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Herein we present a critical overview of the major technologies used to measure Abeta levels in vivo and place these within the context of drug development for Alzheimer's disease.

Monitoring the amyloid beta-peptide *in vivo – caveat emptor*

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As a wave of 'disease modifying' (DM) therapies for Alzheimer's disease (AD) progresses towards the later stages of clinical development, an evaluation of our ability to measure relevant pharmacodynamic effects of such therapies is warranted. Reducing accumulation of amyloid beta (Abeta)-peptide in the brain parenchyma is the primary objective of most current DM approaches. Although a number of methods are available to measure Abeta in blood, cerebrospinal fluid (CSF) and the cerebrum, putative DM-induced changes in the levels of the peptides may not be fully captured, and the reasons for any such changes are not fully understood. Additional candidate biofluid (tau and isoprostanes) and imaging (MRI, FDG-PET) measures may provide alternative supporting evidence of drug activity and subsequent clinical efficacy in patient populations.

Introduction

Sporadic, or late onset, Alzheimer's disease (AD) is the major form of dementia and is primarily a condition of ageing, with a small number of associated risk factors (e.g. apolipoprotein E genotype, diabetes and cardiovascular disease) [1]. Available evidence indicates that the neuropathological hallmarks of AD, senile plaques (SPs) and neurofibrillary tangles (NFTs), develop in a robust neuroanatomical and temporal pattern that is exclusive to humans [2]. Pre-clinical models (transgenic or otherwise) do not faithfully reproduce this pathology [3], making drug discovery for AD particularly demanding. One of the key challenges that the pharmaceutical industry therefore faces is the selection of the correct enabling technologies to detect pharmacodynamic (PD) effects sensitively and reproducibly during the early clinical development of potential DM therapies, and thus gain confidence in the associated drug mechanism of action (MoA).

The amyloid cascade and DM approaches for AD

The amyloid cascade hypothesis [4] underpins the major strategies of the pharmaceutical industry for development of DM treatments of AD. Briefly, summation of the following strands of evidence places the amyloid beta (Abeta)-peptide as the central causative agent in AD:

(1) Abeta-peptides, whether these be monomeric, oligomeric or fibrillar, are toxic to hippocampal and cortical neurons [5].

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(2) Mutations in the Abeta production machinery and in the amyloid precursor protein (APP) itself [6] are responsible for the vast majority of familial AD cases [7–9].
(3) Increased APP gene dosage in Down's syndrome leads to dementia and amyloid deposition during the 3rd–5th decades [9,10].
(4) Injection of Abeta into animals leads to aggregation and induction of tau hyperphosphorylation and NFT formation, suggesting Abeta as causative of AD pathology [11,12].

Recent findings suggest that Abeta oligomeric species (ABOs) may be the most potent neurotoxic form in AD, and thus one might plausibly rename the amyloid hypothesis as simply the 'Abeta cascade hypothesis' [13]. In any case, current DM strategies focus on aspects of Abeta biochemistry such as peptide production, aggregation or clearance. A summary of potential DM strategies, and associated compounds, that are currently in clinical studies are given in Table 1. A detailed examination of these approaches has been given elsewhere [14].

To progress rapidly Abeta-targeting DM strategies into the clinic, it is important to demonstrate that a compound is acting through its proposed MoA in humans, such as a secretase inhibitor blocking central Aβ production. Not only does this increase confidence in the MoA and allow dose-finding for proof-of-concept (POC) studies, such data would be obtained well in advance of meaningful clinical scores, which for sporadic AD, due to its slow progression, may only be obtained many months after initiation of disease-modifying treatment.

A number of direct approaches are available to measure the Abeta levels associated with the peripheral and central compartments and these include specific immunoassays for measuring soluble pools of the peptides, through to more complex metabolic labelling strategies and amyloid imaging using molecular imaging probes such as ^{11}C-PIB and ^{18}F- BAY94-9172. Other more indirect measures of the neurodegenerative processes associated with AD include central measures of tau/phospho-tau (P-tau) and F_2-isoprostanes, and imaging approaches using MRI and ^{18}F-FDG PET. The remainder of the review will therefore focus on (i) the fundamental aspects of Abeta biochemistry, (ii) methods to measure Abeta *in vivo* in humans and their potential limitations as PD markers and (iii) alternative biomarker strategies that may provide indirect measures of AD pathology and disease progression.

Abeta: biochemistry and physiology

Abeta is a highly polymorphic polypeptide both in terms of its length and its potential to adopt a range of quaternary structures with different biophysical (and potentially neurotoxicological) properties (Figure 1). The peptide is produced in a variety of different lengths (typically ranging from 37 to 42 amino acids long) by a sequence of proteolytic cleavages from APP. Of these, Abeta 1–40 and 1–42 (Aβ40 and Aβ42), are considered central to the development of AD and can each, to varying degrees, form a range of soluble (e.g. dimers and oligomers) and insoluble ordered polymers (e.g. fibrils associated with classical plaques), as well as largely disordered aggregates (e.g. diffuse plaques) [15]. In addition, soluble Abeta has been shown to interact with a wide range of proteins (e.g. ApolipoproteinE (ApoE), ApolipoproteinJ (ApoJ), alpha-2-macroglobulin (α2M), human serum albumin (HSA), receptor for advanced-glycation end products (RAGE) and lipoprotein related receptor protein (LRP)) some of which may serve in central and peripheral transport capacities (outlined in Figure 2) [16]. As discussed in more detail below, this plasticity in the structure and solubility means that neuronally produced Abeta

TABLE 1

Summary of recent DM approaches in clinical trials (data obtained from http://clinicaltrials.gov/ct2/home).

Mechanism	Compound	Development phase	Company	Notes
Abeta clearance	AC-001	Ph I	Elan	Antibody/passive immunisation
	R1450	Ph I	Hoffmann-La Roche	Antibody/passive immunisation
	LY2062430	Ph II	Eli Lilly	Antibody/passive immunisation
	AAB-001 (bapineuzumab)	Ph III	Elan-Wyeth	Antibody/passive immunisation
	AN1792	Ph II (discontinued Mar 2002)	Elan	Vaccination/active immunisation
	V950	Ph I	Merck	Vaccination/active immunisation
	CAD106	Ph I	Novartis	Vaccination/active immunisation
	AFFITOPE AD01 and AD02	Ph I	Affiris GmbH	Vaccination/active immunisation
	Gammagard	Ph II	Baxter	Intravenous IgG injection
	PF04494700 (TTP488)	Ph II	Pfizer	Small molecule antagonist of RAGE
Abeta antagonist	Alzhemed	Ph III (discontinued Nov 2007)	Neurochem Inc	Glycosaminoglycan mimetic
	ELND005	Ph II	Elan	(Formerly known as AZD-103), scyllo-inositol
Abeta production	LY450139	Ph III	Eli Lilly	γ-Secretase inhibitor
	Flurizan	Ph III (discontinued July 2008)	Myriad Pharmaceuticals, Inc.	Selective amyloid-lowering agent, reduces Aβ42
	NGX267	Ph I	TorreyPines Therapeutics	M1 agonist
	E2012	Ph I	Eisai	γ-Secretase modulator
Metal chelating	PBT2	Ph I	Prana Biotechnology Ltd.	Metal–protein attenuating compounds
Other	MK-0677	Ph II	Merck	Growth hormone secretagogue may clear Abeta through IGF1 dependent mechanism

The major driving force for these approaches is the 'amyloid cascade hypothesis' which places the Abeta, as the central, causative disease agent. In response to this a range of interventional strategies aimed at Abeta metabolism are at various stages of clinical development and include approaches aimed at (i) increasing peptide clearance through immunisation, (ii) modulating peptide production (secretase inhibitors) and (iii) inhibiting peptide polymerisation.

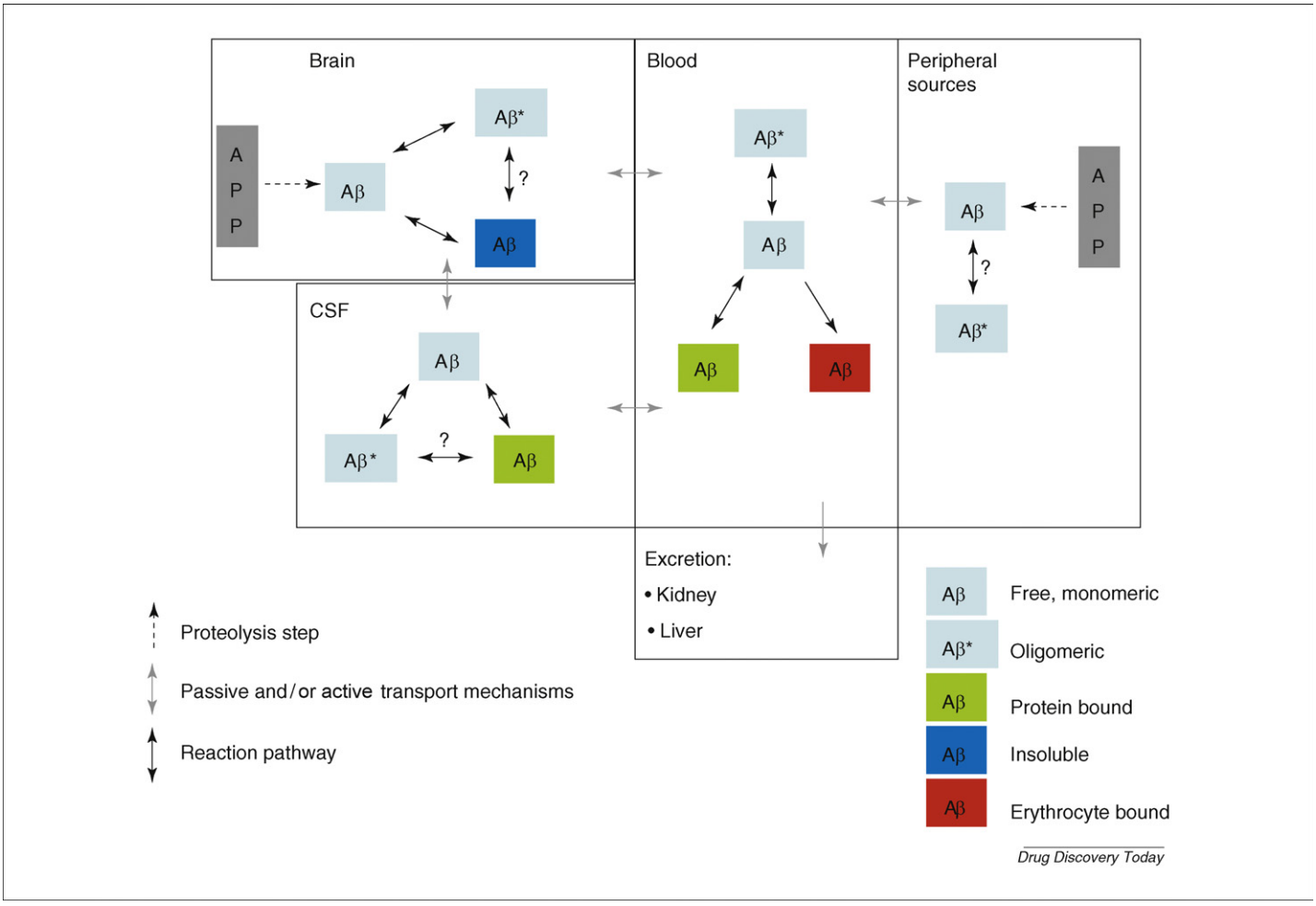

FIGURE 1

Schematic representation of major Abeta pools and potential interrelationships between the central and peripheral compartments. Processing of APP within neuronal cells produces mature Abeta peptide which can remain monomeric or form oligomeric or higher order structures (fibrils and/or diffuse aggregates). Abeta is removed from central compartment either through perivascular drainage or through CSF removal. Abeta is also able to bind to a range of proteins that are also involved in the transport and removal of the peptide. Once in the blood, free Abeta may form additional complexes and may also be cleared via erythrocytes through the formation of immune complexes. Abeta may also be formed in peripheral compartments (e.g. platelets) and tissues (e.g. skeletal muscle). The interplay between these different pathways is poorly understood in both pre-clinical models and humans.

fluxes through a number of different pools (or compartments) and to which additional non-neuronally derived peptide can be added (Figure 1). These complexities present a significant challenge in trying to infer global changes in Abeta metabolism when measuring a single parameter, such as plasma levels.

Methods to measure Abeta *in vivo*

The two biofluids primarily used to measure steady-state levels of Abeta, utilising mainly ELISA based methodologies, are cerebrospinal fluid (CSF) and blood (i.e. plasma and serum). There are a number of common factors to consider before moving on to discuss each pool in more detail.

Sample storage/handling

It is vital that standardised protocols are in place for all aspects of biofluid collection (e.g. timings of sample withdrawal, collection tube types, plasma/serum preparation protocols) and that adequate storage facilities are available to ensure that all samples remain 'fit-for-purpose' (Table 2). For specific details, please refer to the excellent review by Portelius *et al.* [17].

Choice of ELISA protocol/antibodies

The vast majority of studies investigating Abeta levels in either patient populations or clinical studies employ ELISA-based protocols, although there are some examples of gel-based immunoblots [18] and mass spectrometry methods [19]. ELISA-based methods provide a moderately high throughput and typically allow detection down to low picomolar levels. Several different antibody pairs have been described and, most commonly, N-terminal region or mid-region antibodies are used to capture the Abeta peptides and the specificity for either Aβ40 or Aβ42 is provided by the use of a C-terminal specific detection antibody [20,21]. Perhaps, not surprisingly, there has been little standardisation of assay format across both academic and industry-based groups and this in part may explain the variations in Abeta levels observed between cohorts in observational studies.

The Alzheimer's disease neuroimaging initiative (ADNI) [22] has an integrated biomarker arm which it is hoped will provide a route to the methodological standardisation of existing bioassays for potential AD biomarkers. A relevant example of this is the extensive effort to characterise the Aβ42 assay developed by Innoge-

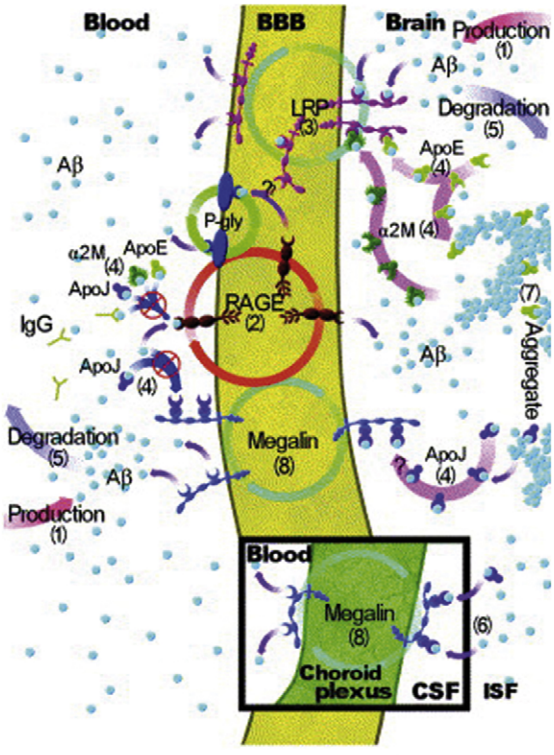

FIGURE 2

Abeta transport mechanisms at the blood brain barrier. A number of mechanisms, orchestrated by Abeta transport proteins, exist to facilitate the efflux and influx of the peptide to the central compartment. These mechanisms have direct implications for the measurement of Abeta in human biofluids, as a large proportion of the peptide will be protein-bound and may be precluded from direct measurement via immunoassay. Reproduced with permission from [87].

netics (INNOTEST beta-amyloid 1–42, antibody pair 3D6 (N-term 1–5) and 4D7A3 (C-term to 42)) [23] both in terms of inter-laboratory reproducibility and standard curve stability. There is little doubt on the specificity of this assay for Aβ42 over any other form of N-terminally or C-terminally truncated peptide. Other companies have developed similar assays with more commonly used antibodies for peptide recognition (i.e. 6E10 or 4G8). A comparison between the INNOTEST and 6E10 based immunoassays indicated that there were no significant differences in Aβ42 levels using the same samples from AD patients, FTLD patients, and healthy controls [24].

Additionally, as Aβ42 levels may be an important PD endpoint in studies, it would be a distinct advantage to elucidate the specificity of the different ELISA formats for the various multimeric forms associated with this peptide. For instance, do 6E10/4G8-based ELISA systems recognise ABOs [25] in agreement with Western blot data? To what degrees are the ABOs recognised by oligomeric specific antibodies, such as A11 [26], also recognised by the ELISA based systems? The need to answer these questions is driven by the revised role of ABOs in mediating neurotoxicity and whether changes in their levels are better correlated with clinical response. Vanderschtile's critical manuscript on the INNOTEST ELISA systems acknowledges the potential confounding variable of ABOs on Abeta measurements [20].

It is reasonable to assume that if ABOs are present in a biofluid sample they would interact to some degree with an anti-Abeta antibody pair and attempts at detecting such species in CSF have been only moderately encouraging. The first published attempt utilised an ultrasensitive BioBarcode assay which employed PCR to increase the signal of oligomers in post-mortem CSF [27] and although successfully able to detect an increased signal in AD cases there has, to date, been no secondary validation of this work. A fluorescence-based assay using labelled 6E10 and 4G8 antibodies has been proposed [28], although these antibodies may have the potential artificially to link Abeta peptides into aggregates, independently of any pre-existing oligomeric species. Finally, the study from Barghorn *et al.* [29] exemplifies the potential risks of using *in vitro*-generated ABOs for assay development as antibodies raised against these preparations were unable to detect CSF derived ABOs. Although it is unclear from the latter example whether the inability to detect CSF ABOs was due to a failure to cross react with native oligomers or sensitivity issues, it nevertheless highlights the difficulties in accurately measuring a discrete Abeta pool.

Protein bound forms of Abeta

In addition to existing in a range of polymorphic states, Abeta has a high capacity to bind to other proteins in the surrounding

TABLE 2

Essential conditions used by our group for handling CSF prior to Abeta measurements using ELISA- or mass spectrometry-methods.

Recommended action	Effect
Take a constant volume of CSF (10–12 ml), with gentle mixing prior to aliquotting and storage at −80 °C	Offsets any potential ventricular:lumbar gradient in CSF Abeta levels
Use polypropylene tubes for sample storage and processing	Reduces loss of Abeta to tubing
Use O-ring lids for tubes	Prevents evaporation of sample during storage
Store CSF samples at −80 °C	Prevents degradation of Abeta
Avoid freeze/thaw cycles – aliquot appropriately for use at source	Prevents degradation of Abeta peptides
Use a consistent protocol for sample thaw	Ensures reproducibility of data across sample sets
Do not use blood contaminated CSF	Prevents contaminating plasma proteins from confounding ELISA data
Avoid addition of protease inhibitors to CSF	Prevents additional background on mass spectrometry analysis, leading to an increase in assay sensitivity

The general principles outlined here can of course be applied to any biofluid.

environment. These interactions may, in part, preclude further aggregation of the peptide and serve as essential events for its transport between the brain and the periphery (Figure 2). Indeed, it has been postulated that a principal reason for the occurrence of senile plaques is the relative paucity of Abeta binding proteins in CSF as compared to blood [30]. CNS extracellular fluid, comprised of both interstitial and cerebrospinal fluid contains between 0.15 and 0.45 mg protein/ml, Abeta peptides making up to ~0.01% of this total.

Proteins such as albumin, ApoE, Clusterin/ApoJ, LRP and Alpha 2 macroglobulin, can bind to Abeta in both CSF and blood. Presumably such protein-bound Abeta is in constant exchange with non-protein bound, neuronally released peptide. A study by Rozga *et al.* applied titrations of Aβ40 and HSA using circular dichroism spectroscopy, obtaining a K_d ~5 μM for the HSA/Aβ40 complex [31]. Much higher affinity interactions have been demonstrated for Aβ40 and ApoJ (~5 nM [32]) and Aβ 40 and 42 with ApoE (both ~10 nM [33]). Moreover, ApoJ appears to mediate the interaction between Aβ40 and LRP-2/megalin, a critical interaction for brain Abeta efflux. Tokuda *et al.* were also able to demonstrate that affinities for Aβ40 and Aβ42 by ApoE3 and E4 were 10-fold higher when the apoprotein was lipidated. In addition, they were able to show that the affinity of the lipidated ApoE3 for Aβ40 was 2–3-fold higher than that for lipidated ApoE4. Although the majority of work undertaken to investigate the functional consequences Abeta interaction with carrier proteins has utilised pre-clinical species [34,35] recent evidence confirms the existence of abundant Abeta–LRP complexes in human CSF and plasma [36]. To date there have been no investigations into the assembly states of Abeta (e.g. ABOs) and their potential to be sequestered by similar carrier proteins.

The differing sensitivities of ELISA systems to detect protein bound Abeta complexes in both blood and CSF are also currently unclear. This is illustrated by a series of studies investigating the distribution of Abeta in plasma. Kuo *et al.* subjected aliquots of plasma to a glass distilled formic acid extraction step, followed by column purification prior to measurement by ELISA [37,38]. Their data indicated the free fraction (i.e. non-protein bound) of Abeta constituted only ~5% of the total amount of peptide present in plasma and that the vast majority of the protein bound peptide was associated with HSA. Further studies have also employed extraction techniques to investigate this cryptic pool of Abeta. Slemmon *et al.* demonstrated that guanidine hydrochloride extraction of plasma resulted in the detection of higher levels of the peptide compared to untreated plasma, although to a lesser degree than observed with formic acid extraction [39].

Although it is possible to format these extraction procedures for high-throughput analysis of clinical samples [40] it appears that each procedure results in a different recovery of total Abeta. There is little value in using extraction procedures if they are not complete – thus of greater value may be the monitoring of Aβ levels associated with specific protein pools of Abeta, such as LRP [36] and/or ApoE/J. This approach would of course require the development of bespoke assays capable of dealing with relatively high numbers of samples and an understanding protein-peptide binding dynamics. The latter point can of course be addressed using in vitro binding techniques and stable isotope labelling strategies [41] to monitor unlabelled and labelled peptide exchange *in vivo*.

Additional CSF specific considerations

CSF physiology

Steady-state measurements of CSF levels of Abeta should represent a key measure for demonstrating a central PD effect of DM agents. Some of the mechanistic factors that contribute to the measured levels of the peptide in CSF are summarised in Figure 1. An often-overlooked mechanism which has a direct effect on lumbar Aβ levels is, however, the existent CSF circulatory physiology (for review see [42]).

CSF is primarily produced by the choroid plexus and its flow is directed through the ventricles, subarachnoid space and parenchyma before entering the venous sinuses, and then onto the general circulation. Changes to CSF circulation have been noted as part of normal ageing but are also associated with a range of neurological diseases (including AD) and may directly or indirectly affect the composition of this biofluid. AD patients share a number of features with individuals suffering from normal pressure hydrocephalus (NPH) [43]. Both patient groups are characterised by a reduction in CSF production rate (from an average 0.36 ml/h to 0.2–0.25 ml/h), increased CSF volumes and, in NPH, there is evidence of a reduced reabsorption at the venous sinuses [44]. Whilst both patient groups share similarly reduced CSF Aβ42 levels the amounts of Tau/P-Tau are increased only in AD (this latter measure perhaps reflects the differing underlying pathophysiologies of the accompanying dementias) [45]. Recent evidence suggests that low CSF Aβ42 levels in AD are associated with the presence of significant SP pathology (as measured by amyloid tracers) [46]. It is not clear, however, whether such an association exists for NPH patients, as histological analyses of cortical biopsy samples indicate that only ~50% of cases possess significant AD-like pathology [47]. This reinforces the concept that multiple mechanisms are involved in determining steady state Abeta levels and that simple correlates (i.e. low CSF Aβ42 = significant SP pathology) or acute changes following drug intervention (i.e. increased CSF Aβ42 = decreased SP pathology) may require more in-depth, and complementary, measures.

A final consideration for CSF measures is the potential change in ventricular volume that may occur during both the disease course and/or in response to treatment. Ventricular enlargement occurs during the clinical progression of AD, as the result of the ongoing cortical atrophy, and leads to a greater volume of CSF. The effect of correcting for this potential dilution of protein levels has been investigated [48] and suggests that there may be a protein-specific effect, at least with respect to using P-tau and Abeta as patient stratification measures. This may be a consequence of the differing ventricular:lumbar ratios associated with these two proteins (1.5:1 for tau and 1:2 for Abeta). It is conceivable, however, that Abeta DM therapies may alter these ratios in a mechanism-specific manner and this may warrant consideration in the analysis plans of clinical data.

Mechanistic interpretations of CSF data from clinical studies

CSF data from, in many cases, ongoing DM therapy studies is available for study (Table 3). Evidently, the inconclusive nature of the steady-state ELISA methods in providing data that clearly demonstrates direct effects on Abeta levels means that they may not be adequate as PD markers. It is, therefore, tempting to ask what the role of these measures is in clinical development plans. In

TABLE 3

Summary of CSF Abeta data from DM trials.

Study	DM-type	CSF Abeta data	Intepretation
[72]	AN-1792; active immunisation	Marginal difference in CSF Aβ42 was observed between antibody responders and placebo recipients at baseline (588 ± 119 pg/ml versus 469 ± 152 pg/ml, respectively; $p = 0.059$).	Possible reduction in Aβ42 – is this due to shift in soluble/insoluble equilibrium towards plaque retention or decreased production/increased central clearance? IHC in encephalitis cases suggests microglial Aβ clearance occurring
[56]	LY-450129; gamma-secretase inhibitor	No CSF Aβ42 difference 4 h after dosing (time of peak plasma decrease in Aβ40) despite maximal dose CSF drug concentrations of 350 nM	Therapy is not active centrally Alternative interpretation is that this could be indicative of a re-balancing of Abeta synthesis and clearance rates to give no change in steady state levels
[85]	R-flurbiprofen: gamma-secretase inhibitor/modulator	Healthy elderly. No CSF Abeta difference between baseline and 21 days post dose with 3 difference dose levels despite adequate CNS penetration	As above
[86]	AF102B; M1 agonist	AD patients Total Abeta levels in CSF decreased in 14 patients by 22%, increased in 3 patients, and were unchanged in 2 patients; the overall decrease in the group as a whole was statistically significant	Decreased production of Abeta supportive of proposed MoA Alternative interpretation is shift in soluble/insoluble equilibrium causing increased parenchymal retention

For all DM trial CSF Abeta data published to date, multiple interpretations are possible, which does not helpfully assist decision making on whether the compound under investigation should progress beyond the current phase of development. Use of metabolic labelling techniques can reduce the number of interpretations by allowing for sensitive measurement of *de novo* synthesis and clearance of central Abeta. Steady-state CSF Abeta measurement, coupled with parenchymal amyloid imaging through PET (sensitivity permitting) could further define the MoA and pharmacodynamic effect of the compound.

the case of the secretase inhibitors/modulators, despite the apparent negative (or 'contrary to expectation') CSF Abeta data, these programs have continued/are continuing, highlighting a disconnect in the role of these measures to provide direct evidence of target engagement/MoA. A large body of data on CSF Abeta levels is likely to be published soon from active/passive immunisation approaches, although again it is unlikely that CSF Abeta measures are providing Go/No Go decision points. Given the plethora of mechanisms that control the presence of the Abeta peptide in the central compartment, and our relative lack of understanding of these mechanisms in humans compared to mice, perhaps this is not surprising.

Non-steady state methods for measuring Abeta in CSF

An advance of potentially great importance for measuring PD effects of DM therapies has been made by researchers at Washington University in St. Louis [41], whereby well-established techniques of stable isotope labelling, mass spectrometry and Abeta immunoprecipitation have been combined to allow monitoring of peptide synthesis and degradation rates in humans *in vivo*. Bateman *et al.* calculated fractional synthesis and clearance rates for Abeta of 8.3%/h and 7.6%/h, respectively, in CSF from healthy human volunteers. This technique brings the following advantages over steady-state level measurements:

(1) Specifically detects *de novo* synthesized Abeta and is thus unlikely to be influenced by insoluble peptide pools.
(2) Monitoring peptide dynamics has the potential to provide an earlier and more sensitive PD readout.
(3) Potential to detect whether metabolic responses (i.e. changes to synthesis and/or clearance rates) are evoked in response to drug treatments, which may offer increased sensitivity over steady-state measurements.

There are, of course, some potential drawbacks with this method; the sample processing and analysis is fairly labour-intensive compared to a steady-state measurement; as discussed above, the use of antibodies may lead to a selection bias in the pool of Abeta analysed; a fully characterised and tested form of stable isotope labelled amino acid is required to ensure reproducibility across batches and sites and finally, there are clinical risks associated with both the placement and the need for an indwelling catheter which will require that the procedure is performed in specialist centres.

As with all biomarker methods, there is a need for validation and refinement of this methodology, but this 'rough diamond' has great potential in demonstrating central PD activity of DM therapies. In addition, this method has the potential to answer key questions surrounding the human metabolism of Abeta, such as rates of peptide synthesis in disease versus non-disease and the effects of ApoE genotype on Abeta clearance, that have, until now, only been tackled using animal models.

Additional blood specific considerations

Due to the relative inaccessibility of the central compartment, blood offers an alternative biofluid for the detection of Abeta peptides. The relative importance of this Abeta pool for PD measures depends not only on the proposed drug MoA, but also on how the data is used within the overall clinical development plan. For example, a number of companies are pursuing a passive immunisation strategy that rely upon the sequestration of plasma Abeta by antibody, and it is the clearance of these immune complexes that drives the efflux of the peptide from the central compartment and prevents its reverse influx into the CNS. In this instance, it is clearly important to be able to detect the formation of the immune complex in the blood and evaluate whether the steady state levels of Abeta are changing in response to the drug, as these are both components of the proposed MoA. The development of modified, or bespoke, assays to determine free Abeta and antibody-complexed Abeta, against the background of the injected biopharmaceutical, may therefore be an important component of the pre-clinical development strategy. Active

immunisation approaches additionally raise the potential for host antibody production to confound ELISA-based measures through epitope masking. This may be especially difficult to control for in pre-clinical studies when full length Abeta peptide is used as the immunogen. However, as second-generation active vaccination approaches are increasingly utilising truncated forms of Abeta this potential risk should be significantly ameliorated.

Although, Abeta can be detected in both plasma and serum, the majority of studies use plasma due to the potentially variable contribution of aggregation-derived peptides to serum measurements. A direct comparison of plasma and serum Abeta measures highlighted not only the inter- and intra-individual variability associated with these measures [49] but also the potential complexity of the relationship between the Abeta pools associated with this biofluid. Abeta levels were determined using a commercial ELISA kit and were ~1.5-2 fold higher in serum versus plasma. The absolute ratios between Aβ42/40 levels were also higher in serum due to higher relative increases in Aβ42 levels. Although, the additional Aβ42 may be platelet-derived (see below), it may also arise from exposure (and/or release) of additional peptide from the plasma protein-bound fraction following clotting.

Interpreting changes to steady state levels is also complicated by uncertainties over what proportion of the blood Aβ pool is derived from neuronal sources. Current knowledge of possible Abeta peptide peripheral sources is limited to platelets and skeletal muscle. Human platelets express full length and partially processed APP, in addition to BACE and presenilin 1, and are capable of producing Abeta [50–52]. The majority of supporting evidence for platelet-induced peptide production has been achieved from *in vitro* studies, and human blood studies suggest platelet activation may not increase Abeta levels [53]. One way forward with area might be to investigate blood Abeta levels in individuals with thrombocytopenia.

Skeletal muscle has been reported to contain ~50 ng/g of Abeta peptides [54] and therefore represents a significant peripheral reservoir of peptide. There is evidence from studies on sporadic inclusion body myositis (sIBM), which is the most common muscle disease leading to severe disability, that muscles produce significant amounts of Abeta. A characteristic feature of sIBM pathology is the accumulation of intramuscle fibre aggregates containing either Abeta or phosphorylated tau [55]. The trigger for Abeta production in this situation is not known, although sIBM is associated with inflammation and oxidative stress, both of which are capable of initiating APP processing events.

Mechanistic interpretations of plasma data from clinical studies

This is of particular interest as we consider data from the gamma-secretase inhibitor LY-450139 studies in humans [56]. Siemers *et al.* demonstrated that plasma Aβ40 levels displayed a maximal decrease 4 h post-dosing but that CSF Aβ42 levels remained unchanged (Table 3). However, supported by data from pre-clinical species, showing a rapid transport of Aβ from central interstitial fluid (ISF) to plasma and a relationship between parenchymal Aβ40 lowering and plasma Aβ40 lowering, the group proposed that the decrease observed in the clinical cohort may still be partially explained by a CNS lowering effect. However, some caution must be taken when extrapolating data from pre-clinical species and other possible mechanisms also need to be fully considered. For example, it is possible to construct a scenario to explain the lack of apparent central activity in humans based on differences in Abeta exchange rates between ISF/CSF/blood and ISF/lumbar CSF. Alternatively, the secretase inhibitor could be acting on a peripheral source of Abeta peptide, platelets for example, which predominantly produce Aβ40 [57].

Parenchymal Abeta as a PD measure: amyloid imaging tracers

Amyloid PET tracers, such as ^{11}C-PIB and SB13 derivatives (^{18}F-BAY94-9172), provide a quantitative *in vivo* measure of the insoluble, cortical Abeta load. The origins and use of theses tracers in AD patient populations have been the subject of a number of review articles [58,59]. Although these tracers target the amyloid-fold associated with fibrillar Aβ, it is unlikely that they are specific for a particular insoluble Abeta pool (e.g. classical plaques, diffuse plaques and cerebral amyloid angiopathy) but rather should be considered as a general marker of Abeta peptide related cerebral amyloidosis [60].

Data from patient cohorts indicates that the maximal cortical uptake of these tracers plateaus at a very early (mild cognitive impairment (MCI)) stage in the disease process and remains fairly stable during disease progression [61–63]. This may seem at odds with the ascribed central role of the neuritic plaque in driving AD until the fact these tracers additionally bind to the other insoluble Abeta pools is factored in. This has significant implications, in terms of attributing any changes (either disease or treatment related) in the cortical uptake of a tracer with a specific type of Abeta-containing lesion.

Although only two ongoing studies (LY2062430 and Gammagard, source http://clinicaltrials.gov/ct2/home) list amyloid imaging as an endpoint, it would be surprising if this technology was not being more widely deployed. No data is, as yet, available in the public domain detailing the sensitivity of this technique to detect regional changes in amyloid load. Histological data associated with the AN1792 study provided evidence that cortical areas can be cleared of senile plaque pathology [64,65], however, whether these microscopic changes translate into the more global measures provided by PET is currently unclear.

An interesting case study combining a potential DM therapy, soluble biomarkers and PIB imaging was reported recently [66] and investigated the acetylcholinesterase inhibitor (-)-phenserine. In addition to its cholinergic effects, pre-clinical data suggested that the drug altered APP processing, leading to reductions in Abeta levels. Although, perhaps limited by the relatively small numbers of participants (10 per arm), the study demonstrated effects (cognitive measures and FDG-PET) consistent with the cholinergic activity of phenserine but could find no strong evidence supporting DM activity. The study highlights not only the importance of combining imaging with biofluid markers but also the inherent risks associated with translating pre-clinical findings associated with DM approaches into the clinical environment.

A small biomarker panel was established to investigate the potential PD effects of phenserine on Abeta metabolism and consisted of Aβ40/42 (CSF and plasma) and CSF measures of the APP fragments, α-sAPP and β-sAPP. PIB scanning was performed at baseline and at 3 and 6 months to measure potential changes to cortical Abeta load. In support of the proposed amyloid lowering

mechanism and, in an ideal world, it may have been hoped to detect a relatively acute lowering of central (and peripheral?) Abeta levels and changes to the α-sAPP/β-sAPP ratio perhaps leading to a longer term reduction in PIB cortical uptake (presumably via dissolution of pre-existing SPs). No significant changes were found associated with either the soluble markers or the imaging data suggesting that, at least at the doses examined in the study, that there was no evidence of the potential DM effect observed pre-clinically.

Perhaps more importantly the study confirmed the previously observed stability in global PIB cortical uptake [67] and that the test/retest stability of these measures was ~5% in the placebo treated group over a three month period, suggesting that this method will only be sensitive to changes of >10–15%. There are risks, however, in generalising this latter point as one patient in the treatment arm displayed a decrease of ~12% in PIB cortical uptake at the 3 month timepoint and there were also patients in both the placebo and treatments ($n = 5$, or 25% of the entire cohort) who showed reductions of >5% at some point during the study. Revision of what level of reduction in tracer uptake constitutes an 'amyloid lowering' proof-of mechanism may, therefore, require careful consideration.

One area where amyloid tracers may have a more direct biomarker role is in patient stratification, particularly when considering individuals with MCI as potential patient population for DM therapies. Data from studies investigating the cortical uptake of PIB and BAY94-9172 [68] indicate that cases with amnestic variant of MCI, and therefore believed to be more prone to conversion to AD, have an enhanced cortical uptake of the tracers. Amyloid imaging could, therefore, be used as an enrichment strategy for patient recruitment or in the sub-analysis of clinical study data.

Indirect measures of DM effects: MRI, FDG-PET and CSF measures of tau and F_2-isoprostanes

MRI

Whole brain and hippocampal volumetric analysis by MRI appears to be sensitive to disease incidence and progression [69]. The majority of published studies comparing patient group data show that hippocampal volume follows the pattern healthy > MCI > AD. In addition, entorhinal cortex volume is decreased in MCI compared to healthy controls [70]. More recently, a longitudinal, voxel-based morphometry MRI study during AD progression has delineated the series of morphological changes that accompanies fast declining cognitive function [71].

A main assumption with using MRI as a PD measure for Abeta targeting therapies is that reducing its parenchymal levels will allow an arrest of neuronal death induced by the peptide and that this will be reflected in a stabilisation of brain volume. Prior to the Elan active immunisation AN-1792 trial, this hypothesis appeared sound. After the trial suspension and analysis of immunisation response, immunohistochemistry and MRI data [72], it was, however, clear that (1) a proportion of patients had responded to the immunisation, (2) antibody response induced the partial clearance of senile plaques and (3) whole brain and hippocampal volume further decreased in antibody responders, contrary to expectations, and this did not correlate with worse cognitive performance. As Siemers *et al.* have previously stated, the apparent disconnect between hypothesis and reality underscores the need to repeat MRI measures in further DM clinical trials [73]. At this point, however, there have been no further published reports of MRI data in such trials.

FDG-PET

^{18}F-labelled fluorodeoxyglucose (FDG) is possibly the best characterised of all PET tracers currently available for use in DM trials. Uptake of FDG parallels the normal cellular uptake of glucose, with subsequent phosphorylation prohibiting further cellular movement. Thus cortical FDG retention is believed to be a robust surrogate marker for neuronal function and is expressed as a cerebral metabolic rate for glucose (CMRglc) [74]. In AD, temporoparietal cortex, posterior cingulate cortex and frontal cortex regions display a hypometabolism that is believed to be reflective of synapse loss, neuronal death and a generalised metabolic deficiency [75]. FDG-PET is sensitive to the differing CMRglc displayed by MCI and healthy individuals [76,77], much like the situation for volumetric MRI. More interestingly, the neuroanatomical physiology of episodic memory, the main memory function first targeted by AD pathogenesis, is confirmed by FDG-PET studies in AD patients undergoing specific episodic memory challenge paradigms. For example, in AD patients a significant correlation between autobiographical memory of the last 5 years and CMRglc in right hippocampal, middle and inferior frontal and middle temporal gyrus [78]. Further evidence is provided by recent data demonstrating a clear correlation between episodic memory, verbal fluency and naming and CMRglc in AD patients [79]

The above evidence suggests that cerebral glucose metabolism may more closely reflect neuronal function than other imaging strategies and thus provide an imaging correlate to clinical cognitive improvement in AD trials. Clinical trials with donepezil and phenserine have both reported treatment-induced effects on FDG uptake and we await with interest further reports from DM trials.

Tau

The existence of tau and P-tau forms in CSF is thought to be due to neuronal death and release of formerly intracellular components into ISF. Alongside Aβ42 levels, tau and P-tau levels differentiate between AD and non-demented controls. Recent studies suggest that increased levels of P-tau combined with low Aβ42 in CSF from subjective cognitive impaired individuals predicts further cognitive decline [80]. Furthermore, MCI patients who converted to AD within a 12-month follow-up period had significantly higher tau and P-tau CSF levels than those who did not convert [81].

In the same way as for FDG-PET, one might argue that using tau/P-tau levels as a PD measure may reflect a biochemical correlate of cognitive function that may be altered by disease modification. A 2-year follow up of the AN-1792 trial revealed that in the limited number of patients who received repeat lumbar puncture, those that had produced antibodies in response to immunisation had significantly lower CSF tau levels than those that received placebo [82]. This result has gained further importance after a 4.5-year follow up on cognitive function in AN-1792 trial patients (Vellas *et al.* ADPD 2007 abstract). Antibody responders showed significantly slower decline on the Disability Assessment for Dementia ($p = 0.015$). Statistically significant differences in favour of responders were also observed on the Dependence Scale and subtests of the neuropsychological test battery ($p < 0.05$). Thus, changes in

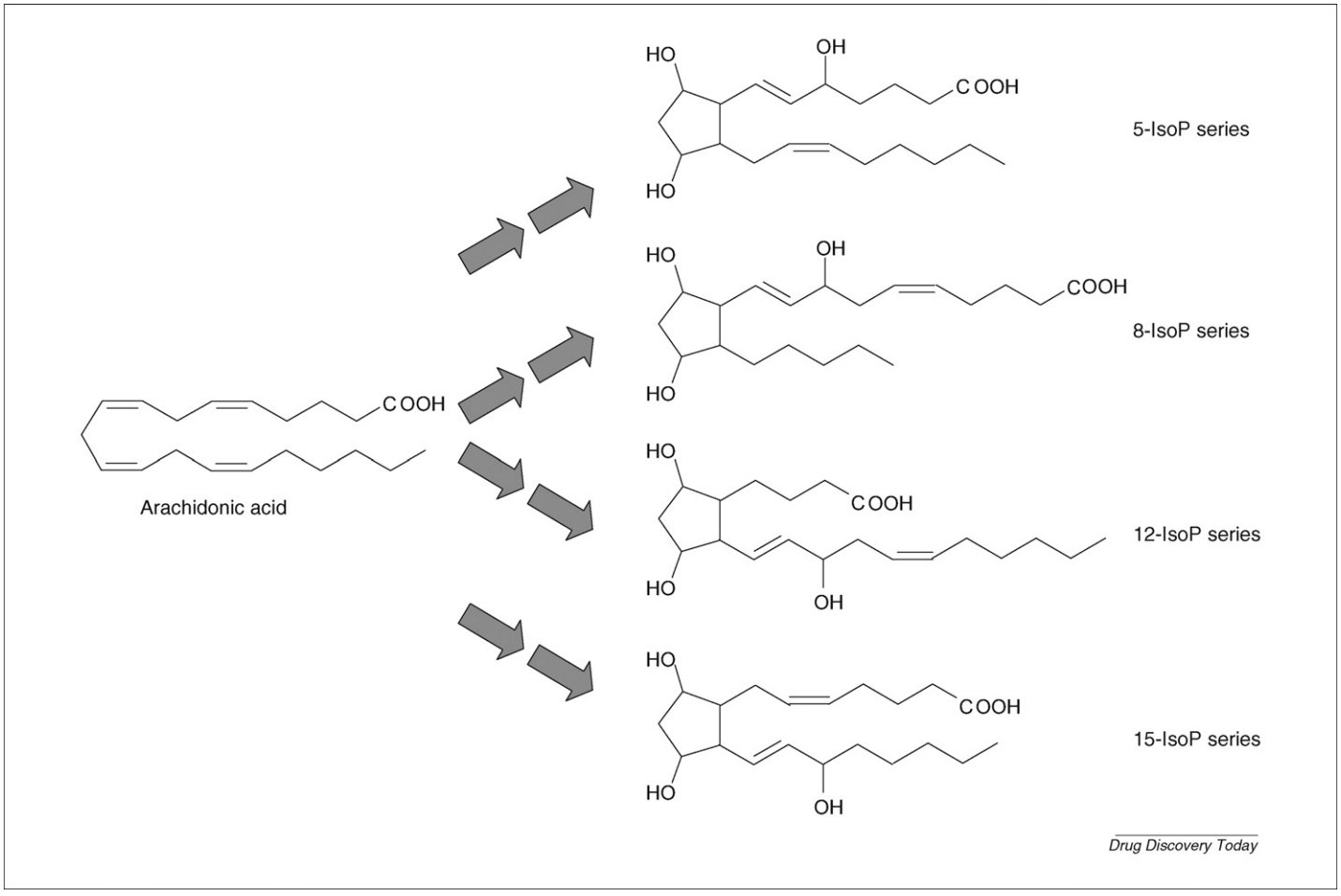

FIGURE 3

Pathways of F_2-isoprostane formation (adapted from [88]). Lipids, such as arachidonic acid, are readily attacked by free radicals, formed *in vivo* during oxidative stress, resulting in the production of a series of stable lipid peroxidation products, termed the isoprostanes (IsoPs). The first class of IsoPs discovered were the F2-IsoPs, so called due to the presence of an F-type prostane ring. The four regioisomers of F2-IsoPs are displayed above, each of which consists of eight racemic diastereomers. Detailed reaction pathways and stereochemistry are not shown for ease of presentation. Measurement of F2-IsoPs in human biofluids is typically performed through the use of GC/LC–MS techniques which allow the detection and quantification (pg levels) of specific series of F2-IsoPs, although this type of analysis is relatively cost and labour intensive. In particular increases in the CSF levels of the 5-IsoP series have been associated with AD [83,84].

CSF tau correlated well with cognitive improvement over placebo in this trial, proving its potential as a key PD marker in this setting.

F_2-isoprostanes

Prostaglandin F_2-like products termed F_2-isoprostanes are formed non-enzymatically as the result of free radical-mediated peroxidation of arachidonic acid and are thought to reflect oxidative stress (Figure 3). AD pathology contains several oxidative stress triggers, such as Abeta, cell damage/death and subsequent glial activation, all of which may contribute to the generation of F_2-isoprostanes. CSF concentrations are significantly elevated in AD patients compared to controls or fronto-temporal lobar degeneration (FTLD) patients [83]. More recently, F_2-isoprostane concentrations have been shown to increase during progression of MCI to AD, and during conversion from normal elderly to MCI [84]. Although this study was underpowered and had slight age differences between the normals and MCI, it does demonstrate the possibility that F_2-isoprostanes change during MCI to AD conversion, a potentially useful attribute of a PD marker for disease modifying approaches.

As yet there are no reports of the effects of DM therapies on F_2-isoprostanes

Conclusions

Clinical development of DM agents can be greatly facilitated by the demonstration, at the earliest possible phase, of drug activity. As discussed above, a variety of methods are available to monitor the peripheral and central levels of Abeta, each with its own set of merits and limitations. Additional indirect measures of neuronal function/health are also available which may provide supporting correlates of drug activity in later phase studies using patient populations. Steady-state measures of Abeta are the result of highly dynamic fluxes of synthesis and clearance which are regulated by multiple mechanisms–as such these measures may be too simplistic for building robust PK/PD relationships across all classes of DM therapy. The data associated with the Abeta production modulators is mixed with compounds demonstrating only modest peripheral effects in the absence of significant central reductions. The recent clinical data associated with flurizan

(http://www.myriad.com/news/release/1170283) raises significant questions regarding how direct measures of Abeta levels are being used to drive forward clinical development and alleviate the considerable risks associated with CNS drug development. More subtle human investigation, such as the use of metabolic labelling strategies, utilisation of non-Aβ measures of DM effects (such as FDG-PET and tau levels), combined with less reliance on pre-clinical data may form a more productive strategy.

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