Clinical Chemistry

ProBNP That Is Not Glycosylated at Threonine 71 Is Decreased with Obesity in Patients with Heart Failure

Article:
Lynley K. Lewis, et al.
ProBNP That Is Not Glycosylated at Threonine 71 Is Decreased with Obesity in Patients with Heart Failure.
Clin Chem 2019;65:1115-24.
http://clinchem.aaccjnls.org/content/65/9/1115

Guest: Dr. Lynley Lewis is a Senior Research Fellow in the Translational BioDiscovery Laboratory of the Christchurch Heart Institute, within the Department of Medicine at the University of Otago, Christchurch, New Zealand.

Bob Barrett: This is a podcast from *Clinical Chemistry,* sponsored by the Department of Laboratory Medicine at Boston Children's Hospital. I am Bob Barrett.

Heart failure is a leading cause of morbidity and mortality worldwide with prevalence expected to increase over the next 20 years. Early diagnosis and optimal management of heart failure are key to reducing its impact. Because B-type natriuretic peptide, or BNP, and amino terminal proBNP, or NT-proBNP, as well as their precursor proBNP, are secreted by the heart in direct proportion to the degree of cardiac dysfunction and clinical severity, measurement of these peptides is now mandated by authoritative international guidelines for the diagnosis and risk stratification of the disease. However, circulating concentrations of both BNP and NT-proBNP are reduced by obesity, and this phenomenon is one of the key weaknesses of the diagnostic performance of the natriuretic peptides in heart failure.

A paper appearing in the September 2019 issue of *Clinical Chemistry* shows that obesity is associated with decreased concentrations of proBNP that is not glycosylated at threonine at position 71 of the peptide. Decreased proBNP substrate amenable to processing could partially explain the lower NT-proBNP and BNP concentrations measured in obese individuals, including those presenting with heart failure.

The lead author of that paper is Dr. Lynley Lewis, a Senior Research Fellow in the Translational BioDiscovery Laboratory of the Christchurch Heart Institute within the Department of Medicine at the University of Otago, Christchurch, New Zealand. Dr. Lewis is our guest in this podcast. So, Dr. Lewis, what is O-glycosylation and why are peptides glycosylated?

Dr. Lynley Lewis Well, the first thing to note is that glycosylation is not the same as glycation. Glycation is the passive chemical addition of glucose to amino acids, is irreversible and can lead to advance glycation end products, which is detrimental

to health. In contrast, O-linked glycosylation is the enzyme catalyzed addition of a modified sugar molecule to either threonine or serum residues in a protein and occurs after the protein has been synthesized. So, it's called a post-translational modification, or PTM.

There are two types of O-glycosylation: the first is the addition of N-Acetyl-D-galactosamine, or GalNAc, and it's catalyzed by a family of GalNAc-transferases. The resulting glycans are often called mutant-type O-glycans, and the sugar chains can be diverse and extended. GalNAc is abundant on many extracellular and secreted proteins. This type of glycosylation has been shown to be present on proBNP, the peptide we studied.

The second type of O-glycosylation is the addition of N-acetylglucosamine or O-GlcNAc, which is catalyzed on O-GlcNAc-transferase, often called OGT. These sugars are not thought to be extended and can be removed by another enzyme O-GlcNAcase, or OGA. I'm not aware of any studies that have assessed proBNP for O-GlcNAc. Glycosylating a peptide can alter its stability and function. Both glycation and glycosylation are increased with obesity and both are predicated in patients with diabetes. Current belief seems to be that O-glycosylation might be beneficial in the short-term in many pathological states including heart disease, but toxic long term. In the heart, it has been speculated that the acute increase in glycosylation is an adaptive response to protect the heart from injury, whereas prolonged, persistent activation e.g. from a high sugar or fat diet is maladaptive, and may contribute to cardiac dysfunction.

In the case of proBNP, the peptide that we studied, glycosylation is thought to provide stability to the proBNP molecule. There are 7 high abundant glycosylation sites at the NT-proBNP end of proBNP. Six of these are in the central region of the peptides, and one of those at threonine 71. Glycosylation at the threonine 71 position of proBNP prevents proBNP being processed into the cardio protective BNP and its congener NT-proBNP.

Bob Barrett: There are many methods that can be used to measure peptides in the circulation. Why was the antibody approach used rather than mass spectrometry?

Dr. Lynley Lewis: Plasma samples contain many different compounds, and with mass spectrometry, samples need to be extracted to remove a lot of these, with the extracts then being further separated by high performance liquid chromatography prior to injection onto the mass spec. Extraction recovery can be variable, glycosylation can be labile, and deglycosylation procedures, which are often necessary, can reach variable

levels of completeness. Amino assays are relatively cheap, and allow high throughput of samples, and as our laboratory has many years of experience in amino assay development, this is a technique we know well. With amino assays, diluted plasma can often be added directly to the assay tube without the requirement for sample extractions, particularly in 2-site assays. Also, using antibodies raised against the non-glycosylate peptides means that only those peptides that are not glycosylated that will be recognized by the antibody and measured. The antibodies are not able to bind if the sugar groups are present. So, if the peptide is glycosylated at this site, it won't be detected by the antibody and so it won't be measured. This process then removes the requirement for deglycosylation procedures.

For our study, we developed three 2-site amino assays for proBNP. All these assays have a common capture antibody that binds to the proBNP ring structure at the C-terminal end of proBNP, i.e. at the BNP end. We paired this C-terminal antibody to three different signal antibodies, which gave us three different proBNP assays. In our total proBNP assay, the signal antibody binds to a non-glycosylated region of proBNP and it can recognize both glycosylated and non-glycosylated proBNP.

In our non-glycosylated T-71 assay, we used an antibody that will bind to the T-71 region only if it is not glycosylated. And in our central non-glycosylated assay, we used a single antibody that will only bind at the central region between amino acids residues 31 to 39 as not glycosylated.

We can then use these assays to measure the levels of each of these proBNP types in our patient samples, and can therefore determine a glycosylation profile for proBNP in these regions in each sample without extraction and without the glycosylation procedures. When we measure these in concert with NT-proBNP or BNP, we can calculate ratios, which gives us the gauge of the proBNP processing rates in an individual. However, there are many other factors that can also influence proBNP processing, particularly the concentrations and activities of the enzymes that cleave proBNP.

Bob Barrett: Dr. Lewis, why is assay specificity so important?

Dr. Lynley Lewis: Well, it's very important to know exactly what is being measured in an assay to be able to correctly interpret the results and perhaps, to work out what it means clinically. When we developed our assays, we wanted to be able to distinguish between different glycosylation states of proBNP. If we just used our total proBNP assay, the result would indicate how much proBNP was being secreted. In our

obese vs. non-obese heart-failed patients, we found the circulating levels of total proBNP were similar.

However, when we looked at a centrally non-glycosylated assay, our measured levels were very low indicating that most of the proBNP was likely to be glycosylated in this region. So, if we had just used this assay to look at proBNP levels, we would have assumed erroneously that there was not much proBNP circulating. Now, we know that proBNP is cleaved between residues 76 and 77 to form bioactive BNP in the NT-proBNP portion, and that this cleavage does not happen when proBNP is glycosylated at threonine 71, due to the sugar molarity preventing the cleavage enzyme binding to its active site. The same happens with an antibody, it cannot bind to its usual site if this site is glycosylated. Therefore, by using an assay specific to the non-glycosylated threonine 71 site, we only measure proBNP that was not glycosylated at threonine 71.

And we were able to show that the concentrations of proBNP not glycosylated at threonine 71 were higher and much more variable than the concentrations of proBNP not glycosylated in the central region, which we believe indicates that glycosylation is differentially regulated between the central and the T-71 sites on this peptide. Also, high concentrations in the non-gly T-71 assay, indicates that circulating proBNP is not glycosylated at T-71 and is, therefore, available for processing into bioactive BNP.

By having a suite of assays to look at the total and glycosylated areas of proBNP can give us some information not just on the circulating levels, but also on the ability of these peptides to be processed to form the bioactive BNP.

Bob Barrett: Do you have any more examples of where assay specificity can be important?

Dr. Lynley Lewis: We all know that BNP and NT-proBNP assays are used to assist in the diagnosis or prognosis of heart failure. For example, a clinician may order a BNP result to help in the diagnosis of heart failure. What the clinician will assume they are finding out is how much bioactive BNP the patient has in his circulation, but the BNP assay not only measures the cardio protective BNP 1 to 32, it also measures the BNP's precursor molecule proBNP and metabolizes both proBNP and BNP.

So, the apparent protective effect of bioactive BNP levels will be overestimated as proBNP is much less bioactive than BNP. This leads to the BNP paradox where the biological activity of BNP is reduced, yet circulating levels are high. And if a repeat sample is done on a patient, estimated BNP

levels increase. It is not known whether this is due to the ProBNP precursor increasing or the active peptide increasing.

In another example, in the NT-proBNP assay, as I mentioned previously, most of the circulating proBNP is highly glycosylated in the central region. This region which is contain on the end terminal proBNP portion of proBNP, remains highly glycosylated when proBNP is processed into NT-proBNP and BNP.

In fact, it's been reported that at least 80% of circulating NT-proBNP is glycosylated centrally. Common commercial assays for measuring NT- proBNP contain an antibody to the central regions and therefore the centrally glycosylated peptides are often not measured because glycosylation blocks finding of the assay antibodies. This likely leads to an underestimation of the amount of circulating NT-proBNP. Sometimes this is important clinically and sometimes not. But with any test, interpretation of the result is helped with increased knowledge of the underlying science.

Bob Barrett: So, doctor, why is this research important?

Dr. Lynley Lewis: Well, the naturetic peptides, including BNP and NT-proBNP, have been used for many years in the diagnosis and prognosis of heart failure. However, from the early days, it was observed that obese people had lower BNP and NT-ProBNP levels than was expected for the condition of their heart.

Different theories had been postulated to explain this, but none have been completely accepted. For example, there are reports showing decreased activity of BNP and increased clearance of it and adipocytes for the pep cells. So, this was put forward as an explanation for decreased levels of BNP with obesity. However, when proBNP is in informatically cleaved, NT-proBNP is formed at the same time as BNP. And NT-proBNP is also decreased with obesity, but it is not cleared in the adipocytes. So, the mechanism for reduced levels must be from another source. O-glycosylation levels generally had been shown to be up regulated with obesity. And in our paper, we have shown that the levels of glycosylation at threonine 71 of proBNP are increased with obesity. Increased glycosylation of threonine 71 would lead to decreased processing of proBNP and thus, decreased levels of both BNP and NT-proBNP and so could, at least in part, explain the lower levels of these peptides in obese people.

Bob Barrett: Well, finally Dr. Lewis, how can these results be translated into clinical practice?

Dr. Lynley Lewis: One of the debates that surrounds the BNP and the obesity story is whether the clinical cut off for ruling out heart failure should take the patient's weight or BMI into account, particularly with the knowledge that people with higher BMI had lower than expected BNP levels. But if we don't understand the underlying physiology, it is difficult to apply an appropriate cut off value. We may find for example, that specific processing rates are linked to different subgroups of heart failure. Perhaps people with greater BMI and increased proBNP processing respond differently to treatment, or have a different long-term prognosis than people with greater BMI and decreased proBNP processing rates, or the other way around. So, the more we know about the mechanisms that control proBNP processing or other processing pathways, the more likely it is that targeted treatments can be developed to improve clinical outcomes.

Bob Barrett: That was Dr. Lynley Lewis, a Senior Research Fellow in the Translational BioDiscovery Laboratory of the Christchurch Heart Institute within the Department of Medicine at the University of Otago, Christchurch, New Zealand. She has been our guest in this podcast with *Clinical Chemistry*. I'm Bob Barrett. Thanks for listening.