

Faster diagnosis? Chlorinated lipids in sepsis

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October 2019—Chlorinated lipids have been shown to be new potential biomarkers for sepsis, and continuing research into their role could lead to faster diagnosis, said David A. Ford, PhD, of Saint Louis University School of Medicine, at this year's AACC annual meeting. Dr. Ford, a professor of biochemistry and molecular biology, discovered chlorinated lipids in 2002, and at the AACC meeting he shared recent research on the association between chlorinated lipids and lung injury and death in sepsis patients.

"Can we define new biomarkers?" he asked at the start of his talk. "And do they have a functional role in mediating some of the injury—the multiorgan failure—that we see during sepsis?"

Multiorgan failure during sepsis is the result of dysregulated blood and endothelial interactions that lead to microcirculatory collapse. The National Institutes of Health in 2014 expanded the number of investigators invited to research microcirculatory dysfunction, Dr. Ford said. He and co-principal investigators Jane McHowat, PhD, professor of pathology at Saint Louis University School of Medicine, and Ronald Korthuis, PhD, professor of medical pharmacology and physiology at the University of Missouri School of Medicine, have been studying the role of chlorinated lipids in sepsis, and their work has been funded by \$1.78 million in NIH grants.



Dr. Ford

"In particular, there are bloodborne elements interacting with the endothelium that cause injury. That's where we got involved in this problem—investigating the microcirculatory dysfunction that leads to organ failure and ultimately death in these patients."

In respect to the microcirculatory collapse, Dr. Ford and colleagues have been exploring "whether the polymorphonuclear cells—the neutrophils—had a role in generating novel compounds, whether these novel compounds could be biomarkers, and whether they could also participate in an injury," he said.

Dr. Ford's laboratory first zeroed in on the role of myeloperoxidase, the predominant protein in neutrophils, which is unleashed when a neutrophil is activated resulting in the production of hypochlorous acid or bleach. "Your white blood cells are making bleach" to destroy bacteria that are taken up by the neutrophil, Dr. Ford said. "It engulfs and destroys them with the strong oxidant hypochlorous acid."

His laboratory was further interested in whether there were biomolecules in the neutrophil and surrounding tissue that could be targeted by hypochlorous acid to lead to other novel compounds. "We are mining for new biomarkers and mediators," with chlorinated lipids as a targeted approach and then also performing unbiased analyses.

"We're doing experiments in our lab that are untargeted," he said, using high-resolution mass spectrometry to find molecules. "Once you find those, you can make them your new targets. Then you can plug them into biological assays and see whether they have an effect." Computational modeling then can be performed with machine learning. "The whole process is considered a systems biology approach."

Dr. Ford and colleagues discovered early on that bleach created by the neutrophils can target the vinyl ether bond of a phospholipid called plasmalogen. Plasmalogens, a molecular subclass of phospholipids, are the most abundant phospholipids in the plasma membrane of many cells, including those of the heart, endothelium, and brain. "The

brain, heart, and vascular system are highly enriched with plasmalogens, which have a vinyl ether linkage at the *sn*-1 position of the glycerol backbone of the phospholipids," he said.

The vinyl-ether bond of the plasmalogen is responsible for generating chlorinated lipids.

"The molecules that we found in my laboratory are chlorinated fatty aldehydes [2-CLFALD] and chlorinated fatty acids [2-CLFA] that get liberated from the vinyl ether linkage of the plasmalogens," Dr. Ford said. These chlorinated lipids, which are usually 16 or 18 carbons in length, are a deleterious side product of the inflammatory response of the neutrophil.

In a separate study, Dr. Ford and Nuala Meyer, MD, MS(MSTR), and Jason Christie, MD, MSCE, of the Division of Pulmonary and Critical Care Medicine, University of Pennsylvania Perelman School of Medicine, showed a link between 2-CLFA and acute respiratory distress syndrome in adult ICU patients who were suspected of having sepsis. The study was conducted as part of a larger University of Pennsylvania study called the Molecular Epidemiology of Sepsis in the ICU (MESSI) cohort.

"We found that the 2-chlorofatty acids in the plasma of these patients were elevated in septic patients who developed acute respiratory distress syndrome," Dr. Ford said. "They were also elevated in patients who did not survive."

Plasma was collected at day zero from patients who were brought into the ICU and verified to have sepsis based on blood culture test results (Meyer NJ, et al. *JCI Insight*. 2017;2[23]:e96432).

"We then wanted to find out whether they [chlorinated lipids] had a role in causing injury at the endothelial layer in the microcirculation," Dr. Ford said. Dr. Korthuis, of the University of Missouri, performed a technique called intravital microscopy, which makes it possible to visualize white blood cells rolling across the vasculature. "You can measure whether white blood cells are rolling and adhering to the microcirculation. Dr. Korthuis superfused the chlorinated fatty acid on the mesenteric microcirculation and saw increased leukocyte rolling as well as leukocyte adhesion" to the endothelium.

"The aldehyde also had an effect," Dr. Ford said. However, "The fatty acid that doesn't have chlorine, palmitic acid, had no effect on leukocyte rolling or on adhesion."

After determining that chlorinated lipids affect endothelial cell surface adhesion, they wanted to see whether they could look at adhesion molecule expression and whether in cell models that would have an effect on neutrophil, platelet, and endothelial function, he said. "We also looked at whether these chlorinated lipids cause netosis."

In a study published last year, Dr. Ford and coauthors described data that suggested a "novel role for 2-CLFA as a lipid mediator" of the formation of neutrophil extracellular traps in neutrophil death, or netosis (Palladino END, et al. *Arch Biochem Biophys*. 2018;641:31-38).

Dr. Ford also shared his laboratory's study of endothelial cells, which used click chemistry with a synthesized analog of 2-chlorohexadecanoic acid (2-CLHA) to examine the subcellular location of the 2-CLFA in coronary artery endothelial cells (Hartman CL, et al. *J Lipid Res*. 2018;59[1]:113-122).

"We synthesized a molecule that had an alkyne at the methyl terminal of the chlorofatty acid. This allows us to perform click chemistry. We incubated this molecule with cells and then clicked the alkyne to an azide, and this azide had a reporter on it," Dr. Ford said. The azide reporter was TAMRA (tetramethylrhodamine), and using fluorescence microscopy they could then see where the chlorofatty acid goes in the cell.

"We found that the chlorolipids co-localize with P-selectin, von Willebrand factor, and Cox IV. These co-localizations indicate that the chlorolipid is in the Weibel-Palade bodies, because that's what stores P-selectin and von Willebrand factor in the endothelium, as well as the mitochondria (Cox IV) of the endothelial cells," he said.

Weibel-Palade bodies contain P-selectin, VWF, and angiopoietin-2, and they change from a rod shape to a circular

shape under activation. “Whenever we treated cells with chlorolipid, they went to this round shape to indicate that the Weibel-Palade bodies got activated,” Dr. Ford said.

The molecules inside the Weibel-Palade bodies perform significant roles during sepsis: The selectins increase neutrophil adherence to the endothelium, VWF causes platelets to adhere, and angiopoietin-2 increases permeability of the endothelial barrier, allowing for the formation of an edema.

Other experiments also showed that 2-CLFA increases the surface expression of P-selectin on endothelial cells. “We treated endothelial cells either with palmitic acid, chloropalmitic acid, or the click analog of 2-chloropalmitic acid,” Dr. Ford said. Under those conditions, P-selectin surface-expressed on those cells. Similar experiments showed that “along with the P-selectin surface expression, we see neutrophils adhering to the endothelium. We also see von Willebrand factor release in a similar sort of graph, and platelets adhering.” The laboratories of Dr. Ford and Dr. McHowat measured leakiness of the endothelium using an electric cell-substrate impedance sensing-resistance measure across the monolayer of endothelial cells. “The chlorolipids caused the endothelium to get leaky.”

Dr. Ford and colleagues also looked at the impact of chlorolipids on netosis. “Phorbol ester is a well-known stimulant to cause netosis,” he said. Myeloperoxidase is released and sticks on the neutrophil DNA that is extruded during netosis. “DNA is released during netosis and it binds to the heme protein, which is myeloperoxidase,” he said.

“After 90 minutes of stimulation, phorbol ester causes very little netosis. But the chloropalmitic acid elicits netosis at 90 minutes and also at 180 minutes. The latter—180 minutes—is when we saw the netosis with the phorbol esters.”

In summary, Dr. Ford said his laboratory’s targeted approach for chlorinated lipids showed that with leukocyte activation there is a respiratory burst, and plasmalogens get targeted by the bleach made by myeloperoxidase, liberating chlorinated fatty acids and chlorinated fatty aldehydes.

“We’ve shown they cause endothelial surface adhesion molecule expression that leads neutrophils and platelets to adhere to the endothelium,” Dr. Ford said. “We’ve seen this in both isolated cells as well as in intravital microscopy work” performed by Dr. Korthuis.

“That ultimately is going to lead to organ failure.”

Their studies have shown that netosis occurs when neutrophils are treated with chlorinated lipids, he said. “Many think of netosis as being an event to capture microbes, and that can be a great thing to happen during sepsis. But we envision with netosis that microbial killing is insignificant compared to neutrophil phagocytosis of microbes. When you have netosis, you’re going to plug the microcirculation, which will lead to further organ failure during sepsis.”

“Additionally, we have shown that these chlorinated lipids are predictive biomarkers of sepsis outcomes with death and acute respiratory distress syndrome,” and the studies are ongoing, Dr. Ford said.

Dr. Ford addressed a concern about the stability of 2-chlorofatty acid for clinical laboratory testing purposes.

“Fortunately, the chlorinated fatty acid is pretty stable. We are doing studies where we’ve spiked a chlorofatty acid in the plasma and measured it after six months, 12 months. We’re up to five years now, and we don’t see a drop with storage at -80°C for frozen plasma.”

A more pressing concern is the cleanliness of the preparation of the plasma, he said. “Often, someone will send us plasma, and we can tell without extracting it that something is wrong because everything is red. Hemolysis is a huge issue that we run into. To me, that is a bigger issue compared to 2-chlorofatty acid stability in our analyses.”□

Amy Carpenter Aquino is CAP TODAY senior editor. Dr. Ford presented in the same AACC session as Dr. Isbell; [see story](#).