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Mass Spectrometry Techniques for Metabolomic Studies of Aging and Age-Related Diseases

By

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Chapter 9

Age is Just a Number: Quantifying Molecules in Whole Blood to Better Understand Healthy Aging

Abstract

Aging is a process that affects everyone, but there is much still to learn about how aging proceeds and why it is so different from person to person. Through the power of analytical tools like chromatography and mass spectrometry, I have spent much of my graduate career studying one molecule in particular, spermidine, that seems to support healthy aging. We began this project with the intention of better understanding the relationship between spermidine and mean cell volume, a value describing the size of one's red blood cells. Although our results were somewhat inconclusive, we feel that our study still has merit, and we plan to further investigate this relationship with a new sample cohort in the future.

Introduction

This chapter is a unique part of my dissertation, aimed at explaining a portion of my research to non-scientists. I wrote this in part because I believe that science should be accessible; after seeing how a lack of scientific understanding drove misinformation during the heat of the COVID-19 pandemic, I was even more impassioned to continue encouraging dialogue between scientists and the general public. I also wrote this chapter so that I could share it with all the non-scientists in my life, in hopes that they would understand just a little of what I have been doing over the past five years. I am grateful to the Wisconsin Initiative for Science Literacy (WISL) at UW-Madison for helping me create this chapter, and to Professor Bassam Shakhashiri, Elizabeth Reynolds, and Cayce Osborne for their feedback, support, and the creation of this initiative, which helps inform the public about what all we do here at UW-Madison, one chapter at a time.

The purpose of aging research

To many, the idea of aging research may be a bit off-putting. Maybe it evokes imagery of man's

search for immortality—the fountain of youth, or the Holy Grail, for example. Although a select few aging researchers may seek to extend the human lifespan, aging research more often focuses on increasing the "health span," a term defined loosely as the amount of time one lives without disease.

Much like lifespan, health span can vary widely from person to person; while some people experience a decline in their health in their 60's or 70's, spending much of their later years in an assisted living facility, others are able to live independently well into their 90's, or even 100's. When you ask the oldest people the secret to their longevity, it is often something you normally wouldn't attribute to a healthy lifestyle. The oldest woman to ever live, Jeanne Louise Calment, attributed her long life to olive oil, port wine, chocolate, and cigarettes (she only quit smoking at age 117). The world's oldest person until 2016 was Susannah Mushatt Jones, who swore that bacon was the key to her longevity. Jessie Gallan, Scotland's oldest living person until her death in 2015, simply said that her secret was avoiding men.

We know that smoking, drinking, and eating unhealthy foods can have an adverse effect on your health, and yet, these supercentenarians lived in relatively good health far beyond the average lifespan with no changes in their lifestyle. By studying the mechanisms of aging, scientists can better understand why health span varies so widely, and ultimately help people extend it to live healthier lives for longer.

What is a biomarker? What makes a good biomarker?

Biomarker is a portmanteau for "biological marker." As defined by the World Health Organization (WHO), a biomarker includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological."

Biomarkers can include typical measurements taken at a primary care visit, like blood pressure or heart rate, but the field of bioanalytical chemistry is particularly interested in molecules that can be found within the body.

Biomarkers are most often used in the context of disease. Usage of biomarkers typically falls into one of three categories: screening and/or diagnosis, prognosis, or monitoring. In any of those categories, a good biomarker must, at the very least, be able to clearly distinguish between the groups that are being studied. It will ideally be unique to only one disease, although that can be difficult to achieve; you can imagine that while cancer and Alzheimer's Disease (AD) may change the body in very different ways, producing different biomarkers, AD and another similar disease like frontotemporal dementia may affect some of the same biochemical pathways. The earlier a biomarker begins to change in disease progression, the better it will serve patients, as earlier diagnosis can lead to earlier treatment and, ultimately, a more positive outcome.

While biomarker discovery is still a work in progress for many diseases, there are several biomarkers already in use, some that you may have encountered before. One such biomarker is a protein called glycated hemoglobin, or HbA1C. HbA1C is the most commonly used biomarker to diagnose both prediabetes and diabetes. Hemoglobin is the protein that gives blood its red color; when glucose builds up in the blood, it will bind with hemoglobin, creating HbA1C. Because red blood cells have a three-month lifespan, an HbA1C test can tell a doctor the average level of glucose in their patient's blood not just that day, but over the previous three months. Besides being used for diagnosis, HbA1C can also be used to manage diabetes through regular A1C tests.

Metabolites as biomarkers

Your genetics determine a lot about you—your eye color, for example, or your blood type. Traits

determined by your genetics make up your genotype, a term used to describe the complete genetic makeup of an organism. Your metabolism is only partially determined by your genotype because the metabolites in your body also fluctuate with all sorts of external changes. Exercise, diet, medications, general lifestyle – all these things can affect what metabolites are present in your body, as well as their concentrations. These observable changes are part of your phenotype; a phenotype includes not just characteristics that are part of your genotype, but also those that are not fully determined by genetics, like weight, physique, and concentrations of metabolites.

Aside from some physical traits, your genetics can also influence whether you develop diseases. Some diseases, like cystic fibrosis or muscular dystrophy, are entirely determined by genetics; if your doctor suspects that you may have a genetic condition, they can sequence your genome and look for small variations in your DNA that are known to be associated with that condition. Other diseases, however, including most age-related diseases, are determined by a combination of both genetics and environmental effects. If you want to know whether you will develop AD, for example, you could have your genome sequenced to see if you have one of the genetic risk factors, but unlike with purely genetic diseases, lacking a genetic risk factor does not preclude you from developing AD anyway.

Genomics (the study of genes) can only tell you what *should* happen based on your genetic code. Amazing scientific breakthroughs have come from the field of genomics, but in diseases that are not 100% genetic, this field of study can only go so far. Proteomics (the study of proteins) can take that research one step further. Proteins are large biomolecules that consist of multiple amino acids, and the blueprints your body needs to make these molecules can be found in your genes. Whether a protein is made, and how much of it is made, depends on whether a gene is expressed; while information that comes from genetics is static, proteins can be more dynamic, reflecting what is

actually occurring in the body. But again, science hits a roadblock, because while protein concentrations are useful to understand, it is difficult to know from this information what function these proteins play.

Metabolomics (the study of metabolites) has an advantage over proteomics in that it is the ultimate downstream result of every prior level of "omics." Genes encode proteins, which in turn regulate metabolites. In addition, metabolites can change due to environmental effects; for example, after you exercise, a metabolite called lactic acid tends to be increased. In that way, metabolite concentrations can reveal a person's systemic response to external factors like drugs, nutrition, and lifestyle, while also reflecting genetic factors. Metabolism often changes very early in disease progression, sometimes even before symptoms start to appear. In AD, for example, the way the brain produces energy starts to shift as early as decades before any noticeable cognitive changes.[1]

Metabolomics brings many unique challenges, many of which are in part due to the more recent development of metabolomics as a field, compared to more well-established fields like genomics and proteomics. Proteins vary in their size and length, but all proteins are comprised of some combination of the same 20 amino acid building blocks. In contrast, while certain metabolite classes can have structural similarities, metabolites as a whole share no overarching motifs. If you think of identifying molecules like putting together a puzzle, metabolomics is as if you had no reference picture on the box.

Additional challenges arise thanks to the sheer number of metabolites that exist; there are over 100,000 metabolites that have been detected in humans, all of which vary in size, biological concentration, structure, and stability. This variation can make them extremely difficult to study, especially in experiments that are untargeted, meaning they are focused on identifying as many metabolites as possible. Some metabolites may be extremely sensitive to temperature or light, for

example, which makes them more likely to degrade before analysis than other more robust molecules. This can lead to artificial changes in metabolite concentrations, where a lack of a certain metabolite is due to sample storage, rather than a biological change in the patient.

What is mass spectrometry?

Mass spectrometry (MS) is a valuable tool for both biomarker discovery and quantitation. MS as a technique is both very *sensitive*, able to detect low levels of biomarkers in samples, and *selective*, with the power to distinguish between very similar molecules. MS is very complex, but a mass spectrometer can be broken into three general pieces: the ionizer, the mass analyzer, and the detector.

In a mass spectrometer, a sample is introduced through an ionizer. A mass spectrometer is very powerful, but it cannot detect molecules unless they are charged. This is where the ionizer comes in. An ionizer can be operated in either positive mode, where it adds a positive charge to a molecule, or in negative mode, where molecules gain a negative charge. In my research, I typically use a technique called electrospray ionization, or ESI. ESI is a soft ionization technique, in that it adds enough energy to ionize molecules, but not so much that it blasts them apart. In ESI, the sample is sprayed out of the tip of a capillary that is charged to an extremely high voltage, so that the molecules in that spray leave the capillary with a charge (**Figure 1**).

The ionized sample next enters the mass analyzer, where the mass spectrometer can separate and identify molecules. In the mass analyzer, ions are separated based on something called their mass-to-charge ratio, or *m/z*. The *m/z* is, simply put, the mass of the molecule (m) divided by the charge it has from the ionizer (z). Big molecules like proteins can carry multiple charges, but my research mainly focuses on small molecules that can only hold one charge. Because of this, the *m/z* for any

molecule I care about is the mass of the molecule, divided by one. The *m/z* is one characteristic we can use to identify a molecule, because every element used to build a molecule has a specific mass; when the masses of all the elements in a molecule are added together, you get the mass of the whole molecule.

Unfortunately, although there are 118 elements on the periodic table to choose from, the human body is 99% comprised of only six elements: oxygen, hydrogen, nitrogen, carbon, calcium, and phosphorus. That means that there are a finite number of elements that are typically found in biological molecules, so some molecules are made of the exact same elements, just in a different order. This means they will also have the exact same mass. We call these molecules isomers (**Figure 2**), and they can make metabolomics extremely complicated, because although mass analyzers are very powerful, they cannot distinguish between two molecules with identical masses. To get more information that we can use to identify different molecules, we have the option of pairing more than one mass analyzer together. After molecules leave the first mass analyzer, they are hit with a big surge of energy. This breaks them into pieces that get sent to a second mass analyzer, where the mass of those individual pieces can be measured. Those pieces are matched back to the original mass, called the precursor, and with the precursor mass and the fragments, the molecule can be identified.

At the end of the mass spectrometer is the detector. This is where the molecules' mass is detected, and the number of ions can be counted. This information is reported out on a mass spectrum (**Figure 3**). On the x-axis is the *m/z*, and the y-axis shows the intensity; the ions that have the highest intensity are the ones that are present in the highest abundance.

One of the biggest advantages MS brings is the ability to couple MS instrumentation with a separation technique. MS is a powerful technique, but with the human metabolome (all metabolites

detected in humans) containing over 100,000 metabolites, even a technique as powerful as MS cannot detect, identify, and quantify every metabolite at the same time. Rather than sending every molecule in a sample into the MS at the same time, adding a separation technique like liquid chromatography (LC) can make it easier to see every molecule. You can think of it a bit like that famous scene from *I Love Lucy*, where Lucy is wrapping chocolates that are coming out on a conveyor belt. At first, there is a manageable number of chocolates coming out, and she is able to wrap each one individually. As they start to come out in higher quantities at the same time, she starts to miss some, and rather than each of them getting wrapped, some are eaten, some are thrown behind her, and some get stuffed into her hat. The mass spectrometer can react the same way when too many molecules are being analyzed at the same time; instead of each one being recorded, some can be missed.

There are many different forms of LC, but they all work in a similar fashion; to explain, I will use the most common form of LC, reversed phase LC (RPLC), as an example (**Figure 4**). In all LC separations, a sample is first injected onto a column, which is a long tube full of material that can help separate different compounds; this material is called the stationary phase. In RPLC, the stationary phase is hydrophobic, meaning it repels water. As the sample passes through the column, hydrophobic molecules are going to be more attracted to the stationary phase than others, and these molecules will stick to the stationary phase rather than just flowing through with the solvent, or mobile phase. The mobile phase will start hydrophilic (attracts water), so hydrophilic molecules will prefer to stay in the mobile phase, leaving the column first in a process called eluting. Elution occurs when a molecule is adequately dissolved in a mobile phase and exits the column. The mobile phase changes on a gradient, becoming increasingly more hydrophobic to encourage molecules to go into the mobile phase. When the mobile phase is suitably attractive, they will come

off the stationary phase and elute. You can think of LC a bit like a bar crawl—if you send a group of people out to go from bar to bar, they won't all return home at the same time. People who aren't drinkers will leave first (these are the hydrophilic molecules that are not retained). Those who want a drink or two before they go home will trickle out after that, like the somewhat hydrophobic molecules. Finally, those who like to stay out into the wee hours of the morning will leave last, just like the most hydrophobic molecules in RPLC. Even though they all arrived at the bar at the same time, the time at which they leave corresponds to how much they are attracted to the bars (the stationary phase).

Using MS to study aging

In the field of biomarker research, there are typically two main areas of study: biomarker discovery (finding new molecules to detect diseases), and biomarker quantitation (measuring exact amounts of molecules that have already been discovered). In my research, I mainly focus on biomarker quantitation; biomarkers are often in low concentrations in the body, so I work to push the boundaries of analytical chemistry, developing techniques to improve quantitation and make these biomarkers viable options for clinical application.

I use mass spectrometry to better understand how the human metabolism changes in response to aging and disease. I am particularly interested in blood metabolomics, for a few key reasons. For one, blood is a particularly accessible source of biomarkers. Blood tests are a common part of a yearly physical, so if new blood biomarkers are identified, they can be easily incorporated into regular medical care. Blood draws are also easily done at most clinics, making a blood-based biomarker panel more accessible for rural or low-income clinics that may not have access to more expensive equipment like MRIs or CT scans. Blood is also interesting because it flows between different organs and systems, transporting molecules between them. Studying this crosstalk will

help scientists have a better understanding of what roles these molecules may play in health and disease.

The role of spermidine and mean cell volume in aging

To better understand aging, many studies have been done to investigate the metabolic profile of older adults. Many of these studies have identified the same molecule of interest: spermidine. This molecule is found in every living organism, and it has been shown to have all sorts of incredible effects on the body; in fact, it is sometimes referred to as a "miracule." One of the main ways spermidine helps the body stay healthy is by supporting a process called autophagy.[2]

You can think of cells like a small factory — different organelles in the cell have different functions, like the nucleus, which directs cell function, or the mitochondria, which is, of course, the powerhouse of the cell. Even though they have different roles in the cell, they all come together with the overall goal of keeping the cell working properly. Just like machines in a factory, organelles can become defective or stop working completely. Instead of shutting the whole factory down, a factory worker would simply remove the machine parts that are defective, save the pieces that still work, and replace the broken parts to get that machine back up and running. In a cell, that repair cycle is called autophagy. Autophagy is how a cell removes damaged cell parts, like misfolded proteins or organelles that are no longer functioning properly, recycling them into functioning parts if they can or removing them if they are beyond repair. Without autophagy, your cells would be littered with broken cell parts, and this would make it harder for your cells to perform all the duties they need to do to keep you healthy. Spermidine induces autophagy, encouraging cells to reduce cellular clutter and keep working properly.

Something that is particularly interesting about spermidine is that the concentration of spermidine

in our blood changes as we age. The older we get, the less spermidine can be found in our blood. This is a phenomenon that can be seen in other animals as well, and it makes sense based on how our cells change with age; older adults tend to have more age-related diseases, many of which can be associated with decreased or impaired autophagy. Fascinatingly, adults that live to extreme ages (100 or older) don't always follow this trend. A study done in 2012 found that while the trend was observable between the first two age groups they studied (31-56 year olds and 60-80 year olds), the third group of adults aged 90-106 actually had higher spermidine levels than you would expect.[3] This study suggests that spermidine may not simply be a measure of health and age, but might actually be associated in some way with extreme longevity.

Another biological value that changes with age is mean cell volume, or MCV. This is a value that describes how large your red blood cells are, and it's expressed in femtoliters (an extremely small volume, as you'd expect). As we age, our average MCV gets bigger. This can be attributed to a few biological changes but is mainly thought to be related to red blood cell lifespan. Red blood cells are larger when they are younger, and they tend to have shorter lifespans in older adults, leading to an overall larger red blood cell size on average. The general trend seen in MCV also has an interesting shift in extreme ages, with centenarians having smaller red blood cells than expected for reasons that aren't fully clear (**Figure 5**).[4] To start building a better understanding of changes in health in the aging process, characterizing the relationships between MCV and age, spermidine and age, and potentially spermidine and MCV could lead to fascinating new discoveries.

The goal of my main research project in graduate school was to develop a method to quantify spermidine in human blood samples with known MCVs. This is no easy feat, for several reasons. First, while blood is a very interesting sample matrix, it is also incredibly complicated to work with. Blood transports metabolites and proteins around the body, which means that it is full of all

sorts of molecules that can complicate analysis. Sometimes molecules can compete for charge in the ionizer of the mass spectrometer; we call this a matrix effect, and it can make it difficult to study complex matrices like blood. Aside from matrix effects, spermidine itself is very small and extremely hydrophilic, which means that it isn't inherently amenable to some of the tried-and-true LC techniques like RPLC.

To tackle this difficult project, rather than trying to work with spermidine as it is naturally, we chose to change the structure of the molecule to make it easier to analyze. This is a technique called derivatization, where we modify the molecular structure to change the chemical characteristics of a molecule. In our case, we chose to use dansylation (**Figure 6**). This process changes the inherent hydrophobicity of spermidine, making it stay longer on the stationary phase and therefore much easier to analyze using traditional RPLC.

For all three analytical methods, we used a particularly useful MS-based technique called multiple reaction monitoring, or MRM (**Figure 7**). This technique makes use of tandem MS by tracking precursor-fragment pairs. With MRM, rather than detecting and quantifying every molecule in a sample, I can tell the MS to look for a specific precursor *m/z* and fragment pair. This helps cut through a complex sample, because rather than recording a complex spectrum of every molecule in the sample, the instrument will only measure and report out the pairs that I request.

Results and future steps

To quantify a molecule in a biological sample, it is important to first generate a calibration curve. You might be familiar with calibration in the context of baking; ovens often have to be calibrated in order to be sure the temperature inside the oven matches the temperature you set. To calibrate an oven, you could set it at 350 °F with a thermometer inside the oven to track the real temperature.

Once it is preheated, if you check the thermometer and see that it is reading 325 °F, you could then adjust the oven settings to ensure that the set temperature matches the temperature inside the oven.

To calibrate in this project, we used a similar molecule to spermidine, 1,6-diaminohexane, as an internal standard. An internal standard gives analytical chemists a frame of reference when they are quantifying an unknown. I can add a known amount of my internal standard to the samples and use that to calculate how much of my unknown is present. In a way, an internal standard is like the thermometer in oven calibration; just how I am confident that my oven thermometer is measuring the right temperature, I know exactly how much internal standard is present in my sample, because I added it in myself.

A calibration curve can be made by using standards (pure versions of your molecule of interest purchased from a chemical supplier); the internal standard will be the same concentration in every calibration point, but the spermidine concentration is varied to see what signal can be measured at different concentrations (**Figure 8**). When the signal is plotted vs. the concentration of the standard, a line can be generated. This line can then be used to calculate how much spermidine is in an unknown sample of whole blood. After extracting the spermidine from whole blood, we are left with a volume of blood extract containing spermidine and other small molecules. We can then add in the same amount of internal standard that we added in each point of the calibration curve. The internal standard serves as a frame of reference that allows us to calculate how much spermidine is in the blood extract.

In total, we extracted and quantified the spermidine from 26 blood samples from 26 different individuals, with MCVs ranging from 61 to 114 fL. We expected to see a negative relationship between the two, such that as MCV increased, spermidine decreased. Instead, we found that there was no correlation between the two values in the sample cohort we analyzed (**Figure 9**). The

results were not what we expected, but I think there is no better study to discuss in this chapter than my spermidine research, because it highlights the entire scientific process, including what happens when things don't go according to plan.

We believe that where our study went wrong was not having ages for the samples included in this sample set. These results suggest that if there is a relationship between MCV and spermidine concentration, it is not age independent. This hypothesis is supported by results from a previous study done by our collaborator for this project, Dr. Tom Raife. In that earlier study, he analyzed the entire metabolome of whole blood of 18 twin pairs (36 people total). Spermidine was detected and quantified in that study, so following the inconclusive results of this project, he re-analyzed the twin study data and looked at the correlation between spermidine and MCV. For the entire cohort, he saw a similar lack of correlation between spermidine and MCV. However, when he reduced the sample set to just those within a narrow age range (18-22 years old), there was a much stronger negative correlation, more like what we were expecting to see in our current study (**Figure 10**).

Our results, as well as the plot generated from the old twin study dataset, suggest that we should apply our quantitative method to a new sample set with both known MCVs and ages. Ideally, this sample set would be comprised of samples from subjects of only one age, or from a narrow range of ages. I hope to be a part of that study, should a new sample cohort come our way, but in my new research lab, I will be focusing my efforts more on disease research, with Alzheimer's disease and diabetes as my main focus.

Conclusion

Although the results of this study were not exactly what we expected, they still suggest that our

hypothesis that spermidine and MCV are negatively correlated could have some merit. In fact, our inconclusive results could be even more interesting than what we expected, as they suggest that age plays a more significant role in this correlation than we thought. In science, there is an expectation that only good results are published, making failure feel even more frustrating when all the published studies seem to be working just fine. It's important to keep in mind that behind every successful paper are a dozen failed experiments, all of which are key to driving research towards discovery.

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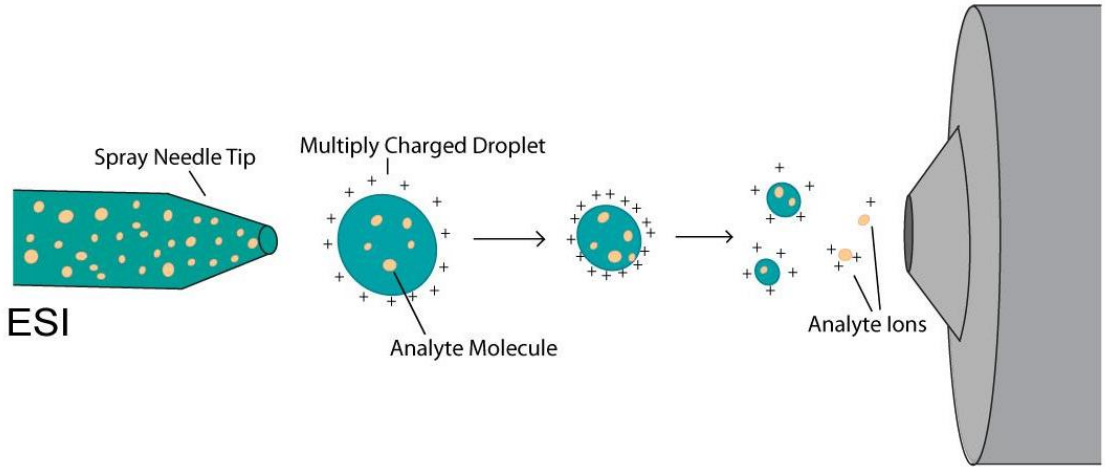

Figure 1: Diagram depicting electrospray ionization (ESI). One of the most commonly used soft ionization techniques is ESI. The sample is sprayed out of a needle tip that has voltage applied. The droplets that come out of the tip are charged. As they move towards the inlet, the solvent the sample is in evaporates off, leaving just ions that can be analyzed.

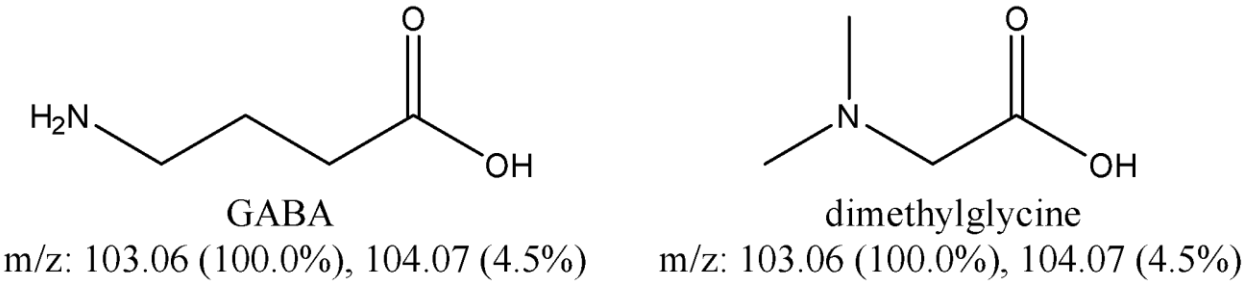

Figure 2. Two isomeric molecules, gamma-aminobutyric acid (GABA) and dimethylglycine. These molecules are visually different, but to a mass spectrometer, they look the same because of their identical *m/z*. We can use tandem MS to break these molecules into pieces; the pieces they break into are different, helping scientists tell them apart.

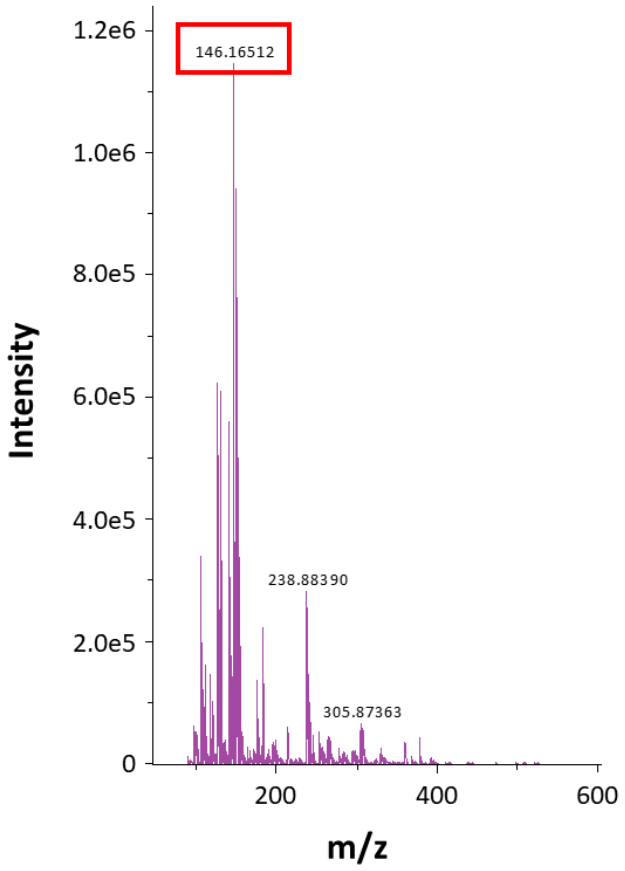

Figure 3. An example mass spectrum. In a mass spectrum, the x-axis shows the m/z, while the y-axis gives a value of intensity. The most intense peak corresponds to the molecule that is most abundant in the sample you are analyzing; in this case, the m/z highlighted with a red box corresponds to spermidine, a molecule of interest for my research.

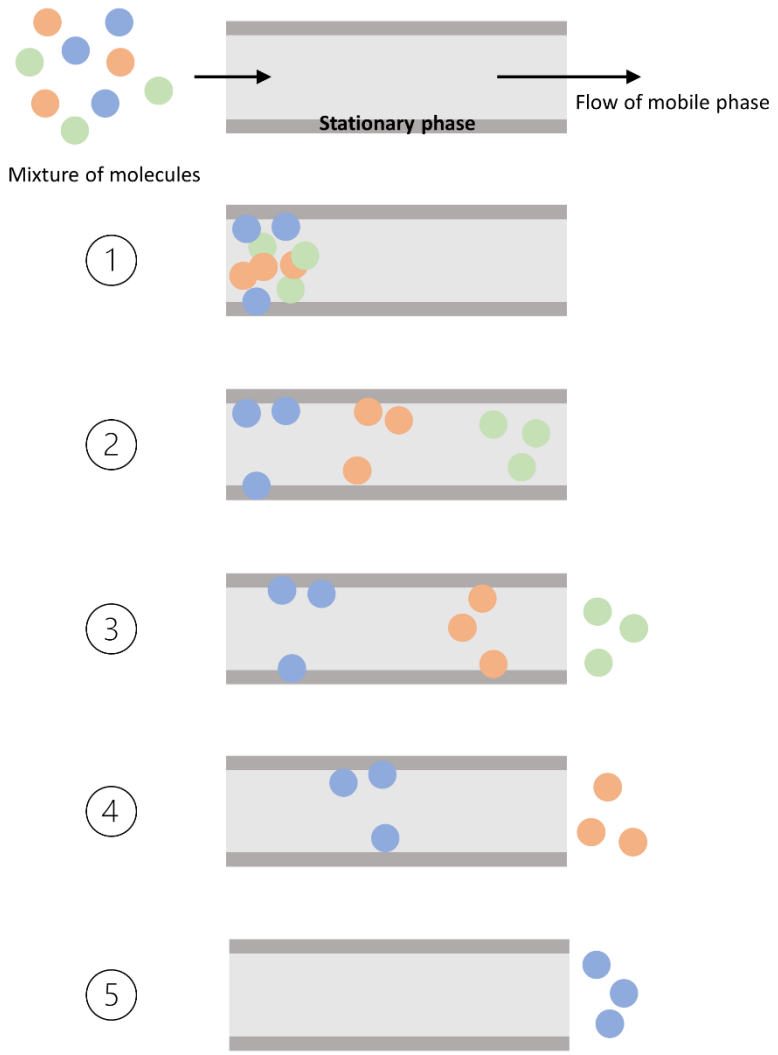

Figure 4. Reversed phase liquid chromatography (RPLC). In RPLC, the stationary phase is hydrophobic (water repellant), and the mobile phase starts hydrophilic (water attractive). First, a sample is introduced to the LC column. As mobile phase flows through, molecules in the sample will begin to separate based on their hydrophobicity. The first molecules that elute are the most hydrophilic (green), which will stay in the hydrophilic mobile phase. As we increase the hydrophobicity of the mobile phase by adding in more hydrophobic solvent, moderately hydrophobic molecules (orange) will elute. By the end of the gradient, the mobile phase is mostly hydrophobic, so very hydrophobic molecules (blue) will be eluted from the column.

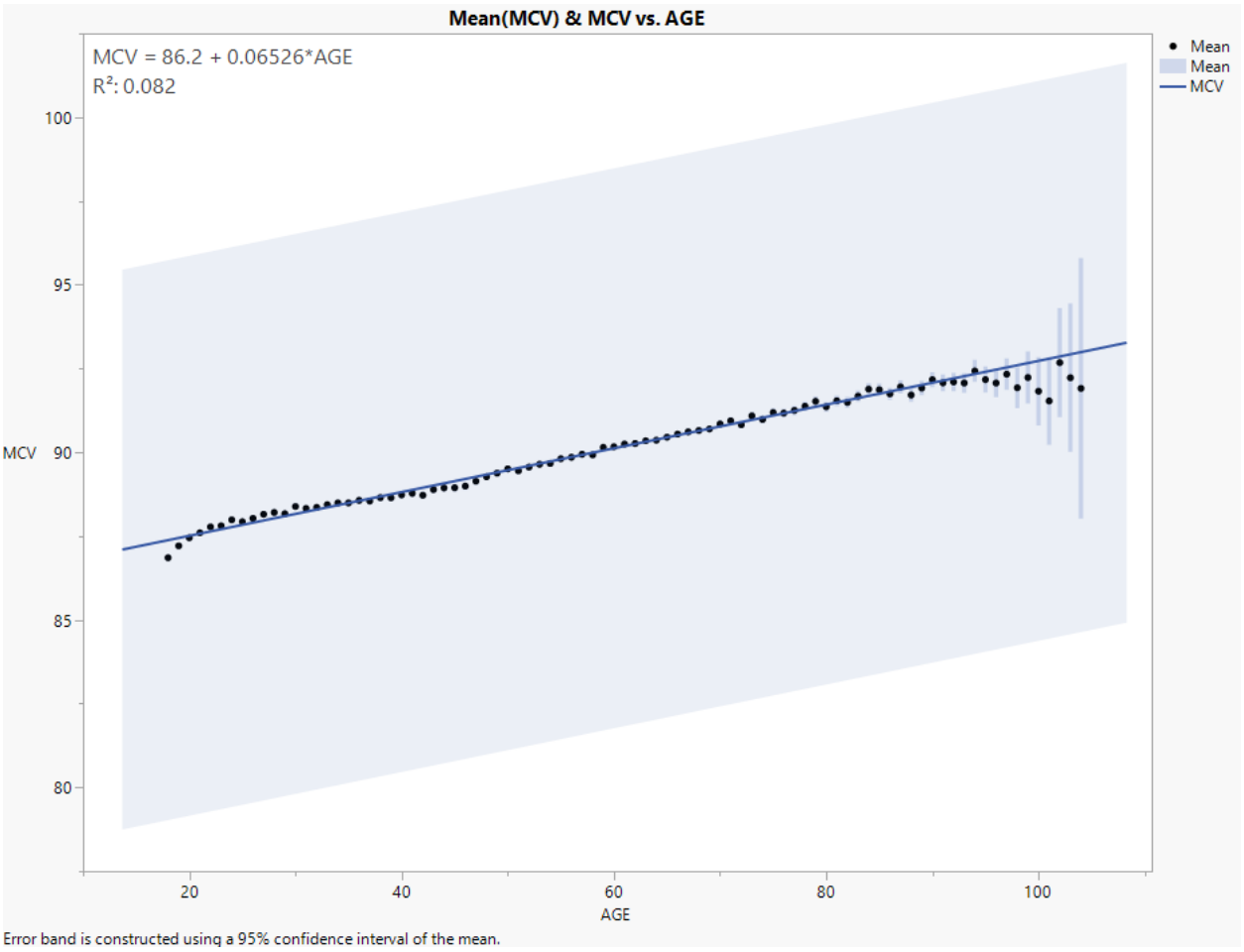

Figure 5. Plot showing mean cell volume (MCV) versus age. The relationship between MCV and age is well-characterized, with MCV typically increasing with age. In the more extreme ages, however (90-110), the trend loses linearity, as these older adults have smaller red blood cells than what would be expected based on the trend.

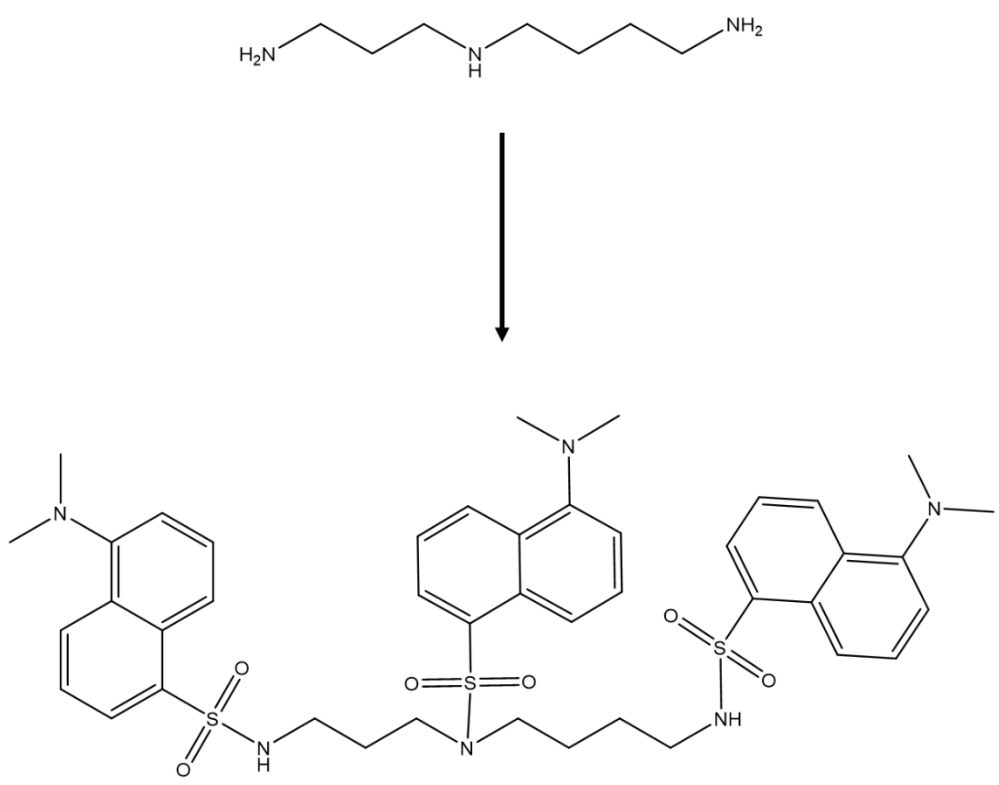

Figure 6. Spermidine (top) being converted into triply-dansylated spermidine (bottom). Adding a dansyl group to every amine on a spermidine molecule takes it from being a very small, hydrophilic molecule and turns it into a much larger, hydrophobic molecule. This means that rather than immediately eluting in an RPLC-based separation, it will be retained on the stationary phase, allowing for clean and reproducible separation.

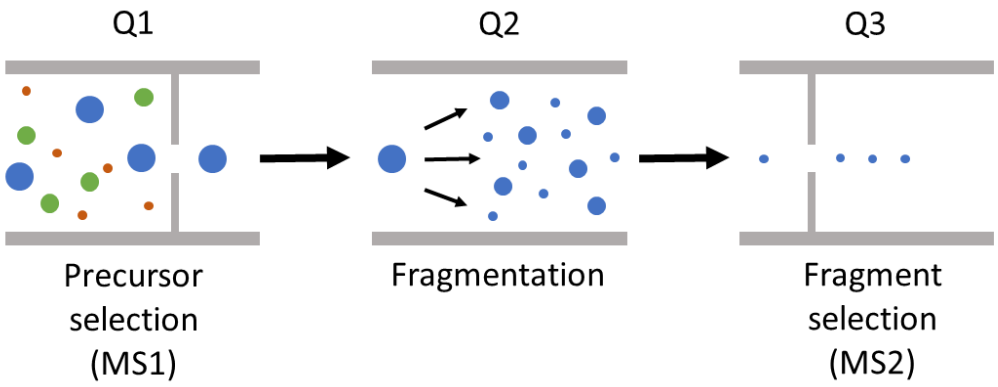

Figure 7. Multiple-reaction monitoring (MRM). MRM is a way to quantify specific molecules of interest in an otherwise complicated sample. This is often done using a specific kind of mass spectrometer called a triple quadrupole. The first quadrupole is a mass analyzer, where the precursor *m/z* (the mass of the intact molecule) is measured. The precursor of interest is selected and moves to the second quadrupole, where it is hit with a burst of energy and fragmented. The third quadrupole is a second mass analyzer, where the fragment *m/z* (the mass of the pieces) is measured. The precursor/fragment pairs can be input into the mass spectrometer, which searches for those pairs during the analysis. Only signal from those pairs will be reported out, significantly simplifying data analysis.

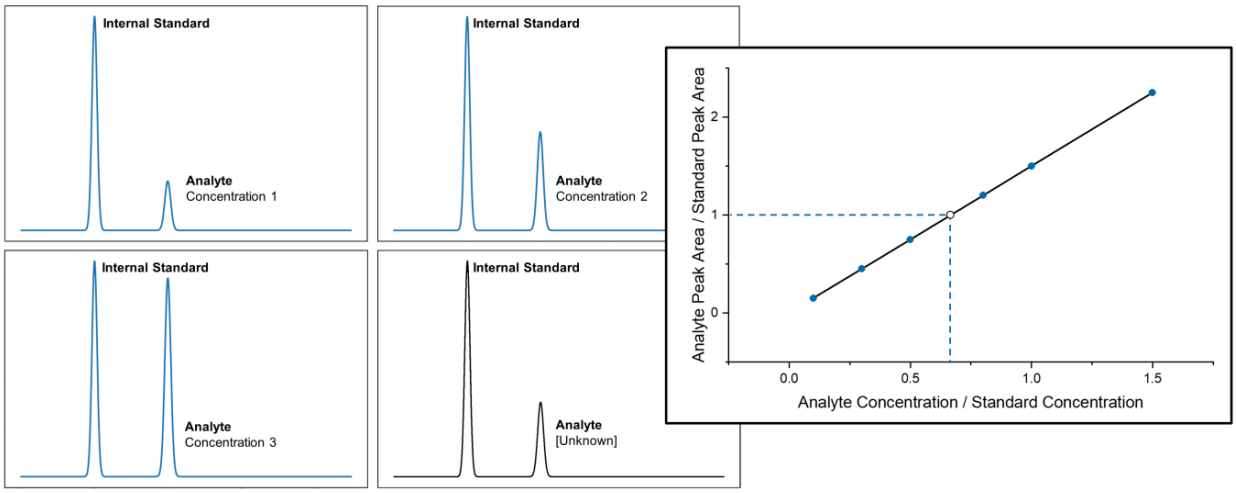

Figure 8. Example showing how a calibration curve is generated. For this project, we used an internal standard to build our calibration curve. To make the points, we hold the internal standard (in our case, 1,6-diaminohexane) constant, but vary the concentration of spermidine standard (shown in blue above). We can generate a calibration curve by plotting the concentrations of the standards against the signal generated in the mass spectrometer. When analyzing an unknown sample, we are able to add the same amount of internal standard to the unknown, and we can use the signal generated and the calibration curve to calculate the concentration of spermidine in our unknown sample. Courtesy to Cameron Kaminsky for this figure.

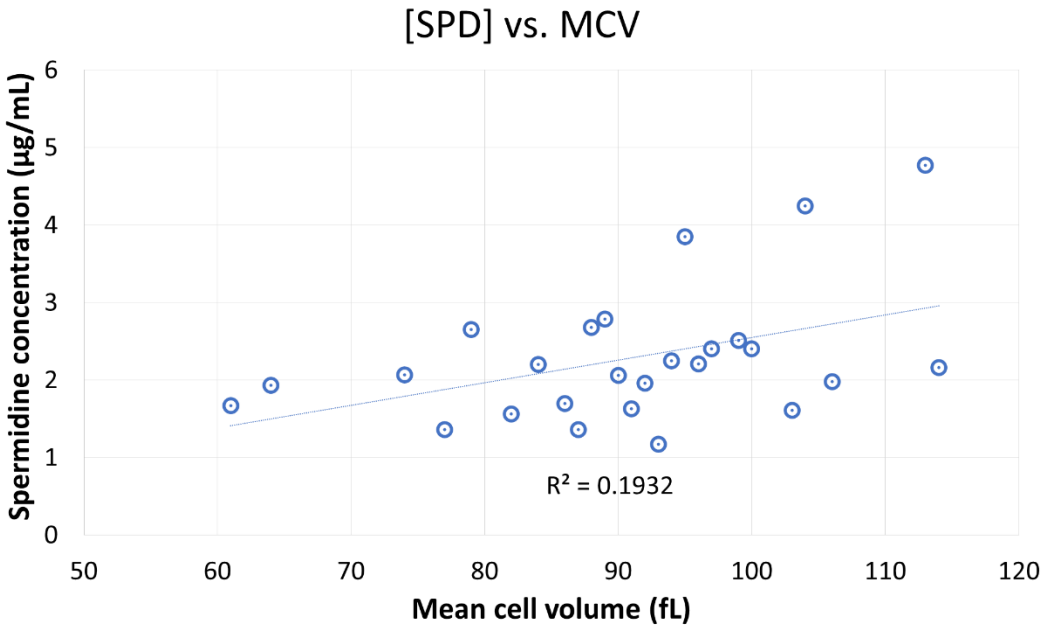

Figure 9. MCV values plotted versus spermidine concentration. Based on what is known about MCV and spermidine, we expected to see a negative correlation. Instead, we saw that there is no correlation, suggesting that age plays a much larger role in this relationship than we initially thought.

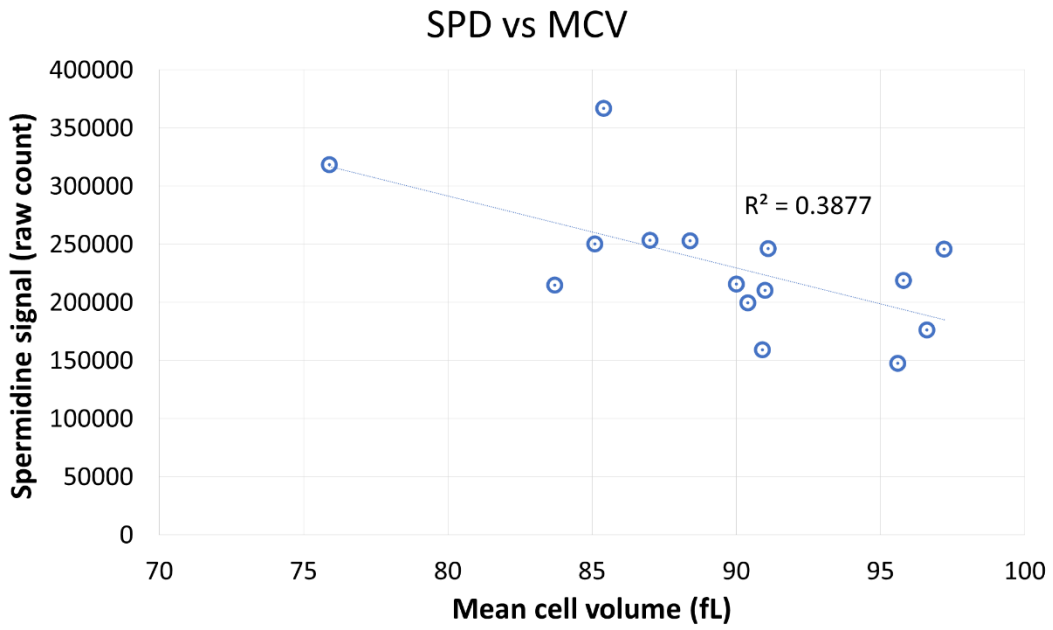

Figure 10. MCV values plotted versus spermidine signal in a narrow age range. Data from a previous study of whole blood collected from twins was re-analyzed following the results of this study. A similar lack of correlation between spermidine and MCV was observed in the entire cohort, but when a smaller age range was examined (18-22), a stronger correlation in the expected direction could be seen.