetrix). Amplified cDNA quantification was performed with  
20 a NanoDrop (Thermo Fischer Scientific) and quality checked with  
21 the Agilent 2100 Bioanalyzer. Fragmentation of cDNA (5.5 µg) and  
22 hybridization were performed following Affymetrix protocols. The  
23 arrays were read with a confocal laser (GeneChip Scanner 3000 7G,  
24 Affymetrix). Then, CEL files were generated using the Affymetrix  
25 GeneChip Command Console (AGCC) software 3.0. The obtained  
26 data were normalized with Affymetrix Expression Console software  
27 v1.3.1 using the RMA statistical algorithm. Genes differentially ex-  
28 pressed were identified by Student's test (unequal variance) compar-  
29 ing groups, including triplicates samples. Only genes with  $P < .05$  and  
30 a fold change of at least 1.3 were considered. Functional annotation  
31 of these differentially expressed genes was performed using the  
32 DAVID functional annotation tool (<https://davis.ncicrf.org>). The raw  
33 and normalized data have been deposited into the GEO database  
34 and are available under the accession number: GSE134204.

35 For MYL9 RT-qPCR, one reverse transcription per biopsy was  
36 performed using the PrimeScript RT Reagent kit (Takara). We also  
37 performed two technical duplicates of qPCR using a SYBR Premix  
38 ExTaq II (Takara) on an AriaMx Real-Time PCR system (Agilent  
39 Genomics) using the following oligonucleotides: MYL9f 5'-CAC-CA  
40 G-AAG-CCA-AGA-TGT-CC-3' & MYL9r 5'-TTG-AAA-GCC-TCC-TTA  
41 -AAC-TCC-3'. Results were normalized to the expression level of the  
42 TBP (TATA Binding Protein) gene. Relative quantification was calcu-  
43 lated using the  $2^{-\Delta\Delta C_t}$  quantification method.

## 2.6 | Histological and immunohistochemical analyses of the biopsies (neck skin)

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49 Skin biopsies were fixed in buffered formalin for 24 hours, embed-  
50 ded in paraffin, and cut into 5µm-thick transversal sections. After  
51 deparaffination, the sections were used for immunohistochemical  
52 analysis of CD31 (Platelet and Endothelial Cell Adhesion Molecule 1)  
53 or MYL9 (Myosin Light Chain 9). Citrate and microwave unmasking

was performed for MYL9 labeling. CD31 was labeled using a rab-  
bit polyclonal anti-CD31 antibody (1:80, Ref. M0821, Dako) and  
MYL9 using a rabbit polyclonal anti-MYL9 antibody (1:100, Ref.  
HPA059197; Sigma-Aldrich). All incubations with primary antibod-  
ies were performed overnight at 4°C in PBS buffer supplemented  
with 3% of BSA. After inhibition of endogenous peroxidase by  
hydrogen peroxide, slides were incubated with the secondary anti-  
body, a mouse anti-rabbit IgG, coupled with peroxidase (K4003,  
Dako) and allow to incubate with its substrate: diaminobenzidine  
(K3468, Dako). Mayer's hematoxylin was used as counterstaining.  
Slides were finally analyzed using a Leica DM2000 optical micro-  
scope equipped with a Leica DFC420C digital camera and the image  
acquisition tool LAS v4.2 (Leica). The number of vessels positive for  
CD31 and MYL9 was counted for each section, and the density was  
expressed as vessel number by µm<sup>2</sup>.

## 2.7 | Statistical analysis

Results are expressed as mean ± standard deviation. Normal dis-  
tribution of data was assessed using a Shapiro-Wilk test ( $\alpha = 0.01$ ).  
Real and perceived ages were compared using the unpaired *t* test.  
Unpaired *t* test was also used to analyze the statistical relevance  
of expression changes for the microarray experiment and RT-qPCR  
results. For the cardiovascular and mechanical sensitivity controls  
of the microcirculation experiments, comparison of results between  
the beginning and the end of the experiment within a group of simi-  
lar perceived age were performed using the paired *t* test. Results  
from clinical scoring, instrumental measurements, and microcircula-  
tion reactivity—including controls—were compared using unpaired *t*  
test and analysis of covariance (ANCOVA) with the real age value  
as a covariate. Unlike the *t* test, which does not differentiate the  
impact of the real age and the perceived age of the two groups, the  
ANCOVA test makes it possible to remove, statistically, the indirect  
effect of the real age in order to analyze the impact of perceived age.  
Results were considered significant for  $P < .05$ .

## 3 | RESULTS

### 3.1 | Clinical and instrumental studies of facial skin color show no differences between subjects looking five years younger than their age and those looking five years older

Clinical and instrumental characterization of facial aging was real-  
ized on subjects whose average perceived age shows, at least, a 5-  
year difference with their real age. Such criteria led to a first group of  
8 women looking 5 years younger than their real age. Their real age  
is  $68.75 \pm 3.01$  years old while their perceived age is  $62.44 \pm 2.85$ ;  
this group shows a highly significant age difference ( $P = .0008$ ) of  
 $-6.31 \pm 1.47$  years between real age and perceived age. The second  
group corresponds to women looking 5 years older than their age.

It includes 14 women whose average real age is  $59.57 \pm 3.01$ , while their perceived age is  $67.43 \pm 3.74$ . This is a  $+7.86 \pm 1.94$  years age difference that is highly significant ( $P < .0001$ ). The perceived age of the two groups is different ( $P = .0038$ ), as is their real age ( $P < .0001$ ). It is also to be noted that the subjects looking older than their age are, in fact, younger than the group of subjects looking younger than their age. Thus the real age of subjects impacts the measures. As shown in Table 1,  $P$ -values issued from unadjusted unpaired  $t$  test and from ANCOVA (age-adjusted test) are different. We choose the ANCOVA test because we want to focus on the perceived age.

To initially characterize these two groups' subjects, we studied their facial skin color since it might be related to their cutaneous microcirculation. Results from clinical scoring (Table 1) show that the glow complexion is higher ( $+30.6\%$ ,  $P = .002$ ) for subjects looking younger than for subjects looking older than their age. The other clinically evaluated parameters—the pink complexion, the beige complexion, and the uniformity of the complexion—are similar in both perceived age groups.

This absence of difference is also observed when performing instrumental measurements (Table 2):  $L^*a^*b^*$  color parameters are similar for subjects looking younger and for those looking older than their real age.

### 3.2 | Subjects looking five years younger than their real age show higher microcirculation reactivity than those looking five years older than their age

To find if a relationship exists between perceived age and generalized cutaneous microcirculation, we analyzed the reactivity of the skin microcirculation in response to local heating in a non-photoexposed skin to focus on the internal aging (forearm skin). Basal skin blood flow and basal cutaneous vascular conductance (calculated as skin blood flow/systolic arterial blood pressure) were not different between the group of subjects looking younger than their real age and the group looking older (Figure 2, Table 3). In contrast, differences were found between the two groups of subjects regarding the skin's microcirculation reactivity in response to local heating (Figure 2, Table 3). In both groups, local heating at the level of the forearm induces biphasic vasodilation with a fast response peak followed by a plateau that reaches a maximum value after 20 minutes. While the peak occurs at the same time in both groups, the amplitude

of the peak is 41% higher ( $P = .027$ ) in the group of subjects looking 5 years younger than in the group of subjects looking 5 years older than their real age. In addition, the amplitude of the plateau is 26% higher ( $P = .038$ ) in the group of subjects looking younger than in those looking older than their real age.

Results show that the blood flow 3 to 5 cm away from the heated zone is stable prior, during, and after the experiment and is not different between the two groups (Figure 2). Basal systolic and diastolic arterial pressures are not different between the two groups ( $p$  ANCOVA = 0.961) and remain stable in the two groups through the experiment (Supplementary information S3), indicating cardiovascular stability.

Basal cardiac frequency is also similar in both groups, while it decreases during the experiment (Supplementary information, Table S3), due to the prolonged bed rest in a quiet room. In addition, measures of cutaneous temperature in the vicinity of the heated zone show good thermal stability (Supplementary information S3). Finally, the cutaneous mechanical perception threshold tests revealed no peripheral sensory neuropathy in any subjects (Supplementary information S3).

### 3.3 | Transcriptome analysis of skin biopsies from women of various perceived age reveals 123 modulated transcripts that are annotated

To identify molecular markers that are differentially expressed according to perceived age, we analyzed the 12 samples showing the largest difference between their perceived and their real age. This transcriptome analysis included the 6 subjects looking the youngest, their real age being  $68.50 \pm 3.02$  years old while the average perceived age given by the experts was  $62.67 \pm 2.73$  years old. This group's subjects show a highly significant difference ( $P = .0057$ ) of  $-5.83 \pm 1.20$  years between their real age and their perceived age. The analysis also included the 6 subjects looking the oldest with a real age of  $59.00 \pm 2.37$  years old and a perceived age of  $68.58 \pm 3.72$  years old, therefore showing a significant age difference of  $9.58 \pm 1.59$  years ( $P = .0003$ ). The real age of the two groups is different ( $P = .0001$ ), as is their perceived age ( $P = .0106$ ), and the subjects looking younger are in fact older than the group of subjects looking older.

We performed a comparative transcriptome analysis between skin biopsies from these two groups. Student's  $t$  test and fold change

**TABLE 1** Clinical scoring of facial skin

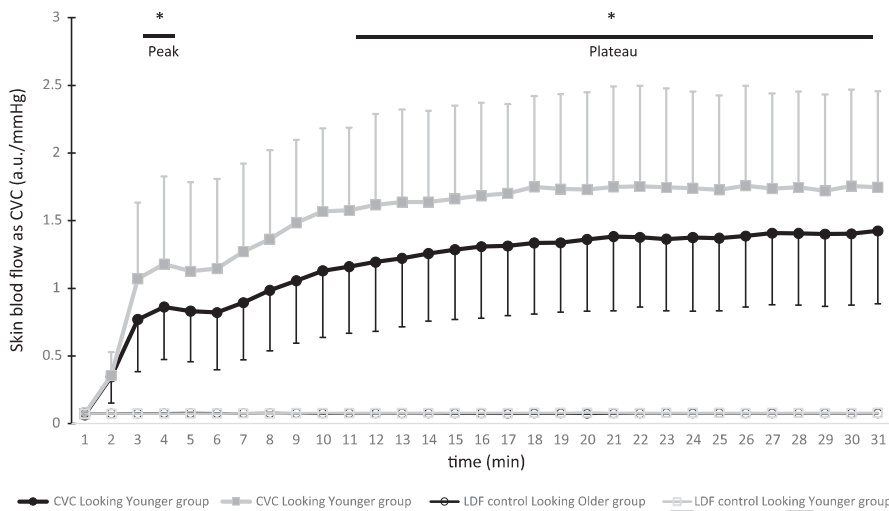
	Subjects looking older than their real age	Subjects looking younger than their real age	Unpaired $t$ test ( $P$ -value)	ANCOVA ( $P$ -value)
Pink complexion	$2.56 \pm 1.45$	$3.88 \pm 1.02$	.034	.958
Beige complexion	$3.45 \pm 1.67$	$3.38 \pm 1.55$	.924	.113
Uniform complexion	$7.39 \pm 1.72$	$7.62 \pm 1.72$	.762	.283
Glowing complexion	$5.03 \pm 0.74$	$6.57 \pm 1.06$	.0007	.002

Note: Results are expressed as mean  $\pm$  standard deviation. Complexions are graded on a 0 to 15 unstructured scale.  $P$ -values are the results of an unpaired  $t$  test and an analysis of covariance (ANCOVA) using the real age as a covariate. Results considered significant ( $P < .05$ ) are in bold.

**TABLE 2** Measured parameters of facial skin color

	Subjects looking older than their real age	Subjects looking younger than their real age	Unpaired t test (P-value)	ANCOVA (P-value)
L* color parameter	62.70 ± 2.06	61.13 ± 2.78	.146	.345
a* color parameter	11.93 ± 1.47	12.44 ± 1.73	.474	.292
b* color parameter	17.56 ± 1.69	17.82 ± 1.36	.724	.754

Note: Results are expressed as mean ± standard deviation. L\*, a\*, b\* values are expressed on a 0 to 100 scale. P-values are the results of an unpaired t test and an analysis of covariance (ANCOVA) using the real age as a covariate.

**FIGURE 2** Cutaneous blood conductance in response to local heating in the group of subjects looking 5 y older than their real age and in the group of subjects looking 5 y younger than their real age**TABLE 3** Microcirculation reactivity in forearm skin

	Subjects looking older than their real age	Subjects looking younger than their real age	Unpaired t test (P-value)	ANCOVA (P-value)
Baseline value	7.46 ± 3.26	9.52 ± 5.36	.272	.595
Baseline CVC	0.064 ± 0.027	0.073 ± 0.043	.576	.576
Peak CVC	0.90 ± 0.11	1.27 ± 0.23	.115	.027
Plateau CVC	1.38 ± 0.15	1.75 ± 0.26	.201	.038

Note: Results are expressed as mean ± standard deviation. Skin blood flow is expressed in arbitrary units (a.u.) and cutaneous vascular conductance (CVC) in a.u./mmHg. P-values are the results of an unpaired t test and an analysis of covariance using the real age as a covariate. Results considered significant ( $P < .05$ ) are in bold.

(FC) threshold identified 185 transcripts upregulated in biopsies of subjects looking younger than their real age compared to those looking older than their real age (Supplementary Information S4). Eighty-nine of these sequences correspond to non-annotated transcripts, 5 correspond to microRNAs, and only 87 correspond to annotated mRNA transcripts. Statistical analysis also identified 27 annotated mRNA transcripts, 4 microRNAs, and 54 non-annotated sequences that are downregulated in biopsies of subjects looking younger than their real age compared to those looking older than their real age (Supplementary information S5).

Bio-informatic functional analysis of the 123 annotated transcripts and microRNAs that are modulated reveals only one significantly enriched function: intermediates filaments, keratin type ( $P = 1.1 \times 10^{-5}$ ). Indeed, four genes involved in the synthesis of type

I keratin (*KRT31*, *KRT33A*, *KRT33B*, *KRT43*) and two type II keratin synthesis genes (*KRT75*, *KRT85*) are repressed in the skin of women looking younger. This might reflect a specific epidermal differentiation status even if these keratins are more specific to hair follicles structures. We also individually examined the other modulated transcripts' putative involvement by direct interrogation of the PubMed database with the gene name and the following keywords: microcirculation, vascular, vessels, and endothelium. This enabled us to identify several modulated transcripts potentially related to vascular functions such as *THBS1* encoding Thrombospondin 1 (FC: -1.33,  $P = .021$ ), *MYL9* encoding Myosin Light Chain 9 (FC: -1.34,  $P = .007$ ) and miR-126 (FC: -1.39,  $P = .03$ ) a microRNA expressed in vascular endothelial cells. These two genes and the microRNA are downregulated in biopsies of subjects looking younger than their real age.

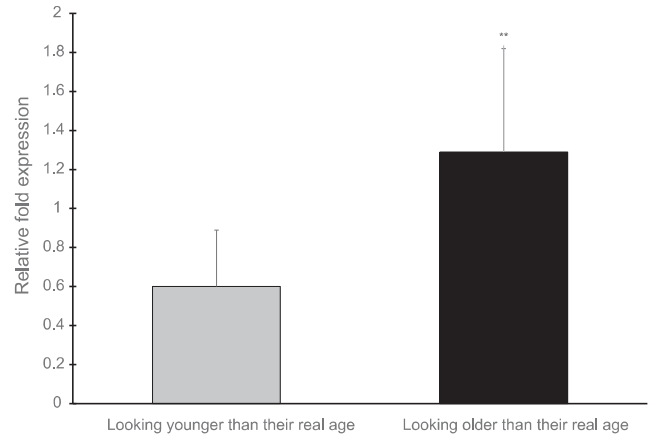
We also identified transcripts potentially associated with a vascular function and that are induced in women looking younger than their real age: *PTGER3* encoding Prostaglandin E Receptor 3 (FC: 1.44,  $P = .02$ ) and *STRN* encoding the Striatin, Calmodulin-Binding Protein (FC: 1.32,  $P = .03$ ).

Among these modulated genes, we particularly focused on *MYL9* that is induced upon aging in arterial tissues (Shehadeh et al, 2011). We confirmed by real-time qPCR that *MYL9* transcript was repressed by 2 folds ( $1.29 \pm 0.53$  vs.  $0.60 \pm 0.29$ ,  $P = .0183$  - Figure 3) in women looking younger than their real age compared to women looking older.

### 3.4 | Immunohistochemical labeling of MYL9 and CD31 are highly variable

To further study *MYL9*, we performed an immunohistochemical analysis on thin sections of skin biopsies. This analysis was performed on the biopsies of 12 subjects, 6 looking younger and 6 looking older than their real age. The subjects looking younger have a real age of  $67.33 \pm 2.58$  years old while their perceived age is  $64.42 \pm 2.85$  years old, therefore showing an age difference of  $-2.92 \pm 0.79$  years is at the limit of significance ( $P = .093$ ). The 6 subjects looking older than their real age were  $60.67 \pm 3.39$  years old while their perceived age is  $67.58 \pm 3.42$ ; a significant age difference of 6.92 years ( $P = .006$ ). In the case of these groups of subjects, the real age of the two groups is different ( $P = .003$ ), but their perceived age is not ( $P = .113$ ).

The analysis of *MYL9* was performed in parallel with that of *CD31* (Cluster Differentiation 31 or Platelet Endothelial Cell Adhesion Molecule 1), a protein present at the surface of thrombocytes, monocytes, macrophages, and neutrophils. *MYL9* is also present in the intercellular junctions of endothelial cells, therefore, labeling blood vessels. Due to the variability of both labeling (Figure 4), samples were analyzed independently from the perceived age. Indeed, several samples present different degrees of matrix alterations that induce or cause changes in the vascular endothelium or detachment of the perivascular matrix as shown by *CD31* labeling, that can combine with more or less developed vasculitis. In addition, *CD31* labeling shows that the density, morphology, and distribution of vessels also significantly differ according to the skins analyzed. The number of vessels positive for *CD31* was counted for each section and the analysis of these data confirmed a substantial heterogeneity among the different samples (data not shown). In the majority of samples, the vessels present in the dermal matrix are mainly capillaries and venules/arterioles of small diameters. Some samples however show some sections of arterioles/venules of moderate diameter. At the vascular level, *MYL9* labeling is also heterogeneous with only a few positive vessels per section. Therefore, the quantification of the labeled vessels was very uncertain and did not show any significant difference among samples (data not shown). Although weak and limited to the deep dermis in most samples, labeling of *MYL9* could be observed in the intima, consisting only of endothelial cells, and



**FIGURE 3** RT-qPCR validation of *MYL9* transcript downregulation in biopsies from 6 subjects looking older than their real age (in black) compared to biopsies of 6 subjects looking younger than their real age (in grey). *MYL9* expression is normalized to the expression of *TPB* (TATA) Binding Protein, and results are shown as the mean of the  $2^{-\Delta\Delta C_t} \pm$  standard deviation.  $P = .0183^{**}$  (unpaired t test)

sometimes in the media, composed mainly of smooth muscle cells. In the epidermis, the labeling of *MYL9* is mainly cytoplasmic.

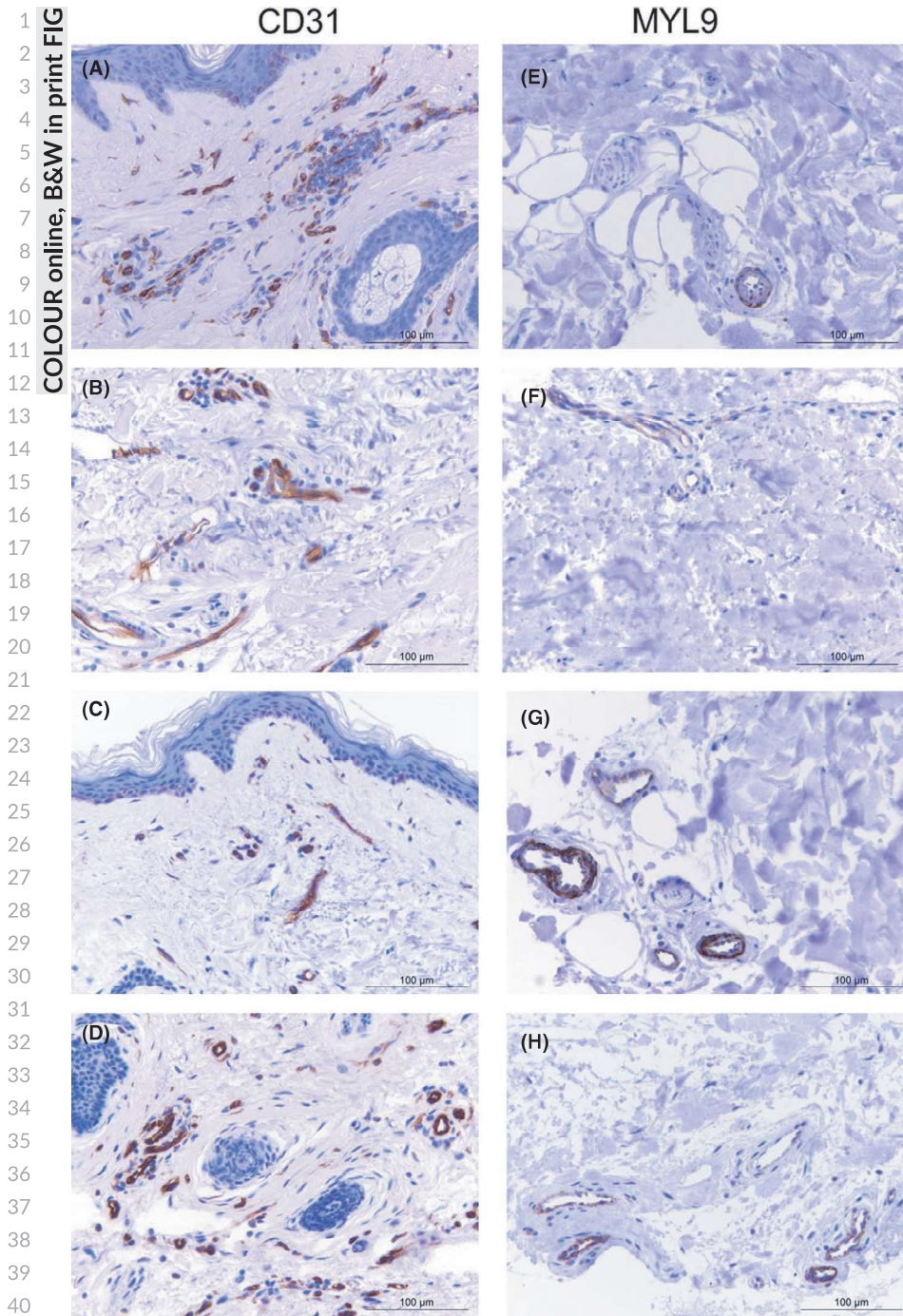
## 4 | DISCUSSION

So far, studies focusing on perceived age essentially aimed at identifying key parameters influencing age perception, revealing a prominent role for wrinkling, sagging, and loss of color homogeneity.<sup>22-26,29-31</sup> Our goal was to move one step further and identify biological markers of perceived age in this work. To do so, we focused on a known age-related skin alteration—the deleterious effect of aging on cutaneous microcirculation—reasoning that differences could lead to changes in blood flow and, therefore to discrepancies in the supply of nutrients to the skin that could affect perceived age.

As a first approach to cutaneous microcirculation in women of different perceived ages, we studied facial skin color. Indeed, skin color mainly results from light absorption/reemission by the skin's two main chromophores: melanin, and hemoglobin.<sup>32</sup> Nevertheless, no differences in instrumental measurements were observed between women looking younger or older than their real age. The only difference we found is that women looking younger than their real age present a higher glowing complexion than those looking older. This glowing complexion does not directly relate to color. Contrary to instrumental measures performed on limited areas of the skin, the glowing complexion is a global parameter that considers the luminosity and the reflection of the skin. It might reflect a better skin health.

Our study's most striking result is the significantly higher microvascular heat-evoked reactivity in a non-photo-exposed skin (forearm) of women looking younger than their real age than of women looking older (assessed in the facial skin). Moreover, the intermediate group with people looking about their age shows a





**FIGURE 4** Representative microscopy images of the IHC labeling of CD31 (left panel) and of MYL9 (right panel). (A), (E) subject 25 and (B), (F) subject 27; subjects looking younger than their real age. (C), (G) subject 15 and (D), (H) subject 18; subjects looking older than their real age. Scale bar: 100 µm

42 microvascular reactivity between the 2 others (Supplementary in-  
43 formation S2) even if there is no significant difference between the  
44 looking older group and "about their age" group results, neither be-  
45 tween the looking younger group and the "about their age" group.  
46 This result is even more compelling that the women looking younger  
47 than their real age are the oldest. Indeed, their average real age is  
48 almost 10 years older than that of the group of women looking older  
49 than their real age. This result is also specific to the cutaneous mi-  
50 crocirculation since monofilament results show no difference in tac-  
51 tile perception, demonstrating the absence of sensory neuropathy.  
52 This heat-induced vasodilatation mostly studied in the forearm skin  
53 is mediated by neurovascular mechanisms<sup>33,34</sup> where nerve fibers

lead to vasodilation through the activation of downstream second  
messengers that include NO.<sup>35</sup> Despite greater sensory afferent  
nerve densities in facial skin, the initial CVC peak to local heating  
is similar in non-glabrous and glabrous skin.<sup>36</sup> This supports that  
the magnitude of the local heating-mediated vasodilation observed  
in the forearm skin would have been similar in the facial skin. With  
aging, vasculature presents reduced NO bioavailability due to de-  
creased synthesis of NO through age-related decreases in L-arginine  
and BH4 availability. There would also be increased NO degradation  
through oxidant stress. Our results demonstrated that, between 55  
to 75 years old, women who look younger have a better microcircu-  
latory function in non-photo-exposed area, which exclude the role

of photo-exposition in this mechanism. These results suggest that maintaining microcirculatory health could participate in maintaining general skin health. Implementing strategies leading to better microcirculatory health and measuring its impact on skin perceived age might be a path of future researches.

The microarray analysis did not provide us any further clue on the underlying mechanisms that would explain the observed difference in the heat-evoked vasodilatation. Globally, it led to the detection of a rather low number of significantly modulated transcripts (123 annotated transcripts including microRNAs), and most of these transcripts exhibited modest fold changes (from 1.3 to 2.18) between women looking younger or older than their real age. The high individual variability inside the 2 groups was probably one explanation of this modest transcriptional response. Nevertheless, we identified few modulated transcripts that can be directly or indirectly related to aging or microcirculation. This is the case of the microRNA miR-126, which expression level decreases in the skin interstitial fluids of elderlies while it is secreted by dermal endothelial cells of young skins where it promotes angiogenesis.<sup>37</sup> Mir-126 is then potentially involved in the skin's vascular function, but the functional consequences of its modulation in total skin samples need to be further clarified.

The other interesting candidate microarray identified is MYL9, a protein that, in rat, increases in the blood vessels of older individuals.<sup>38</sup> The fate of MYL9 in human aging is not known. However, we can assume that, if similar, repression of this gene in the group of women looking younger than their real age—but chronologically older—makes it a better candidate to be a molecular marker of perceived age and could reflect some specific property of the skin microcirculation in these individuals. Indeed, Shehadeh et al.<sup>38</sup> show that the ectopic expression of MYL9 in rats causes endothelial dysfunction that could explain the increased arterial constriction of aged vasculature. Endothelial dysfunction in the aorta of older rats is also associated with a decreased vasorelaxation due to a decline in NO and in endothelium-derived hyperpolarizing factor.<sup>39,40</sup> If these sole elements are not enough to directly link the lower amount of MYL9 in the blood vessels of younger-looking women and their higher microcirculation reactivity, further experiments to explore the role of MYL9 in humans would be of interest.

In conclusion, the most striking characteristic of perceived age we identified is the forearm skin's vascular reactivity, which is improved for subjects looking younger than their real age. Still, the few differences we identified, suggest that the difference between real and perceived age is probably not correlated to strong molecular features. They would also suggest that the skin's response to physical stimulation or external stress might be a better estimator of the heterogeneity of biological aging.

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#### CONFLICT OF INTEREST

CG, AB, A.N, and KV are full-time employees of Clarins; a major company specialized in the design, manufacturing, and marketing of cosmetic products.

#### AUTHOR CONTRIBUTION

CG, AB, A.N, and KV designed the research study, analyzed the data and wrote the paper. AG and JD wrote the paper. JL and BF designed the study, performed the research, analyzed the data and wrote the paper.

#### DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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