The role of endogenous bromotyrosine in health and disease

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Abstract: Bromotyrosine is a stable by-product of eosinophil peroxidase activity, a result of eosinophil activation during an inflammatory immune response. The elevated presence of bromotyrosine in tissue, blood and urine in medical conditions involving eosinophil activation has highlighted the potential role of bromotyrosine as a medical biomarker. This is highly beneficial in a paediatric setting as a urinary non-invasive biomarker. However, bromotyrosine and its derivatives may exert biological effects, such as protective effects in the brain and pathogenic effects in the thyroid. Understanding of these pathways may yield therapeutic advancements in medicine. In this review, we summarise the existing evidence present in literature relating to bromotyrosine formation and metabolism, identify the biological actions of bromotyrosine and evaluate the feasibility of bromotyrosine as a medical biomarker.

Key words: Bromide; Bromotyrosine; Dehalogenase; Eosinophil; Eosinophil peroxidase; Halogen; Inflammation; Myeloperoxidase; Peroxidasin; Thyroid

1. Introduction

3-Bromotyrosine (Br-Tyr) is a compound generated by the halogenation of tyrosine residues in plasma or tissue proteins. Br-Tyr synthesis is primarily catalysed by the enzyme eosinophil peroxidase (EPO). Eosinophil activation causes the release of EPO and subsequent production of Br-Tyr as a stable by-product. EPO, a haem peroxidase, is the most abundant cytoplasmic granule protein present in eosinophils. It uses hydrogen peroxide and a halide or pseudo-halide to catalyse the formation of cytotoxic hypohalous acids which is involved in the immune response against microorganisms and multicellular parasites (1). EPO catalyses the oxidation of bromide ion by hydrogen peroxide and produce a brominating agent that reacts with amine groups and aromatic rings of biomolecules (1–3). The major bromide products of Br-Tyr and 3,5-di-Bromotyrosine (di-Br-Tyr) are detectable following EPO-induced protein oxidation. The

presence of free Br-Tyr in blood or urine is the result of enzymatic degradation of these brominated proteins. Free Br-Tyr undergoes metabolism and leads to the formation of brominated and de-brominated metabolites. In humans, the correlation between eosinophil activation and Br-Tyr formation identify Br-Tyr as a potential biomarker for monitoring eosinophil related inflammatory conditions such as asthma. The aim of this review is to identify the biological actions of Br-Tyr, to evaluate the feasibility of Br-Tyr as a medical biomarker, and to review and consolidate the evidence present in literature relating to Br-Tyr and its constituents.

2. Absorption, Distribution and Physiological Roles of Bromide

Bromide is readily absorbed in the gut via paracellular pathway with a bioavailability of ~96% (4). It is mainly distributed in the extracellular fluid with a volume of distribution of ~ 0.3 L/kg (5–7). It has slow excretion rate due to a marked tubular reabsorption in the kidneys. Thus, the half-life sodium bromide after oral administration has been reported from days to weeks in different studies (7,8). The physiological role of bromide, unlike other halogens, was unknown until very recently (9). However, the interaction of bromine and biological systems were known for over a century. Bromide can interfere with neural signalling and was used for management of epilepsy. In this section, we overview the biological roles of bromine/bromide *in vivo* (figure 1).

2.1. Anti-epileptic agent

Historically, the first mention of bromide's application as an anti-epileptic agent was described in a Lancet paper in 1857, by Sir Charles Locock, Queen Victoria's obstetrician. The use of potassium bromide over the course of 14 months was reported to have successfully cured catamenial epilepsy in a group of 15 women, excluding 1 patient (10). The use of bromide subsequently saw widespread acceptance as an anti-epileptic agent up until the early 1900s, when more advanced drugs such as phenobarbitone superseded it. Bromide was found to enhance GABA-activated currents in cultured neurons at therapeutic concentrations (millimolar

range). This leads to hyperpolarisation of cerebral neurons and potentially control epileptic seizures (11).

Up until the discovery of phenobarbitone in 1912, potassium bromides were the only treatment available for epilepsy. Due to the lack of alternative, bromide was consistently used despite its toxic side effects which were exacerbated by frequent exposure to the drug. Negative effects on the skin, gastrointestinal tract and nervous system are observed in patients suffering from bromism (bromide toxicity) (12). Specifically, symptoms of bromism include skin lesions (bromoderma), gastrointestinal irritation, confusion, irritability, headache, anorexia, emotional lability, fatigue, memory loss, insomnia, disorientation, depression, mydriasis, weakness, gait disturbances, tremor and hyperreflexia (13). Although it is no longer the first choice, bromide still remains as a tertiary choice for paediatric treatment of epilepsy. The use of potassium bromide can be safe and effective with salt loading, diuresis facilitated excretion and round the clock pharmacological monitoring. Moreover, bromide is still widely used as an anti-epileptic agent for canines. However, the effects of bromism are still evidenced both in canines and across other animal species.

2.2. The role of bromide in assembly of collagen IV scaffolds

Bromide was previously thought to have no known endogenous physiological function. However, it was recently established as an essential trace element in the assembly of collagen IV scaffolds in tissue development and architecture (9). Collagen IV scaffolds are stabilized by enzymatic formation of sulfilimine bonds between methionine (Met) and hydroxylysine (Hyl) residues to form a covalently reinforced collagen trimer (9). This sulfilimine crosslinked collagen IV scaffold provides mechanical strength, interacts with growth factors for signalling and serves as ligands for cell surface receptors. This facilitates the function of the basement membrane in epithelial signal transduction and mechanical structure, morphogenesis tissue repair and guidance of pluripotent cells in tissue engineering.

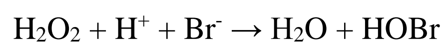
The haem peroxidase enzyme peroxidasin catalyses the formation of sulfilimine crosslinks through the formation of hypobromous and hypochlorous acid (HOBr and HOCl). Though *in*

vitro studies highlight the preference of bromide as a co-factor for peroxidasin over chloride, whether this preference is maintained *in vivo* remains unknown. *In vitro* investigations revealed that sulfilimine bond formation in collagen IV scaffolds is mediated by the formation of a methionine halosulfonium intermediate (HSI) at Met^{93}. Selectivity of the hypohalous acid is determined by the respective end-products mediated by the HSI reaction. HOBr forms a bromosulfonium intermediate which predominantly reacts with the amine group on Hyl^{211} to form the sulfilimine crosslink. This is due to the propensity of the bromosulfonium species to generate smaller partial charge transition states that favour orbital-controlled reactions. Conversely, HOCl forms a chlorosulfonium intermediate which predominantly reacts with water to form methionine sulfoxide which is an uncrosslinkable product. This is due to the propensity of the chlorosulfonium species to generate highly polar transition states that favour charge-controlled reactions. Sulfilimine bond formation proceeding through the bromosulfonium intermediate is also thermodynamically favourable over the chlorosulfonium intermediate, which encounters an unfavourable energetic barrier (9). Studies in bromide deficient Drosophila show altered basement membrane and tissue morphology, aberrant embryogenesis, larval mid-gut defects and lethality. However, where bromide deficiency was reversed, normal development was restored, suggesting that bromide is critical for basement membrane assembly and epithelial tissue development (9).

Bromine has not previously been considered an essential trace element physiologically. Therefore, bromide supplementation has not been considered for use in therapy. Its use in therapy has many potential applications, notably in patients with bromide dietary deficiency. Patients with end-stage renal disease have low serum bromide levels due to dialysis and can also benefit from this treatment (14). Moreover, smokers may also benefit from bromide supplementation as they have elevated serum thiocyanate (7) which inhibits sulfilimine bond formation, even with normal serum bromide levels.

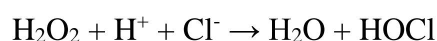
2.3 The role in innate immunity

Neutrophils and eosinophils are both white blood cells that play a role in host defence mechanisms. Neutrophils primarily ingest and kill small microbes through a phagolysosome mechanism whereas eosinophils target parasites and large invading pathogens. However, they can both trigger a respiratory burst which mediates the destruction of pathogens and host tissue alike through the formation of bactericidal reactive species. These reactive species are formed by the intermediary action of myeloperoxidase (MPO) and EPO with halides (15). EPO is encoded by the gene EPX which has an open reading frame of 2106 base pairs. It shares similarities with other mammalian haem peroxidases, MPO, lactoperoxidase and thyroid peroxidase. The most common feature of these peroxidases is the existence of two covalent ester bonds between the haem group and the protein in the functional enzymes. However, MPO is unique in having an additional sulfonium linkage between Met^{243} and the haem group (16,17). MPO and EPO have a variety of substrates they can oxidize, including the halides and pseudo-halide (e.g. thiocyanate and nitrite). The normal blood concentrations of substrates are 0.1 M chloride, 0.02-0.1 mM bromide and 0.1-0.6 µM iodide. While both peroxidases can utilize all halides in the blood, chloride is the primary substrate for MPO whilst bromide is the primary substrate for EPO (1,18). The mutant of MPO wherein haem-linked Met^{243} was mutated showed the lack of preference to use chloride ion, suggesting a role for MPO's unique structure in its substrate specificity (16). Nevertheless, both EPO and MPO catalyse the oxidation of bromide ions by hydrogen peroxide to generate hypobromous acid (HOBr) through the oxidation of bromide (2).



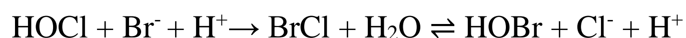
$$H_2O_2 + H^+ + Br^- \rightarrow H_2O + HOBr$$

It has also been suggested that the production of HOBr via the MPO-dependent pathway is partly due to the reaction of hypochlorous acid (HOCl) with bromide and the formation of bromine chloride (BrCl) as an intermediate (19).



$$H_2O_2 + H^+ + Cl^- \rightarrow H_2O + HOCl$$

(This reaction is catalysed by MPO)



$$HOCl + Br^- + H^+ \rightarrow BrCl + H_2O \rightleftharpoons HOBr + Cl^- + H^+$$

In the aqueous environment, HOBr exists in equilibrium with the ionized form ($HOBr \rightleftharpoons H^+ + OBr^-$). Since the pKa for HOBr dissociation is 8.7, HOBr has higher concentration than its ionized form in physiological pH. HOBr a strong oxidant with potent antibacterial/antiparasitic properties. Gaut et al. found that low micromolar concentrations bromide boosted the ability of the MPO/H_2O_2/chloride system to kill *Klebsiella pneumonia* (20). This raises the possibility that bromide could augment oxidative killing mechanisms of neutrophils. While neutrophils are central to host defense against microorganism, eosinophils have a crucial role in host defense against eukaryotic and multicellular parasitic infection. It appears that bromide promotes the anti-parasitic activity of eosinophils. Nogueira et al., demonstrated that EPO/H_2O_2/bromide system plays a significant role in defense against the parasite, *Trypanosoma cruzi* (21). They reported that EPO-coated parasites could be killed in a cell-free system by the addition of bromide/H_2O_2 (21). These reports suggest a role for bromide in innate immune response against both prokaryotic and eukaryotic parasites. Although HOBr formation is crucial for innate immune response, excessive or misplaced generation can damage host tissues, and this has been implicated in several human inflammatory diseases (18). Pioneering work of Slungaard and Mahoney in 1991 proposed that bromide ions mediate the cytotoxicity of EPO in an experimental model of endocarditis (22). Since then many studies have demonstrated the involvement of bromide-dependent mechanisms in pathophysiology of asthma and other inflammatory disorders (18).

3. Interaction of HOBr and Tyrosine Residues in Proteins

Little information is available on the reactions of HOBr with proteins and other biological molecules. The amino acid side-chains were found to be significantly more reactive than the backbone amide groups. As of late, it has been shown that the reaction of HOBr with proteins results in bromination of aromatic amino acid residues, including formation of Br-Tyr and di-Br-Tyr (figure 2). These products have been used as specific markers of HOBr reactions *in vivo*,

with these species detected at elevated levels on bronchoalveolar lavage proteins and proteins isolated from sputum obtained from patients with asthma (15).

Brominated tyrosine is found to be formed by a fast-direct reaction of HOBr with the aromatic ring of tyrosine and a slower reaction involving bromoamines formed on the protein. The original investigations into formation of Br-Tyr and 3-Chloro-tyrosine (Cl-Tyr) by mammalian peroxidases were studied by Wu et al. They demonstrated that these halogenated aromatic amino acids were produced by isolated MPO and neutrophils at physiological concentrations of bromide and chloride (3). However, the levels of Br-Tyr detected were considerably higher with EPO and eosinophils. This suggests that Br-Tyr and Cl-Tyr may serve as selective “molecular fingerprints” to identify sites of tissue injury by leukocyte peroxidases, EPO, and MPO, respectively. However, data from Sentilmohan et al. suggest that MPO-dependent oxidation of bromide is pH dependent, with a significant rise in the yield of HOBr formation at pH above 7 (23). The ability of MPO to produce more Br-Tyr than Cl-Tyr under physiological conditions disallows Br-Tyr from being a specific biomarker of EPO. Consequently, Br-Tyr should rather be considered only as a specific post-translational modification of proteins formed by the action of reactive brominating species.

This overall reaction results in bromination of proteins. The latter part of this review focuses on the validity of Br-Tyr as an inflammatory biomarker. In fact, increased formation of Br-Tyr has been shown in different inflammatory disorders such as asthma (15), rhinosinusitis (24), eosinophilic esophagitis (25) and eosinophilic vasculitides (26).

4. Br-Tyr Metabolism

Both free and protein-bound Br-Tyr can be measured in plasma and body fluids (e.g. bronchoalveolar lavage) (15). To make sampling non-invasive, some investigators have measured Br-Tyr in urine samples and showed that the urinary level of unmetabolized Br-Tyr is elevated in patients with eosinophilic activation (25–27). However, it is likely that free Br-Tyr undergoes metabolism in the liver and other peripheral organs. Therefore, unmetabolized urinary Br-Tyr may not be the best marker for non-invasive assessment of Br-Tyr formation *in*

vivo. In 2016, the urinary metabolites of Br-Tyr were identified in rats (28). Injection of deuterium-labelled Br-Tyr into male Sprague-Dawley rats was associated with excretion of a novel brominated metabolite: 3-bromo-4-hydroxyphenylacetic acid (Br-HPA) as well an unmetabolized Br-Tyr. Br-HPA accounted for $0.43 \pm 0.04\%$ of Deuterium-labelled Br-Tyr, with $0.12 \pm 0.02\%$ of Deuterium-labelled Br-Tyr being unchanged in the urine (28). This showed that Br-Tyr metabolism occurs in rats and its metabolites can be quantified. As shown in figure 3, the process of formation of Br-HPA involved decarboxylation and deamination of Br-Tyr similar to metabolism of other tyrosine derivatives such as 3-nitrotyrosine (Nitro-Tyr) and Cl-Tyr (29–31).

In addition to deamination and decarboxylation processes, Mani et al. found that an intraperitoneal injection of di-Br-Tyr in rats gives rise to formation of Br-Tyr and tyrosine *in vivo* indicating that Br-Tyr undergoes debromination (28). It was further found that 1.3% of deuterium-labelled Br-Tyr had been de-brominated to 4-hydroxyphenylacetic acid (HPA), a major metabolite of tyrosine, suggesting that de-bromination had occurred *in vivo*. They further tested this *in vitro* using human hepatocellular carcinoma (HepG2) cells by incubating them in deuterium-labelled Br-Tyr and analysed its incorporation into cellular proteins. They detected deuterium-labelled tyrosine but not deuterium-labelled Br-Tyr. This suggests that Br-Tyr does not undergo further incorporation into cellular proteins. However, the presence of deuterium-labelled tyrosine indicates that de-bromination of Br-Tyr occurs, where the resulting tyrosine product can be re-introduced in the process of protein synthesis (28). This inspired investigations into whether iodotyrosine dehalogenase 1 (DEHAL1) - a thyroidal enzyme that deiodinases 3-Iodotyrosine (I-Tyr) and 3,5-di-Iodotyrosine (di-I-Tyr) - could also de-halogenate free Br-Tyr. It was shown that DEHAL1 could de-brominate Br-Tyr and di-Br-Tyr *in vitro*. Overall, such findings demonstrate that de-halogenation of Br-Tyr is a major metabolic pathway controlling the levels of free Br-Tyr *in vivo*. This is illustrated in figure 3.

5. Biological Activity of Br-Tyr and its Derivatives

Although Br-Tyr has mainly been used as a biomarker to monitor leukocyte activation, there are studies to show that Br-Tyr *per se* interacts with biological systems and exerts some function. Evidence for the existence of Br-Tyr in serum proteins was first reported by Firnau and Fritze in 1973. After complete hydrolysis of desalted blood serum labelled *in vivo* with $^{82}Br^-$, bromine was detected in the amino acid fraction which was found to be Br-Tyr (32). Free halogenated tyrosine is not usually incorporated into protein structure. It is possible to use genetic engineering to incorporate free Cl-Tyr into protein structure (33). However, there is no such report for Br-Tyr. With our current understanding of bromination reactions *in vivo*, protein-bound Br-Tyr should be considered only as a specific post-translational modification of proteins formed by the action of reactive brominating species. The physiological importance of bromination of proteins on their function is not well understood. It has been shown that tyrosine halogenation can decrease the pKa of the phenol group in tyrosine (34). Perhaps if tyrosine is brominated, it changes the pKa of the -OH group on the phenolic ring. This might affect tyrosine phosphorylation and phosphorylation dependent signaling. At least in theory de-bromination restores the pKa balance to ensure protein function and phosphorylation-dependent signaling can occur efficiently. However, a possible role for bromination and de-bromination of protein-bound tyrosine in cell signaling awaits further investigation. Up until now, most investigations on the interaction of Br-Tyr and physiological systems are focused on free Br-Tyr, which is summarized as follows.

5.1. Effects on the thyroid function

The function of the thyroid is to produce thyroid hormones that modulate production, energy utilization and growth. In the biosynthetic process of thyroid hormone synthesis, one particular step involves the de-iodination of I-Tyr and di-I-Tyr by DEHAL1 as shown in figure 4. In this step, liberated iodide is subsequently reused for further thyroid hormone synthesis. Any disruption in the process of de-iodination can lead to depletion of iodine storage and eventually hypothyroidism (35). A recent study has indicated that both Br-Tyr and di-Br-Tyr can inhibit

de-iodination of iodotyrosines through reversible competition (28). Lamooki et al. used *in silico* simulation to analyse multiple degrees of DEHAL1 inhibition, and though patterns of inhibition differ mathematically (36), it was found that overall, DEHAL1 inhibition resulted in impaired thyroid function.

By-products of the inflammatory cascade, particularly tyrosine derivatives (Br-Tyr, Cl-Tyr and Nitro-Tyr) can compete with I-Tyr and di-I-Tyr, reversibly inhibiting DEHAL1 activity. Consequently, patients with systemic inflammation often present with thyroid hormone abnormalities. This has a particular implication in pregnancy, where the foetus is entirely dependent on the mother's thyroid hormone supply during the first trimester. Thyroid disorders during this period of time are associated with maternal and foetal complications during gestation and sequelae post-delivery (37). Overall, Br-Tyr formation can potentially impair thyroid function due to competitive inhibition of DEHAL1. Further research is necessary to shed light on the extent of thyroid dysfunction that might be mediated through Br-Tyr and related compound.

5.2. Inhibition of inotropic glutamate receptors by di-Br-Tyr

Glutamate is an excitatory neurotransmitter that acts on ionotropic AMPA, NMDA and kainite receptors and metabotropic glutamate receptors to regulate brain signaling activity. During cerebral ischemia, elevated levels of glutamate can cause excitotoxicity and subsequently death of nerve cells. Whilst neuroprotective anti-glutaminergic agents exist, they can only target a single subtype of glutamate receptors or glutamate release, allowing other pathways of excitotoxicity to remain intact.

di-Br-Tyr was found to produce a selective, potent and reversible depression of ionotropic glutamate receptors in hippocampal and cortical neuronal cultures from mice (38). Its multi-pathway targeting allows it to concurrently mediate depression of glutaminergic synaptic transmission through the following mechanisms: firstly, direct competition for the glycine-binding site of NMDA receptors. Secondly, direct competition for the glutamate-binding site AMPA and kainite receptors. Thirdly, attenuation of glutamate release. These effects were first

demonstrated in the aromatic amino acid phenylalanine, of which di-Br-Tyr is a derivative of (38). Kagiyama et al. reported that di-Br-Tyr attenuates glutamate transmission by similar mechanisms as phenylalanine, but with greater potency. More importantly this endogenous compound does not alter heart rate, blood pressure, atrioventricular nodal and intraventricular conduction in isolated heart which indicates its selectivity for neural ionotropic receptors (38). Whilst its neuroprotective and anti-glutaminergic effect has been demonstrated both *in vitro* and *in vivo* in mice, such mechanisms require further investigation in humans. Regardless, di-Br-Tyr remains a therapeutic candidate to mitigate the consequences of ischemic stroke and further neurological disorders involving overactive glutamate receptors.

5.3. Diverse physiological function of Br-Tyr derivatives

Since Morner reported isolation of di-Br-Tyr from marine sponges in 1913 (39), more than 280 bromotyrosine-derived alkaloids have been extracted from marine invertebrates (40). These brominated alkaloids consist of brominated tyrosine and tyramine units that are linked together through side chains or the aromatic ring. It appears that these Br-Tyr derivatives are part of defence mechanisms in marine sponges. They are able to interact with a variety of biological targets in the host organisms such as acetylcholine esterase, protein tyrosine phosphatases, nuclear factor-k B, calcium channels, histamine receptors, DNA methyltransferase, histone deacetylases and mycothiol S-conjugate amidase (41–50). Thus, these Br-Tyr derived marine bioactive compounds are important for drug discovery programs as well as development of antifouling materials. Biosynthesis and biological activities of these compound are reviewed elsewhere (40,50) and are out of scope of this review article.

6. Br-Tyr as a Biomarker

Elevated levels of Br-Tyr have been described in a variety of medical conditions. In this section, we summarise the evidence of Br-Tyr formation and its potential role in the pathophysiology of a variety of diseases reported thus far.

6.1. Asthma

Atopic asthma is a condition characterized by excessive eosinophil recruitment and activation, which is thought to play a role in promoting inflammatory injury in the pathogenesis of asthma. Consequently, activation of EPO results in oxidative damage of proteins through bromination of tyrosine residues. This results in elevated levels of Br-Tyr, as observed in asthma patients. Such an observation of covalent modification of biologic targets in human health or disease has led to Br-Tyr being implicated as a non-invasive biomarker for asthma and inflammatory diseases (15).

Investigation undertaken by Wu et al. found that endobronchial biopsy specimens from atopic asthma patients had comparably more eosinophils and EPO activation as opposed to the control group, though baseline Br-Tyr levels in bronchoalveolar lavage proteins were comparably similar (15). However, upon exposure to an allergen challenge, atopic asthma patients experienced a 10-fold increase in bronchoalveolar lavage protein-bound Br-Tyr. Whilst Br-Tyr in this situation seems to be a candidate for a biomarker in asthma exacerbation, further investigation is still required to ascertain its specificity and if this pattern is also seen in non-atopic asthma exacerbations. Similarly, Aldridge et al. investigated Br-Tyr levels in sputum proteins of stable asthmatics and controls, revealing a 3-fold median increase of Br-Tyr levels and a 2-fold median increase of 3,5-di-Br-Tyr levels in stable asthmatics as compared to controls. They also showed an elevated concentration of EPO in sputum of stable asthmatics compared to controls, which strongly and significantly correlated with Br-Tyr levels. This study supports the findings by Wu et al., and further confirms the role of EPO and its production of reactive bromo species as a common feature of asthma (51).

A subsequent study by Wedes et al. aimed to investigate the efficacy of urinary free Br-Tyr as a non-invasive biomarker to monitor asthma and predict exacerbations (52). This would be the monitoring method of choice for paediatric asthma due to its non-invasive nature and the ease of urine sample collection as opposed to sputum sample collection. The results of their study showed that Br-Tyr levels correlated significantly with asthma exacerbation. Patients with high

Br-Tyr levels were 18.1-fold more likely to have inadequately controlled asthma and 4-fold more likely to experience an exacerbation. The data collected highlights predictive capability of Br-Tyr for paediatric asthmatic episodes (52).

Further studies by Cowan et al. have also found urinary Br-Tyr to predict clinical responsiveness to inhaled corticosteroid therapy (53). Br-Tyr levels decreased in response to inhaled corticosteroid therapy. However, there was no correlation between the level of decrease in Br-Tyr and clinical responsiveness to the corticosteroid therapy. This presents itself as a limitation in specificity of Br-Tyr as a biomarker for clinical responsiveness to inhaled corticosteroid therapy. Nevertheless, this is still significant in the context of a personalized treatment regime, as clinical responsiveness to inhaled corticosteroids can be variable between different patients (53).

Overall, Br-Tyr is an effective biomarker for the evaluation of asthma. It has potential applications in controlling and monitoring asthma and perhaps predicting future exacerbations and clinical responsiveness to inhaled corticosteroid therapy on a personalised basis. Urinary Br-Tyr has been the focus, with it being a non-invasive biomarker, although studies are yet to investigate its metabolites (Br-HPA) and how well it can evaluate asthma, thus future investigations are needed.

6.2. Sinonasal inflammation

Chronic rhinosinusitis is a condition involving the prolonged inflammation of the sinuses. Sinonasal polyposis is a condition involving the presence of benign polyps within the nasal cavity and sinuses. Both chronic sinusitis and sinonasal polyposis are inflammatory sinonasal conditions. They are primarily characterized by eosinophilic infiltration of the sinus mucosa, where elevated tissue levels of Br-Tyr may be observed. Therefore, identification of specific mechanisms of oxidative protein modifications may serve as an objective index of chronic sinusitis and sinonasal polyposis activity (24).

Citardi et al. measured tissue and serum markers of tyrosine halogenation as indices of EPO-catalysed and MPO-catalysed pathways (24). Their findings indicated that tissue levels of Br-

Tyr were significantly higher compared with the control group for both chronic sinusitis and sinonasal polyposis. However, serum levels of Br-Tyr showed no significant differences in both chronic sinusitis and sinonasal polyposis groups compared to their controls. Interestingly, no differences were detected for tissue or serum Cl-Tyr and Nitro-Tyr in patients with sinonasal inflammation either. Chronic rhinosinusitis patients had local enrichment of Br-Tyr in their sinonasal mucosa. This localised tissue level of Br-Tyr was 24-fold higher than serum levels. In contrast, healthy controls had a 12-fold increase in localised tissue levels of Br-Tyr compared to serum levels (24). This finding suggests that there is an ongoing baseline of oxidant-related stress under normal conditions and may be indicative of normal innate immune function in the upper respiratory tract. There is limited mention as to whether the potentially different baseline of oxidant stress in individual subjects should be considered as a determinant of an inflammatory condition.

This study suggests that the high levels of Br-Tyr in the inflamed sinus mucosa tissue of chronic rhinosinusitis and sinonasal polyposis patients is linked to the presence of activated eosinophils. Br-Tyr is produced through the EPO-catalysed oxidative pathway. As a result, Br-Tyr may potentially be used to characterise such conditions.

6.3. Cystic fibrosis

Cystic fibrosis is a genetic mutation that is autosomal recessive, characterized by mutations in the cystic fibrosis transmembrane regulator gene (CFTR). This results in altered epithelial transport of halides and water, causing increased mucus viscosity, reduced mucociliary clearance which decreases antibacterial immune capabilities within the respiratory tract. Saude et al. used nuclear magnetic resonance (NMR) to identify the primary product of MPO/EPO activity in cystic fibrosis sputum samples. They found that Cl-Tyr was the predominant product which can be accounted for by the elevated neutrophil count. However, Br-Tyr and di-Br-Tyr formation was also detected by NMR. Saude et al. endorse NMR as a high resolution, rapid and efficient potential diagnostic tool for metabonomic analysis of disease phenotype (54).

Whilst Br-Tyr levels were found to be statistically significant between cystic fibrosis patients and control group, Saude et al. did not find a correlating increase in eosinophil cell counts between the groups (54). Although they postulated that eosinophil activation accounts for the increased Br-Tyr levels, there is limited discussion of whether Br-Tyr production from neutrophils could account for this increase. Saude et al. also showed that the majority of Cl-Tyr produced is due to neutrophil activation and not macrophage activation. There was a strong negative correlation between macrophage MPO activity, and the Cl-Tyr produced. It should be noted that correlation does not equal causation. Therefore, further investigations are needed to enforce the specificity of Br-Tyr markers against EPO activity in activated eosinophils. There is a need for greater understanding of immune cell behaviour in cystic fibrosis.

Similarly, Thomson et al. extracted bronchoalveolar lavage samples from children which showed a highly elevated neutrophil count consistent with other studies (55). The bronchoalveolar lavage samples showed both high levels of intermediate HOCl and HOBr corresponding to Cl-Tyr and Br-Tyr. However, detection of EPO in bronchoalveolar lavage samples were not detected. It is possible that undetected EPO activity could account for Br-Tyr production. However, the results from Thomson et al. supports neutrophil MPO activity as the primary source of Br-Tyr. This also suggests that neutrophil MPO activity can produce a range of oxidants during an inflammatory response (44).

Furthermore, Xu et al. made an important discovery about the protective antioxidant nature of endogenous thiocyanate against MPO activity linked diseases i.e. cystic fibrosis atherosclerosis and neurodegeneration (56). CFTR, although primarily known as a chloride ion channel, is also able to conduct thiocyanate ions which can limit harmful accumulation of hydrogen peroxide and hypochlorite through the following pathways.

(1) Thiocyanate is oxidized by lactoperoxidase in the airways to tissue-innocuous hypothiocyanite, using hydrogen peroxide in the process.

(2) Thiocyanate competes with chloride ions for MPO utilization, limiting hypochlorite production.

(3) Thiocyanate can act as a reducing agent in the presence of hypochlorite without catalysis. Due to the similar nature of MPO and EPO, it is highly likely that EPO-induced lung injury can also be reduced through therapeutic supplement of endogenous thiocyanate (56).

Though the direct link between CFTR mutations and onset of lung injury is unclear, thiocyanate release by epithelial cells is noticeably decreased in cystic fibrosis patients due to missing CFTR activity. The absence of adequate thiocyanate results in an accumulation of hypochlorite and hydrogen peroxide produced by MPO after onset of infection and inflammation. This in turn leads to self-propagating lung injury and self-destruction of immune cells, escalating injuries to host tissue. Thick mucosal secretions blocking the submucosal glands also predispose cystic fibrosis patients to infection, optimizing the environment for infection to establish and then propagate.

Where the plasma thiocyanate levels of the general population lie between 10 µM and 140 µM, it was found that the cytotoxicity of MPO in airway epithelial cells, neuroblastoma cell lines, pancreatic β cell lines and aortic endothelial cells is attenuated once the concentration of thiocyanate reaches 100 µM. Insufficient thiocyanate levels can hence cause cystic fibrosis patients to present with exaggerated MPO/EPO induced lung injury (56).

Further investigation of MPO monitoring and product specificity may yield functionality in diagnosis and treatment assessment designed to prevent or limit cystic fibrosis lung disease.

6.4. Eosinophilic esophagitis

Br-Tyr is a product of eosinophil activation and has been investigated as a biomarker of eosinophil activation in eosinophilic diseases. Eosinophilic esophagitis (EoE) is a chronic disease that is immune or antigen mediated. it is characterised by excessive eosinophil activation and inflammation of the oesophagus which leads to oesophageal dysfunction. The gold standard for diagnosis and monitoring of eosinophilic esophagitis is through eosinophil counts from oesophageal biopsies. Biomarkers for eosinophil degranulation are strongly associated with symptoms. However, they are invasive as a blood sample needs to be taken which can be distressing and inconvenient especially for young children. Therefore, a non-invasive diagnostic

and monitoring tool is still an unmet medical need for the evaluation of eosinophilic esophagitis disease (25).

In 2016, it was found that urinary protein-bound Br-Tyr in creatinine normalised urine was a useful non-invasive clinical marker for the evaluation of eosinophilic esophagitis (25). A mass spectrometry method was used to measure urinary Br-Tyr levels in creatinine normalized urine - the 'Eosinophil Quantitated Urine Kinetic' (EoQUIK). The study found that the median normalized Br-Tyr levels increased 93- fold in eosinophilic esophagitis patients compared to non-atopic controls and 13-fold in comparison with atopic controls. Moreover, cut off thresholds of 17pg of Br-Tyr per 400mg of creatinine yielded 100% specificity and a negative predictive value of 100% for non-atopic controls. A threshold of 145pg yielded 90% specificity and 79% negative predictive value for atopic controls (25). This shows that the difference in eosinophil degranulation between the groups was accurate and could be used as a diagnostic monitoring tool for evaluation of eosinophilic esophagitis. Although, perhaps a larger validation study is needed due to the small sample size used. Nevertheless, there is still promise for urinary Br-Tyr as a biomarker for EoE. Given the fact that taking oesophageal biopsy is invasive, urinary Br-Tyr may provide a non-invasive test for monitoring patients with EoE.

Additionally, if urinary Br-Tyr was greater than 20pg per 400mg of creatinine, this increased the risk of the patient having eosinophilic esophagitis by 4.8-fold (95% confidence interval) when compared to non-atopic controls, after controlling for race and sex. This further supports that urinary Br-Tyr can be used to help evaluate eosinophilic esophagitis disease. However, the study measured only protein-bound Br-Tyr levels, which may be inaccurate considering proteins do not pass through the kidney's filtration barrier in high quantities. Collection of enough protein for hydrolysis and protein-bound Br-Tyr measurement requires high volume of urine, which may not be easy to collect from children. The study has not reported the level of unmetabolized free urinary Br-Tyr or its metabolites (e.g. Br-HPA) in the urine. Therefore, further investigation is needed and may show a better marker of eosinophil activation in EoE.

6.5. Chronic enteropathy in canines

Chronic enteropathy is a persistent inflammatory disease that effects the gastrointestinal tract and is thought to involve the infiltration of eosinophils in the gastrointestinal tract. Chronic enteropathy diagnosis involves the exclusion of gastrointestinal tract parasites and other diseases e.g. pancreatitis and exocrine pancreatic inefficiency. Chronic enteropathy is also classified based on the patient's response to the type of treatment e.g. food responsive diarrhoea, antibiotic responsive diarrhoea and steroid responsive diarrhoea. Steroid responsive diarrhoea can be sub-classified into: eosinophilic gastroenteritis and lymphocytic-plasmocytic enteritis. Serum Br-Tyr in canines has been found to be elevated in some cases of chronic enteropathy, which may be a marker for eosinophil activation in the gastrointestinal tract (57).

Although, Sattasathuchana et al. found that there was no relationship between peripheral eosinophil count and the presence of eosinophils in gastrointestinal tissues (57). They did find that serum Br-Tyr concentrations were higher in canines with eosinophilic gastroenteritis, lymphocytic-plasmocytic enteritis and pancreatitis in comparison to the controls. Whereas exocrine pancreatic inefficiency concentrations were not significantly different in comparison to the controls. This shows that chronic enteropathy does in fact involve eosinophil activation. However, only serum Br-Tyr was analysed. Perhaps faecal or urinary Br-Tyr could assess gastrointestinal tract inflammation to a higher level of accuracy. However, the study still shows that serum Br-Tyr levels are elevated in chronic enteropathy cases and could have the diagnostic potential to differentiate between eosinophilic gastroenteritis and lymphocytic-plasmocytic enteritis. This needs to be investigated further.

Nevertheless, Sattasathuchana et al. did investigate further the clinical potential of serum Br-Tyr in food responsive diarrhoea and steroid responsive diarrhoea canine cases (58). Although, there was no association between peripheral eosinophilia in food responsive diarrhoea, steroid responsive diarrhoea and the control groups and no further association between peripheral eosinophilia and serum Br-Tyr. This shows that peripheral eosinophilia is not a good diagnostic tool for canines with food responsive diarrhoea and steroid responsive diarrhoea. Conversely,

serum Br-Tyr concentrations in canines with steroid responsive diarrhoea was higher in comparison to the food responsive diarrhoea and control groups. Moreover, serum Br-Tyr concentrations were higher in food responsive diarrhoea canines than in the controls. This suggests that serum Br-Tyr does have the diagnostic potential to differentiate between food responsive diarrhoea and steroid responsive diarrhoea. Although, there was no association between the 'canine chronic enteropathy clinical activity index' (a tool used to investigate the prognostic outcome of canines with chronic enteropathy) and serum Br-Tyr. Further investigations of both variables may be useful to further evaluate the diagnosis and prognosis of chronic enteropathy. Yet, serum Br-Tyr still has the clinical potential to differentiate between food responsive diarrhoea and steroid responsive diarrhoea and perhaps can evaluate the progression of chronic enteropathy too. However, neither of the studies have evaluated the clinical potential of Br-Tyr's metabolites (i.e. Br-HPA) in diagnosis and monitoring chronic enteropathy, thus further investigations are needed.

6.6. Eosinophilic vasculitides

Vasculitides is a group of disorders resulting in inflammatory damage to various sized blood vessels caused by leukocyte migration. This is further classified into eosinophilic vasculitides such as Churg-Strauss syndrome and non-eosinophilic vasculitides. Vasculitis can also be an underlying accompanying feature in vasculitis diseases and rheumatoid arthritis. Churg-Strauss syndrome is characterized by hyper eosinophilia. This means that urinary Br-Tyr could be considered as a selective marker of EPO-catalysed oxidation. Br-Tyr reflects clinical disease activity alongside other urinary markers such as eicosanoid and Cl-Tyr (26). Higashi's et al. findings show urinary concentration of Br-Tyr as elevated during acute Churg-Strauss syndrome exacerbation and a decrease during clinical remission (26**).** Though Br-Tyr is cited as a candidate marker for eosinophil activation, there is limited discussion in how to obtain Br-Tyr from urinary samples, where it is assumed Br-Tyr are a specific sole candidate for eosinophil activity. Although, attempts have been made to identify Br-Tyr and Cl-Tyr as preferential products of eosinophils and neutrophil/monocytes respectively. Due to the lack of

understanding and identification of major urinary metabolites of Br-Tyr, the study falls short of directly associating Br-Tyr to the pathogenesis of vasculitides. Therefore, further investigation is required to identify causation.

6.7. Other Medical Conditions Associated with Br-Tyr Formation

6.7.1. Diabetes mellitus

Diabetes is a metabolic disorder characterized by hyperglycaemia where oxidative stress forms a proponent of the pathogenesis of diabetic complications. Since Br-Tyr levels have been detected in patients with diabetes, there is potential for Br-Tyr to be used as an inflammatory biomarker in diabetes (48).

This allows for consideration of Br-Tyr as an inflammatory biomarker, where elevated urinary levels of Br-Tyr are observed in patients with diabetes compared to the control groups, indicating an activated immune system in diabetics (59). The urinary quantification of modified tyrosine residues appears to be a useful, non-invasive, non-pain inducing marker of oxidative stress *in vivo*. However, the detailed generation mechanism and specificity of Br-Tyr remains unknown and the metabolic process of excretion of modified tyrosine in urine is still unclear. Therefore, further studies are required for assessment.

Subsequent findings by Asahi et al**.** affirm the presence of Br-Tyr as an oxidative stress marker in both lipopolysaccharide (LPS)-treated rats and human urinary samples from diabetic patients (60). Though elevated levels of Br-Tyr were detected on day 2, elevation in levels of 8-halogenated 2'-deoxyguanosines (8-halo-dGs) also produced by MPO were observed even earlier than modified tyrosine residue. This suggests that 8-halo-dGs may be more suitable potential biomarkers of early inflammation. Further investigations are required to determine if the prevalence of specific MPO derived compounds are better correlated or suited to a specific stage of inflammation and if alternate oxidized halogenated proteins may be a better biomarker than oxidized halogenated tyrosine. Furthermore, a detailed mechanism for the formation of halogenated, nitrated and oxidized products *in vivo* has not yet been fully established.

6.7.2. Sepsis

Mice models of polymicrobial sepsis mirror the inflammatory conditions present during widespread infection that may be closely representative to infections in human subjects. This demonstrates that the generation of oxidized tyrosine residues in extracted lavage originates from MPO and EPO activity during immune response. This establishes causation between production of chlorinating and brominating intermediates (Cl-Tyr and Br-Tyr) with MPO in the context of bacterial infections and its bactericidal role (20).

Gaut et al. measured Cl-Tyr and Br-Tyr levels in lavage extracts between MPO deficient and wild type mice. Both of the mice models had undergone intraabdominal infection and sepsis through blind-ended cecum ligation and puncture (CLP) (20). The cellular response to sepsis in both groups of mice were comparable with predominant neutrophil response. However, MPO deficient mice were recorded to have increased mortality with no subjects surviving more than 5 days. In contrast, wild-type control mice saw significantly improved mortality rates of 63% surviving more than 5 days and 38% surviving more than 7 days.

Generation of halogenating intermediates largely increased during acute inflammation, with lavage fluid showing 16-fold increase in levels of Cl-Tyr and 6-fold increase in levels of Br-Tyr. During sepsis, there was an increase in Cl-Tyr and Br-Tyr levels in wild type mice. Conversely, MPO deficient mice showed insignificant increases in Cl-Tyr and a 59% decrease in Br-Tyr during sepsis. This indicates that MPO produces chlorinating and brominating intermediates *in vivo* during inflammation. Furthermore, the established presence of EPO confirms that MPO has a multi-substrate oxidation capability. The ongoing EPO activity means that there is a less significant decrease in Br-Tyr compared to Cl-Tyr between the mice groups. Remarkably, the bromination pathway still optimally occurs at physiological concentrations that are 1,000-fold to 10,000-fold lower than Cl- concentrations. This preference of bromide over chloride may have physiological relevance but this has not yet been established. Likewise whether there is a difference in the halogenation process of bound proteins as opposed to free

amino acids is unclear. Whether or not the severity of inflammation affects the uniformity and repeatability of production of halogenated tyrosine residues also requires further investigation.

6.7.3. Vasospasm in subarachnoid haemorrhage

Cerebral vasospasm is a major delayed complication of subarachnoid haemorrhage (SAH) and results in development of cerebral ischemia and neurological deficits. Inflammation, reactive species and MPO activity promote neutrophil involvement which acts as a major factor in cerebral vasospasm pathogenesis. Elevated levels of Cl-Tyr, Br-Tyr and Nitro-Tyr in the cerebrospinal fluid suggest a role of MPO in the development of a pro-oxidant cerebral vasospasm inducing state (61). Provencio et al. reported that neutrophil percentage in the cerebrospinal fluid days prior to the onset of vasospasm is an independent predictor of vasospasm in SAH patients. Furthermore, Cl-Tyr and Br-Tyr levels in the cerebrospinal fluid were significantly greater in patients with SAH. This supports a mechanistic role for MPO in formation of reactive species in SAH (61). This finding substantiates previous reports on modulation of vascular tone by MPO through nitric oxide oxidation (62). However, further investigations are necessary to explore the feasibility of targeted interventions towards MPO and prevention of vasospasm in SAH.

7. Measurement of Br-Tyr in Biological Samples

A variety of different techniques have been used for the quantification of Br-Tyr in tissue and biological fluids. Conventional HPLC methods (with UV or fluorescence detector) can be used for detection of Br-Tyr in simple solutions (3,28). However, the sensitivity and specificity is not high enough for accurate measurement of Br-Tyr in biological specimens such as urine, blood and tissues. It appears that mass spectrometry is the most specific method for detection of this small molecule. In order to increase reliability of mass spectrometry-based methods, the isotope dilution approach has been employed. ^{13}C labelled or deuterium labelled internal standards can be synthesised to enhance sensitivity and specificity of detection (18). Table 1 summarises the available methods of quantifying Br-Tyr in biological samples.

An efficient and reliable method for producing sufficient quantities of standard compounds is necessary to measure Br-Tyr and di-Br-Tyr formation in biological samples, Several methods that employ an enzymatic synthesis of di-Br-Tyr using peroxidase have been reported, as have chemical methods for di-Br-Tyr synthesis (3,28,63). Tilley et al. developed a non-enzymatic preparative-scale method of synthesizing di-Br-Tyr and Br-Tyr (63). A simple protocol for synthesis and purification of Br-Tyr and di-Br-Tyr is described in appendix 1. This method has been usefully used for synthesis of stable isotopes of Br-Tyr (e.g. ^{13}C-lablled) that are commonly used in mass spectrometric analysis of biological samples. Although Br-Tyr is considered as a stable molecule *in vitro*, its stability in different biological matrices in humans has not been investigated systematically. It is known that Br-Tyr is stable for up to 8, 30, and 180 days after incubation in canine serum at 4°C, -20°C, and -80°C (57).

8. Conclusions

Br-Tyr is of significant interest as it has been shown to exert pathogenic effects on thyroid function through reversible DEHAL inhibition based on *in vivo* and *in silico* modelling and neuroprotective effects on the brain through inhibition of inotropic glutamate receptors based on *in vivo* and *in vitro* modelling. Further investigation of such biological effects in humans could elucidate enhanced understanding in treatment of thyroid deficiency and the role of Br-Tyr as a therapeutic candidate for ischemic stroke and neurological disorders.

Current literature has yet to establish a role for the bromination or de-bromination of protein bound tyrosine, though it remains a possibility that it confers a modulating effect on cell signalling. Conversely, unbound Br-Tyr remains a strong potential biomarker for pathophysiology involving leukocyte activation, particularly in eosinophil related inflammatory conditions such as asthma. Br-Tyr levels are heavily tied to EPO and MPO activity in leukocytes and are notably elevated in such medical conditions, but further ascertaining of causation with pathophysiology is required to establish the specificity of Br-Tyr. At the current stage the lack of detailed mechanistic specificity suggests Br-Tyr as a monitoring biomarker for inflammation as opposed to a diagnostic biomarker.

Acknowledgments. We would like to pay our respects to the original author of this paper, Mariam Sabir (1998 – 2017) who passed away in October of 2017 to epilepsy. Mariam was a very bright student studying Applied Medical Sciences at University College London. She channelled great passion and positivity in everything she did, academically, humanely and in all aspects of her life, with the goal of making an impact in medicine and science, to ease what suffering she could in humanity. Her enthusiasm and dedication to research led to her starting this paper in the first year of her university studies whilst maintaining outstanding grades. Mariam never stopped epilepsy from allowing her to pursue the life she wanted and was a girl who was determined to make the most of what she had.

This work was supported by a grant from Royal Free Charity and the Wellcome Trust, UK (Grant no. 075748/Z/04/Z).

Declaration of interest statement. The authors declare no conflicts of interest.

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Appendix 1.

Synthesis of ^{13}C-labelled Br-Tyr

Materials:

- 10mM ^{13}C-Tyrosine
- 99.9% chloroform
- Potassium Permanganate 10mM ($KMnO_4$),
- Sodium Bromide (NaBr) 10mM
- 1M HCl

In a glass tube, add 1ml of the above compounds in the following order:

- Chloroform
- Potassium permanganate solution (10 mM)
- Sodium Bromide solution (10 mM)
- HCl (1 M)

Mix well until the chloroform layer becomes yellow then remove the supernatant. Add 1ml ^{13}C-Tyrosine to the yellow chloroform layer. Mix the solution well until two clear layers appear then remove the supernatant. The products can be purified using HPLC with on a C18 column. This can be done using a gradient of 10% acetonitrile containing 0.1% (v/v) TFA (trifluoroacetic acid) (solution A) and 70% acetonitrile containing 0.1% (v/v) TFA (solution B). Initial conditions were 100% solution A, changing to 20%:80% solution A/solution B over 30 min, then to 100% solution A from 30 to 35 min. The fractions containing ^{13}C-Bromotyrosine can be identified by their retention times and characteristic UV spectra. Fractions containing ^{13}C-Bromotyrosine are pooled and freeze-dried under vacuum.

Figure legends:

Figure 1. The interaction of Bromide with physiological systems. Bromide has multiple functions through formation of reactive bromo species to interaction with ion channels (e.g. GABA-sensitive chloride channels). HOBr is formed by the action of different peroxidases (e.g. EPO, MPO and peroxidasin) and is the main reactive brominating species at neutral pH.

Figure 2. Chemical structure of 3-Bromotyrosine and 3,5-di-Bromotyrosine.

Figure 3. The metabolism of 3,5-di-bromotyrosine and 3-Bromotyrosine. DEHAL1 de-brominates di-Br-Tyr and Br-Tyr back to tyrosine. tyrosine, Br-Tyr and di-Br-Tyr are metabolised by tyrosine decarboxylase and monoamine oxidase to their respective hydroxyphenylacetic acid (HPA) derivatives.

Figure 4. The recycling pathway of iodide and its role in formation of 3-Iodotyrosine (I-Tyr), 3,5-di-Iodotyrosine (Di-I-Tyr), T3 and T4. Br-Tyr inhibits DEHAL1 and potentially disrupts iodide recycling.

Table legend:

Table 1. Analytical methods used in measurement of bromotyrosine (Br-Ty) in biological samples.

Figure 1

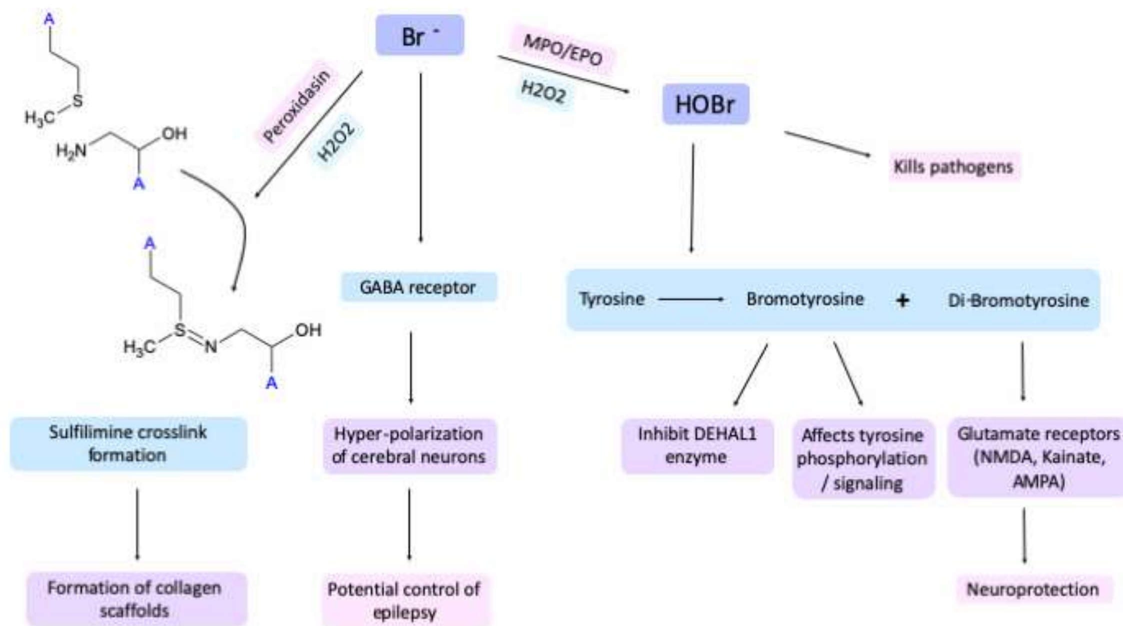

Figure 2

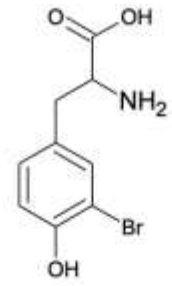

Figure 3

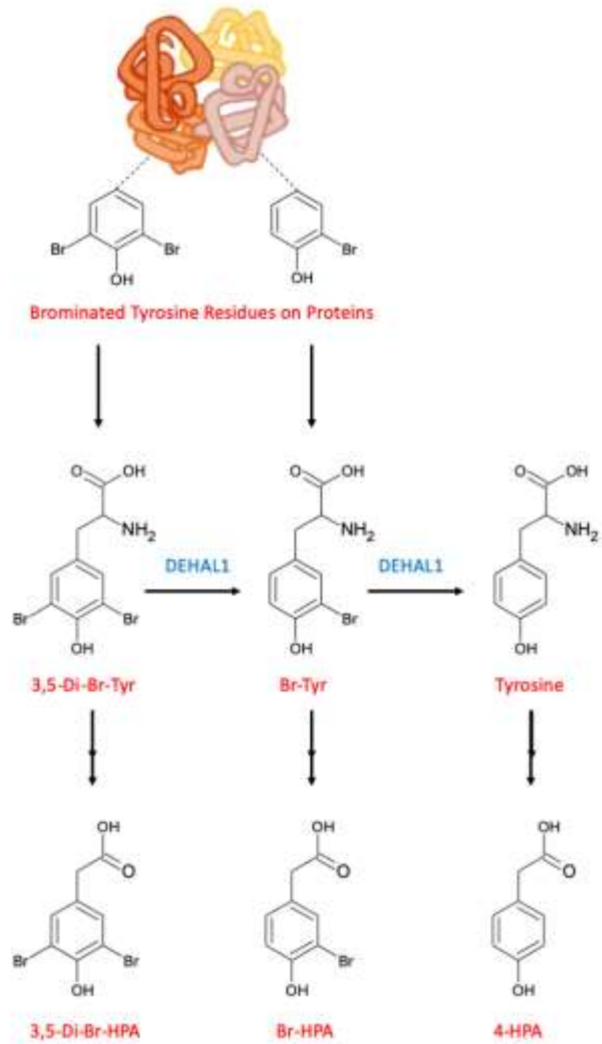

Figure 4

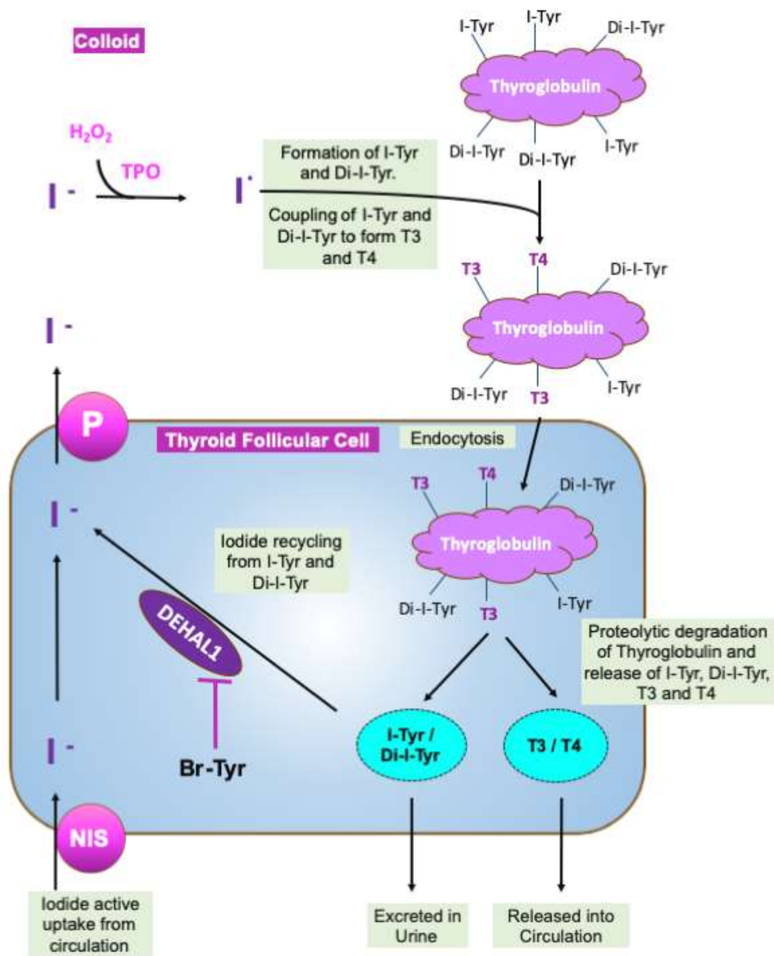

Table 1

Method	Advantages	Disadvantages
GC/MS: Gas chromatography/ Mass spectrometry (15,20,28,64)	• Technique is two orders of magnitude more sensitive than LC-MS and is hence more effective for analysis of trace amounts of halogenated tyrosine species. • Mass spectrometry provides specific structural information and allows the use of stable isotopes as internal standard • Multiple biomarkers can be quantified in a single biological sample.	• Derivatization is required which might involve hi temperature and acidic conditions, potentially forming artificial tyrosine derivatives.
LC/MS: Liquid chromatography/ Mass spectrometry (20,52)	• Samples do not have to be derivatized prior to analysis. • Mass spectrometry provides specific structural information and allows the use of stable isotopes as internal standard • Multiple biomarkers can be quantified in a single biological sample.	• Technique is two orders of magnitude (100-fold) less sensitive than GC-MS when used with microbore HPLC columns, and poses the problem found in previous studies where plasma values la below the detection limit

		• Requires a long liquid chromatography equilibrat time to ensure reproducible retention times, meaning analysis may take ~90 minutes per samp
Capillary electrophoresis (65)	• Fast separation of tyrosine compounds allowing rapid analysis of large number of samples. • Low sensitivity	• Buffer must be specific and optimum conditions met, if not separation of tyrosine adducts can fail.
Immunochemical approach (66)	• Can visually show the localization of modified tyrosine.	• Not suitable for rigid quantification and chemical identification of modified tyrosine; semiquantitative. • Cross-reacting but structurally distinct molecules can confound results.
High resolution NMR: Nuclear Magnetic Resonance Spectroscopy (54)	• Can rapidly analyze mixtures at the molecular level without requiring separation	• Relatively low sensitivity