Title: Discussion of future directions and current knowledge after 8EWLM session on resolution of Inflammation

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INTRODUCTION

As displayed in Table 1, during the 8EWLM meeting, there was one session with three invited speakers on topics relating to the biology of specialised pro-resolving mediators of inflammation (SPMs). At the end of the presentations, there was a 30 min general discussion about current issues in research on SPMs. The speakers had before the conference agreed to recording of the panel discussion which was moderated by Drs Rådmark and Dahlén. This was carried out and transcribed by the authors of this report. Text in italics represent additions or comments during review of the typescript. As indicated, part of the discussion was in response to questions from the audience. For the aid of readers not attending the meeting, we have added six references to publications mentioned during the discussion.

It may be concluded that there were different opinions expressed concerning which SPMs that reliably have been identified in human samples. The meeting chairs proposed the formation of an expert group for consensus concerning guidelines and common practise in the bioanalysis.

Table 1: Presenters in the session Resolution of inflammation

Charles Serhan, Brigham and Women's Hospital-Harvard Medical School, Boston, US: Pro-resolving lipid mediators are novel signals for tissue regeneration
Marc Dubourdeau, Ambiotis SAS, France: Resolution lipid signature in different physiological and pathological contexts
Dieter Steinhilber, Goethe Universität, Frankfurt, Germany: Pro-resolving lipid mediator formation in leukocytes

DISCUSSION

Sven-Erik Dahlén (SED): We have half an hour now for general discussion. We have received several specific questions that we hope to bring up, but I thought we should start with a high level perspective on some areas for future research as outlined in Table 2. The work by in particular Charles Serhan and co-workers have provided us with a toolbox containing a lot of new compounds that can be studied for pharmacological effects. Today we have heard a rhapsody of such data and we will continue to hear more during the course of the meeting about pharmacological effects. There is no doubt that the reported effects are exciting.

What really is the issue for debate is what the effects mean in terms of the doses, and the endogenous levels. In order to understand what this means in

medicine, which is closer to my heart than mouse data, I really think we have to come into what has been started in the cellular studies, namely, to look at the levels of different classes of lipid mediators also *in vivo*. In what context they are formed and so forth. We discussed this during a pre-meeting with the speakers that one of the most unmet needs in this field is to study metabolism *in vivo* of the SPMs, as indicated on the slide. I mean, similar to the classical experiments John Oates, Garret FitzGerald and others did when parent prostaglandins were injected to find out how they are metabolised, so you have markers to monitor for the *in vivo* production in humans. The basal production is probably less interesting than the production in disease states at the time when these compounds are hypothesised to be important regulators. That is what one need to look into in tissue samples, maybe in the circulation etc.

Table 2: Text from slide shown at the introduction of the discussion.

Some areas for further research: ▪ Metabolism of SPMs and identification of biomarkers to monitor *in vivo* production in humans ▪ Pharmacologic control of SPM biosynthesis Steroids, NSAIDs, FLAP inhibitors, biologics etc ▪ Expression and regulation of key enzymes and receptors in human tissues

So I wonder if any of the three speakers want to comment on this. Are you aware of efforts in this direction because I really think that could be helping us forward.

Charles Serhan (CS): Yeah, I mean you raise an extremely important point, but I need to point out to you there are 38 human clinical trials in Pubmed.com on SPMs and there are many many groups [*other than my own*] that have reported SPM production in human samples. The key in all those are several weeks of increased Omega3 fatty acid intake. For example, in these studies, there is at the minimum 3 weeks of taking supplements of EPA/DHA and those are peripheral blood samples.

SED: The point of this meeting is to really catch the enthusiasm for continued research on SPMs, could we therefore agree that a hight priority would be to develop more knowledge about the metabolism of SPMs and to be able to do *in vivo* production in humans? One reason I say, as a physiologist, is really that as clearly shown here, by Dieter and by yourself Charlie, there are cell specific profiles, but *in vivo* you have the interaction with tissue, between various cells and that's what counts to really understand this.

CS: Absolutely.

SED: If we go to the second question [*on my slide*] then, [*about the influence of pharmacologic interventions on biosynthesis*], I find only scanty evidence in the literature, although there are some recent papers, but in order to put this into perspective, I think we need to understand better how conventional interventions within inflammation affect these pathways. Steroids, the NSAIDs, FLAP-inhibitors etc. I don't know what [*the speakers*] say about this? For

example, I will show data tomorrow on how NSAIDs block lipoxin formation in human mast cells.

Dieter Steinhilber (DS): Well, of course, I mean, if you look at prostaglandins, they are [*generally*] pro-inflammatory, but prostaglandin E_2 also carries a very important anti-inflammatory activity. In principle, it gives the impression also that if you look at the 5-LOX it generates pro-inflammatory mediators, for sure, but if it is involved also in stopping the inflammation, there are questions, what are the driving anti-inflammatory resolution compounds? You [*Charlie*] have the papers together with Jesper [*Haeggström*] showing, for example, if you take LTA_4 hydrolase inhibitors you get a shift towards SPM formations. I think that there are many opportunities to manipulate the system and to go from the pro-inflammatory to the anti-inflammatory [*pathways*]. I think the traditional NSAIDs not only block the onset, but also the resolution of inflammation.

SED: I would like to know specifically, what will two weeks of oral steroid treatment do in patients with the formation of SPMs? Is there any information of that? Because, that's what you use in the clinic.

CS: Yeah, but I think I want to go back, Sven-Erik, and address another point you made [*The first in Fig 1*] of knowing what to measure in particular in a human compartment to have a better understanding of local and distal metabolites. The peripheral blood profiles are measured in most labs, and one that I'm particular impressed with is the group from Switzerland [*1*]. In that setting, they used total parenteral nutrition (TPN), and as in our hospital in the ICU, this Omega3-supplementation gives very [*clear SPM detection*]. So substrate availability is, I think, critical. But most importantly, when you talk about resolution, the definition of resolution, which I tried to emphasise to you all today, is quantifying the neutrophil loss in the tissue. That component of tissue histology, and the composition of the inflammatory exudate-characterize the resolution phase.

I was happy that Marc showed the classical Willoughby paper, because he pointed out, in the early symposiums [*on inflammation 50-60 years ago*], that you cannot have a "willy-nilly" definition of resolution. It was critical, in fact, that people were on the same page of definitions so we did write along with prominent members in the resolution field at the time, a consensus-report published in 2007 [*2*]. It defined the terms in resolution, and so, seeing a particular resolving mediator doesn't necessarily mean you're in the resolution phase. It's all about where and when. And we know that from the prostaglandin work, so I think we have to, as Bengt [*Samuelsson*] said, and I quote, "We really have to look at [*what*] they [*did*] before us". We just can't take the systems that we know and impose them here, because the limiting step, as I see it, and what I tried to emphasise today in my presentation, is the potential for precision nutrition. Now, I don't doubt anything Dieter showed in his systems, but the questions for us is how to make those *in vitro* systems more physiologic and more physiologically meaningful, as I see it. Because our mission is to bring people to the field of lipid mediator research, because it is exciting, and it is fascinating.

SED: I think we agree on that, I think now, in the interest of the audience we should go to some of the questions and try to have short answers you in the panel, please.

Olof Rådmark (OR): I would just like to come back to this pharmacological control of SPM biosynthesis and if I recall correctly various NSAIDs can be regarded as resolution toxic, because they can have a bad effect on SPM formation.

CS: Yes.

OR: Would that be due to reduced PGE_2, I mean PGE_2 is supposed to induce this switch of the lipid mediator spectrum and is that due to increased biosynthesis of the enzyme 15-LOX-1 or is it due to activation of the 15-LOX-1 or is it due to translocation of the 15-LOX-1 to the unknown subcellular compartment which Oliver has talked about.

CS: Right, I'll find very quick answers to all these important questions. Our very first paper where we had resolution in the title, we found that in resolution of parasite infection, PGE_2 and LXA_4 were acting together. It was published in 2000 and, again to emphasise the point, it depends on where and when. There is another beautiful recent paper published in Science Translational Medicine showing that NSAIDs disrupt resolution [*3*]. It shows persistence of lower back pain when NSAIDs are being used, because they're disrupting resolution. Very powerful [*study*]. Right, we have all these alternatives, it depends on the [*involved*] cell type too what the major impact of the NSAID is. We found that there is a sort of paralysis of the inflammatory exudate, and the ability of those macrophages to carve away the apoptotic neutrophils. That means collateral tissue damage and more inflammation.

Marc Dubourdeau (MD): If I can add something. I think that the main issue that indeed we're facing may be that we are not in agreement about the biological models and of the way to see resolution. Because we speak about different cellular models with maybe different cellular compositions and with different media that can have an impact on cellular differentiation. When we work on macrophages, we know that macrophages are very plastic cells. It is quite complicated to know how to differentiate cells and to ascertain that mediator release from these cells really reflect M1 or M2. As it stands, we often use calcium ionophore, but we know that calcium ionophore is not sufficient to increase the level of phosphorylation and some people use PMA, because first it will induce phosphorylation and then an induction of calcium into the cells. There is also PLA2 which is important in producing these mediators. So, this is really the first time for me to have cellular models and animal models on which we agree.

It's also about the method that we use to measure SPMs. In all our labs, we have different ways to measure SPMs. We use mass-spectrometry. But there are some tricky things. So if we want to test [*a hypothesis about SPMs*], it is important that we agree on what will be the cell-type and what will be the measurement. This may be an answer to the second point and if we want to go to human, the problem is the same. When we do blood sampling, some people use EDTA-tubes while other researchers use heparin tubes or something else.

That will have an impact too on the measurement. And at the end, in our Center in Toulouse, we know that when sampling is completed, there is a window of one hour at least where we don't know where the sample is. I mean it can be on the bench or on the cart to be delivered to the analytical platform and so on. During that time the sample may become modified. So, we have many problems to face, but I think that by discussing and by comparing what we do, I'm sure that we'll succeed in finding the solution, but really, it's some points that we shall discuss deeply to be sure that we discuss the same thing.

SED: Thank you Marc, I think that's really helpful, and it really brings me to the elephant in the room. I mean, there is an undercurrent here, there is an issue, there are ongoing debates about how to measure these compounds. My view is that the only way to resolve this is to do exactly as Charlie suggested, namely, to have a consensus group that meets to discuss the analytical strategy. I mean, this is not rocket science, but it's [*a matter of*] good mass-spectrometry. And then different labs should use the same method, they could exchange samples from studies, you could, for example, have blinded samples that you send to different labs. I have seen this done in other disputes during my career and it often resolves issues quite well, to do something like that. That is just an idea to move this forward. As already mentioned, I'm not so interested in the mouse studies, I'm interested in measurements in humans and to be able to know [*the levels in disease*]. As Charlie said, how is it in the synovial tissue in RA, how is it in intestine and in the lungs? What metabolites could we measure in the urine to estimate whole body production, and things like that. So, I think that would really be [*great progress for the field*] if some of you guys that do these measurements could meet and sit together to plan studies.

Nils Schebb (from the audience): I think that it's an excellent suggestion that we should compare our methods. That's actually what we did in 2018, when I and a colleague went to Desmond Dalli's lab and compared our methods and, in the end, we had consensus. We know why we can't detect these compounds with established methods and Charlie~~s~~ and Desmond can with alternative methods. This is because we have different definition of the sensitivity and other criteria.

CS: Hi Nils. So that's very important, but it's also very important to know where to look. You have very nice [*analytical*] methods, but you should be aware that peritoneal dialysis of humans is a very chronic inflammation condition on its way to fibrosis. Yet in that setting you have found lipoxins and some resolvins, but not all. So, this is really the point, when studying an inflammatory exudate, it is of interest to determine if that exudate is from tissue in resolution, or not. Whereas Garret FitzGerald, in his study [*4*] using LPS challenge found, in peripheral blood, MaR1 and Protectin D1, as far as I recall. Even at very high, what I would consider, toxic doses [*of fish oil*]. I don't prescribe taking 12 grams, I mean, that's a diarrhea-inducing dose. And so, where and when [*SPMs are measured*] is really critical and then there are very nice studies on human placenta work where they profile all of the D-series resolvins, so the tissue origin is critical. And then having each step along the way to make the balance, you and I agree on that. And one issue that we forget about is the tissue culture media which is devoid of n-3 fatty acids.

Garret FitzGerald (GF, from audience): So, Charlie. You've given me the introduction to respond to your critique about our paper?

CS: Yes.

GF: Thank you. So, first of all, the doses of EPA that we choose, Charlie, are based on the fact that we have shown in placebo-controlled trials that these were the doses that were tolerated and reduce blood pressure in hypertensive people [*5*]. And they were doses that were also shown, which needs to be considered, to have effects in terms of platelet function [*6*]. So that was the rationale for the doses. We used two different doses – a low dose as well, and we saw no dose-response relationship with any of the SPMs. Yet we saw, dose-related relationships with trienoic prostaglandins formation, trienoic isoprostane formation and the phospholipid shift in red cell membranes. Then, again to bias things in your favour, when people were loaded with EPA, we gave them LPS. Again we saw all the things you would expect during the inflammatory phase and in the resolution phase, in terms of cytokine formation etc, but we saw no SPM formation. Which comes back to Nils' point; we do take very different approaches to analysis of these compounds. We use assays with limits of detection, we showed our chromatograms, we showed what our baseline was in terms of signal-to-noise and as you know, in your approach you don't rely on (at least, in the approach that you published largely, peak-detection, you don't show chromatograms. You show processed spectra with processed peaks. Now I would suggest a really great advancement in the field here would be to show the original chromatograms and from those chromatograms to show the derived, processed, peaks. And that's the sort of data we don't have in the literature from your methodology.

CS: No, wait a second. That's not my methodology. This is a toolbox we introduced. There are, as I said, 38 clinical trials from multiple groups around the world that have used the resolvins.

GF: What does that have to do with the analytical methods? The fact that people perform clinical trials? None of the clinical trials have got to phase 3, that is showing efficacy.

CS: No, no, no, don't go there in that room. Because those are defined clinical trials by NIH-criteria which means that they're taking supplements. And in all those studies, people show chromatograms. That's a matter of style if you want to show individual traces (analyte chromatograms) from multiple reaction monitoring (MRM) acquired data. That's fine. I don't have any arguments with that, but the point that I'm coming back to is that the definition of resolution, that you just used, is not what we're talking about. And I understand, there is a lot of interest in Europe around EPA for the cardiovascular effects, for commercial reasons, but I think that there is an enormous potential for studying agonists of resolution to help, in many other clinical scenarios, and that's my mission.

GF: I applaud the clinical mission and I think there is a very different issue here in terms of [*which pharmacological actions*] chemicals synthesised based on these structures may have, and in which biological context that may be desirable. It's an open question and I'm open to any answer, I think it's a noble

thing to look to see if they do. [*However, it is*] a different question whether these compounds are formed endogenously in sufficient amounts to drive resolution of inflammation.

CS: Well, the point that you make about sufficient amounts is very important.

GF: [*Answering that question*] depends on the methodology employed and that's where the integration of noise in the chromatograms [*is an issue*]. Showing which peaks have been selected from those chromatograms needs to be provided so the [*origin of*] derived clean peaks that are shown in processed data in so many papers is exactly what we need, to resolve this issue.

CS: What's the question?

SED: Well, it's more validation of the data, and more transparency, I think that is what is being asked for. This is an ongoing important debate to resolve.

Now, time is over for this session, we have two more exciting days of this conference. In my mind, this session was an excellent start. We can see that there are differences in opinion, but we can also see that this is a really thrilling field that you have pioneered Charlie. I think it's time now for more investigators to get involved, to apply different methods, and I really would welcome the formation of a consensus-group on analytical methods so we can go into the clinic and understand this area much better, because the pharmacology, that we see, as Garret said, is provoking.

Chairmen's final comment during editing:
In this discussion it was briefly touched upon some issues regarding formation and functions of the specialized pro-resolving mediators. Written questions were handed in from the audience, but time did not permit these to be discussed. Some of the questions are in relation to two of the discussion headlines in Table 2 are listed below in Table 3. There was no submitted question concerning the pharmacologic control of SPM biosynthesis, but this was to some extent discussed during the session. We believe that the unanswered questions in Table 3 indicate topics that merit for further research and discussions at future meetings.

Table 3: Selection of unanswered questions from the audience (see also Table 2)

1) *Metabolism of SPMs and identification of biomarkers to monitor in vivo production in humans*

- Why is SPM measurement so controversial - is it sample preparation or the MS setting? After all the compounds seem to be so biologically effective.
- How come most labs don´t report on SPM detection?
- In what clinical situations have SPMs been studied/used?
- Are SPMs endogenous factors?
- Has anyone stimulated monocytes/macrophages with apoptotic neutrophils and not detected SPMs?
- The levels of SPMs are usually very low in vivo, particularly in human subjects. If the levels are too low to activate their proposed receptors, how could they be considered as SPMs? By definition, SPMs are supposed to be produced during inflammation and trigger resolution. If their levels are too low, how could they trigger resolution then?
- Is there a clear separation of "resolution" and tissue regeneration? PGE_2 shows effects promoting tissue regeneration, but won´t be considered as one of the SPMs?
- Since LMs are considered paracrine mediators would you agree that measuring, in particular, SPMs needs to be done in tissues in favour of serum and other bodily fluids, and therefore overcome detection problems?
- What is the consensus on SPM levels in plasma? Can CTRs (MCTR1-3, PCTR1-3) be reliably found in plasma?
- Since lipid mediators are known to be secreted from innate immune cells. Why some of the groups measured oxylipins just in the cell pellets and not in the supernatant?

2) *Expression and regulation of key enzymes and receptors in human tissues*

- Are SPMs formed in ALOX5 knockout mice? What about Alox15 and Alox15b knockout mice?
- What about soluble phospholipases? New evidence on triggering inflammation driven by extracellular vesicles ….
- Dr Serhan showed data with M2 macrophages. Has it been differentiated between M2a and M2c (the latter is TGFß treated) as the latter has very different phenotype?
- When it comes to stem cells, which are the main drivers of tissue regeneration, it seems that there´s vey few data showing any effects of "SPMs" on stem cell recruitment/activation?

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