SCIENTIFIC REPORTS

SUBJECT AREAS:
LIPIDOMICS
LIPIDS
MASS SPECTROMETRY
BIOCHEMISTRY

Received
13 May 2011

Accepted
8 June 2011

Published
27 June 2011

Correspondence and requests for materials should be addressed to I.A.B. (igor.butovich@utsouthwestern.edu)

Comparative HPLC-MS^n analysis of canine and human meibomian lipidomes: many similarities, a few differences

Igor A. Butovich[1], Anna M. Borowiak[2] & J. Corinna Eule[2]

[1]University of Texas Southwestern Medical Center, Department of Ophthalmology and the Graduate School of Biomedical Sciences, Dallas, TX, [2]Klinik und Poliklinik für kleine Haustiere, Freie Universität, Berlin, Germany.

The aim of this study was to evaluate the lipidome of meibomian gland secretions in canines (cMGS) – a common pet and laboratory animal – and to compare it with that of human MGS (hMGS), to determine whether canines could be used as a valid experimental animal model in studies of the biochemistry and physiology of the human ocular surface in general, and of the Meibomian glands in particular. The MGS of both species were evaluated using HPLC in combination with atmospheric pressure chemical ionization ion trap mass spectrometry. The main lipid classes found in cMGS were very long chain cholesteryl esters, wax esters, (O-acyl)-omega-hydroxy fatty acids (OAHFA), and cholesteryl esters of OAHFA. The lipidomes of cMGS and hMGS were found to be qualitatively similar, which implies similar biosynthetic and biodegradation pathways in canines and humans. However, some quantitative differences between the two were observed.

Human meibomian gland secretions [hMGS, also known as *meibum*[1]] are the main source of the lipids that form the outermost layer of the tear film (TF) called the tear film lipid layer (TFLL). The TF covers the entire ocular surface and plays various beneficial roles in ocular physiology[2, 3]. The biochemical composition and structure of the human TF are complex[4, 5]. Because the TF has multiple sources of its components, and because of its ever-changing nature (in part, due to constant blinking which causes physical mixing of TF components), evaluation of the TF is a formidable task. Obvious ethical concerns limit the scope of the in vivo experiments that are feasible with the human ocular surface. Thus, a side-by-side comparison of TF and MGS samples collected from humans and laboratory animals may help in finding a better animal model of the human ocular surface. It may also provide an interesting and important insight into the mechanisms of the TF and TFLL stabilization in different species. This information could also be useful in guiding selection of treatments for domestic animals [who can also suffer from dry eye-like conditions[6]], from the arsenal of prescription and over-the-counter treatments available for humans. Finally, information on the biochemical composition of animal meibum is interesting from biochemical and phylogenetic points of view.

In 1976, Baron and Blough[7] were the first to report results of lipid analysis of bovine MGS. Three years later, a preliminary analysis of rabbit samples was published by Tiffany[8], followed by a more detailed report from the same group[9]. Systematic comparative studies of human and animal meibum were started by Nicolas Nicolaides et al. in the late seventies of the 20th century[10] and continued in that laboratory until the late eighties[11]. The studied animals included steer, rabbit, and bovine[11–18]. A comprehensive review of the work done with animal meibum goes far beyond the scope of the current manuscript. However, we want to emphasize that a perfect match for hMGS has yet to be found in the animal kingdom. A recent comparative lipidomic analysis of human and animal meibum [Butovich, I.A. & Millar T.J., *Invest. Ophthalm. Vis. Sci.* 50:ARVO E-abstract 2545 (2009)] yielded results that demonstrated that all tested animals (varying from domesticated cat to bovine to marsupials to rabbit) had lipidomes which differed substantially from each other and from human. Most of the studied animals are known to have extremely long interblink intervals[19]. The interblink interval in dogs, however, is much shorter [between 8 sec for puppies and 25 to 30 sec in adults[19]], which is closer to the human interblink interval than that of many other animals. Moreover, dogs have often served as laboratory animals in other physiological and biochemical studies, including studies in the area of physiology of the ocular surface in general, and of the meibomian glands in

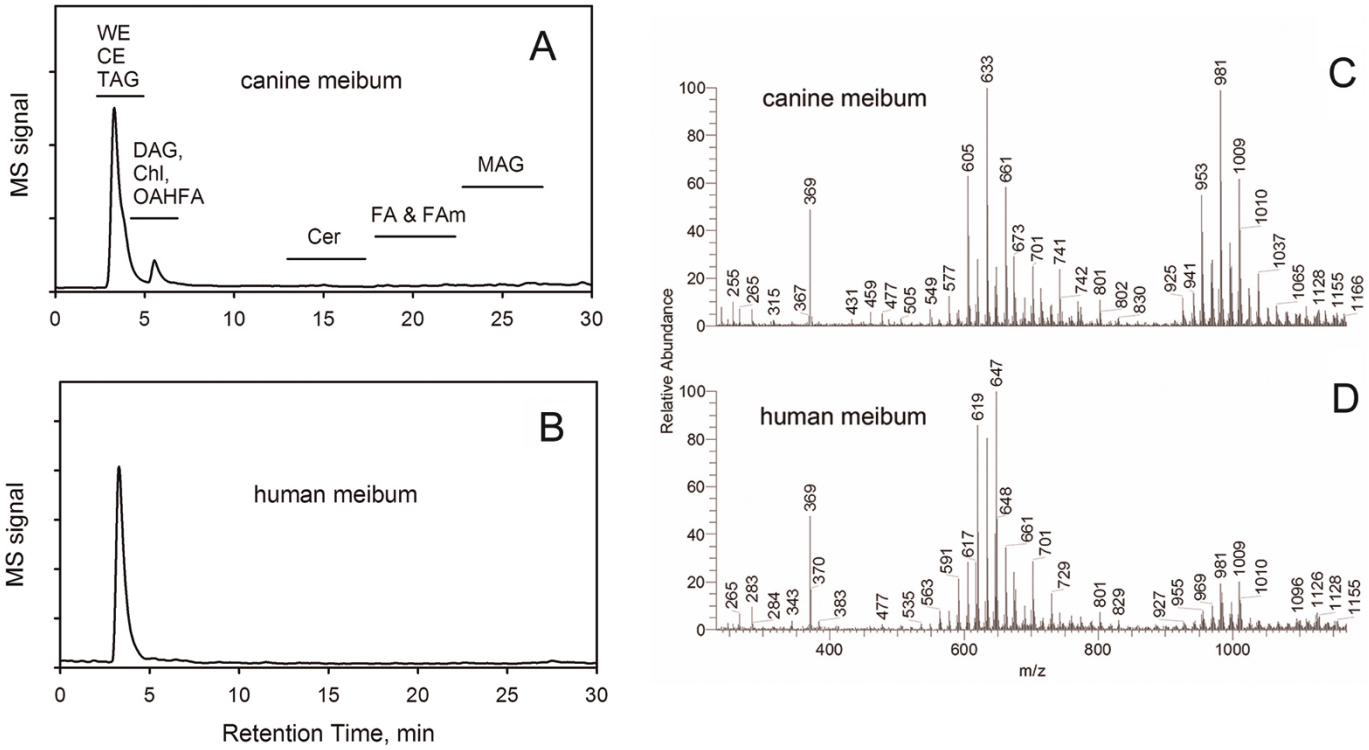

Figure 1 | Normal phase total ion chromatograms and mass spectra of canine (Panels A, B) and human (Panels C, D) meibum. Canine meibum was dissolved in the *n*-hexane:propan-2-ol solvent mixture (1 : 1, v/v) to make ~0.1 mg/mL stock solution, of which a 4 μL aliquot was injected and analyzed as described in Materials and Methods. Retention times of authentic lipid standards are marked with horizontal bars. Human meibum was analyzed in a similar fashion. Mass spectra (Panels B and D) were taken in positive ion mode (PIM) using an APCI MS ion source. The corresponding chromatographic peaks from Panels A and C were averaged between 3 and 10 minutes, the noise was subtracted using the areas 0 to 3, and 10 to 20.

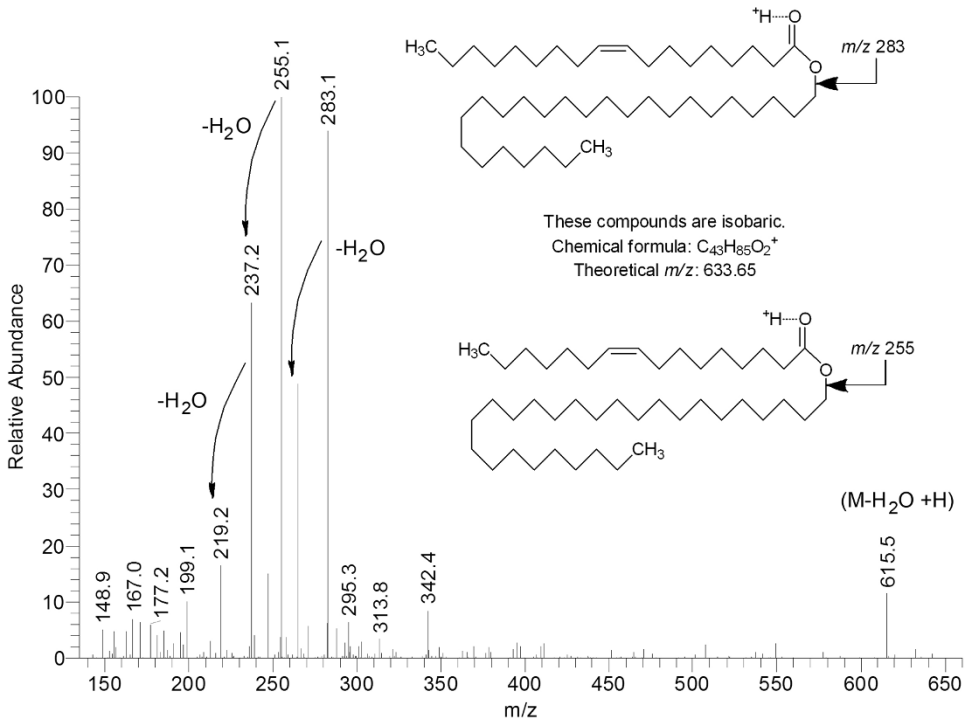

Figure 2 | Structural elucidation of canine wax ester *m/z* 633 in a collision induced dissociation PIM APCI MS experiment. Parent ion *m/z* 633 was fragmented in an APCI MS^2 experiment *m/z* 633@37V→product ions. The two structures shown are those of two isobaric compounds that were identified on the basis of their fragmentation patterns.

particular. In more recent studies, the frequency of blinking in dogs has been measured to be 3 to 5 blinks per minute, and a blink in one eye was accompanied by a blink in the other about 85% of the time. However, it was not unusual for a restrained dog to blink 10 to 20 times per minute[20]. Another study indicated a normal mean canine blink rate of ~12 per minute[21].

The canine TF break-up time (TFBUT) was measured to be 23±8 sec, with a range of 10 to 35 sec[22,23]. These numbers are slightly higher than those of humans (for whom a 5 to 10 sec inter-blinking interval is the norm), but are still not as dramatic as those for guinea pigs and rabbits (a popular choice in dry eye research) who blink rarely if at all[19]. Considering that the lipid composition of MGS is believed to be a critical factor in TF stability[2–5], we speculated that canine MGS (cMGS) should be biochemically close to the human one, which would justify further use of these animals as experimental models in studies of ocular biochemistry in general, and in dry eye studies, in particular. Thus, the goal of this study was to determine the level of similarity between human and canine meibomian lipidomes by means of high performance liquid chromatography and atmospheric pressure chemical ionization ion trap mass spectrometry (HPLC-MS), as described earlier for human samples[24–30].

Results

Between 0.1 and 0.2 mg of anhydrous canine meibum was collected from each subject. As with humans, normal phase HPLC (NP HPLC) analyses of canine samples, conducted in positive ion mode (PIM), produced a rather featureless total ion chromatogram (TIC) with just one main and a second minor HPLC peaks (Fig. 1A). No detectable HPLC peaks were observed in the areas of chromatograms where authentic monoacylglycerols (MAG), normal and very long chain ceramides (Cer and VLC-Cer), and fatty acids (FA) and fatty acid amides (FAm) eluted.

The main NP HPLC peak had a retention time (RT) of 3.2–3.5 min. This was the characteristic RT of wax esters (WE), triacyl glycerols (TAG), and CE[25]. A similar TIC was observed for a human meibum sample (Fig. 1B). However, canine meibum did produce another (though minor) HPLC peak, with RT between 5 and 6 min. This secondary HPLC peak had a RT indicative of free cholesterol (Chl), diacylglycerols (DAG), and (O-acyl)-omega-hydroxy fatty acids (OAHFA)[26]. Human MGS samples have been demonstrated to have such lipids as well. However, the levels of Chl, DAG, and OAHFA present in hMGS were apparently lower than those detected in cMGS, thus resulting in a more noticeable HPLC peak with RT 5–6 min in the latter case.

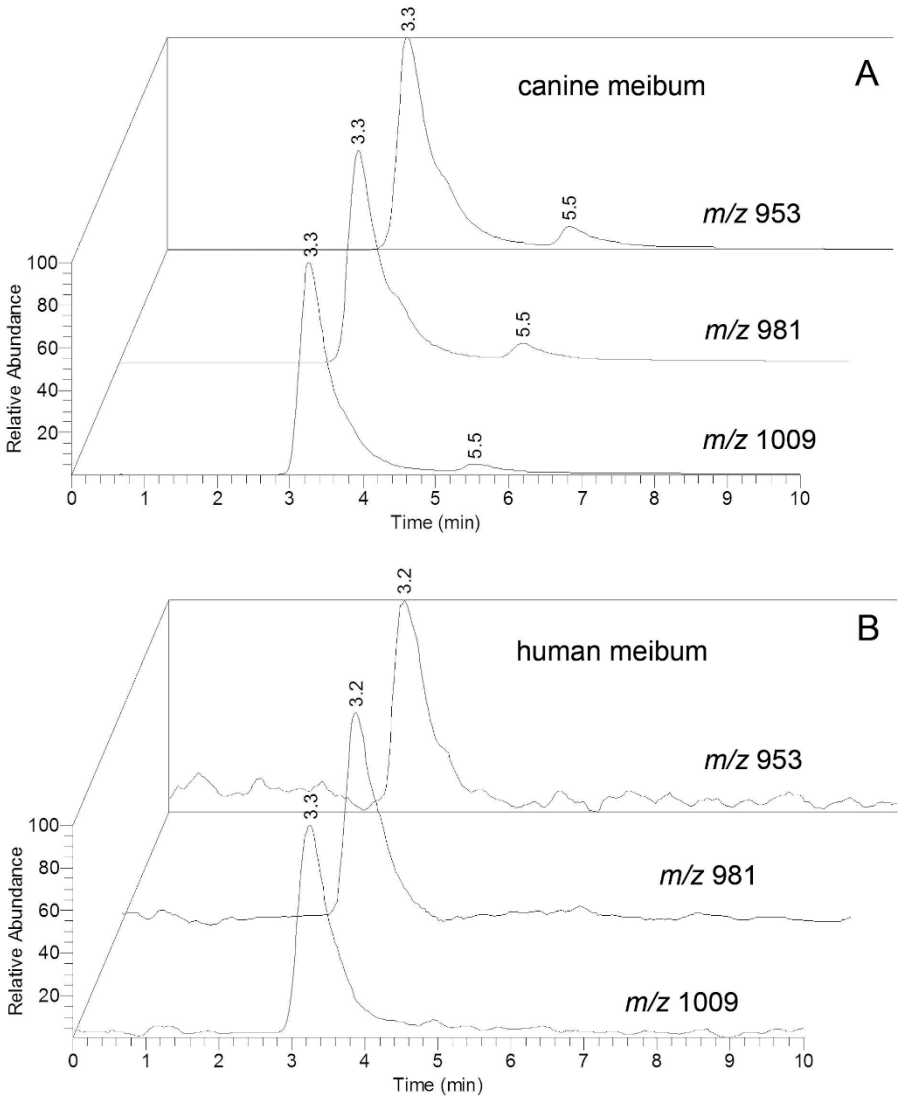

Figure 3 | Comparative normal phase HPLC-PIM APCI MS analyses of unknown lipids *m/z* 900-1100 present in canine (Panel A) and human (Panel B) meibum. Three lipid species are shown (*m/z* 953, 981, and 1009). Compared with human samples, the canine samples consistently showed a much higher relative presence of these compounds. The canine samples also showed the presence of isomeric and/or isobaric compounds with the same *m/z* ratio.

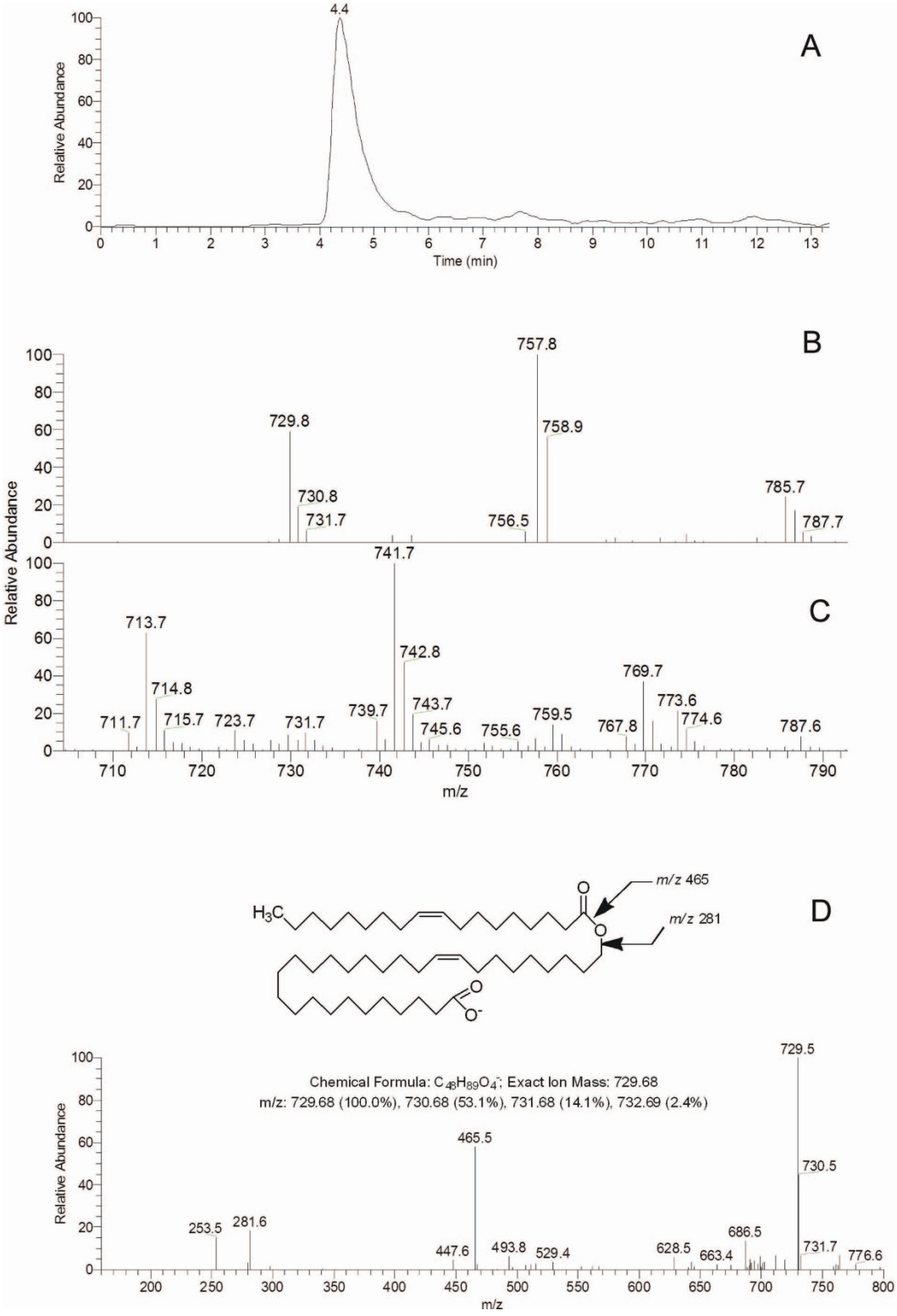

Figure 4 | Structural elucidation of OAHFA detected in canine meibum. *Panel A.* Total ion chromatogram of canine meibum recorded in NIM in a normal phase HPLC experiment. Only one major peak with a retention time of 4.4 min was detected. *Panel B.* The averaged NIM mass spectrum of the HPLC peak with RT of 4.4 min. *Panel C.* The averaged PIM mass spectrum of the HPLC peak with RT of 4.4 min. *Panel D.* Parent ion *m/z* 729 was fragmented in a NIM APCI MS^2 experiment *m/z* 729@cid37V→product ions. The fragmentation pattern of the compound is exactly the same as the one reported earlier for a human compound *m/z* 729 (26). There were two coeluting isobaric isomers detected, namely O-oleoyl-30-hydroxytriacontenoate and O-palmitoleoyl-32-hydroxydotriacontenoate. The former isomer produced product ions *m/z* 281 and 465, while the latter produced ions *m/z* 253 and 493. The structure and fragmentation of O-oleoyl-30-hydroxytriacontenoate are shown.

To evaluate the biochemical composition of canine meibum, its TIC were divided into 1-min sections and analyzed section by section as described in detail in our previous publication[25]. Based on the RT of authentic lipid standards of a WE, TAG, and CE nature[24–30], no meibum analytes were expected to elute during the first 2 to 3 min of the experiment. Thus, this portion of the chromatogram produced only an injection/solvent peak and was used to subtract constant chemical noise generated by the solvent from the signals of the lipids in the rest of the chromatogram.

The main canine NP HPLC peak with an RT value of ~3.3 min produced the mass spectrum shown in Fig. 1C. This spectrum resembled the spectrum of human meibum (Fig. 1D) in many ways. The canine sample produced a wide range of lipid signals that were essentially the same as the human ones. Among these major detected components were the signals *m/z* 369 (a characteristic fragment of both Chl and CE), 551 to 673 [long chain WE, observed as $(M+H)^+$ ions], and a group of signals *m/z* 900 to 1100, which have previously been observed in humans, but have yet to be positively identified and quantified in either of species.

When an extracted ion chromatogram of cMGS ion *m/z* 369 was plotted (not shown), it became clear that there were two HPLC peaks with two totally different RT (thus, two different types of Chl-containing molecules) in each of the tested samples. As we mentioned earlier, the first (and the major) peak coeluted with a range of authentic CE (C_{16}- to C_{24}-CE; RT ~3.3–3.5 min), while the second peak had a RT indicative of free Chl (~6.6 min). The same pattern was invariably observed for human samples.

Canine compounds *m/z* 551 to 673, previously identified in human meibum as oleic-acid based WE with extremely long-chain fatty alcohol component[26, 28, 29], coeluted with each other, thus replicating the results of the human meibum analysis (not shown). The chemical nature of the canine signals *m/z* 551–673 was evaluated in MS^n experiments as we described previously. The compounds produced a characteristic ion of oleic acid *m/z* 283 $(M + H)^+$ and an ion of palmitoleic acid *m/z* 255 $(M+H)^+$ along with their dehydration product ions *m/z* 237 $[(M+H\text{-}H_2O)^+]$ and 219 $[(M+H\text{-}2H_2O)^+]$ (Fig. 2). The results for only one sample compound *m/z* 633 are shown. However, other WE detected in canine meibum showed the same pattern: a portion of $C_{16:1}$-WE was always present as an isobaric component. These WE compounds were indistinguishable from those described for human compounds and authentic WE[26, 28] with the main difference being a larger presence of palmitoleic acid-based WE in canine samples (typically, up to a 1 : 1 molar ratio of $C_{16:1}$ to $C_{18:1}$ forms of isobaric WE) compared to their human counterparts[29].

The last major (and a very diverse) group of canine signals *m/z* 900–1200 had a more complex elution pattern, consistently showing two to three isobaric components that eluted at distinctively different retention times (note a major peak with RT of 3.3 min, a shoulder with RT of 4 min, and another peak with RT of 5.5 min; Fig. 3A). The

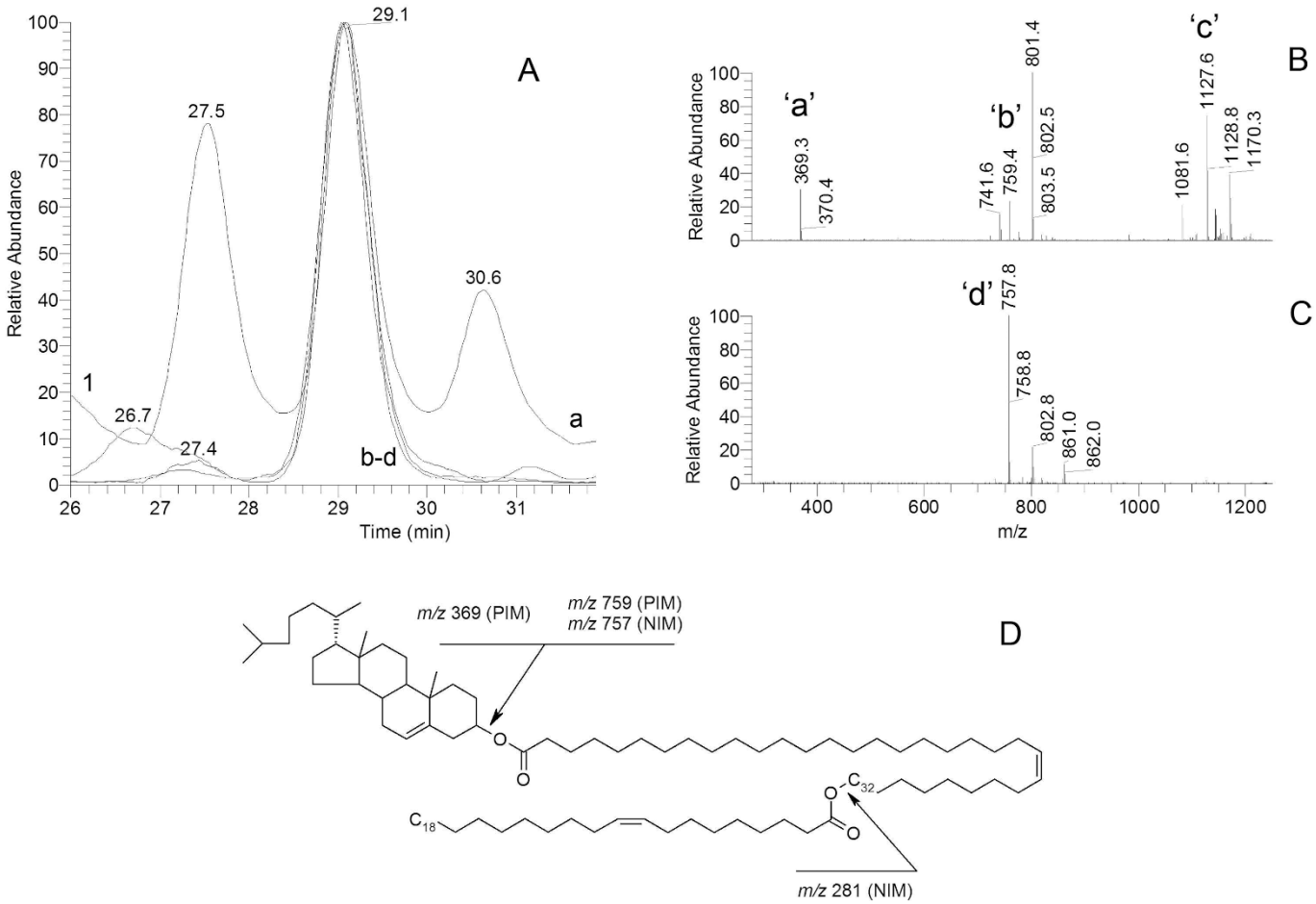

Figure 5 | Elucidation of the structures of canine CE-OAHFA using reversed phase HPLC-APCI MS. *Panel A.* Reversed phase HPLC-APCI MS traces of ions *m/z* 369 (a), 759 (b), and 1127 (c) recorded in PIM, and of ion *m/z* 757 recorded in a separate experiment in NIM. Note that the retention times of all the ions are the same (29.1 min). *Panel B.* An averaged mass spectrum of the peak with RT of 29.1 min. *Panel C.* Averaged NIM mass spectrum of the peak with RT of 29.1 min. *Panel D.* The proposed structure and characteristic fragments of one of the canine CE-OAHFA, CE of O-oleoyl-32-hydroxydotriacontenoic acid (*m/z* 1127 in PIM). The same compound has also been identified in human samples.

human samples, on the other hand, were more homogeneous with respect to these signals and typically showed only one major HPLC peak for each of the signals, with a possibility to develop a shoulder and/or a small satellite peak (Fig. 3B). Also noticeable in human samples was the lower ratio of these compounds to other lipids (Fig. 1). At this time, their chemical nature remains unknown for both canine and human meibum.

Another apparent difference between the canine and human WE is the relative ratio of odd and even-chained alcohols: canine meibum has a higher ratio of oleic-acid based WE with odd numbers of carbons in their saturated alcohol moieties (mostly, C_{23} and C_{25}), while human meibum shows a higher abundance of even-numbered alcohols (with C_{24} and C_{26} being the most prominent).

The secondary canine NP HPLC peak had a RT of 4 to 6 min which was close to that of DAG, Chl, and OAHFA[26, 30]. As discussed above, the presence of Chl was too low to account for this HPLC peak. When analyzed in PIM, the peak produced a complex pattern of analytes with *m/z* above 700, with the most prominent ions being 713, 741, and 769. These MS signals have also been found in the human samples, albeit in smaller quantities incapable of producing a noticeable TIC HPLC peak. When the traces of these ions were plotted as extracted ion chromatograms, their retention times and *m/z* values were found to be the same for both canine and human meibum. Earlier we showed that human meibum is rich in OAHFA[26, 29]. When the canine samples were analyzed for the presence of OAHFA in negative ion mode (NIM) HPLC-MS experiments, their major ions $(M-H)^-$ were anticipated to produce ions with *m/z* values of 729, 757 and 785, similar to human preparations. These compounds were indeed detected in canine meibum, and even in the same apparent ratios as in human samples [compare Fig. 4 and[26, 29]]. Importantly, the RT values of the $(M-H)^-$ ions *m/z* 729, 757, and 785 were the same as those of ions *m/z* 713, 741, and 769 detected in PIM. Therefore, it was reasonable to assume that the trio of major ions *m/z* 729, 757, and 785 were related to ions *m/z* 713, 741, and 769, which all derived from a series of homologous OAHFA with molecular masses 730, 758, and 786 Da. This prediction was indeed confirmed in subsequent MS experiments. The structure of canine OAHFA with molecular weight of 730 was elucidated in NIM MS^2 experiments (Fig. 4D). These MS^2 fragmentation experiments clearly demonstrated that, as with human OAHFA *m/z* 729[26, 29], the canine compound was present as a mixture of at least two coeluting isobaric compounds, namely O-oleoyl-30-hydroxytriacontenoate and O-palmitoleoyl-32-hydroxydotriacontenoate. In PIM, two co-eluting related ions were detected: an $(M + H)^+$ ion *m/z* 731 and an $(M - H_2O + H)^+$ ion *m/z* 713 (not shown). In MS^2 collision-induced (cid) fragmentation experiment m/z731@cid37V, ion *m/z* 731 produced a dehydration product ion *m/z* 713 as a major product. Thus, combining the HPLC and MS data, this unknown compound was tentatively identified as a mixture of O-oleoyl-30-hydroxytriacontenoic acid and O-palmitoleoyl-32-hydroxydotriacontenoic acid. The same patterns were observed for compounds 758 Da (O-oleoyl-32-hydroxydotriacontenoic acid/O-palmitoleoyl-34-hydroxytetratriacontenoic acid) and 786 Da (O-oleoyl-34-hydroxytetratriacontenoic acid/(O-palmitoleoyl-36-hydroxyhexatriacontenoic acid) (not shown), corroborating our hypothesis about similarities between canine and human meibum.

A related group of compounds, namely CE of OAHFA (CE-OAHFA), was also observed in the samples of both species. Their identification was based on the coelution of RP HPLC peaks detected in PIM that produced ions $(M+H)^+$, $(M + Na)^+$, $(M + K)^+$, $(M-Chl + H)^+$, and a spontaneously formed product ion $[(Chl - H_2O + H)^+$ (*m/z* 369)[24, 27]], as well as the corresponding peaks of OAHFA product ions $(M - H)^-$ and $(M - Chl - H)^-$ in NIM. To verify the structural assignments, MS^n experiments with major species of this class of compounds were also conducted. The results for a compound with molecular mass 1127.1 ($C_{77}H_{138}O_4$) are shown in Figs. 5 and 6. In PIM, the compound was registered as ion *m/z* 1127.9 $[(M + H)^+]$, while in NIM an ion *m/z* 1126.0 $[(M - H)^-]$ was detected. A series of major characteristic ions was detected in both modes. In PIM, ions *m/z* 369, 741, 759, 801, 1127, and 1170 were observed. The initial chromatographic characterization of the compound revealed that these ions, as well as ions *m/z* 757 and 1126 detected in NIM experiments, all co-eluted producing HPLC peaks with RT of 29.1 min regardless of the detection modes (Fig.5, panel A). This observation allowed us to hypothesize that they derived from the same compound. The fragmentation pattern of ion *m/z* 369 was identical to that of cholesterol and cholesteryl esters[24, 27]. The results of NIM fragmentation experiments substantiated this idea: ion *m/z* 1126 (Fig. 6) did produce critically important ions

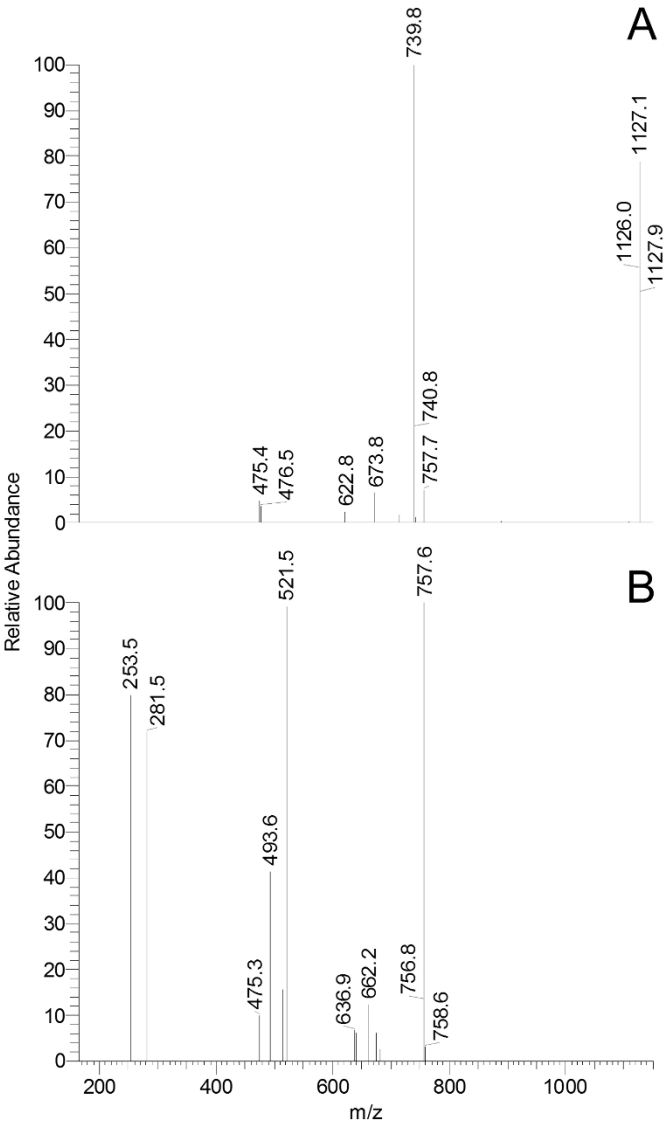

Figure 6 | NIM MS^2 and MS^3 fragmentation studies of canine CE-OAHFA *m/z* 1126. *Panel A.* Ion *m/z* 1126 was fragmented in an MS^2 experiment at a collision- induced dissociation energy of 38V (isolation width 1.2 amu). Note the formation of an ion *m/z* 757, shown in Figure 5, and of its dehydration ion *m/z* 739. *Panel B.* Ion 757 was fragmented in an NIM collision-induced experiment to produce ions *m/z* 253, 281, 493, and 521. Similarly to the data presented in Figure 4, these ions indicated the presence of two isobaric OAHFA that had palmitoleic and oleic acid as acylating components. Thus, these compounds were designated as O-oleoyl-32-hydroxydotriacontenoic and O-palmitoleoyl-34-hydroxyteratriacontenoic acids.

m/z 757 [(M – H – $C_{27}H_{44}$)$^-$, loss of cholesteryl fragment], its dehydration product *m/z* 739 [(M – H – $C_{27}H_{46}O$)$^-$, loss of cholesterol], and ion *m/z* 475 ($C_{32}H_{59}O_2$). The latter, with all likelihood, was derived from ion *m/z* 757 due to the loss of an oleic acid fragment (loss of a molecular mass of 282). The following NIM experiment [*m/z* 1126@cid38V→*m/z* 757@cid38V→*m/z* (493+281) + (521 + 253)] confirmed this conclusion: the fragmentation pattern of ion *m/z* 757 was consistent with a mixture of two isobaric compounds, O-oleoyl-32-hydroxydotriacontenoic acid (described above) and O-palmitoleoyl-34-hydroxytetratriacontenoic acid. Thus, the ion *m/z* 1127 was determined to be produced by a mixture of two isobaric compounds, cholesteryl esters of O-oleoyl-32-hydroxydotriacontenoic acid and of O-palmitoleoyl-34-hydroxytetratriacontenoic acid. These two compounds have identical theoretical monoisotopic masses of 1127.06, which results in a $(M+H)^-$ ion with a theoretical *m/z* 1128.07 (detected *m/z* 1127.6, Fig. 5), and a $(M-H)^-$ ion with a theoretical *m/z* 1126.06 (detected signals *m/z* 1126.0 and 1127.1). Adjacent peaks with RT of 27.5 and 30.6 min were identified as O-oleoyl-30-hydroxytriacontenoyl-Chl (*m/z* 1099) and O-oleoyl-34-hydroxytetratriacontenoyl-Chl (*m/z* 1155), respectively (major isoforms are indicated). Other members of the family of CE-OAHFA were also identified in the same fashion.

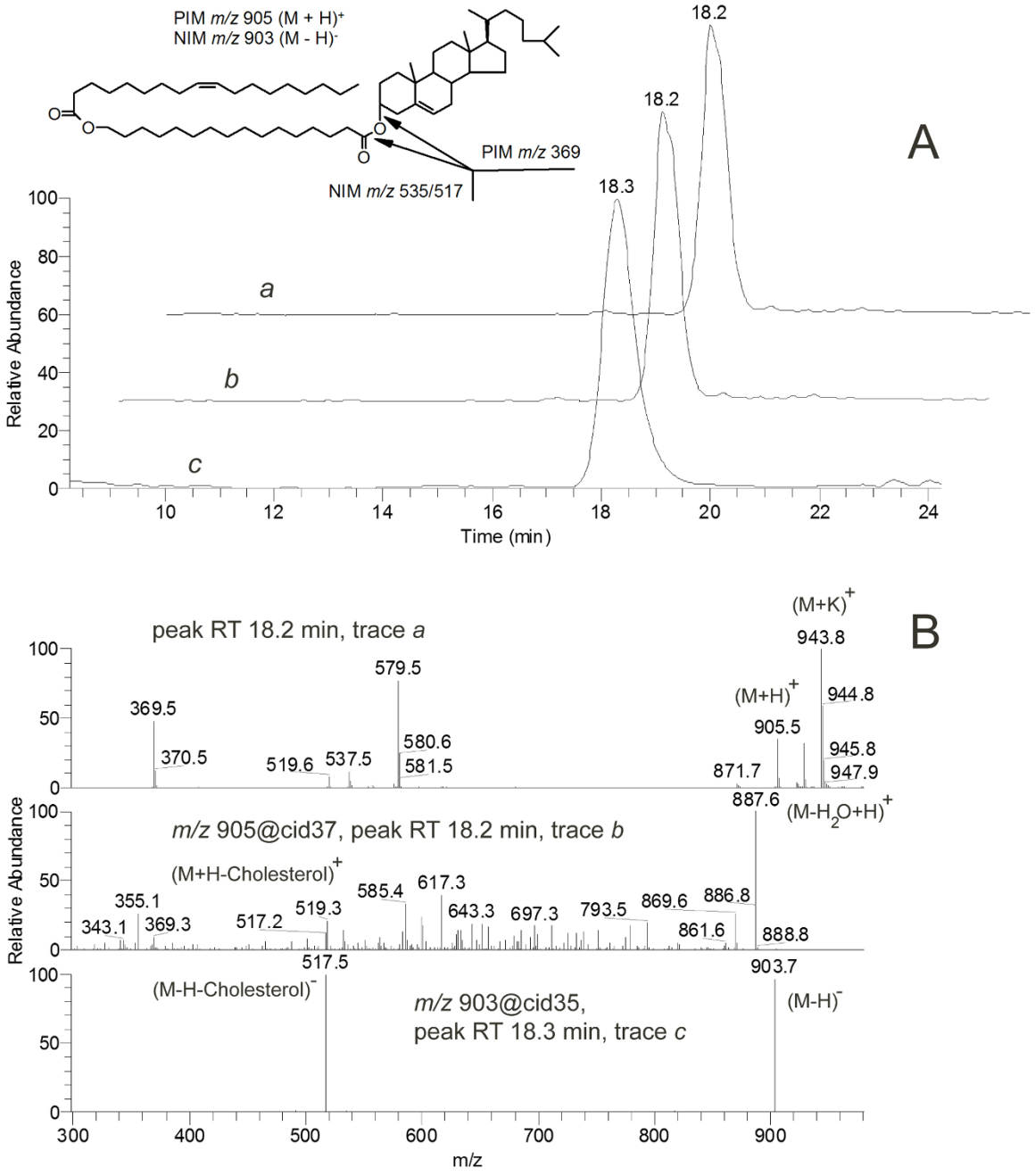

Figure 7 | Reverse phase HPLC MS^n analyses of the cholesteryl ester of O-oleoyl-16-hydroxypalmitic acid in PIM and NIM. *Panel A*. Three reverse phase chromatograms of the compound are shown. Trace ***a*** – extracted ion chromatogram of the $(M+H)^+$ ion *m/z* 905 (full scan, PIM). Trace ***b*** – HPLC trace recorded in a PIM MS^2 experiment (*m/z* 905@cid37→product ions). Trace ***c*** – HPLC trace recorded in a NIM MS^2 experiment (*m/z* 903@cid35→product ions). *Panel B*. Trace ***a*** – full MS of the compound in PIM. Trace ***b*** – MS^2 spectrum of the compound in PIM (*m/z* 905@cid37). Trace ***c*** – MS^2 spectrum of the compound in NIM (*m/z* 903@cid35). The spectra of the corresponding averaged HPLC peaks with retention times of 18.2–18.3 min are shown.

To verify the structural assignments of CE-OAHFA, a standard compound – a cholesteryl ester of O-oleoyl-16-hydroxypalmitic acid (monoisotopic mass 904.82) – was synthesized and studied by HPLC-MS^n (Fig. 7). Note that the monoisotopic masses of its $(M\text{-}H)^-$ and $(M+H)^+$ ions are *m/z* 903.82 and 905.83. The experimentally observed $(M\text{-}H)^-$ and $(M\text{-}H_2O+H)^+$ signals were, correspondingly, 903.7and 887.6. Because of its shorter ω-hydroxy fatty acid chain (C_{16} in the standard vs. C_{30}-C_{34} in meibomian CE-OAHFA), the standard compound expectedly had a shorter retention time (around 18±1 min) than those of the corresponding meibomian CE-OAHFA (between 26 and 31 min). However, it ionized and fragmented essentially the same way the meibomian lipids did. Importantly, this compound was visible in NIM as an ion with an *m/z* value of 903 $(M - H)^-$, and in PIM as ions *m/z* 905 $(M + H)^+$, 927 $(M + Na)^+$, 943 $(M + K)^+$, and 987 $(M + CH_3COOH + Na)^+$. In PIM, the spontaneously in-source generated product ions were ions *m/z* 537 $[(M + H - C_{27}H_{44})^+$, loss of cholesteryl moiety], *m/z* 519 $[(M + H - C_{27}H_{46}O)^+$, loss of cholesterol], and 579 (acetic acid adduct of ion *m/z* 519). In an MS/MS experiment *m/z* 903.7@cid35V conducted in NIM, the important characteristic fragments of the standard were ions *m/z* 535 $[(M - C_{27}H_{44} - H)^-$, i.e. loss of the cholesteryl moiety] and *m/z* 517 $[(M - C_{27}H_{44} - H_2O - H)^-$, i.e. dehydrated ion *m/z* 535). In a PIM MS/MS experiment *m/z* 905.7@cid47V, the detected characteristic fragments were ions *m/z* 887 $(M + H - H_2O)^+$, 869 $(M + H - 2H_2O)^+$, 519 $(M - \text{cholesterol})^+$, 369 ($C_{27}H_{44}$, i.e. cholesteryl fragment) and 355 (loss of an additional methylene group).

The last group of compounds evaluated in this study was CE. These were analyzed using reverse phase HPLC (RP HPLC) instead of NP HPLC because the former more effectively separated individual CE species on the basis of the lengths of their fatty acid chains[24, 27]. Canine meibum was found to contain the same CE as human meibum, though the ratios of their individual CE components were not identical (Fig. 8). The single most abundant CE in cMGS was a $C_{25:0}$-CE, with $C_{24:0}$-CE and $C_{26:0}$-CE being present in much smaller quantities. In hMGS these three CE were detected in comparable quantities. Nonetheless, a bell-shaped distribution of CE of different molecular weights, ranging from $C_{16:1}$-CE to $C_{34:1}$-CE and centered around a $C_{25:0}$-CE, was characteristic of both biological species.

The meibum lipidomes of all 10 tested dog breeds were found to be qualitatively similar and differed only in the relative quantities of a few lipid classes (Fig. 9A). The lipids detected in a sample from a Tibetan mastiff are presented in Fig. 9B. Clearly, the mastiff sample closely resembles an average cMGS sample. The major lipid species observed in cMGS are summarized in Table 1. A similar approach has been previously used to establish a baseline for hMGS studies. It has been demonstrated that the intersample variability of the human samples was of the same magnitude[30].

Discussion

The HPLC-MS experiments described above allowed us, for the first time, to establish a comparative baseline for lipidomic analysis of canine meibum. They also showed that the major individual lipid species found in canines were structurally related to those of human meibomian gland secretions. The absence of exact chemical standards for OAHFA and CE-OAHFA made it impossible to determine their actual concentration in meibum. However, this did not preclude us from comparing human and canine samples side-by-side. The main conclusion that can be drawn from the results of this project is that canine meibum is biochemically close to, but not identical with, the human one. This similarity becomes apparent upon a side-by-side inspection of the mass spectra of both the excreta, and is reinforced by results of a structural evaluation of the detected compounds. The major lipid classes identified in the canine meibum in these experiments were very long chain CE, WE, OAHFA, and CE-OAHFA [or ω-Type I-St, using the classification of Nicolaides and Santos[16]]. These are the same types of molecules that have previously been found in human and steer samples[16], and in a range of other animals, including rodents, bovine, and marsupials (Butovich IA, et al. *Invest. Ophth. Vis. Sci*, 2009; 50:ARVO E-abstract 2545), gorilla (Wojtowicz JC, et al. *Invest. Ophth. Vis. Sci.*, 2009;

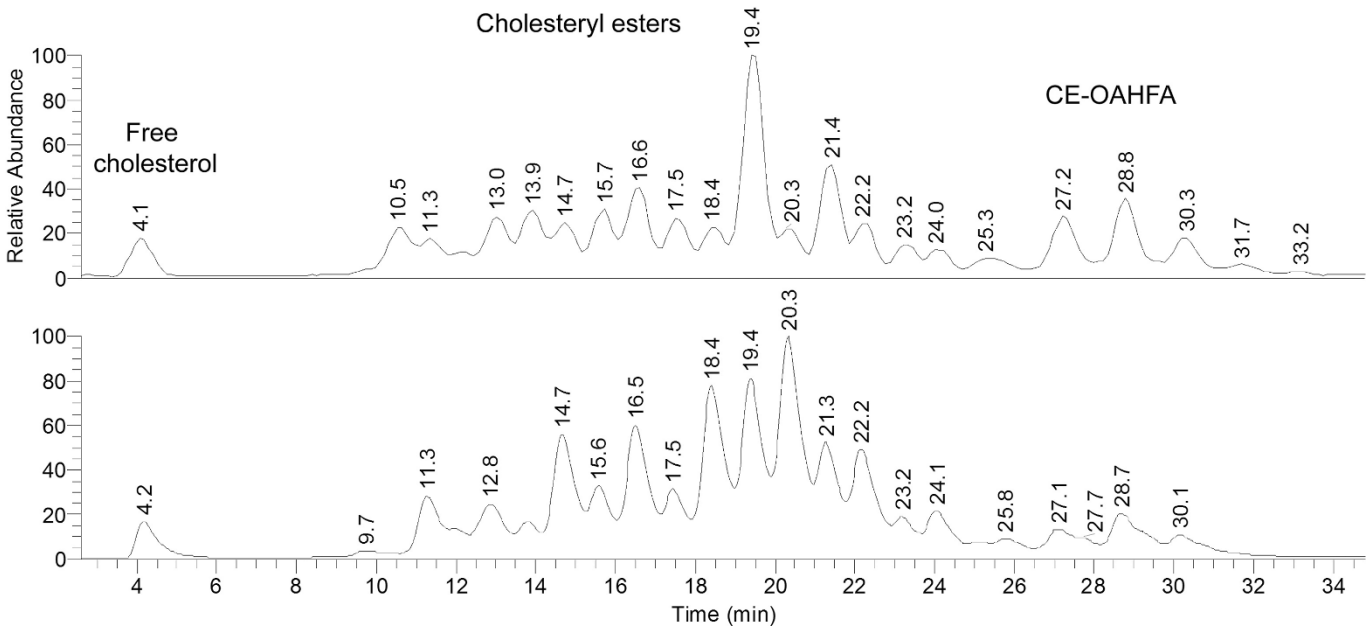

Figure 8 | Comparative reversed phase HPLC-PIM APCI MS analyses of CE present in canine and human meibum. The traces of ion *m/z* 369 were extracted from total ion chromatograms of canine (upper trace) and human (lower trace) meibum recorded in PIM. The following major Chl-containing lipids were identified in both types of samples: peak RT 4.1(±0.1) min – free cholesterol; 10.5 min – $C_{16:1}$-CE; 11.3 min – $C_{18:1}$-CE; 12.8 min – $C_{20:1}$-CE; 13.9 min – $C_{19:0}$-CE; 14.7 min – $C_{20:0}$-CE and $C_{22:1}$-CE; 15.7 min – $C_{21:0}$-CE; 16.5 min – $C_{22:0}$-CE and $C_{24:1}$-CE; 17.5 min – $C_{23:0}$-CE; 18.4 min – $C_{24:0}$-CE and $C_{26:1}$-CE; 19.4 min – $C_{25:0}$-CE; 20.3 min – $C_{26:0}$-CE and $C_{28:1}$-CE; 21.3 min – $C_{27:0}$-CE; 22.2 min – $C_{28:0}$-CE and $C_{30:1}$-CE; 24.1 min – $C_{30:0}$-CE and $C_{32:1}$-CE; 25.3 min – $C_{34:1}$-CE; 27.2 min – $C_{18:1}$-(O-$C_{30:1}$)-Chl; 28.8 min – $C_{18:1}$-(O-$C_{32:1}$)-Chl; 30.2 min – $C_{18:1}$-(O-$C_{34:1}$)-Chl.

51:ARVO E-abstract 4169), and dogs (Eule JC et al. *Invest. Ophth. Vis. Sci.*, 2010; 51:ARVO E-abstract 4159). The existence of these compounds has been re-confirmed by Chen et al[31], though no authentic standards were used in the latter paper to confirm structural assignments of the observed MS signals.

The surprising finding that CE-OAHFA can be observed and studied in NIM MS experiments (Figs. 5–7) greatly facilitated their analyses as it allowed us to align chromatograms of the samples obtained in PIM and NIM to crosscheck the retention times of the analytes. This, in turn, facilitated product identification by reducing (or eliminating altogether) the odds of detecting false positives caused by co-eluting isobaric compounds of other types. The mechanism of ionization of CE-OAHFA in NIM is unknown at this moment, and its exploration goes beyond the scope of this paper. However, one can speculate that ionization of CE-OAHFA in NIM occurs through an intramolecular interaction of one of its carbonyl groups with one of its methylene groups, or at a C2 position of either of the fatty acid chains in the CE-OAHFA, which also involves one of the carbonyl oxygen atoms of CE-OAHFA.

In our hands, even the unknown canine compounds with *m/z* values 900–1100 produced patterns resembling those of human meibomian gland secretions (Fig. 1), though their relative amounts are higher in canines than in humans.

As with human meibum, accurate quantitation of the detected lipids is impossible at this time due to the lack of standards of many compounds needed to generate their respective calibration curves. It is absolutely necessary to use individual calibration curves for each of the compounds that are to be quantified because different classes of

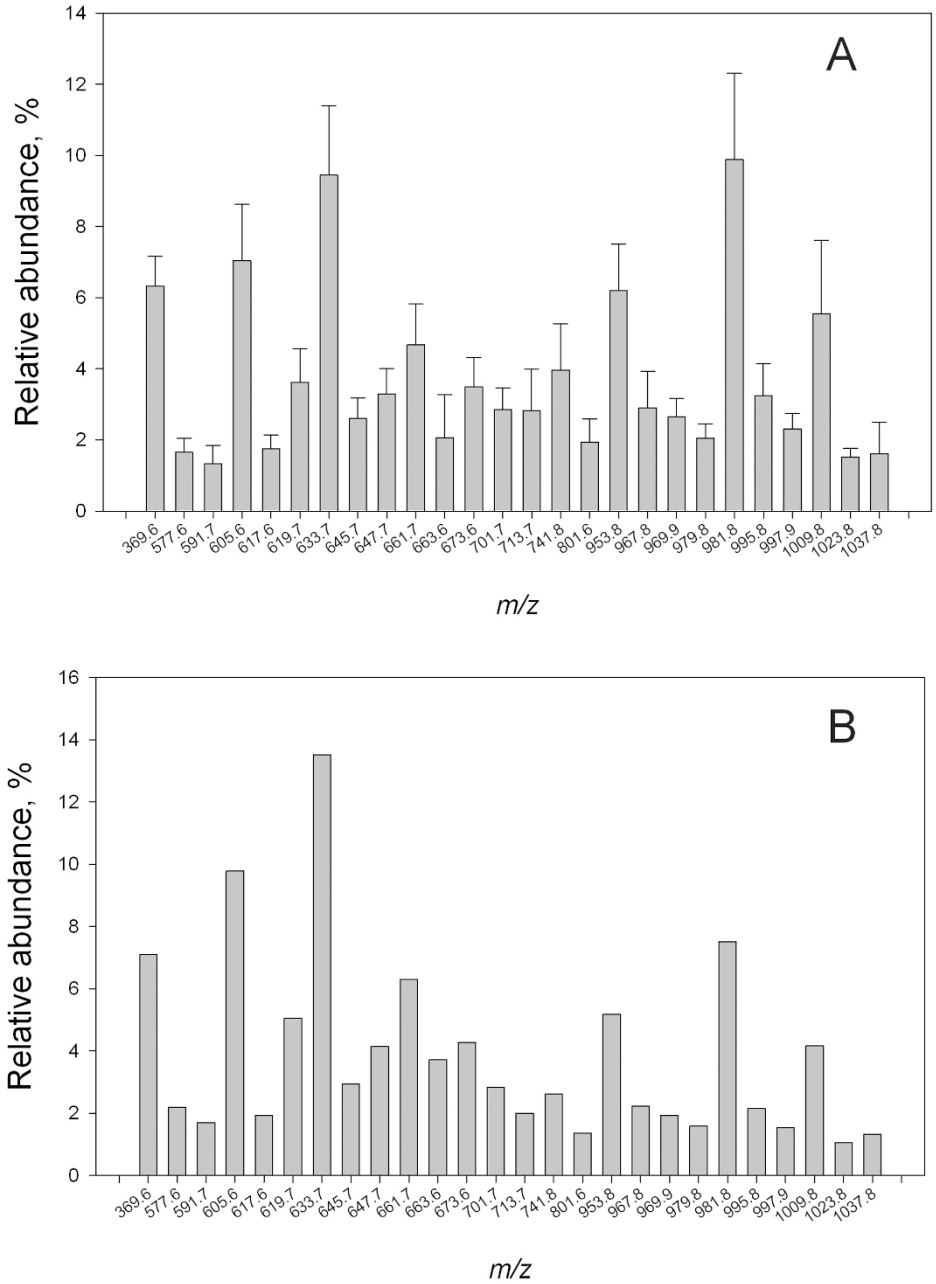

Figure 9 | PIM APCI mass spectrum of major lipid species detected in canine meibum samples. *Panel A.* Twenty-six major lipid signals detected in the samples collected from ten canine breeds have been averaged. Initially, each sample was analyzed individually by HPLC-MS in triplicates. Thirty major signals were compared between the breeds, and the 26 signals found in every breed were then averaged and plotted. Four signals were attributed to sporadic contaminations and/or unknowns. The results are presented as Median ± Standard Deviation (n = 10). The sum of all 26 signals is 100%. *Panel B.* For comparison purposes, the PIM APCI MS of the Tibetan mastiff is shown.

Table 1 | Canine and human meibomian lipids detected in the PIM and NIM APCI HPLC-MS^n experiments[1]

Meibomian lipids detected in the PIM HPLC-MS experiments

Experimental *m/z*[2]	Proposed molecular formula $(M + H)^+$	Theoretical monoisotopic *m/z*	Compound
369.58	$C_{27}H_{45}$	369.35	Chl, all CE
577.64	$C_{39}H_{77}O_2$	577.59	$C_{21:0}$-$C_{18:1}$-WE
			$C_{23:0}$-$C_{16:1}$-WE
591.68	$C_{40}H_{79}O_2$	591.61	$C_{22:0}$-$C_{18:1}$-WE
605.64	$C_{41}H_{81}O_2$	605.62	$C_{23:0}$-$C_{18:1}$-WE
			$C_{25:0}$-$C_{16:1}$-WE
617.65	$C_{42}H_{81}O_2$	617.62	$C_{24:0}$-$C_{18:2}$-WE
619.67	$C_{42}H_{83}O_2$	619.64	$C_{24:0}$-$C_{18:1}$-WE
			$C_{26:0}$-$C_{16:1}$-WE
633.66	$C_{43}H_{85}O_2$	633.65	$C_{25:0}$-$C_{18:1}$-WE
			$C_{27:0}$-$C_{16:1}$-WE
645.67	$C_{44}H_{85}O_2$	645.65	$C_{26:0}$-$C_{18:2}$-WE
647.68	$C_{44}H_{87}O_2$	647.67	$C_{26:0}$-$C_{18:1}$-WE
			$C_{28:0}$-$C_{16:1}$-WE
661.67	$C_{45}H_{89}O_2$	661.69	$C_{27:0}$-$C_{18:1}$-WE
			$C_{29:0}$-$C_{16:1}$-WE
663.63	Unknown/contamination		Unknown/contamination
673.65	$C_{46}H_{89}O_2$	673.69	$C_{28:0}$-$C_{18:2}$-WE
689.63	$C_{47}H_{93}O_2$	689.72	$C_{29:0}$-$C_{18:1}$-WE
701.72	$C_{48}H_{93}O_2$	701.72	$C_{30:0}$-$C_{18:2}$-WE
713.74	$C_{49}H_{93}O_2$	713.72	$C_{31:0}$-$C_{18:2}$-WE
731.66 and 713.72	$C_{48}H_{91}O_4$ and $C_{48}H_{89}O_3$	731.69 and 713.68	$C_{18:1}$-(O-$C_{30:1}$) OAHFA
759.65 and 741.69	$C_{50}H_{95}O_4$ and $C_{50}H_{93}O_3$	759.72 and 741.71	$C_{18:1}$-(O-$C_{32:1}$)-OAHFA
787.70 and 769.77	$C_{52}H_{99}O_4$ and $C_{52}H_{97}O_3$	787.75 and 769.74	$C_{18:1}$-(O-$C_{34:1}$)-OAHFA
900–1100	undetermined	undetermined	unknowns
1099.17	$C_{75}H_{134}O_4$	1099.03	$C_{18:1}$-(O-$C_{30:1}$)-Chl
1127.05	$C_{77}H_{138}O_4$	1127.06	$C_{18:1}$-(O-$C_{32:1}$)-Chl
1155.21	$C_{79}H_{142}O_4$	1155.09	$C_{18:1}$-(O-$C_{34:1}$)-Chl

Meibomian lipids detected in the NIM HPLC-MS experiments

Experimental *m/z*	Proposed molecular formula $(M - H)^-$	Theoretical monoisotopic *m/z*	Compound
729.80	$C_{48}H_{89}O_4$	729.68	$C_{18:1}$-(O-$C_{30:1}$)-OAHFA
757.84	$C_{50}H_{93}O_4$	757.71	$C_{18:1}$-(O-$C_{32:1}$)-OAHFA
785.81	$C_{52}H_{97}O_4$	785.74	$C_{18:1}$-(O-$C_{34:1}$)-OAHFA

Meibomian cholesteryl esters detected as ions m/z 369 (PIM) and acetic acid adducts of their FA moieties (NIM, shown below)

Experimental *m/z*	Adduct $(FA + acetate)^-$	Theoretical monoisotopic *m/z*	Compound
301.20	$C_{15:0} + Ac^-$	301.24	$C_{15:0}$-CE
313.19	$C_{16:1} + Ac^-$	313.24	$C_{16:1}$-CE
329.26	$C_{17:0} + Ac^-$	329.27	$C_{17:0}$-CE
341.16	$C_{18:1} + Ac^-$	341.27	$C_{18:1}$-CE
357.08	$C_{19:0} + Ac^-$	357.30	$C_{19:0}$-CE
369.07	$C_{20:1} + Ac^-$	369.30	$C_{20:1}$-CE
383.00	$C_{21:0} + Ac^-$	383.32	$C_{21:0}$-CE
396.99	$C_{22:1} + Ac^-$	395.32	$C_{22:1}$-CE
413.03	$C_{23:0} + Ac^-$	413.36	$C_{23:0}$-CE
425.06	$C_{24:1} + Ac^-$	425.36	$C_{24:1}$-CE
441.00	$C_{25:0} + Ac^-$	441.39	$C_{25:0}$-CE
453.25	$C_{26:1} + Ac^-$	453.39	$C_{26:1}$-CE
455.12	$C_{26:0} + Ac^-$	455.41	$C_{26:0}$-CE
469.35	$C_{27:0} + Ac^-$	469.42	$C_{27:0}$-CE
481.01	$C_{28:1} + Ac^-$	481.43	$C_{28:1}$-CE
497.00	$C_{29:0} + Ac^-$	495.44	$C_{29:0}$-CE
509.24	$C_{30:1} + Ac^-$	509.46	$C_{30:1}$-CE
523.23	$C_{31:1} + Ac^-$	523.47	$C_{31:1}$-CE
537.19	$C_{32:1} + Ac^-$	535.47	$C_{32:1}$-CE

[1]Compounds were detected in both types of samples. Major isobaric compounds are shown. Other isobaric forms of listed WE, OAHFA, and CE are likely to exist
[2]Average of three to five measurements
Ac^- – acetate; Chl – cholesterol; CE – cholesteryl esters; OAHFA – (O-acyl)-omega-hydroxy fatty acids; WE – wax esters. An example of labeling the cholesteryl esters of OAHFA: $C_{18:1}$-(O-$C_{30:1}$)-Chl is (30-O-oleoyl)-triacontenoic acid.

compounds may produce MS signals of different intensities even if the compounds are present in the same molar amounts. This means that the most intense signal in a spectrum does not automatically identify that compound as the most abundant species in the mixture due to differences in ionization efficiencies of different molecules. This is not a unique deficiency of mass spectrometry: the same rule applies to every analytical technique with the exception of gravimetric analysis. Indeed, molecular absorptivity (ε_m) in ultraviolet, visible range, and infrared spectroscopies, or the quantum yield in fluorescence spectroscopy, or staining ability of dyes in thin layer chromatography are all compound-specific. Experiments are in progress to obtain the quantitative information on canine meibomian lipidome.

Nevertheless, it is possible to compare various canine samples with each other and with human samples in terms of the *relative* amounts

of individual lipids *if* the conditions of the analyses are kept identical, preferably in side-by-side experiments, as they were compared in our studies. Our experiments have demonstrated that canine samples from various breeds are similar, but not identical to each other (manuscript in preparation). The major difference observed was in the relative amount of unknown compounds *m/z* 900–1100 (Fig. 1). Canine meibum has a relatively larger proportion of OAHFA (minor HPLC with RT 4–6 min, Fig.1A) than human meibum. Assuming that OAHFA, whose proposed role in the human TF is to form an amphiphilic barrier between the highly nonpolar majority of meibomian lipids and the aqueous layer of the TF, are the major amphiphilic lipids found in human meibum, this observation could be related to a somewhat higher stability of canine TF, and their slightly longer interblink intervals.

An interesting observation has been made with regard to the relative intensities of WE signals in dogs and humans. A highly reproducible pattern of WE signals *m/z* 605↑, 619↓, 633↑, 647↓, 661↑ for canine meibum and an inverse pattern 605↓, 619↑, 633↓, 647↑, 661↓ for human MGS have been observed in each and every tested sample. The difference is apparently based on peculiarities of the respective lipid biosynthetic pathways in canines and humans. The relative ratio of the WE may be regulated by the substrate specificity of the enzymes involved in WE biosynthesis or by the relative availability of acetyl-CoA and propanyl-CoA in the meibomian gland, or by one of a number of other reasons. At this moment, we do not know which biosynthetic step is critical for producing this outcome, and whether the corresponding fatty alcohols are straight or branched. However, in previous publications on animal and human meibum many fatty alcohols and fatty acids were reported to be branched[16, 18, 32–34]. We expect these and other questions to be answered in future experiments.

The observed similarities of the lipidomes of dogs and humans might have a connection with the previously reported similarities of their TF stability measured as TFBUT[22, 23]. Indeed, depending on race, an average TFBUT of a healthy, non-dry eye human population varies from below 10 to at least 20 sec with the range extending from 5 sec to as high as 35 sec[35, 36]. These numbers are well within those observed for dogs of various breeds and age[22, 23]. Canine ocular structures have been shown to produce soluble and membrane-bound mucins[37], just like human ocular tissues. The pH values of aqueous tears of dogs [around 7.2[23]] and humans [about 7.4[38]] are also similar. Thus, the overall environment in which canine meibum forms a TFLL is similar to that of humans.

In conclusion, it seems that because of apparent biochemical similarities between meibomian gland secretions of dogs and humans, dogs could be used as a suitable animal model for human meibomian gland, tear film, and dry eye studies, at least in those concerned with lipid metabolism. The large presence of various CE-OAHFA in canine meibum, many of which have also been found in humans though in much smaller quantities, has facilitated the structural characterization of CE-OAHFA and helped to advance our understanding of the lipid biochemistry of human and animal ocular surface.

Methods

All reagents used in the study were of analytical grade, or better. Organic solvents of HPLC or spectroscopy grades were products of Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Muskegon, MI). Lipid standards, listed in our previous publications[24–30], were manufactured by Sigma-Aldrich and Nu-Chek Prep, Inc. (Elysian, MN). Cholesteryl ester of O-oleoyl-16-hydroxypalmitic acid was synthesized from O-oleoyl-16-hydroxypalmitate [described earlier[26]], and free cholesterol. The former was initially converted into O-oleoyl-16-hydroxypalmitoyl chloride in a reaction with thionyl chloride, and then the product was used to acylate free cholesterol. The cholesteryl ester of O-oleoyl-16-hydroxypalmitic acid was isolated by RP-HPLC using a protocol described earlier[24, 26, 27]. Only stainless steel, platinum, glass, and Teflon® were ever in contact with collected samples.

Ophthalmic evaluation of the canine subjects was performed at the Freie Universität in Berlin using slit lamp biomicroscopy, interferometry, meibometry, Schirmer's tear tests, and blood chemistry including Chl, triglyceride and thyroid globulin. Canines with health problems and ocular abnormalities were excluded from the study. The animals were treated in accordance with the Guidelines of the Freie Universität for research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In order to establish a baseline for our compositional cMGS studies, which would be representative of an "average" cMGS, meibum specimens from dogs of 10 different breeds (1 to 9 years old, both genders) were studied. Studied canines included Belgian shepherd crossbreed, dachshund crossbreed, do khyi, German sheep dog crossbreed, giant schnauzer, husky, poodle crossbreed, poodle, schnauzer crossbreed, and a terrier crossbreed. A detailed report on the similarities and differences of these 10 breeds will be published elsewhere. Meibum was soft-squeezed from meibomian glands and collected using a spatula as described for humans[25]. The meibum samples were quantified gravimetrically using a microbalance. The samples were stored in glass vials at subzero temperatures in a dry state. Then, the samples were evaluated using ion trap atmospheric pressure chemical ionization HPLC-MS as described before[24–30]. Just before the analyses, the samples were reconstituted in 1 mL of hexane:propan-2-ol solvent mixture (1 : 1, v/v). Because of the sheer amount of labor needed to conduct the analytical HPLC-MS experiments, the Tibetan mastiff (do khyi) meibum was used for structural characterization of most of the lipids. Its chemical composition was found to be very close to the "average" canine meibum determined for ten studied breeds of dogs, which is discussed in the Results section. However, selected types of lipids (e.g. sterols and steryl esters) were analyzed for every breed, and were found to be structurally identical.

Briefly, two different types of HPLC experiments were conducted – normal phase and reversed phase HPLC. The NP HPLC analysis was optimized to separate *lipid classes*, while RP HPLC was designed to separate the lipids on the basis of their overall *hydrophobicity*.

The NP HPLC analysis of the samples was performed on a Diol silica gel column (3.2 × 150 mm, 5 μm particles, manufactured by Phenomenex, Torrance, CA). The mobile phase was an isocratic mixture of *n*-hexane (95%, by vol), propan-2-ol (4.9%, by vol), and glacial acetic acid (0.1%, by vol). The column was equilibrated at 30°C. The flow rate was maintained at 0.3 mL/min. Between 1 and 10 μL of the meibum stock solution (100 μg/mL) was injected. The RP HPLC analysis of canine meibum was conducted on a Hypersil C18 silica gel column (2.1 × 150 mm, 5 μm particles, ThermoFisher, San Jose, CA) in a *n*-hexane-acetonitrile-aqueous acetic acid gradient solvent system as described earlier[24, 26, 27]. In both procedures, the analytes were monitored and identified mass spectrometrically in the range of mass-to-charge (*m/z*) ratios of 150 to 2000 in the PIM (unless stated otherwise) using the atmospheric pressure chemical ionization technique.

The results were plotted as total ion chromatograms of all detected *m/z* signals, and/or as extracted chromatograms of particular ions of interest with specific *m/z* values. In certain cases, to improve the sensitivity of the analyses, single ion monitoring of ions of interest was used, e.g. of ions *m/z* 369 of Chl and Chl esters (CE)[24, 27, 28].

For comparison purposes, three samples of human meibum from three healthy donors (two males 49 and 28 years old, and one female, 33 years old) with no ocular pathologies were analyzed alongside the canine samples under exactly the same experimental conditions. The tenets of the Declaration of Helsinki were adhered to during the collection, storage, and analysis of human samples. The procedures were approved by the University of Texas Southwestern Medical Center Institutional Review Board. The canine and human samples were compared with authentic lipid standards, where possible.

1. Nicolaides, N., Kaitaranta, J. K., Rawdah, T. N., Macy, J. I., Boswell, F. M., & Smith, R. E. Meibomian gland studies: comparison of steer and human lipids. *Invest. Ophthalmol. Vis. Sci.* **20,** 522–536 (1981).
2. Tiffany, J. M. The normal tear film. *Dev. Ophthalmol.* **41,** 1–20 (2008)
3. Ohashi, Y., Dogru, M., & Tsubota, K. Laboratory findings in tear fluid analysis. *Clin. Chim. Acta.* **369,** 17–28 (2006).
4. King-Smith, P. E., Fink, B. A., Hill, R. M., Koelling, K. W., & Tiffany, J. M. The thickness of the tear film. *Curr. Eye Res.* **29,** 357–368 (2004).
5. Butovich, I. A. The Meibomian puzzle: combining pieces together. *Prog. Retin. Eye Res.* **28,** 83–498 (2009).
6. Barabino, S., Chen, W., & Dana, M. R. Tear film and ocular surface tests in animal models of dry eye: uses and limitations. *Exp. Eye Res.* **79,** 613–621 (2004).
7. Baron, C., & Blough, H. A. Composition of the neutral lipids of bovine meilbomian secretions. *J. Lipid Res.* **17,** 373–376 (1976).
8. Tiffany, J. M. The meibomian lipids of the rabbit. I. Overall composition. *Exp. Eye Res.* **29,** 195–202 (1979).
9. Tiffany, J. M., & Marsden, R. G. The meibomian lipids of the rabbit. II. Detailed composition of the principal esters. *Exp. Eye Res.* **34,** 601–608 (1982).
10. McFadden, W. H., Bradford, D. C., Eglinton, G., Hajlbrahim, S. K., & Nicolaides, N. Application of combined liquid chromatography/mass spectrometry (LC/MS): analysis of petroporphyrins and meibomian gland waxes. *J. Chromatogr. Sci.* **17,** 518–522 (1979).
11. Nicolaides, N., Santos, E. C., Smith, R. E., & Jester, J. V. Meibomian gland dysfunction. III. Meibomian gland lipids. *Invest. Ophthalmol. Vis. Sci.* **30,** 946–951 (1989).
12. Jester, J. V., Nicolaides, N., & Smith, R. E. Meibomian gland studies: histologic and ultrastructural investigations. *Invest. Ophthalmol. Vis. Sci.* **20,** 537–547 (1981).
13. Nicolaides, N., & Ruth, E. C. Unusual fatty acids in the lipids of steer and human meibomian gland excreta. *Curr. Eye Res.* **2,** 93–98 (1982).

14. Nicolaides, N., Santos, E. C., & Papadakis, K. Double-bond patterns of fatty acids and alcohols in steer and human meibomian gland lipids. *Lipids.* **19,** 264–277 (1984).
15. Nicolaides, N., Santos, E. C., Papadakis, K., Ruth, E. C., & Muller, L. The occurrence of long chain alpha, omega-diols in the lipids of steer and human meibomian glands. *Lipids.* **19,** 990–993 (1984).
16. Nicolaides, N., & Santos, E. C. The di- and triesters of the lipids of steer and human meibomian glands. *Lipids.* **20,** 454–467 (1985).
17. Jester, J. V., Nicolaides, N., & Smith, R. E. Meibomian gland dysfunction. I. Keratin protein expression in normal human and rabbit meibomian glands. *Invest. Ophthalmol. Vis. Sci.* **30,** 927–935 (1989).
18. Kolattukudy, P. E., Rogers, L. M., & Nicolaides, N. Biosynthesis of lipids by bovine meibomian glands. *Lipids.* **20,** 468–474 (1985).
19. Blount, W. P. Studies of the movements of the eyelids of animals: Blinking. *Quart. J. Exp.Physiol.* **18,** 112–125 (1927).
20. Gum, C. G., Gelatt, K. N., Esson, D. W. Physiology of the Eye. *Veterinary Ophthalmology.* 4th edn. (ed. Gellat, K. N.) Blackwell Publishing, Ames, Iowa, 149–182 (2007).
21. Carrington, S. D., Bedford, P. G. C., Guillon, J. P., & Woodwards, E. G. Polarized light biomicroscopic observations on the precornial tear film in dogs. *J. Small Anim. Pract.* **28,** 605–602 (1987).
22. Saito, A., & Kotani, T. Estimation of lacrimal level and testing methods on normal beagles. *Vet. Ophthalmol.* **4,** 7–11 (2001).
23. Saito, A., Izumisawa, Y., Yamashita, K., & Kotani, T. The effect of third eyelid gland removal on the ocular surface of dogs. *Vet. Ophthalmol.* **4,** 13–18 (2001).
24. Butovich, I. A. Fatty acid composition of cholesteryl esters of human meibomian gland secretions. *Steroids.* **75,** 726–733 (2010).
25. Butovich, I. A. Lipidomic analysis of human meibum using HPLC-MSn. *Methods Mol. Biol.* **579,** 221–246 (2009).
26. Butovich, I. A., Wojtowicz, J. C., & Molai, M. Human tear film and meibum. Very long chain wax esters and (O-acyl)-omega-hydroxy fatty acids of meibum. *J. Lipid Res.* **50,** 2471–2485 (2009).
27. Butovich, I. A. Cholesteryl esters as a depot for very long chain fatty acids in human meibum. *J. Lipid Res.* **50,** 501–513 (2009).
28. Butovich, I. A., Uchiyama, E., & McCulley, J. P. Lipids of human meibum: mass-spectrometric analysis and structural elucidation. *J. Lipid Res.* **48,** 2220–2235 (2007).
29. Butovich, I. A. On the presence of (O-acyl)-omega-hydroxy fatty acids and of their esters in human meibomian gland secretions. *Invest. Ophthalmol. Vis. Sci.* **52,** 639–641 (2011).
30. Butovich, I. A., Uchiyama, E., Di Pascuale, M. A., & McCulley, J. P. Liquid chromatography-mass spectrometric analysis of lipids present in human meibomian gland secretions. *Lipids.* **42,** 765–776 (2007).
31. Chen, J., Green-Church, K. B., & Nichols, K. K. Shotgun lipidomic analysis of human meibomian gland secretions with electrospray ionization tandem mass spectrometry. *Invest. Ophthalmol. Vis. Sci.* **51**, 6220–6231 (2010)
32. Joffre, C., Souchier, M., Gregoire, S., Viau, S., Bretillon, L., Acar, N., Bron, A. M., & Creuzot-Garcher, C. Differences in meibomian fatty acid composition in patients with meibomian gland dysfunction and aqueous-deficient dry eye. *Br. J. Ophthalmol.* **92,** 116–119 (2008).
33. Duerden, J. M., & Tiffany, J. M. Lipid synthesis in vitro by rabbit Meibomian gland tissue and its inhibition by tetracycline. *Biochim. Biophys. Acta.* **1042**, 13–18 (1990).
34. Harvey, D. J.,& Tiffany, J. M. Identification of meibomian gland lipids by gas chromatography-mass spectrometry: application to the meibomian lipids of the mouse. *J. Chromatogr.* **301,** 173–187 (1984).
35. Briggs, S. T. Tear breakup time in normal Saudi population: age and gender. *Intl. Contact Lens Clinic.* **25,** 137–141 (1998).
36. Elliott, M., Fandrich, H., Simpson, T., & Fonn, D. Analysis of the repeatability of tear break-up time measurement techniques on asymptomatic subjects before, during and after contact lens wear. *Contact Lens Anterior Eye.* **21,** 98–103 (1998).
37. Hicks, S. J., Carrington, S. D., Kaswan, R. L., Adam, S., Bara, J., & Corfield, A.P. Demonstration of discrete secreted and membrane-bound ocular mucins in the dog. *Exp. Eye Res.* **64,** 597–607 (1997).
38. Fischer, F. H., & Wiederholt, M. Human precorneal tear film pH measured by microelectrodes. *Graefes Arch. Clin. Exp. Ophthalmol.* **218,** 168–170 (1982).

Acknowledgements

This work was supported in part by an NIH grant R01EY019480 (to IAB) and an unrestricted grant from Research to Prevent Blindness (New York, NY).

Author contributions

IAB was responsible for the analytical part of the experiments, human meibum collection and evaluation, and writing the manuscript. AMB performed the canine meibum collection and participated in the evaluation of canines. JCE was responsible for the selection of canine subjects, their ophthalmic evaluation, and contributed to writing and editing of the manuscript.

Additional information

Competing financial interests: To comply with the Journal's policy on duality of interests, IAB would like to declare that this work was supported solely by his NIH grant R01EY019480. IAB has a sponsored research contract with Alcon Laboratories (USA), and a consulting agreement with Pfizer (USA). The manuscript is based solely on the results obtained within the framework of his current NIH R01 project, and is not related to any other projects in his laboratory. JCE and AMB declare no financial interests.

License: This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivative Works 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

How to cite this article: Butovich, I.A., Borowiak, A.M. & Eule, J.C. Comparative HPLC-MS^n analysis of canine and human meibomian lipidomes: many similarities, a few differences. *Sci. Rep.* **1**, 24; DOI:10.1038/srep00024 (2011).