Cagrilintide: turning the tide, by your side

By Primum Non Nocere

Most of us here have researched all manners of GLP meds. Some have reached maximum dosages, and some have even stacked, yet still, they stall. Enter Cagrilintide.

With a completely different pathway than the GLP meds, it has been a boon to turn the tide against stalls. Like a sidecar to your cool GLP motorbike, the stall stood no chance.

But now there's a swirling controversy about the safety of Cagrilintide, specifically about the pH of reconstituted Cagri, the formation of fibrils, and whether it poses a serious health risk.

I usually like to mention articles and references at the very end of a write-up, but in this case, it is easier to mention the articles first because we'll be going back to them repeatedly throughout this.

The studies are posted at the end of this write-up, so go there now, open those links in separate tabs/windows, and then come back up here.

So the articles are:

ARTICLE 1:
The first link is an original publication of how they went from amylin and pramlintide (an older version of amylin analogue) to finally coming up with the final version of Cagrilintide (Codename: AM833). It's a pretty dry read but if you go through it, you'll see that Novo Nordisk (NN) tested not 1, not 2, but 27 different versions of "cagri"! They had to find the best analogue molecule that balances the solubility, stability, actual human half life, potency/efficacy, etc. And how they finally chose Analogue 23 as the winner. From hereon, for the sake of simplicity, this article/link/publication will be called ARTICLE 1.

ARTICLE 2:
The second link is somewhat related. This describes another attempt by NN to test another amylin analogue codenamed NN1213. While this isn't directly related to the current Cagri AM833, this study is useful as a comparator of a particular piece of data regarding formation of the feared "fibrils", which is sometimes referred to as High Molecular Weight Particles (HMWP). Also from my understanding, NN1213 development is currently on hold as NN chose to go with AM833 for now. From hereon, let's refer to this as ARTICLE 2.

ARTICLE 3:

Next is an article about ThioflavinT (ThT) test, which is a commonly used method to detect fibril formation, as a reference when we discuss fibril formation and testing. From hereon this will be called ARTICLE 3.

ARTICLE 4:
The fourth link is the Boogeyman, the source of this whole controversy. It is the patent filing describing the preparation of cagri and sema, and why they chose to separate the two peptides instead of combining it into 1 single mixture. This was filed in 2021. @Daringer 🫁 noted that when he read this, some parts of it are almost like word-salad, and that's because the original filing document was in Chinese! That's because it was originally filed in Taiwan/Taipei. So what you're reading is the English translation. From hereon this will be called ARTICLE 4.

ARTICLE 5:
The fifth link is yet another patent filing for cagrisema, but this time they're describing how they were able to COMBINE the two peptides, and they did it at a higher pH 5.8. They also did degradation test of Cagrilintide and Semaglutide at 37 Celsius under different mixtures at pH 4.0 and pH 6.0. This was a newer patent filing in 2023, filed originally in English so it's easier to read than the previous patent filing above. From hereon it will be called ARTICLE 5.

ARTICLE 6:
The next link is an article about amylin pharmacology and Alzheimer's pathogenesis, and how they may or may not be related, and the complexity of amylin effect on the disease process. From hereon this will be called ARTICLE 6.

ARTICLE 7:
The seventh link is simply a primer of amyloid fibril, describing what it is, the general properties, and how it is suspected to contribute to the disease process. This will be referred to as ARTICLE 7.

ARTICLE 8:
And the last link is just an old experiment that showed how macrophages with lysosomes help in getting rid of amyloid fibrils, and this will be ARTICLE 8. It's a pretty old publication from 1971 so it's in the form of scanned pdf, and sometimes you have to refresh multiple times to get those scanned pages up.

Ok so that's a pretty damn long intro. Now let's roll.

The current controversy, if I may summarize it briefly is like this:

PREMISE 1: Cagri needs to be reconstituted at pH 4, otherwise it will degrade and form fibrils.

PREMISE 2: When fibrils are injected SQ, it will find its way all over your body such as your brain tissue.

PREMISE 3: The fibrils that are deposited in the brain tissue cause Alzheimer's.

In short, the concern was: going from premise 1 to premise 3, if you reconstitute your Cagri at pH above 4, are you putting yourself at high risk for Alzheimer's?

That's a lot of leap and presumption, and the problem is not that these 3 premises are all wrong. Complete falsehoods are easy to disprove. The problem is that these are half-truths, oversimplifications, and things taken out of context, which is the type of fallacy most difficult to unscrew. We have to go back to basic biochemistry, then cross over to medicine, to be able to untangle this.

First open your tab for ARTICLE 1.
Amylin is naturally produced in your body together with insulin (they are co-secreted by the B cells of the pancreas). Amylin is prone to aggregate and form 'fibrils', so they made initial modification of its backbone with proline substitutions when they came up with Pramlintide, the previous amylin analogue. Problem is, Pramlintide is short-acting and needs 3 shots per day with meals, which is inconvenient, so they want to develop a long-acting one. You can read the nitty gritty details there (basically adding a lipid moiety to allow reversible albumin binding in human plasma to prolong the half-life, similar to how they prolong the half-life of GLP-1 drugs). But this lipid modification also makes it somewhat more prone to form fibrils, so they had to make further changes by adding "salt bridges" in the structure to address this and at the same time still preserving the solubility and efficacy. They started with (I think) 800 different versions and after in vitro studies and modeling, they narrowed it down to 27 different versions of "Cagri". They tested these for potency & receptor binding affinity (see Table 3) and solubility (Table 4).

And finally... We arrive at the heart of the matter: they tested the stability using ThioflavinT (ThT) lag test and peptide recovery (Table 5) with a Physical Stress Test. Let's go slightly deeper because this is important.

First, what is amyloid fibril? This is a good time to open ARTICLE 7 for reference. A peptide or protein is composed of a string of amino acids, which has its own primary, secondary, tertiary, and quaternary structures. The primary structure is simply the amino acid sequence, that's it. The secondary structure is where we are interested. Once the protein/peptide is synthesized with its relevant amino acid sequence, the backbone tends to take a shape of either alpha-helix (spiral) or beta-sheet (more flat), see the attached photo below for better visualization. The problem child here is the beta-sheet. When peptides assume the beta sheet flat form, they sometimes cross-link with more beta-sheet peptides. So they link and pile on top of each other forming the "Nucleus" or "seed" of the fibril. Then these seeds/nuclei grow/elongate by recruiting even more of the beta-sheet peptides on their wings, until they finally mature into a big linear &

relatively flat large molecule that we know as amyloid fibril. These fibrils are large and heavy (on rare occasions they can be so large and can be seen with regular microscopy), thus they are also called High Molecular Weight Protein/Peptide Particles (HMWP). So when you see the term HMWP in the tables or sentences of the articles I posted, in this context, it is referring to the fibrils.

By the way, amyloid fibrils is a general umbrella term, it can be composed of different things, such as amylin, amyloid beta, apolipoproteins, immunoglobulins/antibodies, and even insulin.

How do you detect these fibrils? Enter the ThioflavinT lag test. Now you can go to ARTICLE 3 if you want additional reading. ThioflavinT is a yellow-to-orange dye that can bind to fibrils, and once they bind they can be fluorescent, and the intensity of the fluorescence corresponds to the amount of fibrils, which you detect using spectrophotometry.

Now remember that Cagri analogue candidates backbone were all modified similarly to Pramlintide, specifically to minimize its tendency to aggregate and form fibrils, so if you just let them sit around undisturbed, it's going to take a looooong time before you start seeing any

fibrils. Nobody's got time for that, especially not Big Pharma, gotta make some money from this investment! Also, remember part of their patent filing includes the stability of the dissolved compound, so for example semaglutide has 2-year expiration from the manufacturing date, and insulin has maybe 1 year? Are they supposed to let their meds sit around on the lab refrigerator for 1-2 years? That's supremely impractical.

So we borrow the concept of cooking with a pressure cooker, speed things up. What you do is, you put these compounds under duress, force them to break down, force them to form fibrils! In fact this is industry standard.

There are 3 variables:

- **Chemical duress:** they chose 2 values, the so-called "optimal" pH 4.0, and "stress" pH 7.5
- **Temperature duress:** they chose 37 Celsius (98.6 F)
- **Mechanical duress:** I would argue this is the most important part: they put these compounds in a centrifuge and spin them at 960 rpm with 1mm amplitude. Headspinning. And this is very important. This severe mechanical agitation and compression is crucial in trying to make the peptides/proteins shatter or misfold and eventually cross-link their way to the eventual fibrils.

This is the so-called "stress storage condition". Novo Nordisk put the Cagri analogues under all these duress at the same time, for 45 hours non-stop, and they use spectrophotometry to detect ThT fluorescence every 20 minutes to see if there are any fibrils. If you open ARTICLE 1, scroll down to Table 5. The "lag time" is the time when the ThT test starts detecting fibrils, in other

words, longer lag time is better. And the "recovery" is the amount of peptide recovered from the solution after the stress test.

After all that, 2 compounds emerged as the best candidates: analogues 22 and 23. They both have excellent lag times and recovery at pH 4. But analogue 23 certainly beats out 22 at pH 7.5. And after further consideration of other factors (potency in human, half-life, peptide recovery, etc), Novo Nordisk decided that analogue 23 is the champion! The Michael Jordan of Scandinavia.

So now let's concentrate on analogue 23. NN knew from the pramlintide data that pH 4 is best, and it shows in the stress test, it has a lag time of 45 hours and peptide recovery of 96%. And in pH 7.5 the lag time is 41 hours, with recovery of 76%.

Now you say, wait.... 76%, does that mean the solution now contains 24% fibrils? No. Most of the "lost" peptide during the stress test are simply broken down molecules, only a small portion turn into fibrils. And for that, let's now open ARTICLE 2.

ARTICLE 2 describes yet another attempt by NN to find an amylin analogue, with a parent compound called NN1213, and this time they tested 21 different variations of this NN1213. Now go to Table 6 and Table 9, where they put the stress test report with ThT lag time and recovery. And this time they actually measured the HMWT (fibril) content!!

What do you notice there between the "recovery" and the "HMWT"? What I noticed were:

1. THERE IS NO CORRELATION between "Recovery" amount, and "HMWT" amount. Meaning, whether you recover 90% or 20% of the original peptide, it does NOT predict how much HMWT (fibril) you have.
2. Most analogues only have around 1-3% HMWT at the end of the stress test, so where did the rest of the lost peptide go? As mentioned before: degraded and broken down, but only a small portion of them turn into fibrils.

But then you say, well that's conjecture, and yes fair enough. So let's go to the last part of the chemical analysis before we go to the medical side. It's time to open ARTICLE 4 and ARTICLE 5.

ARTICLE 4 is the original Taiwanese patent filing that was the source of this controversy. In this formulation, they decided to separate Cagrilintide and Semaglutide, for the concern that Cagrilintide may not be stable enough at much higher pH, and that's an understandable concern, given that Cagrilintide backbone is similar to Pramlintide (having 3 proline substitutions to reduce the risk of aggregation and fibril formation). At pH 7, Cagrilintide can still undergo

deamidation of asparagine residues just like Pramlintide, which causes instability. So they decided to just stick to pH 4 for Cagri. But that's not the end of the story...

In 2023, there is a newer patent filing, open ARTICLE 5. Then go to table 2. Again it shows that pH 4 is best for Cagri, so that's non-debatable. But... Apparently adding Hydroxypropyl-beta-cyclodextrin (HP-B-CD) is significantly protective against Cagrilintide breakdown at pH 6 even at 37 Celsius. Read that again, 37 Celsius (98.6 F). That's a warm body. At 21 days, there was 75% Cagri remaining and only 0.5% HMWP/Fibril. That's like carrying your reconstituted Cagri vial in your armpit for 21 days straight. So once again for those sitting at the back, what's the magic word? REFRIGERATE!

And if you want to nerd it out further, keep reading that ARTICLE 5, you'll see how they play around with different things in the formulation, the effects of toxicity agents, surfactants, different buffers, etc.

BONUS POINT: The next time someone disses mannitol "filler", show them this article and scroll down to tables 10 & 11, it will show you that mannitol is the best tonicity agent for Cagri and Sema (and probably other peps too). That should shut them up.

So the bottom line of the chemistry part of this writeup is: sure reconstituted pH 4.0 may be best for Cagrilintide but higher pH is ok too, as long as it's not extreme (again just me personally, I'm very comfortable with anything pH 6.5 or below, maybe even pH 7 if my storage condition is good and I'm not stretching it ridiculously like 5 months or longer).

Now let's go to the medical side!

This is actually the easier part.

Fibril this, fibril that, fibril everywhere...

What's the problem with these fibrils? Once these proteins or peptides transform into fibril form, they become very stable. So stable in fact that they are stiff, nonfunctional, and insoluble, that they are deposited in the tissues where they are formed.

So how does the brain end up with some amylin fibril deposits? As mentioned, amylin is co-secreted with insulin into your bloodstream, and the original form of amylin is small and soluble, travelling all over, able to cross the blood-brain-barrier (BBB) and bind to its receptors in the brain cells, among other organs. If you have too much circulating amylin including in your brain tissue, some of them will eventually clump together WITHIN the brain to form the amylin fibrils and get deposited there. Let me say this one more time: the amylin fibrils in the brain are formed WITHIN the brain.

Amylin fibrils that are formed outside the brain are insoluble and cannot travel long distances, and they will get deposited in the tissues where they are formed. So even if your Cagri is in such a bad shape that it has so many fibrils in it, if you inject it SQ, it will stay there and potentially causing differing degrees of ISR, some mild, some severe to the point of tissue necrosis. But they don't magically teleport themselves into your brain.

Of note, just as a medical addendum, while beta-amyloid protein has been implicated in Alzheimer's (and even then it's still not fully understood how, nobody is 100% sure now that this is the true cause of Alzheimer's), amylin fibrils role in Alzheimer's is much more questionable. Only more recently that researchers are looking more into it and there's a lot of unanswered questions and questionable associations. But that's beside the point. What's important right now is that, no, your fibril deposit under the skin is not about to find its way to your brain. These fibrils usually possess some resistance to enzyme-mediated breakdown, but your immune cells (especially your macrophages that contain lysosomes) can help in "digesting" and slowly removing these fibrils, as seen in the experiment shown on ARTICLE 8. So obviously you don't want to load your skin with a ton of amyloid fibrils, but the amounts we get from reconstituted peps are frankly quite small. As always, the dose makes the poison. Yes, even pharma-grade peptides contain minute amounts of fibrils. Heck, even your own body makes very small amounts every single moment but your body can keep pace getting rid of them in low amounts.

Whew, that was a long one. Let's recap.

- **Is pH 4 best for Cagri?** Sure! If you want to strive for that and are confident about your ability to tinker with buffered

 solutions, nobody will stop you. Just be careful about the amount and type of buffer you use, more is not always better.
- **Am I going to sweat it and try to go for pH 4?** Personally I won't. Personally I'm comfortable with anything below pH 7. That being said, it may not be a bad idea to test the pH of your reconstituted peptide occasionally, a good raw and lyophilization facility would have included buffers in their finished products, which should keep the reconstituted peptides in the acidic range for Cagrilintide.
- **Am I going to refrigerate my reconstituted Cagrilintide?** ABSOLUTELY. But don't freeze. Be cool, not cold.

Lastly, in the wise words of @TessaM: do not play badminton with your pep vials.

ARTICLE 1:

https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00565

ARTICLE 2:
https://pubs.acs.org/doi/full/10.1021/acs.jmedchem.4c00022

ARTICLE 3:
https://www.tandfonline.com/doi/full/10.1080/13506129.2017.1304905

ARTICLE 4:
https://patents.google.com/patent/TW202140063A/en

ARTICLE 5:
https://patents.google.com/patent/WO2023187067A1

ARTICLE 6:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9886804/#:~:text=The%20metabolic%20peptide%20hormone%20amylin,to%20Alzheimer's%20disease%20(AD).

ARTICLE 7:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2634529/

ARTICLE 8:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2047489/?page=1