generated fragmentation products of more complex lipids. In addition, the HPLC-MS methods adopted in our laboratory are designed to provide quantitative results, as we use standard curves to quantify the analytes where possible (see, e.g., our recent paper on CEs[6]). Our HPLC-MS approach used for FFA quantitation showed that their total content in normal human meibum, though varying from sample to sample, was typically <0.1% (weight/weight).

Thus, the FA signals reported by Chen et al. most likely were in situ–generated artifacts—namely, products of the spontaneous in-source fragmentation of more complex meibomian lipids such as CEs, TAGs, and di- and tri-esters. This observation is critical for our understanding of the chemical composition of normal human meibum and the role of FFAs in tear film physiology and pathology.

Another related problem seems to be the presence of a mixture of isobaric C16:1- and C18:1-based WEs reported by Chen et al. According to their report (see Fig. 3 of their paper online), C16:1-WE could be present in quantities approaching those of isobaric C18:1-WE. This was not the case in our HPLC-MS experiments, in which the palmitoleic acid–based WEs were indeed detected, but in quantities typically below 10% of their C18:1-based counterparts. In fact, the typical presence of C16:1-WE was so small that an analysis was deemed to be unnecessary at the time. Again, the HPLC step greatly facilitated identification of the WEs. Thus, the claimed observation of the intense product peaks of C16:1-WE could be partly a result of the presence of unknown isobaric compounds (not necessarily of the WE nature) with palmitoleic acid in their structures that had not been chromatographically separated from WE before the MS analysis.

A few minor comments concern mostly the diligence in referring to the recent work done in the area. (1) The FA composition of meibomian CEs described by Chen et al.[1] closely matched the one that had already been described in an earlier paper on the topic,[6] where exactly the same major saturated FAs (C24:0, C25:0, and C26:0) were reported and quantified. (2) The possible role of OAHFAs, exactly as described by Chen et al., had already been described in a recent review.[8] Unfortunately, these two papers were not referenced and discussed by Chen et al.

In conclusion, it seems that the experimental approach described by Chen et al., though very informative, still has limitations that can lead to inadvertent but still erroneous interpretation of the data: In this case, a 10- to a 100-fold overestimation of the presence of endogenous FFAs and a large overestimation of the presence of C16:1-WE in normal human meibum.

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Author Response: On the Presence and Role of Polar Lipids in Meibum

The truth often comes from debating. We are glad to discuss the issues Dr. Butovich et al. has raised about our paper.[1] We recognize Dr. Butovich's contribution to the identification and quantitation of wax esters and cholesteryl esters. However, it is important to gather information on all the major species in meibomian lipids.

The first comment was regarding the origin of the free fatty acids (FFAs) that we observed in normal human meibum. Dr. Butovich suggested that the FFAs we reported were from in-source decay of complex lipids, such as cholesteryl esters, triacylglycerols, and di- and triesters. We also thought about this when reading the paper of Butovich,[2] published recently (made available on the publisher's website on May 12, 2010, 1 month after the submission of our manuscript). We agree that the relative intensities of the FFAs of C20 to C28 chain length appear to match those of the cholesteryl esters with the same fatty acid moiety compositions (Fig. 3 of our paper[1]) and are somewhat similar to the fatty acid compositions of the cholesteryl esters reported by Butovich.[2] It is probably true that these fatty acids mainly originate from cholesteryl esters. It is also possible that considerable amounts of C18:0, C18:1, and C16:1 FFAs are from the dissociation of diesters or triacylglycerols. Currently, we are working on an alternative way for determining the amount of FFAs.

The second comment is about the amount of C16:1-based wax esters. We agree that for some wax esters (e.g., wax esters with chain length longer than C43), C18:1 is the dominant fatty acid moiety and that the C16:1-based wax esters are less than 10% of their C18:1-based counterparts. However, for meibum samples with wax esters of more than 40 different isobaric *m/z* values, the relative intensities of C16:1- and C18:1-based wax esters vary significantly. Depending on the *m/z* values of the wax esters, the quantities of some C16:1-based wax esters can be even greater than the isobaric C18:1-based wax esters (e.g., C40:2, C40:1, and C41:1), and their analysis should not be deemed to be "unnecessary." Overall, as we indicated in our paper, the relative amounts of C18:1- and C16:1-based wax esters were consistent with a previous report in which they accounted for 57.39% and 11.66% of the total wax esters, respectively.[3] In addition, the fragmentation patterns show that the structures of these isobaric species are those of wax esters and are very likely difficult to separate from their C18:1 counterparts with the resolution of HPLC separation that Dr. Butovich used. In fact, the extracted ion chromatograms of two wax esters in meibum as shown in a paper by Butovich et al.[4] (Figs. 3D, 3F; the latter corresponds to the same wax ester species as indicated in Fig. 3 of our paper) display peaks

broader than those of the synthetic C18:1-based wax ester standards, indicating the co-elution of more than one species of similar structure.

It is clear that this is a subject area in which new information is rapidly appearing in the literature. The first reference[2] that Dr. Butovich mentioned was available online on the publisher's website May 12, 2010, a month after our manuscript was submitted. As for the second reference regarding (*O*-acyl)-omega-hydroxy fatty acids [OAHFA], as early as May 3, 2009 at the ARVO Annual Meeting, we proposed the idea that polar lipids include the elemental compositions for these previously unidentified peaks (not using the specific term OAHFAs), 3 months and 18 days before the review paper by Butovich[5] was published online (August 4, 2009, submission date unknown), whereas the other research paper mentioned[4] was submitted May 18, 2009. Dr. Butovich, as well, did not cite our report in either paper. More important, the OAHFAs are very likely from in-source decay of the ω-type I-St diesters, similar to the apparent fatty acids observed from the dissociation of cholesteryl esters. The model proposed by Butovich[5] requires further testing.

In summary, we agree that the FFAs present in meibum, as measured using electron spray ionization mass spectrometry could be from in-source decay, and the amount may be an overestimate when contrasted to nonmanipulated meibum. However, when measured in this way, any group repeating this experiment would find a similar result. We disagree that the C16:1-based wax esters were overestimated.

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