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Human Meibum Chain Branching Variability with Age, Gender and Meibomian Gland Dysfunction

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Abstract

NMR spectroscopy was used to measure hydrocarbon chain branching variability in meibum with age, gender and Meibomian gland dysfunction (MGD). A cohort of 65 meibum donors without dry eye and 31 donors with MGD was studied. Heteronuclear 2D NMR was used to confirm CH₃ resonance assignments allowing us to positively identify 23 of the 27 proton resonances of cholesteryl ester and ¹H resonances due to straight chain, iso-branched and anteiso-branched hydrocarbons. Meibum from donors without dry eye contained 57 ± 1 % straight-chains, 23 ± 1 % iso-branched chains and 20 ± 1 % anteiso-branched hydrocarbon chains. Compared with meibum from donors without dry eye, meibum from donors with MGD contained less, 50 ± 2 % straight-chains, more, 32 ± 2 % iso-branched chains and the same amount, 18.0 ± 0.07 %, of anteiso-branched hydrocarbon chains. Meibum hydrocarbon chain branching did not change with age between 22 and 68 years of age, nor was it influenced by gender. Based on previous studies, one would expect anteiso-branched chains would contribute to lowering the phase transition temperature of meibum, decrease the elasticity of the tear film lipid layer and increase the molecular area and spreading of meibum on the surface of the eye. Although we speculate that the observed differences in iso-chain branching do not influence tear film stability or rheology, the speculation has yet to be tested.

Keywords

Age; Branching; Dry eye; Lipid; Meibum; Meibomian gland dysfunction; NMR

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CONFLICTS OF INTEREST

None of the authors has a conflict of interest

1. INTRODUCTION

A thin film of lipids derived from meibomian glands in the eyelids covers the surface of the tear film. Although it forms only 0.3 % of the thickness of the tear film,^{1,2} the lipid film serves to: prevent the overflowing of tears, lubricate, improve refraction, inhibit evaporation, physically stabilize the tear film, degrade mucin clots, provide antibacterial activity, and suppress light.³⁻⁷ A measure of tear film stability is the amount of time it takes for tears to break up following a blink (TBUT).⁸ On average, TBUT decreases with age from over 35 s in infants to ~8 s in adults.⁹⁻¹³ With meibomian gland dysfunction, TBUT has been found to be even shorter (below 5 s) and this leads to dry eye symptoms.¹⁴⁻¹⁷

NMR spectroscopy has been a valuable tool for the evaluation of the lipid composition in the ocular lens¹⁸ (reviewed in 19), and human meibum lipid.²⁰⁻²⁶ Confirmation of NMR resonance assignments is critical to the interpretation of the changes in the NMR spectra of meibum with age, gender, race and dry eye. Inverse heteronuclear 2D experiments were performed to make NMR resonance assignments for the spectra of human meibum. The two major heteronuclear 2D NMR experiments are heteronuclear multi-bond correlation spectroscopy (HMBC), used to observe long-range interactions over distances of two to three bonds, and heteronuclear single quantum coherence (HSQC) used to determine the protons resonances that are associated with specific carbon resonances.²⁷ The technique involves the transfer of magnetization from the proton to the heteronucleus (in this case carbon 13) and then back to proton, the more sensitive nucleus. The technique can discern between CH₃ and CH moieties and CH₂ moieties. HSQC has been applied to the ester region of the NMR spectra of meibum between 5.5, and 3.9 ppm,^{26,28} applied to the squalene resonances of meibum near 5.2 ppm,²⁹ and applied to the CH₂/CH₃ region of meibum from 2.6 to 0.6 ppm including hydrocarbon chain branching.²⁶

It has been known for decades²⁹ and more recently confirmed^{26,30} that the hydrocarbon chains of meibum are significantly branched. Changes and variability of meibum hydrocarbon chain branching with age, gender, race or dry eye has not been studied. Hydrocarbon chain branching can have a significant influence on hydrocarbon chain fluidity and packing. For example, the anteiso-branched hydrocarbon, 14 methylpamitic acid, melts at a much lower temperature between 39.5°C and 40°C compared with the straight chain hydrocarbon, palmitic acid, that melts between 61°C to 63°C and the iso-branched hydrocarbon, isopalmitic acid, which melts between 61.8°C and 62.4°C, Figure 1 (www.chemspider.com).

In the current study, we examined the CH₃ NMR resonance region of a pool of meibum from infants and adolescents using HMBC and HSQC in which the ester region was previously studied using only HSQC NMR.^{28,29} In the current study of a pool of infant meibum, we used HSQC, and for the first time, HMBC, to confirm resonance assignments for cholesterol ester CH₃ moieties and hydrocarbon moieties associated with wax and cholesteryl esters: straight chain, iso-branched and anteiso-branched CH₃. We quantified meibum hydrocarbon chain branching in a cohort of 65 donors between the ages of 13 and 88 years-old and a cohort of 31 donors with meibomian gland dysfunction.

II. MATERIALS AND METHODS

A. Materials and Methods

Materials—Cholesterol, 14 methylpamitate, palmitate, isopalmitate, CDCl_3 , cyclohexane- d_{12} , tetrahydrofuran (THF) and methanol (MeOH) were obtained from Sigma-Aldrich (St. Louis, MO).

B. Collection and Processing of Human Meibum

Written informed consent was obtained from all donors. Protocols and procedures were reviewed by the University of Louisville Institutional Review Board as well as the Robley Rex Veterans Affairs Institutional Review Board. All procedures were in accord with the Declaration of Helsinki.

Donors for branched chain analysis were grouped into three cohorts: C_{n500} , donors without dry eye who donated meibum that was analyzed previously (M_{n500}) using a 500 MHz NMR; C_{MGD500} , donors with meibomian gland dysfunction (MGD) who donated meibum that was analyzed previously²¹ (M_{MGD500}) using a 500 MHz NMR and C_{n700} , donors without dry eye who donated meibum that was analyzed using a 700 MHz NMR (M_{n700}).

A cohort of infants/children without dry eye, $C_{child700}$, donated meibum $M_{child700}$, for 1D and 2D studies using a 700 MHz NMR.

Collection and processing of human meibum for C_{n500} and C_{MGD500} was made on the same cohorts as published previously²¹ and copied below.

Cohorts C_{n500} and C_{MGD500} —Meibomian gland expression was done by compressing the eyelid between cotton-tipped applicators with strict attention to avoid touching the eyelid margin during expression. All four eyelids were expressed and about 1 mg of meibum was collected per individual for direct spectroscopic study. The expressate was collected with a platinum spatula and immediately spread onto the AgCl window and into 0.5 mL of tetrahydrofuran/methanol, 3:1, volume:volume (THF/MeOH) in a 9-mm micro vial with a Teflon cap (Microliter Analytical Supplies Ind., Suwanee, Georgia). All samples were frozen under argon gas until analysis. Analyses were performed within three weeks of collection of the sample. Storage of the sample on AgCl windows for over two months under argon did not affect the sample.²⁵ Prior to NMR analysis, the THF/MeOH in the micro vial containing meibum lipid (ML) rinsed from the spatula as was evaporated with a stream of argon gas.

After infrared analysis and solvent evaporation, ML was removed from the AgCl window using a series of solvents with different hydrophobicities to ensure that all lipid classes were extracted from the AgCl window. First, the AgCl window was placed with the ML side down, into a 15-mL glass scintillation vial containing 1 mL of hexane and purged with argon gas. A glass vial rather than a plastic one was used in all protocols to avoid plasticizer contamination. The vial was sonicated in an ultrasonic bath (Branson 1510, Branson Ultrasonics, Danbury, CT) for 10 min. The hexane was decanted into the micro-vial containing the ML rinsed from the spatula. The hexane was evaporated under a stream of nitrogen gas. Methanol (1.5 mL) was then added to the scintillation vial containing the AgCl

window and purged with argon gas. The vial was sonicated in an ultrasonic bath (Branson Ultrasonics, Danbury, CT) for 10 min. The methanol was decanted into the micro-vial containing the ML rinsed from the spatula and was evaporated under a stream of nitrogen gas. THF/MeOH (1.5 mL) was added to the scintillation vial containing the AgCl window and purged with argon gas. The vial was sonicated in an ultrasonic bath (Branson Ultrasonics, Danbury, CT) for 10 min. The micro vial containing the extracted meibum lipid was lyophilized for 12 hours to remove trace amounts of organic solvents. Finally, deuterated cyclohexane (0.5 mL) was added to the sample and sonicated (Branson Ultrasonics, Danbury, CT) for 10 minutes in a bath sonicator. The solution was transferred to glass NMR tubes (Sigma Chemical Co, St Louis MO) and NMR spectra were collected.

Cohort M_{n700}—Meibomian glands were gently expressed by pressing the eyelid with a fingertip with strict attention to avoid touching the eyelid margin during expression. All four eyelids were expressed, and approximately 0.5 mg of meibum lipid was collected per individual for direct spectroscopic study. The expressate was collected with a platinum spatula under a slit lamp and the pool of infant meibum was immediately dissolved into 0.5 mL of deuterated cyclohexane in a 9-mm microvial with a Teflon cap (Microliter Analytical Supplies Ind., Suwanee, GA). For the cohort of adults, meibum was dissolved in CDCl₃. Argon gas was bubbled onto the samples to prevent oxidation. The sample in the vial was capped and frozen under argon gas until analysis. Analyses were performed within 3 weeks of collection of the sample.

B. Clinical Diagnosis

Clinical diagnosis was made on the same cohorts as published previously²¹ and copied below.

Subjects were recruited from the Kentucky Lion's Eye Center and the Robley Rex Veterans Affairs Medical Center in Louisville, KY. Normal status was assigned when the subject's meibomian gland orifices showed no evidence of keratinization or plugging with turbid or thickened secretions and no dilated blood vessels were observed on the eyelid margin.

The diagnosis of MGD was made according to the criteria of Foulks and Bron.³¹ Plugging of the meibomian glands of at least 5 out of 10 orifices in the central portion of the upper eyelid was required for diagnosis of MGD. The secretion expressed by the meibomian gland had to be turbid, turbid with clumps, or paste-like. Both, inflammation of the eyelid margin, as evidenced by swelling of the eyelid margin, and 2+ vascular injection of the posterior lid margin, were necessary for diagnosis. The presence of telangiectasia of the posterior eyelid margin was confirmatory of chronic disease but not required for entry. Tear film stability was determined by instillation of sodium fluorescein into the tear film. Tear breakup time was less than 5 seconds for all subjects in C_{MGD500}.

D. NMR Spectral Measurements

Cohorts C_{n500} and C_{MGD500}—Spectral data were acquired (Inova-500 spectrometer; Varian, Lexington, MA). The following parameters were used: eight hundred scans were

acquired with a spectral width of 15 ppm, 60° pulse, 4-K data points, 1.0-second delay time, and 2.049-second acquisition time at 25°C.

Cohort C_{n700}—Spectral data were acquired using a Varian VNMRs 700 MHz NMR spectrometer (Varian, Lexington, MA) equipped with a 5 mm ¹H{¹³C/¹⁵N} ¹³C enhanced PFG cold probe (Palo Alto, CA). Spectra were acquired with a minimum of 250 scans, 45° pulse width, and a relaxation delay of 1.000 second. All spectra were obtained at 25°C. HSQC or HMBC experiments were performed using 512 increments with 16 scans per increment, 45° pulse width, and a relaxation delay of 1.000 second, mixing time of 0.080 second, and a one-bond coupling constant of 140 Hz. Spectra were analyzed using MestReNova software, version 7.1.2–10008 (Mestrelab Research S.L., Santiago de Compostela, Spain). The TMS resonance was set to 0 ppm.

Commercial software (GRAMS 386; Galactic Industries Corp., Salem, NH) was used for phasing, curve fitting and integrating.

III. RESULTS

Donor demographics for the four cohorts are presented in Table 1.

Fifteen resonances were resolved in the CH₃ region NMR spectra of M_{child700} (Fig. 2a). Resonances 1, 2, 3 and 15 (Fig. 2a) corresponded to CH₃ moieties from cholesterol (Fig. 2e); resonances 4, 5 and 6 corresponded to CH₃ moieties from palmitate representative of a straight chain hydrocarbon (Fig. 2d); resonances 7 and 9 corresponded to CH₃ moieties from isopalmitate representative of an iso-branched hydrocarbon (Fig. 2b) and resonances 8, 10, 11 and 12 corresponded to CH₃ moieties from 14 methylpalmitate representative of an anteiso-branched hydrocarbon (Fig. 2c). Assignments for the ¹H-NMR resonances in the CH₃ region are provided in Table 2.

The shifts and intensities of the ¹H's (Fig. 2) and associated ¹³C's from the HSQC (Fig. 3) and HMBC (Fig. 4) spectra of meibum (Fig. 3) allowed us to identify the proton resonances (Table 3) and ¹³C resonances (Table 4). The resonance assignments were confirmed as CH or CH₃ or CH₂ moieties by HSQC (Table 3). The shifts of the resonances were in the expected positions based on published studies,^{26,32–44} and the carbons and protons were logically coupled to other resonances (Tables 2, 3 and 4). The resonances labeled in the HSQC spectra of infant meibum (Fig. 3) were resolved in the ¹³C-NMR spectra (Fig. 5). 1D and heteronuclear 2D experiments allowed us to positively identify 23 of the 27 proton resonances of cholesteryl ester (Tables 3 and 4) and the resonance assignments for human meibum (Fig. 2). Resonances for the protons and carbon associated with cholesteryl ester carbon #11 were not found in our HMBC spectra.

Cholesteryl methyl moieties, carbon #s 18 and 19 (Fig. 1), appear as well resolved singlets at 0.72 and 1.04 ppm in the spectra of M_{n700} (Fig. 2). The ratio of the areas of the two singlet resonances for all our meibum samples was constant 1.1 ± 0.1 (standard deviation) near the expected value of 1. The CH₃ NMR spectral region measured previously using a 500 MHz NMR^{21–24} was also analyzed for the first time in the current study. The NMR spectra of the CH₃ region for meibum measured using a 500 MHz spectrometer was slightly different from

that measured using the 700 MHz spectrometer (Fig. 6). Note that resonances labeled 6, 8, 10 and 11 in Figure 2 were not well resolved in the NMR spectra measured using a 500 MHz spectrometer (Fig. 6). One reason for the differences between the spectra is that the 700 MHz instrument has superior resolving power compared with the 500 MHz instrument. Another reason for the differences between the spectra is that CDCl_3 was the solvent for M_{n700} whereas cyclohexane- d_{12} was the solvent for M_{n500} and M_{MGD500} . Cyclohexane- d_{12} was initially chosen by our laboratory because the first NMR spectra of meibum was measured by another group using cyclohexane- d_{12} as a solvent.²⁰ We found that all of the resonances in the CH_3 region for the lipid standards and meibum in cyclohexane- d_{12} (Fig. 6), were shifted down field by about 0.03 ppm compared with the same lipids in CDCl_3 (Fig. 6b).

With confidence in the NMR resonance assignments, CH_3 branching isomers were quantified for M_{n500} , M_{n700} and M_{MGD500} . The areas for resonances 4 through 14 in Figures 2 and 6 were quantified using a curve fitting algorithm. The areas for resonances labeled 1, 2, 3 and 15, in Figures 2 and 6, assigned to cholesterol CH_3 moieties were directly integrated. These resonances were used to determine the contribution of resonance number 16 to the areas of resonance numbers 7 and 9 (Figs. 2 and 6). The CH_3 resonances associated with the ring structure of cholesterol are identical to the ring structure resonances for meibum cholesteryl esters as they are far from the acyl hydrocarbon chain so that the hydrocarbon chain ester does not influence them. Apart from the cholesteryl ester resonance at 4.6 ppm and the wax ester resonance at 5 ppm the resonances associated with hydrocarbons from wax esters or cholesteryl esters are the same. Thus, we were able to quantify total meibum chain branching and were not able to discern between hydrocarbon chain branching associated individually from wax or cholesteryl esters (Table 5). We calculated that resonance number 16, assigned to cholesterol carbon iso- CH_3 moieties numbers 26 and 27, contributed $46 \pm 4\%$ of the total intensity of the iso CH_3 region.

Despite using different spectrometers and solvents, hydrocarbon chain branching was not statistically different for M_{n500} compared with M_{n700} , $P = 0.87, 0.34, 0.53$ for the percentage of anteiso-, straight- and iso- branching, respectively. The % branching for the samples is provided in Table 5. There was no statistically significant gender (Table 5) differences between any of the parameters measured (Table 5).

There was a significant difference in the percentage of straight and iso-branched chains for meibum from Black donors compared with Caucasians (Table 5). It is interesting that for black donors, the % of chain branching for M_{MGD500} and M_{n500} plus M_{n700} were very similar (Table 5). There were no statistically significant differences in branching between Asians and Caucasians. With only two Hispanic donors, no conclusions regarding branching could be made. Although some of the racial comparisons were statistically significant, the authors suggest that they are tentative due to the small sample size.

IV. DISCUSSION

A wealth of information can be obtained from the CH_2 and CH_3 region of the NMR spectra of human meibum between 0 and 3 ppm.²⁶ Resonances due to saturation, CH_3 moieties, and

cholesteryl esters are found in this region. The shifts of the cholesteryl ester resonances using 1D and 2D HSQC and HMBC were in the expected positions based on other studies,^{39–44} and the carbons and protons were logically coupled to other resonances. This allowed us to positively identify 23 of the 27 ¹³C resonances of cholesteryl ester.

Most of the tentative assignments made previously^{21,23,26} were accurate with the exception that the 0.89–0.95 ppm region that contains overlapping resonances due to cholesteryl moieties in addition to those for wax ester–CH₃ moieties. The resonance at 1.39 ppm in the spectra of meibum pooled from infants is from the –(CDH)– moiety, a contaminant in the deuterated hexane solvent. This resonance was not present in the spectra of meibum from the adult meibum cohort used in the present study as the more conventional CDCl₃ was used as a solvent instead of d-hexane.

Numerous band assignments were confirmed using inverse 2D NMR spectroscopy which may be used to interpret future NMR studies related to meibum in relationship to age, dry eye and dry eye treatments.

The value for the molar percentage of branched chains in meibum measured in the current study by NMR spectroscopy was similar to that of a single pool of meibum hard expressed from adult human cadavers four decades ago using gas liquid chromatography–mass spectrometry (Table 5).²⁹ Mass spectrometry is advantageous over NMR spectroscopy in that individual moieties can be quantitated. For instance, mass spectrometry showed that almost all of the branching occurred in saturated hydrocarbon chains.^{29,30} The disadvantage of mass spectrometry over NMR spectroscopy is that it is more costly and time consuming and there could as many as over “30,000 molecular species” of lipids in human meibum and standards for most of them are needed.⁴⁵ To measure branching by mass spectrometry, all of the many species of each type of branching need to be added together compounding the experimental error. The advantage of NMR spectroscopy is that it is relatively quicker and cheaper than mass spectrometry, standards are not necessary and the sample is not destroyed during analysis, so that mass spectrometry or other biophysical measurements can be made on the same sample. This advantage allowed us to measure branching in 96 meibum samples, one of the largest lipidomics studies to date.

The general observance that iso-branched chains > straight-chains > anteiso-branched chains determined by NMR resonance intensities²⁶ was apparently different than the order of branching measured in the current study, a gas liquid chromatography–mass spectrometry study²⁹ and a gas-liquid chromatography-ion trap mass spectrometry study (Table 5).³⁰ The latter three studies gave the order straight-chains > iso-branched chains > anteiso-branched chains. The reason for the difference is, although the relative resonance intensities of the NMR study²⁶ are in agreement with the current study, a significant amount (40%) of the iso-branched chain intensity arises from the cholesterol moiety of the cholesteryl ester esters and not the hydrocarbons that compose the cholesteryl and wax esters. In the current study, we accounted for the contribution of the iso-CH₃ moieties from cholesteryl esters.

The values of 55% straight- and 25 % iso-branched chains determined using gas liquid chromatography-mass spectrometry²⁹ was almost identical to the NMR results of the current

study (Table 5) which is remarkable considering the small sample/pool size (one) assayed in the previous study²⁹ compared to the sample size of 65 analyzed in the current study.

There were significantly more branched chains for meibum from donors with MGD compared with those that did not have dry eye (Table 5). The differences in branching was mostly due to the percentage of iso-branched chains. The difference could be due to an increase in cholesteryl esters as cholesteryl esters contain less straight chains and more iso-branched chains compared with wax esters (Table 5), however, as only one pool of meibum was examined in the previous study,²⁹ this idea needs to be confirmed. Furthermore, M_{MGD500} contains less cholesteryl esters compared with M_{n500} ^{21,22}

Lipid order or stiffness increases with age between 0 and 25 years of age as tear stability decreases⁴⁷ and lipids from donors with dry eye symptoms and an unstable tear film due to meibomian gland dysfunction (MGD) have the most ordered lipid measured, 47% ordered.⁴⁶ Thus, in context to the increase in lipid order with age the increase in lipid order with MGD is an exacerbation of aging and could contribute to tear film instability.⁴⁷ Changes in lipid order may influence the rheology of the tear film as “An increase in lipid order driven by saturation causes changes in the rheology of human meibum surface films, resulting in stiffer, more elastic films with lower lift off, increased maximum surface area, and an increase in the maximum compressibility modulus^[48,49].”⁴⁷ It is unlikely that the differences in chain branching contribute to the increase in lipid order or tear film stability given the studies discussed in the next paragraph.

Whether branching influences tear film stability or whether it depends on race or dry eye has yet to be determined. Hydrocarbon chain branching is relatively rare in most human organs. It is more common in bacteria and animal surface fats.^{50,51} Bacterial growth and temperature were related to hydrocarbon chain branching.^{52,53} Based on previous studies,^{52–58} one would expect anteiso-branched chains would contribute to lowering the phase transition temperature of meibum, decrease the elasticity of the tear film lipid layer and increase the molecular area and spreading of meibum on the surface of the eye as unsaturation does.^{47–49} However, this is not the case for MGD as anteiso branching was the same for M_{MGD500} versus M_{n500} and M_{n700} . The larger amount of iso-branched chains in M_{MGD500} compared with M_{n500} and M_{n700} is unlikely to contribute to hydrocarbon chain order as iso-branched lipids melt at the same temperature as straight chain hydrocarbons (Introduction). Although we speculate that the observed differences in branching do not influence tear film stability or rheology, the speculation has yet to be tested.

IV. CONCLUSIONS

Fifteen resonances were resolved in the CH_3 region NMR spectra of human meibum. The shifts of the resonances were in the expected positions based on published studies, and the carbons and protons were logically coupled to other resonances. This allowed us to positively identify 23 of the 27 ^{13}C resonances of cholesteryl ester and 1H resonances due to straight chain, iso-branched and anteiso-branched hydrocarbons. Meibum contained 57 % straight-chain, 23 % iso-branched chains and 20 % anteiso-branched hydrocarbon chains. Meibum hydrocarbon chain branching did not change with age between 22 and 68 years of

age, nor was it influenced by gender. Based on previous studies, one would expect anteiso-branched chains would contribute to lowering the phase transition temperature of meibum, decrease the elasticity of the tear film lipid layer and increase the molecular area and spreading of meibum on the surface of the eye. Meibum from donors with MGD had the same amount of anteiso-branched chains, less straight chain, 50 %, and more iso-branched chains, 32 % compared with meibum from donors without dry eye. Whether these differences in branching influence tear film stability or rheology has yet to be determined.

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14-methylpentadecanoic Acid (isopalmitic acid)

14-methylhexadecanoic Acid (14-methylpalmitic acid)

Heptadecanoic Acid

Figure 1.
Structure of standards used to characterize human meibum chain branching.

Figure 2.

NMR spectra of the CH_3 region measured with a 700 MHz spectrometer. Samples were dissolved in CDCl_3 . a) meibum from a Caucasian male 17 years old. b) 14-methylpentadecanoic acid with $-\text{iso}$ CH_3 moieties c) 14-methylhexadecanoic Acid with anteiso CH_3 moieties d) heptadecanoic Acid with straight chain CH_3 moieties d) Cholesterol. Numbers refer to resonances with assignments in Table 1. Cholesterol carbon numbers refer to those labeled in Figure 1.

Figure 3.

NMR heteronuclear single quantum coherence spectra of infant meibum in the CH_3 resonance region. Numbers refer to resonances labeled in Fig. 5. HSQC spectra show correlations between ^1H and ^{13}C spectra indicating which proton resonances are associated with a specific ^{13}C resonance.

Figure 4.

NMR heteronuclear multiple-bond correlation spectra of a pool of human infant meibum. Correlated resonances appear in the same vertical or horizontal line and represent moieties that are 1 to 3 bonds apart on the same molecule.

Figure 5.
 ^{13}C NMR spectra of infant/child meibum. Numbers are resonances identified in the HSQC spectrum (Fig. 3).

Figure 6.

A) NMR spectra of the CH₃ region measured with a 500 MHz spectrometer. Samples were dissolved in cyclohexane-*d*₁₂. i) meibum from a Caucasian male 21 years old. ii) 14-methylpentadecanoic acid with –iso CH₃ groups iii) 14-methylhexadecanoic Acid with –anteiso CH₃ groups iv) heptadecanoic Acid with straight chain CH₃ groups v) Cholesterol. Numbers refer to resonances labeled in Figure 2 with assignments in Table 1.

B) NMR spectra of 14-methylhexadecanoic Acid with anteiso CH₃ moieties. i) measured with a 700 MHz NMR and dissolved in CDCl₃. ii) measured with a 700 MHz NMR and dissolved in cyclohexane-*d*₁₂. iii) measured with a 500 MHz NMR and dissolved in cyclohexane-*d*₁₂.

Table 1.

Cohort Demographics.

	C _{child700}	C _{n700}	C _{n500}	C _{MGD500}
Average age (y)	4 ± 2	29 ± 16	38 ± 16	66 ± 18
Age range (y)	1 to 8	13 to 68	19 to 88	33 to 87
Median Age (y)	4	24	24	73
Gender (% male) (% unknown)	57	57	76	58 26
Race (%) [*]	C (86)	C (79)	C (81)	C (61)
	B (14)	B (11)	A (19)	B (10)
		H (5)		H (3)
		A (5)		? (26)
Number of Donors	Pool of 7	44	21	31

^{*} C is Caucasian, B is Black, A is Asian, H is Hispanic, ? is unknown.

Table 2.

CH₃ region NMR resonance assignments for meibum in Figure 2.

Figure 2 resonance #	Shift (ppm)	CH ₃ Resonance Assignment
1	1.00 to 0.996	Cholesterol Carbon #19
2	0.906	Cholesterol Carbon #21
3	0.897	Cholesterol Carbon #21
4	0.878	Straight-chain
5	0.868	Straight-chain
6	0.858	Straight-chain
7	0.853	Iso-branched
8	0.850	Anteiso-branched
9	0.843	Iso-branched
10	0.839	Anteiso-branched
11	0.829	Anteiso-branched
12	0.821	Anteiso-branched
13	0.799	Not assigned
14	0.789	Not assigned
15	0.663	Cholesterol Carbon #18
16	0.858 to 0.843	Cholesterol Carbon #26 and #27

Table 3.

¹H Comparison of Literature Values for Cholesteryl Ester Shifts with those Measured in this Study Using NMR HMBC and HSQC for meibum from human infants.

Cholesteryl-ester (carbon #) [†]	δ ¹ H Literature* Average $\pm$ STD (ppm)	This Study HMBC (ppm)	δ ¹ H This Study HSQC (ppm)	δ ¹³ C This Study HSQC (ppm)	¹ H to ¹³ C HMBC Couplings (carbon #) [†]	HSQC Assignment
18	0.65 $\pm$ 0.05	0.73	0.72	12.24	12,13,14,17	Not CH ₂
26	0.83 $\pm$ 0.4	0.89			24,25	
27	0.83 $\pm$ 0.05	0.89			24,25	
21	0.91 $\pm$ 0.03	0.97	0.96	19.6	20,22	Not CH ₂
14 α	0.96 $\pm$ 0.081	1.01	1.04	57.78	7,8,12,13,15,18	Not CH ₂
19	1.00 $\pm$ 0.03	1.04	1.04	19.6	9,10	Not CH ₂
225	1.02 $\pm$ 0.03	1.04	1.05	37.11	21,23	CH ₂
17 α	1.07 $\pm$ 0.04	1.12	1.13	57.22	13,14,16,18,20,21	Not CH ₂
24 <i>R</i>	1.08 $\pm$ 0.07	1.12	1.15		23,25	
23 <i>S</i>	1.36 $\pm$ 0.26	1.36			22,24,25	
20	1.37 $\pm$ 0.05	1.39			17,18,21,22	
25	1.48 $\pm$ 0.04	1.45	1.53	28.99	24,27	Not CH ₂

* From 5 citations in citation 11 and citations 9,10,12.

[†] See Figure 1

Table 4.

¹³C Comparison of Literature Values for Cholesteryl Ester Shifts with those Measured in this Study Using NMR HMBC and HSQC for meibum from human infants.

Cholesteryl (carbon #)	$\delta^{13}\text{C}$ Literature* Average $\pm$ STD (ppm)	$\delta^{13}\text{C}$ This Study HMBC, Average $\pm$ STD (ppm)	$\delta^{13}\text{C}$ This Study HSQC (ppm)	¹³ C to ¹ H HMBC Couplings (carbon #)
18	12.1 $\pm$ 0.5	11.1 $\pm$ 0.2	12.2	12 α , 14 α , 16 α , 17 α , 20
21	19.0 $\pm$ 0.5	18.6 $\pm$ 0.2	19.18	17 α , 20, 22S, 22R
19	19.6 $\pm$ 0.5	18.6 $\pm$ 0.2	19.6	1 α , 9 α
25	28.3 $\pm$ 0.5	27.9 $\pm$ 0.3	28.99	23S, 24R, 26, 27
20	36.1 $\pm$ 0.5	36.3 $\pm$ 0.3		17 α , 21, 22
22	36.5 $\pm$ 0.6	36.4 $\pm$ 0.3	37.11	20,21,23R,23S
10	36.8 $\pm$ 0.4	36.5 $\pm$ 0.3		1 α , 4 α , 4 β , 6 α , 9 α , 19R
24	39.4 $\pm$ 1.3	39.4 $\pm$ 0.5		22S, 22R, 23R, 25, 26, 27
27	39.5 $\pm$ 0.6	38.6 $\pm$ 0.0		25
13	42.6 $\pm$ 0.4	42.1 $\pm$ 0.1		11 α , 11 β , 12 α , 12 β , 14 α , 18
9	50.4 $\pm$ 0.5	50.4 $\pm$ 0.1	51.21	7 α , 7 β , 8 β , 11 β , 19R
17	56.5 $\pm$ 0.5	56.5 $\pm$ 0.3	57.22	15 β , 16 α , 18, 20

* From 5 citations in citation 11 and citations 9,10,12.

Table 5.

Molar isomer ratios for meibum from adults compared with literature values.

Sample	Anteiso-Branched	Straight-Chain	Iso-Branched	n
M _{n500} and M _{n700} (%)	20 ± 1	57 ± 1	23 ± 1	65
M _{MGD500} (%)	18.0 ± 0.7	50 ± 2	32 ± 2	31
M _{n500} and M _{n700} verses M _{MGD500} (<i>P</i>)	0.34	0.0003 *	< 0.0001 *	
M _{n500} and M _{n700} Male (%)	21 ± 1	56 ± 1	22 ± 1	37
M _{n500} and M _{n700} Female (%)	19 ± 2	58 ± 2	23 ± 1	22
M _{n500} and M _{n700} , male vs. Female (<i>P</i>)	0.33	0.33	0.50	
M _{n500} and M _{n700} Black (%)	18 ± 5	45 ± 4	37 ± 5	5
M _{MGD500} Black	19 ± 4	43 ± 9	38 ± 11	3
Black vs. M _{n500} and M _{n700} (<i>P</i>)	0.93	0.02 *	< 0.0001 *	
M _{n500} and M _{n700} Asian (%)	24 ± 5	51 ± 4	24 ± 5	5
Asian vs. M _{n500} and M _{n700} (<i>P</i>)	0.41	0.12	0.92	
M _{n500} and M _{n700} , linear regression Correlation Coefficient, R, verses age (<i>P</i>)	0.32 (> 0.1)	0.21 (> 0.1)	0.23 (> 0.1)	
Meibum all moieties (% , citation 29)	19.3	54.9	24.5	1 [†]
Wax Esters (% , citation 26)	11	83	5	14
Wax Esters (% , citation 29)	13	78	9	1 [†]
Cholesteryl Esters (% , citation 29)	30	23	46	1 [†]

Average ± Standard Error of the Mean.

* Statistically Significant

[†] Pool