Translational research on the use of a rapid analytical methodology for detecting acute cardiac ischemia, prior to a heart attack

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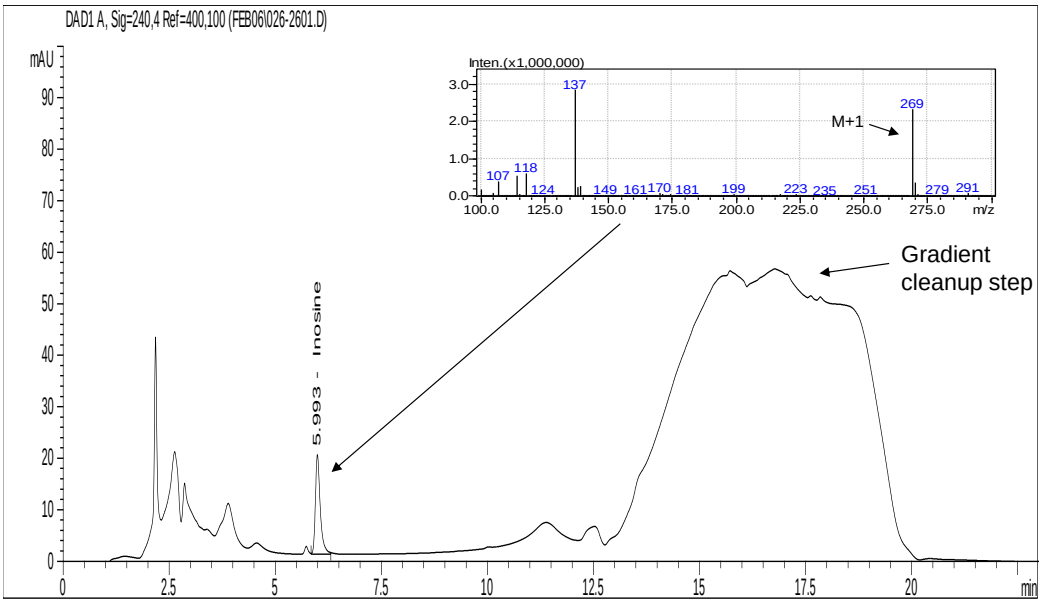
Our research focuses on the critical medical need of a biomarker and analytical method for detecting acute cardiac ischemia, prior to having a heart attack. The urgency of this type of research can best be shared by the following statistics:

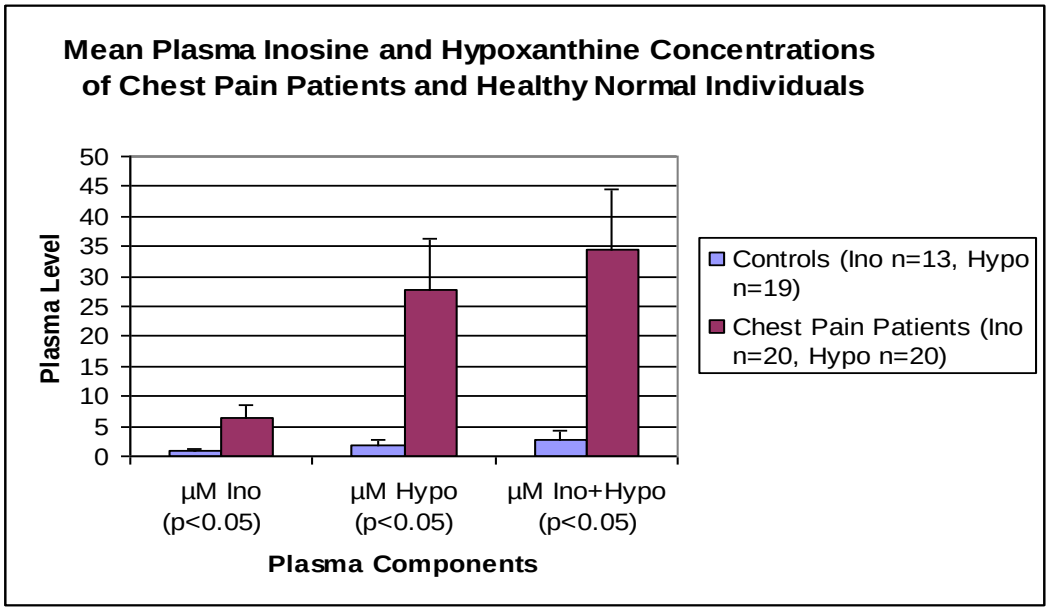
In the United States…

- Every 34 seconds, an individual has heart attack [1];
- Heart attacks may lead to cardiac arrests, which are the leading cause of deaths nationwide [2];
- The mortality rate for those experiencing cardiac arrests outside of a hospital setting is over 92% and this extremely high rate has not significantly improved during the past 30 years [3].

Our translational research starts with basic research using the mouse model [4]. The research objective was to identify potential biomarkers from ex-vivo mouse hearts undergoing conditions of acute cardiac ischemia. Briefly, the mice were anesthetized and sacrificed, with the hearts removed for subsequent experiments. Conditions of acute cardiac ischemia (oxidative stress) were placed upon the hearts, with the Krebs buffer effluent from the heart collected and analyzed using LC-DAD and LC-ESI-MS methods [5,6]. As seen in the following figure, using HPLC with an Onyx C18 monolith column, LC gradient, and DAD and MS detectors, inosine (RT 5.9 min, MW 268.2 Da) was identified and found significantly elevated in the ex-vivo ischemic mouse heart samples, as compared to ex-vivo non-ischemic mouse hearts (controls).



Next, our clinical research focused on measuring plasma levels of inosine and hypoxanthine (inosine metabolite in the bloodstream) from ER non-traumatic chest pain patients. Briefly a quantitative LC-UV method was validated and used for plasma analysis [7], with the following bar chart demonstrating that the ER patients had significantly elevated plasma levels of inosine and hypoxanthine, relative to healthy normal individuals (controls).



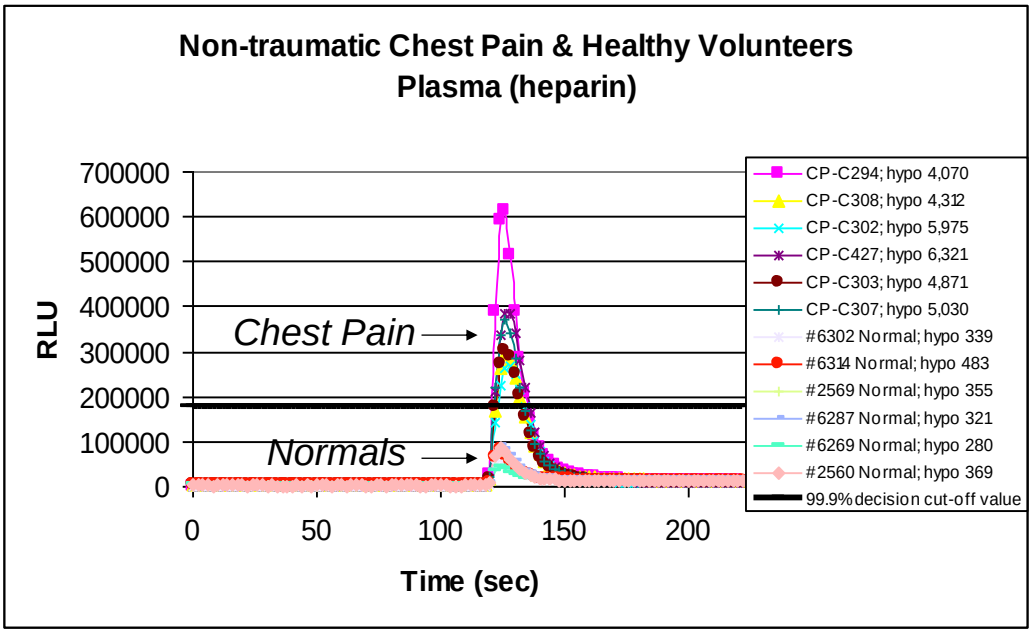
However, as we know that HPLC technology does not easily lend itself for use in an ER environment, we next developed a novel and rapid microplate luminometer test method to measure the plasma levels of inosine and hypoxanthine, in less than 30 seconds [8]. And unlike HPLC systems, the microplate luminometer is relatively inexpensive to purchase and maintain, currently used in many laboratories, and would be easier to train on and operate for compliance in a CLIA certified clinical lab.

This chemiluminescence method has high sensitivity and specificity. Briefly, the test method utilizes biological enzymes purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO), which are specific for enzymatic conversions of inosine and hypoxanthine, respectively. The enzyme PNP converts inosine to hypoxanthine and XO converts hypoxanthine to xanthine, followed by XO conversion of xanthine to the final product uric acid (in human species). Each time XO reacts with 1 mole of hypoxanthine, and subsequently with 1 mole of xanthine, the metabolic by-products of each XO enzymatic turnover is production of 1 mole of hydrogen peroxide (H_2O_2) and 2 moles of superoxide anion radical (O_2 −). Both of these reactive by-products can become substrates for use in chemiluminescence reactions.

To achieve greater sensitivity as low concentrations (1–3 µM) of inosine and hypoxanthine are typically found in human plasma, we used a highly sensitive luminescent photoprotein called Pholasin®. It is a photoprotein isolated from the bivalve

mollusk *Pholas dactylus*, and has been reported to possess a very sensitive chemiluminescent material called lucidalin®, which can react with superoxide anion radical (SAR) and other reactive oxygen species (ROS) such as the hydroxyl free radical [9]. Pholasin is an approximately 34–36 kDa glycoprotein, and has been reported to not possess fluorescent properties [10]. It has been extensively studied for more than 30 years with commercial use patented by Knight Scientific Ltd (Plymouth, UK). The SAR generated by XO enzymatic turnover reacts with Pholasin to generate measurable blue-green light (with a maximum ~490 nm), thus an amplification of signal is observed (1 mole of hypoxanthine can generate 4 moles of SAR). Knight *et al*. cited Pholasin improving chemiluminescence sensitivity by more than 100-fold, in comparison to using lucigenin for the same studies [10]. The reaction of Pholasin with SAR is rapid (flash type technique, typically seconds), and method sensitivity can be further increased with use of a signal enhancer (e.g., Adjuvant-K™ proprietary from Knight Scientific Ltd).

Plasma samples from the non-traumatic chest pain patients and healthy normal individuals are depicted in the following chemiluminescence RLU overlays. As seen in the RLU overlays, the enzymatic reaction and chemiluminescence response to inosine and hypoxanthine in untreated plasma samples is rapid and sensitive. This novel chemiluminescence methodology received a US patent in 2013 [11].



In conclusion, as cardiovascular disease (e.g. acute MI) is a leading cause of mortality and morbidity in the world, a rapid diagnosis and treatment would be important to improving patient outcomes. As recently reported by the CDC, the chances of survival for cardiac arrest victims that occur outside the hospital environment is less than 10%. As there have been no improvements to this low survival rate in the last 30 years, this unmet critical medical need should be high priority for the medical community and

biomarker researchers. Accordingly, biomarker research for the medical condition of acute cardiac ischemia is an on-going process, as there is not one biomarker that is FDA approved and used on a routine basis.

Our clinical studies are underway to evaluate plasma inosine and hypoxanthine levels, in conjunction with cardiac troponin levels, to better understand their diagnostic potential for indicating acute cardiac ischemia, prior to heart tissue necrosis. As our basic research and preliminary clinical studies have demonstrated that inosine and hypoxanthine are potentially good biomarkers for indicating acute cardiac ischemia, the use of this rapidly eluting biomarker and quick screening test can save millions of lives and billions in hospital cost every year. Having global impact, the ultimate goal for this research is to miniaturize the US patented clinical assay for use in a point of care handheld medical device, to be used in the same fashion as the commonly used glucose meter by individuals with diabetes. If successful, this research will help to reduce the frightening CDC survival statistics for out of hospital heart attack victims.