

The β -cell assassin: IAPP cytotoxicity

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Abstract:

Islet amyloid polypeptide (IAPP) forms cytotoxic oligomers and amyloid fibrils in islets in Type 2 diabetes (T2DM). The causal factors for amyloid formation are largely unknown. Mechanisms of molecular folding and assembly of human IAPP (hIAPP) into β -sheets, oligomers and fibrils have been assessed by detailed biophysical studies of hIAPP and non-fibrillogenic, rodent IAPP (rIAPP); cytotoxicity is associated with the early phases (oligomers/multimers) of fibrillogenesis. Interaction with synthetic membranes promotes β -sheet assembly possibly via a transient α -helical molecular conformation. Cellular hIAPP cytotoxicity can be activated from intracellular or extracellular sites. In transgenic rodents overexpressing hIAPP intracellular proapoptotic signals can be generated at different points in β -cell protein synthesis. Increased cellular trafficking of proIAPP, failure of the unfolded protein response (UPR), or excess trafficking of misfolded peptide via the degradation pathways can induce apoptosis; these data indicate that defects in intracellular handling of hIAPP can induce cytotoxicity. However, there is no evidence for IAPP overexpression in T2DM. Extracellular amyloidosis is directly related to the degree of β -cell apoptosis in islets in T2DM. IAPP fragments, fibrils and multimers interact with membranes causing disruption *in vivo* and *in vitro*. These findings support a role for extracellular IAPP in β -sheet conformation in cytotoxicity. Inhibitors of fibrillogenesis are useful tools to determine the aberrant mechanisms that result in hIAPP molecular refolding and islet amyloidosis. However, currently, their role as therapeutic agents remains uncertain.

46

47 **Introduction**

48 Two aspects of the amyloidogenic process are significant for diabetes. Firstly the role of
49 small assemblies (so-called “oligomers”) of Islet Amyloid PolyPeptide (IAPP, also known as
50 amylin) which can have a cytotoxic role in the islet (Westermarck, et al. 2011) and secondly,
51 the degree of replacement of islet cells by the invasive amyloid deposits in Type 2 diabetes
52 (T2DM) (Clark, et al. 1988; Jurgens, et al. 2011). These processes are likely to be linked in
53 T2DM since the process of amyloidogenesis damages cells whatever the mechanism of the
54 IAPP-induced toxic insult. Cell death and toxicity in relation to diabetes is a more apparent
55 and testable end point in animal models than is possible in humans; but it must be
56 remembered that studies *in vitro* and in created animal models *in vivo* are unlikely to model
57 exactly the slow (possibly over decades) process of islet amyloid deposition in T2DM.
58 Moreover, islet amyloidosis and substantial islet cell cytotoxicity is not a universal feature of
59 all patients with T2DM and is likely to be only one of the many factors that contribute to
60 T2DM in humans.

61 Amyloid fibrils are macromolecular assemblies in an elongated fibrillary form which are
62 deposited in different tissues of the body in different disease states. The common feature of
63 the amyloid diseases is that the component molecules have become refolded from their
64 normal conformation as a result of genetic mutations or disease conditions (Glenner 1980)
65 (Chiti and Dobson 2006). Amyloid fibrils share several common structural features despite
66 the wide diversity of proteins and polypeptides that form amyloid *in vivo* or *in vitro*. The
67 inter-molecular cross- β conformation in which β -strands run perpendicular to the long axis of
68 the fibril and interstrand hydrogen-bonds are parallel to the fibril axis is a common feature,
69 as is the long and unbranched nature of the fibrils (Riek and Eisenberg 2016). The term
70 “oligomer” describes assemblies of small numbers of β -strands/molecules (dimers-
71 multimers) at the beginning of the process of amyloidogenesis (Glabe 2006). The protein
72 molecules are non-covalently linked in the polymeric (fibril) state by backbone hydrogen
73 bonding and sidechain sidechain interactions. Thus, amyloid formation differs from most
74 synthetic polymeric molecules which involve covalently linked monomers

75 The biophysical properties of IAPP and proIAPP have much in common with the Alzheimer
76 peptide A β which is the major component of cerebral amyloid plaques in Alzheimer’s disease
77 (AD). The major difference between amyloidosis of these two peptides is that, whereas

increased concentration/decreased clearance of an alternative cleavage product of the larger Alzheimer precursor protein (APP) results in the amyloid plaques in AD (Selkoe 2011) (islet amyloid is formed from the normal sequence of the peptide in Type 2 diabetes. Whether there is decreased clearance of proIAPP/IAPP from the islet spaces in islet amyloidosis has yet to be determined.

The structure of IAPP and fibril assembly

Early in the study of IAPP amyloidogenesis it was observed that differences in the sequence of IAPP within positions 20-29 in rodents and humans correlated with the lack of islet amyloid in rats and mice (Westermarck, et al. 1990); human IAPP (hIAPP) and rodent IAPP (rIAPP) differ in six amino acids in this region (Figure 1). The most important of these differences are the three proline residues in rIAPP at positions 25, 28 and 29 and the His-18 to Arg substitution. Proline disrupts the hydrogen bonding and intra-molecular β -sheet formation and this could potentially explain the lack of islet amyloid in rodent models of diabetes (Betsholtz, et al. 1989). Replacement of His-18 in hIAPP with Arg in rIAPP ensures that the sidechain will be positively charged at all physiologically relevant pH values and decreases the tendency to aggregate and form amyloid (Abedini and Raleigh 2005; Jha, et al. 2014). Work with small fragments of rIAPP and hIAPP have shown that other regions of the molecule can themselves aggregate to form β -sheet rich amyloid structures and thus could interact in the assembly process of hIAPP 1-37. It is now known that the peptide fragments hIAPP 30-37, 8-20 and 10-19 also form amyloid fibrils (Gilead and Gazit 2008; Jaikaran, et al. 2001; Mazor, et al. 2002; Nilsson and Raleigh 1999). The sequence IAPP 30-37 is identical in rodent and human IAPP (Figure 1), suggesting that this region of the rodent sequence has the potential to form β -sheets, but the adjacent proline-rich region in rIAPP and the additional charge at position 18 prevents amyloid formation by the full length rodent polypeptide. Studies with variants of hIAPP have shown that proline substitutions outside the 20-29 region can abolish amyloid formation by hIAPP, and replacement of Asn-14 or Asn-21 can have drastic effects (Abedini and Raleigh 2006; Koo, et al. 2008). Conversely, replacement of residues Arg-18, Leu-23, and Val-26 in rIAPP by the corresponding amino acids found in hIAPP leads to weakly amyloidogenic polypeptide even though it still contains the three proline substitutions found in rodent IAPP (Green, et al. 2003). Collectively these studies and the studies of IAPP derived peptide fragments show that factors other than the sequence within residues 20-29 play an important role in dictating the amyloidogenicity of

hIAPP, none-the-less the sequence within the 20-29 segment is an important amyloidogenic determinate.

Native hIAPP is a positively charged, hydrophobic peptide due to the amidated C-terminus and the lack of acidic residues. In cells and in the circulation hIAPP is considered to be in a so-called “intrinsically disordered” state (Williamson and Miranker 2007). This term refers to the fact that the IAPP molecule does not adopt a rigid globular conformation, but rather samples an ensemble of rapidly converting, partial and less structured conformations. The question is how and when does the conformation change to create cytotoxic refolded molecular structures? There have been suggestions from Nuclear Magnetic Resonance (NMR), crystallography studies of IAPP-maltose binding protein fusions and circular dichroism (CD) studies that, prior to the refolding into a β -conformation (the crucial unknown condition which leads to islet amyloidosis), the molecule adopts a transient α -helical conformation (Abedini and Raleigh 2009; Apostolidou, et al. 2008; Williamson and Miranker 2007; Wiltzius, et al. 2008). Lipids and interactions with membranes can promote the formation of a partial helical state (Apostolidou et al. 2008; Knight, et al. 2006; Williamson, et al. 2009). It is now considered that this helical conformation facilitates the conversion of hIAPP to β -conformation and subsequent intermolecular association.

Several structural models have been proposed for hIAPP amyloid fibres formed *in vitro* and they share common features, although they differ in the details of the assemblies (Luca, et al. 2007; Wiltzius et al. 2008). Each monomer of IAPP forms two β -strands connected by a loop/hairpin region. Monomers stack on top of each other such that the backbone to backbone hydrogen bonds are located between different molecules rather than within a single polypeptide. The fibre is made up of two symmetrical related U-shaped stacks of monomers oriented such that polypeptides in adjacent stacks are orientated anti-parallel to each other (Figure 2). The region IAPP 20-29, although now known not to be the only sequence to regulate fibril formation, is likely to be important for the initial refolding of the molecule into a hairpin shape turn/loop in the region of residues 18-27 (Akter, et al. 2016; Luca et al. 2007; Westermark et al. 1990; Wiltzius et al. 2008) (Figure 2). Different hypotheses concerning the intramolecular stacking of the side chains between two adjacent monomers have been proposed. One model proposes that two monomers in adjacent stacks pack via the side chains of residues 26-32; this forms the building block of the oligomer (Figure 2). The disulphide bridge between residues 2 and 7 at the N-terminal end of the peptide is not part of the β -sheet assembly in this model and remain largely exterior to the longitudinal axis of the protofibril.

(Figure 2) (Luca et al. 2007; Wiltzius et al. 2008). Alternatively, the turn/loop connecting the two β -strands within each monomer had been proposed to occur between residues 18–22, association of the two monomers has been postulated to occur via a steric zipper between the regions residues 20-27 and residues 29-33 (Wiltzius et al. 2008).

The naturally occurring mutation Ser20Gly (S20G) found in a small number of Asian diabetic patients (Lee, et al. 2001; Sakagashira, et al. 1996) has been shown to enhance amyloidosis and cytotoxicity in hIAPP S20G transgenic mice compared to hIAPP native sequence transgenic mice (Meier, et al. 2016), and to accelerate fibril formation *in vitro* (Cao, et al. 2012; Krotee, et al. 2017; Ma, et al. 2001; Sakagashira, et al. 2000). It has been suggested that this mutation creates a different intermolecular energetics at this site which affects β -sheet assembly (Krotee et al. 2017). However, the real question is which part or parts of the molecule and which assemblies of multimers act as the toxic species to instigate cell death.

Amyloid proteins/peptides assemble into amyloid fibrils with a distinct time course which represents different stages in assembly processes (Figure 3) (Chiti and Dobson 2006). Amyloid formation typically proceeds through so called “lag phase”, during which little or no detectable amyloid is present. This leads to a growth phase and culminates in a plateau phase (saturation phase) in which amyloid fibrils are in equilibrium with residual soluble peptide. These stages can be identified *in vitro* by analysis of molecular conformations and β -sheet assemblies and are typically monitored *in vitro* using the small dye thioflavin-T (Abedini, et al. 2016). It is presumed that similar processes would occur *in vivo* but with different time courses depending upon the cellular conditions. The first or lag phase starts with monomeric peptide and, if the conditions are favourable, refolding of monomers and assembly of oligomers; oligomers then assemble into protofibrils/protofilaments which are components of mature amyloid fibrils (Sunde and Blake 1997) (Figure 3). The growth phase represents the elongation of the protofibrils and assembly of the fibrils (Figure 3). Eventually the vast majority of the amyloidogenic molecules are incorporated into amyloid fibrils and no further activity is present; this is the saturation phase (Figure 3). *In vivo*, this last theoretical situation would never occur since the β -cells continue to secrete IAPP and new molecules would be added to existing deposits in the extracellular space or create new sites of amyloid formation in the islets. It has been suggested from *in vitro* experiments that there is little or no cytotoxicity of the mature amyloid fibrils from the saturation phase (Abedini et al. 2016). It is notable that synthetic hIAPP in fibrillar form when added to cultured cells or islets is not

cytotoxic to cells in culture. Extending this *in vitro* finding to the *in vivo* situation, it suggests that the extensive “mature” IAPP amyloid deposits in the extracellular space containing many millions of molecules in fibrillar form are only minimally cytotoxic; newly refolded IAPP monomers/oligomers which are not incorporated into the extensive fibrillary assembly could then be the cytotoxic species.

During the lag phase, the peptide structure appears largely amorphous by transmission electron microscopy (EM) and soluble assemblies ranging from monomers to hexamers have been detected by photochemical induced cross-linking and by ion mobility mass spectrometry (Abedini et al. 2016; Young, et al. 2014). At this stage, unlike material present in the elongation phase, there is little evidence of measureable well developed β -sheet structure and the assemblies could be easily digested with proteolytic enzymes (Abedini et al. 2016; Young et al. 2014). These preparations from the lag phase were cytotoxic whereas samples taken later from the reaction, from the elongation and saturation phases has less or no cytotoxic properties. Parallel investigations with rIAPP and hIAPP peptides with amino acid substitutions indicated that although oligomers were formed, they had limited or no cytotoxic properties (Abedini et al. 2016; Young et al. 2014)

Cytotoxicity, peptide fragments and membrane interactions.

Experimentally, cytotoxicity *in vitro* is determined by addition of different fragments or peptides to cells (usually β -cells) in culture. Alternatively, peptide interactions with model membranes or liposomes *in vitro* can provide data on how peptides would combine with cell surfaces. However, many of the early observations on hIAPP peptides *in vitro* were hampered by the tendency of synthetic hIAPP and its fragments to rapidly form amyloidogenic assemblies *in vitro*. Thus careful handling of the peptide is required to ensure that correct conformations are being investigated at that moment in time (Higham, et al. 2000b). This is a more difficult scenario with cells in culture where hIAPP fibril formation is a dynamic and rapid process; many of the early reported studies were not able to differentiate between the IAPP conformation during the early phases of the oligomeric assembly (when the peptide was first added to culture) and the presence of mature fibrils (at a later time point when the cells died). Measurement of β -sheet structures by Thioflavin T fluorescent assay is not suitable to probe the initial assemblies of IAPP. In addition, most analytical methods such as circular dichroism (CD) or Fourier Transform Infrared Spectroscopy (FTIR) of peptides undergoing molecular structural changes indicates only the overall nature of the intermediates

of fibril assembly in the sample. However, Nuclear Magnetic Resonance (NMR) and Ion-mobility spectrometry–mass spectrometry (IMS-MS) can detect small molecular intermediates (Young et al. 2014) while newly developed two dimensional IR methods can provide residue specific information about the development of β -sheet structure (Kamata, et al. 2014). These methods provide a dynamic view of the fibril formation with appropriate time resolution given the life time of the process (Abedini et al. 2016)

It is now apparent that many different IAPP fragments including components of rIAPP and mutated sequences, will form oligomers. However, not all of these are cytotoxic (Abedini et al. 2016) as measured by immediate responses (not the terminal point of cell death) such as upregulation of proinflammatory markers. In general, the molecular species that has been identified to interact with the cells and elicit a response were those taken from the lag phase and low order oligomeric assemblies with little β -sheet component (Figure 3); the samples containing the mature fibrils in β -sheet conformation (Figure 3) were less cytotoxic (Abedini, et al. 2016). This corresponds to results from other methods of analysis of the early phases of fibrillogenesis (Young, et al. 2014). However, other similar studies have suggested that the toxic species include hIAPP fragments that are already in β -sheet conformation (Krotee et al. 2017).

Although it has proved to be exceptionally difficult to correlate *in vitro* studies with cell biology, fibrillogenesis of many amyloidogenic proteins is enhanced at a synthetic membrane interfaces and this may be linked to an increase in permeability (Figure 4A). The membrane induced self-assembly pathway of any amyloidogenic protein, including IAPP, has yet to be determined and a multitude of conflicting mechanisms have been proposed to explain their membrane activity. The confusion is likely due, in part, to the wide variety of model membrane systems employed for *in vitro* studies. Most existing model membranes are incomplete mimics of the relevant biological membranes; they all lack asymmetry, most lack cholesterol and they often have high levels of anionic lipids. It is not clear how studies with these simplified model membranes translate to the situation *in vivo*. Human IAPP is believed to initially interact with membranes though the N- terminal region (Knight et al. 2006), but the role of individual residues is not understood. It is not known if features that modulate amyloid formation in solution and *in vitro* also do so in the environment of a biological membrane. Not only is the structure of the lipid bilayer and relative concentrations of phospholipids and cholesterol important but also the presence of many membrane associated proteins; these include membrane receptors, ion channels, proteins interacting with

extracellular matrix. Intragranular IAPP will come into close proximity with all of these components on exocytosis of the secretory granule.

It has been shown that interaction of monomeric human IAPP with synthetic phospholipid membranes or bilayers promotes a transient conformational change to a molecular α -helical structure at the membrane interface with the helices lying parallel to the membrane surface (Apostolidou et al. 2008; Knight et al. 2006). Synthetic membranes which contain a significant fraction of anionic lipids significantly accelerate human IAPP amyloid formation *in vitro*, with more highly charged membranes having a larger effect (Zhang, et al. 2017). Most studies of hIAPP-membrane interactions have made use of simple model membranes comprised of pure anionic lipids, such as phosphatidylglycerol (PG) or phosphatidylserine (POPS), or mixtures of anionic lipids with zwitterionic lipids, such as 1- palmitoyl- 2-oleoyl- phosphatidylcholine (POPC). The content of anionic lipid typically ranges from 25 to 100 mole % of the total phospholipids.

Standard model membranes employed in biophysical studies fail to capture the biology of membrane disruption *in vivo*. The β -cell plasma membrane contains a low percentage of anionic lipids, (estimates range from 2 to 13%) and cholesterol and is asymmetric. The outer leaflet is enriched in the neutral lipids and contains a very low percentage of anionic lipids, while the inner leaflet is enriched in phosphatidyl- ethanolamine (PE) and the anionic lipids, POPS and phosphatidylinositol (PI) (Abedini et al. 2016; Trikha and Jeremic 2011). Thus extracellular IAPP will face a membrane with a very low percentage of anionic lipids. Even the inner leaflet contains a lower percentage of anionic lipids than is employed in most model membrane studies. Cholesterol is important for the uptake of hIAPP *in vivo*, (Trikha and Jeremic 2011) but the majority of biophysical studies of human IAPP membrane interactions have used vesicles that lack cholesterol. Some biophysical studies that included cholesterol also employed high concentrations of anionic lipids and these can mask the effect of the sterol. Higher concentrations of cholesterol in model membranes decrease the rate of fibril formation at the surface whereas increased POPS leads to increased rate of fibrillogenesis (Zhang et al. 2017).

In vitro, hIAPP binding to synthetic liposomes induces both membrane leakage (an *in vitro* marker of potential cell damage) and cellular toxicity (Kumar, et al. 2015). However, synthetic rIAPP as well as other hIAPP variants (which are unable to adopt β -sheet conformation and

do not form fibrils nor induce cytotoxicity) induce low leakage with less efficiency than hIAPP *in vitro*, although higher concentrations of rIAPP can induce vesicle leakage that is comparable to that induced by toxic hIAPP even though rIAPP is not toxic under the conditions studied (Cao, et al. 2013a; Cao, et al. 2013b) . The mechanism of IAPP-mediated synthetic membrane leakage is controversial. IAPP induced membrane damage has been proposed to result from a range of different mechanisms some of which can be related to biological systems. It was suggested that hIAPP caused changes in membrane curvature and pore formation of synthetic membranes (Engel 2009; Engel, et al. 2008) and increased membrane conductance has been detected in cells (Casas, et al. 2007; Casas, et al. 2008; Engel et al. 2008; Kaye, et al. 2004; Mirzabekov, et al. 1996) (Figure 4B a). However, it is unclear if these pores are persistent or transient. Other studies have argued for a carpeting, detergent like mechanism removing some parts of the membrane (Figure 4B b) (Lee, et al. 2012). There is also evidence for association of membrane damage with the growth of amyloid fibrils at the membrane surface (Engel et al. 2008). Changes in the composition of the model membranes, including incorporation of cholesterol demonstrated that hIAPP dye leakage from liposomes was more sensitive to membrane composition than amyloid fibril formation at the surface (Zhang et al. 2017). However, the time course of sustained leakage and fibril formation are comparable which is consistent with fibrillogenesis at the membrane surface (Brender, et al. 2011; Cao et al. 2013b; Engel 2009). Taken together these studies suggest that hIAPP fibrils developing at the surface of the cell might exert cytotoxicity and that the membrane composition of β -cells (or other islet cell types) might be critical in susceptibility to external attack by multimeric hIAPP or hIAPP fibrils.

A live cell approach to membrane inter-actions has been made using radioimaging techniques, MIN-6, clonal β -cells and a lipophilic dye, laurdan has been used to indicate changes in membrane fluidity. The data suggest that in the early stages of fibrillogenesis, hIAPP partitioned into the membrane and later mature fibrils can induce cell blebbing and death (Pilkington, et al. 2016). Experiments on cells treated with various preparations of soluble or partially insoluble preparations of IAPP, identified potential membrane channels as sites for action (Casas et al. 2008; Engel et al. 2008; Kaye et al. 2004; Mirzabekov et al. 1996). These oligomeric forms of human, but not rIAPP, were proposed to act via the transient receptor channel (TRPV4) in β -cell membranes to increase influx of calcium (Casas et al. 2008) and to downregulate the intracellular proteasome degradation pathway (Casas et al. 2007). Clustering of hIAPP at the plasma membrane and internalisation of the peptide

has been noted in INS-1 β -cells cultured with hIAPP (Trikha and Jeremic 2011) suggesting that external interaction of the peptide with the membrane could lead to internalisation of fibrils. This in turn could lead to activation of cell death pathways from within the cell.

Where does the cytotoxicity originate in vivo ? Extracellular or Intracellular?

The question of the origin of the insult that results in cell death has been a long debated point. In vivo, does the insult arise from within the cell (as a defect in the pathway of a secreted peptide) or is cell death triggered from the secreted peptide accumulating at an extracellular site and interacting with the cell membrane or a receptor? It is likely that there is more than one mechanism of toxicity and different mechanisms may be more or less effective depending upon the cellular conditions. It is notable that *in vivo*, it is primarily the islet β -cells (from which the peptide is secreted) and not the adjacent α - or δ -cells that are affected by the cytotoxicity. Alpha cells also have higher survival rates when exogenous synthetic hIAPP is applied to intact islet cells (Law, et al. 2010). This suggests that the β -cells (and/ or their plasma membranes) are more susceptible to the effect of hIAPP compared to α -cells and it is not just the process of IAPP peptide secretion from β -cells which differentiates the cell susceptibility.

Intracellular IAPP and fibrils

Most amyloidogenic proteins are secreted proteins/peptides which have undergone considerable post-translation modification, including protein folding within their cells of origin. Some are misfolded during their original production (often as a result of a genetic mutation) and other become misfolded at later stages (Chen, et al. 2015). ProIAPP undergoes post-translational processing in islet β -cells and aberrant reactions at any one of the many events in this pathway could result in conversion to β -conformation and aggregation. This in turn can trigger cytotoxicity and cell death (Figure 5).

ProIAPP in the Endoplasmic Reticulum

In the β -cell, transcribed propeptides/proteins (e.g. proIAPP, proinsulin) undergo molecular folding in the endoplasmic reticulum (ER); disulphide bridges are formed and peptides which adopt well folded structures undergo correct folding into their near final conformation. At this point, there is a strict process of quality control. If there is overproduction of the peptide or an aberrant sequence is translated/transcribed, the misfolded or excess peptide/proteins are

targeted for destruction via the Unfolded Protein Response (UPR). This is a collective name for several signalling pathways which are designed to operate continuously to reduce the effects of abnormal ER protein accumulation on cellular viability (Chen et al. 2015). These processes include detection of overexpression of normal proteins and/or misfolded proteins, signalling for disposal of this excess via the proteasome or lysosomal systems and cellular survival when this regulatory system becomes overburdened. Aberrantly misfolded peptides/proteins are targeted for ubiquitination and move to the cytosol for degradation of the misfolded material via the proteasome. However, this normal process has a limited capacity and if the UPR is overloaded, this can result in ER stress which can have disastrous consequences for the viability of the cells if it is not alleviated by removal of the insult or following activation of other protective systems. Under normal situations, proIAPP expression is regulated through the IAPP gene promoter; this promoter, although glucose responsive, is less sensitive than the insulin promoter (Zhu, et al. 2017), and normal secretion of IAPP is 1-10% of that of insulin. Similarly, production of proIAPP/ IAPP co-ordinately changes with that of insulin in rodents and humans in relation to stimulation with glucose and arginine and in T2DM diabetes (Alam, et al. 1992; Butler, et al. 1990; Kahn, et al. 1990; Kahn, et al. 1998; MacNamara, et al. 2000). Transgenic rodents expressing hIAPP gene are useful models. However, in many of these rodent models many copies of the transgene are expressed; up to 74 copies have been identified in one model (Zhang, et al. 2014). Since these transgenes are under control of the insulin gene promoter, they over-respond to glucose compared to rIAPP in normal wild-type mice; overproduction of the transgenic peptide was as much as x2 in transgenic rats (Matveyenko and Butler 2006) and x5 in transgenic mice (Fox, et al. 1993). Cytoplasmic IAPP fibrils are frequent in circumscribed compartments in the cytoplasm of transgenic animals and in human islets *in vitro* (Paulsson, et al. 2006) (Figure 6A) but not seen in cells of humans with T2DM. It is unclear if these fibrils are situated in the ER or in other cellular compartments.

ProIAPP is also fibrillogenic (Exley, et al. 2010). Increased concentrations of human proIAPP peptide in the ER has been shown to activate the UPR, and target the newly transcribed proIAPP to pathways of degradation. At the same time there is activation of other ER stress responses have been demonstrated (Chen et al. 2015; Westermark et al. 2011) which, if not checked, can lead to cell death. Experiments in transgenic mice have suggested that overexpression of hIAPP, but not the rodent peptide, generates ER stress and activation

of the ubiquitin pathway targeting degradation (Huang, et al. 2007a) suggesting that it is hIAPP but not just the volume of peptide in the ER that is the primary factor. Similar conclusions were drawn from observations on islets from type 2 diabetic subjects *in vitro* although there was no evidence that hIAPP affected proteasome activity or that IAPP aggregates were the cause of these effects (Costes, et al. 2011). The disulphide bridge in proIAPP (and proinsulin) is also formed at this stage by the enzyme protein disulphide isomerase (PDI) which allows correct folding of the peptides. However, it has been shown in hIAPP transgenic mouse islets that overexpression of this enzyme does not reduce hIAPP fibril assembly suggesting that cysteine bridge formation is unlikely to be a critical step for fibril assembly (Montane, et al. 2016).

The human model of overexpression of human ProIAPP/IAPP occurs in sporadic insulinomas. These are adenomas which are usually non-proliferative consisting of cellular β -cell clusters in the pancreas about the size of a large islet. Cells in these tumours have unregulated secretion of insulin/proinsulin (Guettier, et al. 2013); this results in severe and debilitating hypoglycaemia for the patient. Surgical removal of the adenoma is the usual form of treatment. These β -cells also have unregulated secretion of IAPP/proIAPP (Toshimori, et al. 1991) and many contain extracellular and intracellular IAPP amyloid (Clark 1992; O'Brien, et al. 1994a; Toshimori et al. 1991). Although most of the IAPP amyloid is extracellular in these tumours, intracellular accumulations of fibrillar IAPP is found in some cells, often in extensive arrays as seen in transgenic mice (Paulsson et al. 2006) (Figure 6B). Intracellular fibrils were immunoreactive for proIAPP (Paulsson et al. 2006) suggesting that refolding of proIAPP and aggregation of fibrils inside the cell (probably in the ER) had occurred. The cytotoxic nature of the intracellular fibrils in tumour cells cannot be properly assessed since these cells have a different pattern of turnover and lifespan compared to normal islet cells.

Autophagy and IAPP/ ProIAPP in the cytoplasm

Autophagy (“self eating”) is a normal and important process of the cell to remove damaged/dysfunctional organelles and cytoplasmic material which have the potential to be cytotoxic. In the β -cell, this housekeeping activity is also responsible for maintaining the secretory granule population (Halban 1991) and for removal of damaged mitochondria (Ashrafi and Schwarz 2013) and other foreign material in the cytoplasm. Autophagy is also

involved in removal of ER-stress related material (Bartolome, et al. 2014). Observations in hIAPP transgenic mouse islets indicate that intracellular thioflavin S positive material (amyloid fibrils) was bound to p62 (a scaffolding protein essential for targeting proteins for autophagy) suggesting that fibrillary forms of IAPP are recognised by the autophagic system for degradation; in addition, β -cell content of hIAPP was increased in mice deficient in the autophagic machinery indicating that degradation via autophagy usually regulates IAPP (Meier, et al. 2014; Rivera, et al. 2014; Westermarck et al. 1990). Cells containing IAPP fibrils also showed evidence of apoptosis suggesting that either the rescue process in the cell has become overwhelmed or that the proIAPP fibrils are cytotoxic within the cytoplasm (Paulsson et al. 2006). Although fibrils has been identified in these experiments, the molecular structure of proIAPP/IAPP in the cytoplasm and its mechanism of cytotoxicity is a matter for debate (Zraika, et al. 2010).

It has been proposed that prefibrillar, oligomeric hIAPP, “toxic oligomers” are present in the cytoplasm in overexpressing cells. An antibody recognising oligomeric IAPP has identified cytoplasmic material in β -cells of hIAPP transgenic mice and patients with Type 2 diabetes; this pathology of cytoplasmic “toxic oligomers” has been proposed to form an unifying hypothesis for loss of insulin-secreting β -cells in Type 2 diabetes (Gurlo, et al. 2010; Ritzel, et al. 2007; Zhao, et al. 2009). However, all of these findings are based on the binding of a so-called “conformational antibody” which does not recognise an antigenic amino acid sequence but a conformational shape of the molecule (Glabe 2008). These antibodies are useful for identifying small assemblies of IAPP *in vitro*, but their use in biological material and tissue sections is less well defined since specificity could be compromised by recognition of a large variety of molecules with β -conformation and their applicability to IAPP detection has been questioned (Zraika et al. 2010) Whilst there may be some evidence for cytotoxicity of visible extracellular fibrils or clearly identified, intracellular molecular aggregates in hIAPP producing cells *in vivo*, it is unclear that less visible, intracellular “toxic oligomers” could be a major insult in human islets and cells where no islet amyloid is visible, but the lack of good reagents for conclusively identifying hIAPP oligomers hinders such studies. It seems unlikely that minute assemblies of cytoplasmic hIAPP in β -cells which otherwise appear normal and continue to secrete insulin, could be a major causative factor for Type 2 diabetes. This metabolic condition involves many factors both in islets and in the periphery which contribute to the dysregulated insulin secretion; loss of β -cells and islet amyloid

formation are likely to be contributory rather than causative factors for the disease. (Clark and Nilsson 2004)

ProIAPP/IAPP in granules

In the β -cell, translated proIAPP together with proinsulin is transferred to the Golgi apparatus to be packaged into secretory granules (Figure 5). The granules also contain the Prohormone Convertase enzymes PC1 (also known as PC3) and PC2 and the amidating enzyme Peptidylglycine- α -Amidating Monooxygenase (PAM) that are responsible for post-translation modification of proIAPP and proinsulin (Figure 1) (Higham, et al. 2000a; Hutton 1994; Marzban, et al. 2004; Wang, et al. 2001b). Proinsulin processing is defective in T2DM leading to an increased secretion of intact proinsulin and processing intermediates (MacNamara et al. 2000; Temple, et al. 1992; Ward, et al. 1987). hIAPP overexpressing β -cells lacking PC1/3 and PC2, exhibit increased proIAPP associated with increased islet amyloid deposition and islet cell death compared to cells possessing the processing enzymes (Marzban, et al. 2006). Islets from hIAPP transgenic mice lacking PC2 were less viable when transplanted than islets from hIAPP mice with PC2 suggesting that proIAPP is a more toxic form of the molecule than the mature peptide (Courtade, et al. 2017). Just as with human insulinomas, it is likely that intracellular fibrils are formed from the abundant proIAPP in this condition (Figure 5).

Secretory peptides are in a very high concentration in the granule; insulin/proinsulin granule concentrations are estimated to be $\sim 100\text{mM}$ and IAPP concentrations $1\text{-}10\text{mM}$ in man (Kanatsuka, et al. 1989). Granules are at pH 3-5 (Hutton 1994) and the concentration of zinc is estimated to be 20mM (Hutton, et al. 1983). It is therefore surprising that, at this relatively high concentration, proIAPP or IAPP do not become fibrillar in the granule under normal conditions. Fibril-like structures are present in granules of some animal models overexpressing hIAPP (Paulsson et al. 2006) (Figure 7A). However, under normal conditions it is likely that the granule peptides also have influences on each other. Interaction *in vitro* of insulin, c-peptide, proinsulin and zinc with hIAPP have been investigated and shown to have variable effects on IAPP fibrillogenesis; high concentrations of zinc accelerate, but lower concentrations reduce fibril formation (Brender, et al. 2010; Westermark, et al. 1996). Human proinsulin and insulin formed heteromolecular complexes with hIAPP and prevented fibrillogenesis (Jaikaran, et al. 2004; Knight, et al. 2008; Larson and Miranker 2004; Wiltzius, et al. 2009). Thus it seems that under conditions of normal hIAPP gene expression,

molecular buffering capacity in secretory granules where peptide concentrations are high, are effective in preventing β -sheet formation of hIAPP or human proIAPP.

Secretory granules surplus to requirements in the cell are removed and degraded to constituent components via the lysosomal system by a type of autophagy known as crinophagy (Schnell, et al. 1988). The finding of high concentrations of immunoreactive IAPP in human beta cell lysosomes and lipofuscin bodies (Clark, et al. 1989) (Figure 7B) indicates that this is a normal pathway for degradation of granule peptides including IAPP. Lipofuscin bodies are intracellular storage organelles for material in the lysosomal system and since they accumulate with age, also markers for β -cell ageing (Cnop, et al. 2010); cellular material which has not been degraded by lysosomal proteases and lipases accumulates in these bodies. The finding of hIAPP lipofuscin bodies in human cells but not rIAPP in mouse or rat cells suggests that proteolysis of hIAPP is inefficient compared to rIAPP resulting in accumulation over time in man. Although it has been proposed that autophagy is impaired in β -cells of patients with T2DM (Meier et al. 2014; Rivera et al. 2014; Westermarck et al. 1990), there is little evidence for increased autophagic bodies containing hIAPP in cells from diabetic patients (Cnop et al. 2010).

Thus, in the cell, at various stages of post-translational modifications or passage through the secretory granule mechanisms, there is the potential for excess hIAPP to oligomerise and form fibrils. This could result in activation of cell death signals and apoptosis. However, how this relates to IAPP in T2DM remains a critical question. At present there is little evidence for overproduction of IAPP in diabetes (Butler et al. 1990; Eriksson, et al. 1992; Jorgensen, et al. 2000; Kahn et al. 1998; Sanke, et al. 1991). In addition, currently there is no compelling data to suggest that IAPP-related cellular damage could be a pathogenic factor for onset of the disease in man. Moreover, IAPP is not in the list of 39 susceptibility loci for T2DM (Gaulton, et al. 2015; Prasad and Groop 2015).

Extracellular IAPP and fibrils

IAPP is co-secreted with insulin from islet β -cells into the extracellular space in islets by exocytosis of secretory granules (Rorsman and Renstrom 2003). It is assumed that, under normal conditions, IAPP in the secretory granule is in a natively unstructured molecular form and would be released on granule exocytosis in that form. Secreted peptides cross the extracellular islet space to the islet capillaries and enter the circulation. At the point of

exocytosis, the concentrations of IAPP and insulin fall and the ambient pH rises to 7.0 (Obermuller, et al. 2005; Rorsman and Renstrom 2003). Thus the space immediately adjacent to the plasma membrane will transiently contain a high IAPP and insulin concentration together with the other contents of the granule. This change in environment and loss of molecular buffers is likely to have impact on the potential for IAPP to aggregate. The mechanism of, and trigger for oligomerisation in the extracellular space is largely unknown and may be initiated by several different factors. The process of exocytosis involves membrane fusion between the granule and the plasma membrane. SNARE proteins in the membranes are involved in the fusion of the granule membrane with the cell membrane and release of the granule contents (Rorsman and Renstrom 2003). Due to the structural properties of the SNARE transmembrane domains the membrane properties (e.g. viscosity) are likely to be modified during exocytosis and could promote the interaction between hIAPP and the membrane. This could potentially initiate the process of fibrillogenesis at the point of exocytosis (Han, et al. 2017; Hastoy, et al. 2017). In all animal and cell models and human T2DM, IAPP fibrils are found in invaginations of the β -cell membrane. This was first shown in 1973 in islets and islet macrophages from a subject with T2DM (Westermarck 1973). This type of membrane interaction is characteristic of both biosynthetic IAPP (from secreted peptide) in human and transgenic hIAPP mouse islets (Westermarck et al. 2011) and synthetic exogenous IAPP (added to the media) (Janson, et al. 1999) (Figure 8A, B, C). This suggests that oligomeric hIAPP interacts with sites on the cell membrane. These characteristic membrane invaginations are present in animal, human and cellular models including islets from patients with T2DM (de Koning, et al. 1994a; Westermarck 1973; Westermarck et al. 2011) (Figure 8A). At this point, the IAPP fibrils can be visualised arranged in parallel orientation and in close contact with the membrane (de Koning, et al. 1994b; Janson et al. 1999; Westermarck 1973) (Figure 8B,C). In contrast, these invaginations are not present in α -cells adjacent to amyloid deposits (Figure 8A). The association of the fibrils with the membrane is more random and irregular in cells to which synthetic peptide has been added (Casas et al. 2008; Janson et al. 1999). Normally, following exocytosis, the granule membrane is quickly recovered by endocytosis involving clathrin on the cytoplasmic side (Ceridono, et al. 2011). Under normal circumstances, these “omega-shaped” (as seen in EM images) endocytotic secretory vesicles are so quickly recaptured from the cell membrane that they are not apparent even in highly secreting cells. However, the presence of clathrin coated endocytoses is markedly increased in areas of β -cell membrane adjacent to extracellular IAPP fibrils in islets of transgenic mice expressing hIAPP (Figure 8B) or in cultured normal human

islets. This leads to the intriguing possibility that the extracellular IAPP fibrils/oligomers may be arresting complete granule membrane recovery, possibly by retaining an attachment to the membrane or stimulating endocytosis. Such interference with the normal fluidity of the membrane by extracellular fibrils could compromise cellular function and could lead to cytotoxicity. The sections of the membrane influenced by the fibrils are likely to be dysfunction in terms of further granule release or receptor-mediated signalling. This in turn, could affect cellular function and possibly cell viability.

The extracellular surface of the β -cell plasma membrane is coated with glycosylated proteins including heparan sulphate proteoglycans (HSPGs) which is a known ligand for IAPP (Meng and Raleigh 2011; Westermark 1973; Westermark and Grimelius 1973) and also a component of islet amyloid deposits (Castillo, et al. 1998). Enzymatic removal of HSPG (Oskarsson, et al. 2015; Potter, et al. 2015) or blockade of HSPG binding sites (Hull, et al. 2007) reduced islet amyloid formation in hIAPP transgenic mouse or human islets in vitro; this suggests that the proteoglycans are a binding site for IAPP on the outside of the cell. If HSPGs are a primary anchoring site in the extracellular space for monomeric secreted hIAPP then hIAPP/HSPG interactions could lead to a high local concentration of hIAPP and promote aggregation (Westermark 1973)

The role of the extracellular amyloid deposits and extracellular oligomers in cell death has been challenged; studies on transgenic mice expressing hIAPP showed that β -cell death could occur in the absence of visible extracellular islet amyloidosis (Haataja, et al. 2008; Huang et al. 2007a). From this observation, it was concluded that cell death pathways were generated as a result of ER stress in cells overexpressing hIAPP (but not in cells overexpressing rIAPP) rather than IAPP fibril formation outside the cells (Huang et al. 2007a). In humans, one model of massive overexpression of IAPP is the condition of anti-insulin receptor antibodies resulting in severe insulin resistance. This is coupled with overexpression of insulin and extensive extracellular islet amyloid (O'Brien, et al. 1994b). However, the extent of cell death has not been quantified in this condition.

In contrast, the importance of extracellular hIAPP has been demonstrated in a different hIAPP transgenic mouse model with a lower degree of hIAPP expression where there was a close association of the degree of extracellular amyloid deposition and loss of islet cells suggesting that toxicity was related to extracellular islet amyloidosis (Wang, et al. 2001a). In human post-mortem samples the degree of cell death was directly associated with the amount

of extracellular islet amyloid (Jurgens et al. 2011) implicating the fibrils (or the process of forming the fibrils) in cytotoxicity. Since there is little evidence that IAPP is overexpressed in T2DM (Jorgensen et al. 2000) it is more likely that IAPP secreted from the cell becomes misfolded outside the cell and activates cell death pathways by interaction with the cell membrane or membrane bound receptors. Once the misfolded hIAPP is attached to the membrane (or other sites adjacent to the membrane) it could form a nidus for further secreted peptide to bind and aggregate thus creating extracellular fibrils (Jarrett and Lansbury 1993). It is likely that the early misfolded peptide (which is probably the cytotoxic species of IAPP) converts to amyloid fibrils.

Initially, islet amyloid deposits in islets in Type 2 diabetic patients and spontaneously diabetic Macaque monkeys are located in extracellular, pericapillary regions of the islet and specifically between the two basement membranes (one surrounding the islet cells and one adjacent to the capillary)(de Koning, et al. 1993) (Figure 9A). Although, in material taken at the time of autopsy, some of these islet deposits are not adjacent to islet cells undergoing apoptosis in that snapshot of time, it is unclear if cells have died previously. This site of initial fibril formation also suggests that accumulation of the peptide might arise as a result of poor clearance of the peptide into the capillaries and increased concentration in this restricted space. In biological systems, fibrils forming in human or hIAPP transgenic mouse islets *in vitro* are found more frequently between islet cells and in the central regions of islets than on the well-perfused cell surface (Potter, et al. 2010). IAPP immunoreactive material (some fibrillary) accumulates on the basal surface of cells grown on a matrix (Figure 9B) but not on the exposed cell surface. This all suggests that restricted diffusion of the peptide from a more confined space (and increased concentration) could play a part in fibrillogenesis as proposed for A β in AD (Selkoe, 2011 #201). This effect could explain the finding of amyloid in human and hIAPP transgenic mouse islets when transplanted into sites which are poorly perfused such as islets in the liver (Westermarck, et al. 2012) or under the kidney capsule or when encapsulated (Bohman and Westermarck 2012; Udayasankar, et al. 2009; Westermarck et al. 2012); this amyloid compromises their viability and function (Potter et al. 2010).

Cellular Pathways of Cytotoxicity:

It is now evident that the small oligomeric hIAPP species induce cytotoxicity whether the amyloid peptide is exogenously applied (Abedini et al. 2016; Lorenzo, et al. 1994) (either synthetic hIAPP or peptide fragments) or secreted from the cell. Induction of cell death from

intracellular events and from extracellular application appear to follow a final common pathway of apoptosis rather than necrosis. This pathway involves a series of events in the cell which finally results in chromatin condensation, nuclear fragmentation and total loss of cellular function.

Extrinsic apoptotic pathways can be initiated on the outside of the cell largely via Fas/ Fas ligand binding to the cell membrane Fas Receptor. This in turn initiates activation of caspase 8 and subsequently cleavage of caspase3 in the apoptotic cascade. Exogenous application of hIAPP has been shown to activate this pathway and blockade/deletion of the Fas receptor or inhibition of caspase 3 activation reduced hIAPP-induced cytotoxicity (Law et al. 2010; Park, et al. 2017; Zhang, et al. 2008; Zhang, et al. 2003). Further evidence for an inflammatory-type of response to hIAPP has been shown by production of proinflammatory cytokines, including interleukin1- α (Masters, et al. 2010) and interleukin1- β (IL1- β) (Westwell-Roper, et al. 2011). This response was observed in islets *in vitro* with exogenous application of synthetic hIAPP and in cultured islets from transgenic mice expressing hIAPP (Westwell-Roper et al. 2011); blocking the IL1- β receptor reduced production of IAPP-induced production of cytokines suggesting that this was the mode of action of the cytotoxic hIAPP. Moreover, a link has been shown between hIAPP-induced cytokine production and upregulation of Fas; blocking experiments demonstrated that IL1- β released from the cells activated apoptosis via Fas/Fas ligand binding (Park et al. 2017). The molecular complex known as the inflammasome has been implicated in release of cytokines in hIAPP induced cell death (Masters et al. 2010).

The question remains whether this pathway exists in T2DM. Increased β -cell apoptosis is a hallmark of islets in Type 2 diabetes (Butler, et al. 2003). However, the inflammatory pathway is better represented in islet cell death in autoimmune Type 1 diabetes than in T2DM. Increased expression of IL-1 β has been observed in islets in T2DM (Boni-Schnetzler, et al. 2008) and associated with glucotoxicity (Maedler, et al. 2002). These data suggest that inflammation could be a factor in the pathogenesis of Type 2 diabetes (Donath and Shoelson 2011). Macrophages also release cytokines and an increased density of tissue macrophages in islets in Type 2 diabetes has been proposed (Richardson, et al. 2009). Quantitative analysis of macrophage content of human pancreas from Type 2 diabetic subjects has suggested that, at least in some patients, islet amyloid is associated with increased numbers of islet macrophages (Kamata et al. 2014). However, specific analysis of macrophage content of amyloid-containing and amyloid-free islets in spontaneously diabetic monkeys showed no

increased macrophage density associated with islet amyloidosis although macrophages containing immunoreactive IAPP material were identified in islets (de Koning, et al. 1998). Observations in hIAPP transgenic mice have indicated that islet macrophages are associated with increased inflammatory signals in amyloid containing islets (Meier et al. 2014). Thus tissue macrophages may play both scavenger and inflammatory roles in this pathology.

Activation of intrinsic (intracellular) mechanisms of apoptosis do not involve cell membrane receptor signalling. In addition, the cell has the potential to suppress some intracellular pro-apoptotic signalling by activation of anti-apoptotic mechanisms. Anti-apoptotic restoration of cellular homeostasis operates largely via the Bcl-2 family of proteins (Taylor, et al. 2008). The activity of this family of proteins balances the pro- and anti-apoptotic signalling cascades at different steps of protein processing in the cell (Gross and Katz 2017).

This balancing mechanism operates in the ER. The unfolded protein response (UPR) is the first step in protein production that can lead to apoptosis. If there is an excess production of misfolded protein such as hIAPP, a pro-apoptotic pathway is initiated involving activation of PERK (protein kinase like ER kinase) and transcription factor CHOP (C/EBP homologous protein) and related proteins (Gross and Katz 2017). This pathway is also activated in so-called “ER stress” responses, and can develop as a result of other cellular disorders- notably gluco- or lipotoxicity in β -cells and has been demonstrated in relation to hIAPP cytotoxicity (Huang, et al. 2007b). CHOP activated proteins promote apoptosis via inhibition of protective Bcl-2 but the balance of pro- and anti-apoptotic proteins is delicate, can be reversed quickly and can be modulated by external stimuli. The involvement of CHOP in hIAPP-induced cell death and onset of diabetes has been demonstrated in transgenic mice overexpressing hIAPP (Gurlo, et al. 2016; Huang et al. 2007b).

Inhibitors of IAPP fibrillogenesis: a suitable treatment in type 2 diabetes?

Since the discovery of the peptide 30 years ago, there has been considerable interest in development of inhibitors of IAPP amyloid formation for potential treatment of Type 2 diabetes. This has run in parallel (but somewhat behind) ideas for treatment of AD (Westermarck 1972). Some most successful inhibitors of *in vitro* fibrillogenesis are small peptide molecules that interact with hIAPP and modulate the peptide aggregation process. Many of these have been developed and tested both on aggregation and cytotoxicity *in vitro* and in some cases administered to hIAPP transgenic animals with some degrees of successful inhibition of amyloidosis (Potter, et al. 2009; Scrocchi, et al. 2002; Sivanesam, et

al. 2016; Wang, et al. 2014; Westermarck and Grimelius 1973; Wijesekara, et al. 2015). Investigating the mechanism of action of some of these inhibitory compounds has been useful in providing information on the mechanisms of refolding and fibrillogenesis (Cohen, et al. 2006; Young et al. 2014; Young, et al. 2015). However, although many of these compounds have been tested in mice, not all of them would be suitable for clinical use. Alternative inhibitors which could be administered to humans have included some aromatic compounds including resveratrol (a component of red wine) (Evers, et al. 2009; Mishra, et al. 2009; Porat, et al. 2004) and some polyphenols including (-)-Epigallocatechin 3-Gallate (EGCG)(a natural component of green tea); inhibition of fibrillogenesis was accompanied by disaggregation of fibrils and formation of non-toxic oligomers (Engel, et al. 2012; Meng, et al. 2010). Other inhibitors effective *in vitro* are tetracycline, congo red, ruthenium red, and organic designed “foldamers” (Aitken, et al. 2003; Kumar and Miranker 2013; Zhu et al. 2017). Therapeutic drugs used to treat hyperglycaemia have also been tested as inhibitors including thiazolidinediones, metformin and sulphonylureas with varying degrees of success (Fortin and Benoit-Biancamano 2015).

It is evident that in hIAPP transgenic animals and in spontaneously diabetic cats and monkeys, the degree of amyloidosis is directly related to the onset of hyperglycaemia since all diabetic animals have varying but quite severe islet amyloidosis (de Koning et al. 1993; Guardado-Mendoza, et al. 2009; Wang et al. 2001a; Zhang et al. 2014). However, the islet amyloid load of islets in most patients with T2DM is not high and unlike animal models of islet amyloid and diabetes, the degree of amyloidosis is not related to onset or duration of diabetes. Islet amyloidosis is one of the few visible pathological changes in islets in T2DM (Ehrlich and Ratner 1961; Westermarck 1972). The prevalence of islet amyloid (the percentage of islets in a pancreas affected by amyloid) has been assessed as being between <1% to 90% in patients with T2DM ; a human pancreas has been estimated to have approximately 1000 islets. However, in more than 40% of patients with T2DM < 25% of islets are affected (Clark and Moffitt 2007; Jurgens et al. 2011). Moreover, the patients with low prevalence of islet amyloidosis had < 10% of the islet space filled with amyloid (amyloid severity) (Jurgens et al. 2011). Thus, in many patients with established T2DM, sometimes of many years’ duration, little amyloidosis is visible; low degrees of islet amyloid are associated with low percentages of β -cell loss (Jurgens et al. 2011). This would argue against the hypothesis that IAPP and ER stress were a major feature of β -cell death which was in turn a major aetiological factor for onset of T2DM. A more likely scenario is that functional

changes in islet cells are associated with T2DM onset and that IAPP and amyloidosis are a result of these alterations. However, it is known that the distribution of islet amyloidosis in the pancreas is variable and it has been proposed that not all islets respond to a glucose challenge at any one time (Zhu, et al. 2016). It may therefore be important to identify which islets are amyloid-containing (even if only a few are affected) in relation to any functional changes. The real questions are firstly does this level of islet pathology require treatment and secondly how would the efficacy of this treatment be assessed?

Current therapies for T2DM are directed towards optimising the release of insulin and glucagon from islet cells which are largely desensitised to glucose; the end points of this type of therapy are improved glycaemia (improved haemoglobin A1c) and better glucose homeostasis. These factors are critical for long term minimisation of the devastating complications of T2DM such as retinopathy, kidney disease and amputations. Whilst islet amyloid and the associated β -cell destruction will contribute to eventual reduction of insulin secretion, inhibitors for IAPP fibrillogenesis may not be the initial therapy of choice for T2DM unless it can be proved that IAPP and its cytotoxicity has a definite role in the pathogenesis of T2DM. Currently it seems more relevant to focus IAPP research on and working towards understanding the cellular and biochemical factors that promote misfolding/aggregation of IAPP in T2DM than developing anti-amyloid clinical therapies.

In addition, since the role of amyloidosis in onset and progression of T2DM is unclear, the development and assessment of an islet amyloid inhibitor would require relevant end points such as evidence of reduction of islet amyloidosis. Currently, it is not possible to determine which patients have islet amyloid or its rate of deposition. The search for treatments for AD and the progression of cerebral plaque formation have been accompanied by development of methods quantify cerebral amyloid plaques by imaging. Positron Emission Tomography scanning in combination with markers for amyloid and tau (Pittsburg Compound, PiB) (Klunk, et al. 2004) has allowed identification of cerebral plaques and efficacy of treatment regimens. However, at the present levels of sensitivity, this type of analysis for would not be successful for detection of a few islets with amyloid *in vivo*.

In conclusion, hIAPP, if overexpressed, can exert its toxic effects at many intracellular stages of protein production and degradation in β -cells. In T2DM it is more likely that secreted IAPP misfolds outside the cells and the early stages of β -sheet assembly- the small oligomeric molecular species – are responsible for inducing apoptosis and contribute to fibril

formation. These small molecular assemblies of hIAPP are the misfolded assassins of the β -cell in T2DM.

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Figure Captions

Figure 1. Amino acid sequence of proIAPP in different species: ProIAPP is 67 amino acids long in humans, Macaques, cats and dogs but 70 amino acids in rodents and guinea pigs. The propeptide is cleaved by the enzymes PC2 and PC1/3 and further amidated at the C-terminus by peptidylglycine α -amidating monooxygenase (PAM). This results in the the mature 37 amino acid IAPP. The conservation of the amino acid components between the different species is shown in the lower panel.

Figure 2. Structural models of the hIAPP protofibril. A. A β -bend in regions 18-27 results in a horseshoe shaped structure with the N-and C-termini (N-term, C-term) on the same face. There are no intra peptide backbone hydrogen bonds in the structure; instead the backbone hydrogen bonding occurs between adjacent monomers in one of the horseshoe shaped stacks. B. The two stacks are aligned so that two molecules lie antiparallel in the same plane and interact via their sidechains. Protofibrils have a super helical twist, although the β -sheets are still relatively flat by the standards of globular proteins. Protofibrils further assemble into fibrils containing two or more protofibrils (Wiltzius, et al. 2008).

Figure 3: Schematic diagram of amyloid fibril formation from hIAPP. During the initial phase (lag phase) monomeric hIAPP forms oligomers and adopts conformations which differ from the monomeric state. The growth phase involves elongation of protofibrils and assembly into fibrils. A saturation phase, (also called the plateau phase) occurs with synthetic hIAPP in vitro when most of the monomers have become assembled into mature fibrils.

Figure 4: A. Schematic representation of the influence of membranes on the rate of hIAPP fibril formation. Synthetic membrane liposomes (blue line) reduce the lag time and accelerate the growth phase compared to fibrillogenesis in the absence of membrane (red line). B. Diagrammatic representation of potential processes of membrane interactions of hIAPP. (a) hIAPP (red) hydrophobic, monomeric hIAPP adopts a transient helical conformation at the membrane surface which becomes β -sheet leading to oligomer assembly at the membrane. These multimers interact with the lipid bilayer to form a pore. (b) multimers assembled at the membrane bind to components of the outer leaflet of the bilayer and destroy the structure. (c) multimers assembled at the membrane continue to extend into protofibrils and fibril growth occurs with part of the fibril inserted into the membrane and membrane damage.

Figure 5: Diagram of intracellular mechanisms contributing to cytotoxicity of hIAPP in β -cells. With normal IAPP expression (left hand diagram) proIAPP (red) synthesised in the endoplasmic reticulum (ER) is transported to the Golgi through the cytoplasm. If aberrant folding or transcription/translation occurs, misfolded molecules are targeted (unfolded protein response, UPR) by ubiquitination for degradation via the proteasome. proIAPP is packaged in the Golgi, together with insulin and processing enzymes, into secretory granules. Maturation and post-translational processing of the peptide occurs in the granule where proIAPP/hIAPP is largely stabilised by interaction with insulin. Some granules are targeted for destruction via the lysosomal system (crinophagy) and fuse with lysosomes. Granule components, including amino acids and lipids, arising from this degradation process are recycled back for re-synthesis. Human IAPP is partially resistant to lysosomal proteases and is retained in the lysosomal storage compartment (lipofuscin bodies). Overexpression (right

hand side) of proIAPP affects trafficking through the ER, overloading the UPR and creating ER stress which can lead to cell death. High concentrations of proIAPP will aggregate in the ER or cytoplasm forming fibrils. These are removed by autophagy and targeted to the lysosomal system. Overload of this protective mechanism can result in apoptosis. The high concentrations of proIAPP/hIAPP can result in fibril formation in secretory granules. On release from the cell by exocytosis of the granule, hIAPP can interact with the membrane causing cytotoxicity or be deposited as fibrils in the extracellular space.

Figure 6: IAPP and proIAPP fibrils in overexpressing hIAPP transgenic mouse and human insulinoma cells viewed by electron microscopy. A. A bundle of fibrils (f), immunogold labelled for IAPP in cytoplasm of a β -cell of a hIAPP transgenic mouse. B. Arrays of fibrils immunoreactive for IAPP in the cytoplasm of a cell from a human insulinoma. i, insulin granules; amy, IAPP amyloid fibrils m, mitochondrion; n, nucleus. Scale bars A, 500nm; B, 1.0 μ m.

Figure 7: IAPP in insulin secretory granules of a hIAPP transgenic mouse and in a lipofuscin body of a non-diabetic human subject. A. Immunogold labelling with an antibody against hIAPP with N-terminal of proIAPP intact demonstrates fibrillar IAPP/proIAPP in mature insulin granules (i) (with halos) (arrow) and in immature granules (without halos)(arrowhead) suggesting that fibrils are formed at this site from unprocessed proIAPP. Scale bar 200nm. B. Immunoreactivity for IAPP in a lipofuscin body (arrows) of a non-diabetic subject. M, mitochondrion; I, insulin secretory granule. Scale bar 200nm.

Figure 8: Extracellular IAPP fibrils in plasma membrane invaginations of β -cells seen with electron microscopy. A. Islet with extracellular amyloid immunogold labelled for IAPP from a Type 2 diabetic subject. The amyloid (amy) is situated between the capillary (c) and the β -cell (β) and in deep invaginations of the β -cell membrane (arrows). Adjacent alpha cells (α) although in close contact with the amyloid (arrowhead) do not have membrane invaginations or show nuclear (n) signs of apoptosis. Scale bar 1.0 μ m. B. Extracellular amyloid fibrils immunogold labelled for IAPP (amy) from an islet of an hIAPP transgenic mouse. The fibrils lie in invaginations of the β -cell plasma membrane (arrows). Some of these membrane invaginations show thickening characteristic of clathrin coating suggesting an arrested process of membrane recovery/endocytosis (e.g red arrow). m, mitochondrion. Scale bar 200nm.

Figure 9: Localisation of IAPP amyloid fibrils in an islet of diabetic macaque monkey and in hIAPP expressing β -cells cultured on a matrix. A. Extracellular amyloid fibrils in humans and all animal models are initially deposited *in vivo* between islet cells and islet capillaries. Gold labelled IAPP fibrils (amy) are situated between the two basement membranes (arrows) in the islet; one adjacent to the islet capillary (cap) and a second adjacent to the β -cell. i, insulin granule. Scale bar 1.0 μ m. B. Electron micrograph of hIAPP expressing β -cells cultured on a filter culture insert. Section taken through the filter (filter) shows the base of the cells with invaginations containing fibrils gold labelled for IAPP (amy). Some of these invaginations (arrows) have a thickened membrane suggesting clathrin coating. m, mitochondrion; n, nucleus, i, insulin granule. Scale bar 200nm.

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