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Review Article

Validation Status and Recent Advances for the Quantification of 3-Nitrotyrosine as a Biomarker of Oxidative/Nitrosative Stress in Rheumatoid Arthritis

Hamza Boucetta [a, b, #], Imene Adouani [c, d #], Dorothy Owusu Asante [e], Zoulikha Makhloufi [f], Tai-jun Hang [a, b *]

[a] Key Laboratory of Drug Quality Control and Pharmacovigilance (China Pharmaceutical University), Ministry of Education, Nanjing, 210009, China.

[b] Department of Pharmaceutical Analysis, school of pharmacy, China Pharmaceutical University, Nanjing 211198, China.

[c] Department of Pharmacy, Faculty of Medical Sciences, Ferhat Abbas University Setif-1, Setif 19000, Algeria.

[d] Laboratory of biotechnology and genomics in medical sciences, Setif 19000, Algeria.

[d] School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing 211198, China.

[e] Department of Pharmaceutics, School of Pharmacy, China Pharmaceutical University, Nanjing 211198, China.

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Corresponding author: Tai-jun Hang

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Abstract:

Biomarkers have recently been utilized to screen, detect, and diagnose illnesses. Rheumatoid arthritis (RA) is one of several autoimmune-related disorders that could benefit from more accurate and validated biomarkers. According to many meta-analysis studies and clinical trials, oxidative/nitrosative stress and RA activity alter in the same way. Therefore, oxidative biomarkers such as 3-nitrotyrosine (3-NT) may be utilized to evaluate the disease activity. Due to the high complexity of proteins in the matrix of interest and the analytical procedures required for robust quantification, 3-NT is still in its early phases for comprehensive validation and clinical application. Mass spectrometry-based techniques are particularly suitable for the quantification of 3-NT, although they still require more advances for their transformation from biomedical research setting to clinical use. This review will examine the qualification and clinical reliability of 3-NT at first, followed by the validation status of existing mass spectrometry-based methods and other novel techniques, that have received a lot of interest but haven't been applied yet in RA.

Keywords: 3-Nitrotyrosine; Biomarkers; Rheumatoid arthritis; Oxidative/nitrosative stress; Mass spectrometry.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic autoimmune disease (CAID) that develops a remarkable inflammatory response, manifested by excessive infiltration of inflammatory cells like macrophages, neutrophils, and lymphocytes, and in the synovial cavity as well as peripheral blood [1]. Pro-inflammatory cytokines like tumor necrosis factor-a (TNF-α), Interferon γ (IFN-γ) , Interleukin (IL)- 1β, and IL-6 are vital elements in the abnormal transformation of some cells in RA autoimmune activity [2]. Also, the biomolecules created by oxidative/nitrosative stress in the affected joints are important biomarkers for assessing the pathophysiology of RA patients [3]. Radicals such as superoxide (O2• −), peroxyl (ROO•), perhydroxyl (HO_2•), and hydroxyl (HO•) are the most prevalent reactive oxygen species (ROS) identified in affected joints, and reactive nitrogen species (RNS) such as nitrogen dioxide (NO_2) and peroxynitrite (OONO−) [2]. Physiopathologically, RA patients experience destructive and proliferative synovitis as a direct result of ROS and RNS inundation (driving factors) [3]. To put it another way, when the body's antioxidant system and/or the body's ability to eliminate/neutralize free radicals is overworked, a redox imbalance is created, resulting in damage to the joint cells' nucleic acids and proteins as well as their membranes and lipids [2,3]. As we have referred before, RNS play a significant role in the chronicity of inflammatory reactions, leading to cartilage and bone destruction in patients with RA. Therefore, it has been established that oxygen and nitrogen free radicals play equally an essential role in the pathogenicity of RA [4].

Biomarkers of oxidative and nitrogenous stress may be useful in assessing and predicting the evolution of pathologies [2]. Hence, clinical biomarkers and accurate analyzing methods of oxygen and nitrogen-free radical species for joint damage in arthritis are needed for monitoring and evaluating the pathogenic status of RA, as well as comprehending the pharmacologic responses to specific therapeutic interventions [2]. In addition, the use of biomarkers can improve the understanding of RA biological and predict therapeutic response [3]. Serum malondialdehyde (MDA) levels are positively connected to lipid peroxidation, ROS formation and disease activity in general, according to meta-analysis studies and clinical trials supporting the idea that RA's oxidative stress and disease activity change in lockstep [1,5]. These findings suggest that oxidative biomarkers, such as 3-nitrotyrosine (3-NT), could be leveraged to assess RA activity and predict its prognosis [6].

In principle, arthritis has been proved to be associated with the increased intra-articular formation of 3-NT, which may contribute to joint damage. 3-NT is a relatively specific marker of nitrosative reactions damage mediated by nitric oxide (NO−) and its by-products [7]. However, the validation of a biomarker is challenging and depends not only on its qualification and clinical correlation but also on the sensitivity and specificity of the analysis methods employed.

RA 3-NT overproduction pathways and distribution

The generation and formation of 3-NT is mainly caused by peroxynitrite (ONOO−) which in turn is made up of the entire interaction between superoxide (O2• −) and nitric oxide (NO−)[8]. The reaction of nitration is maximal at physiological pH, according to Beckman JS *et al.* [8] and decreases under more acidic or basic conditions [9]. Another pathway involves

heme-containing peroxidases forms $^{\cdot}NO_2$, which will then conduct protein nitration (principally myeloperoxidase (MPO), minatory eosinophil peroxidase, and heme proteins expressed in activated leukocytes) [9]. Peroxynitrite targets other amino acids but tyrosine, such as phenylalanine and histidine [10]. Protein sulfhydryls [9] and tyrosyl residues are the principal targets of peroxynitrite in proteins [9]. Peroxynitrite-mediated reactions are strongly influenced by Carbon dioxide/bicarbonate (1.3 and 25 mM in plasma, respectively)[7], and incite the nitration reaction of aromatic rings as in tyrosine, and even ensure the sustainability of the reaction in the presence of antioxidants. Also, it has been proved that those transition metal ions, either in free form (Cu^{2+}, Fe^{3+}, Fe^{2+}) or as a complex structure involving protoporphyrin IX (heme), increase and accelerate the yield of this reaction [7]. Identifying induced nitric oxide synthase (iNOS) in human rheumatoid synovium suggests that the regulation of the $NO^{\cdot}$ may offer a pathway for prognostic and therapeutic intervention[12]. iNOS is also correlatively related to matrix metalloproteinase (MMP) activity, mediating endothelial dysfunction and decreasing extracellular matrix synthesis [13]. Inhibition of iNOS activity using a non-selective inhibitor such as NG-monomethyl-L-arginine (NMMA) ensues a significant reduction of $NO^{\cdot}$, in turn, a reduced reaction of synovial inflammation and tissue damage in animal models of arthritis were noticed as well [3].

Numerous factors may lead to rising and upregulating the expression iNOS in many cells and tissues, namely bacterial lipopolysaccharide (LPS) contact, and pro-inflammatory cytokines such as interleukin 1 (IL-1), TNFa, or IFN-γ [3]. Klocke *et al.* [14] have demonstrated the protective role of activating xanthine oxidoreductase (XOR), and its final oxidation product uric acid in peroxynitrite induced tyrosine nitration inhibition [14]. Several signal transduction and pathways have been implicated in the induction of iNOS, including NF-kD, p38, and the Jak-STAT pathways [15].

iNOS (source of the nitrating species) and nitrotyrosine-containing proteins distribution between joint's constitutional tissues and autoimmune reaction cells already triggered; in the inflamed synovium as Sandhu *et al*.[16] described is variable and inconstant. Expression of iNOS in synovial lining cells, macrophages, vascular endothelium, and vascular smooth muscle cells was proved [16]. Meanwhile, their expression in the stroma, synovial neutrophils, plasma cells, or lymphocytes has been refuted. Nitrotyrosine-containing proteins, unlike iNOS, were highly elevated in areas of the subsynovium of stroma and, to a lesser extent, in the stroma of the synovial layer, and remarkably low expressed in neutrophil; which may be due to its short half-life in tissues [16]. Protein Albumin, because of its high abundance in plasma and its ability to migrate to synovial cells, is recurrently getting oxidized and nitrated by peroxynitrite, leading to structural changes of both trapped and traveled albumin, making this biomolecule stable, undegradable, and persisting for a long time in blood circulation, on the one hand, and causing an enhance of immunogenicity, on the other [17].

3-NT as RA oxidative/nitrosative stress biomarker

At early stages, the mechanisms through which oxidative/nitrative stress may contribute to articular and systemic inflammation initiation in RA remains vague and uncertain [1]. A recent recapitulative study on RA pathological mechanisms and modern pharmacologic therapies by Guo et al.[18] elaborated that ROS/RNS play an essential role in two well-determined stages (out of four) during the establishment of this

disease. The first stage starts by the infiltration of leukocytes to the synovial area and followed by stimulation of pro-inflammatory mediators secretion in synovial fluid, and then an enhance in interactions of fibroblast-like synoviocytes (FLSs) with innate immune cells including neutrophils that secrete ROS which may further aggravate the inflammation and osteoclastogenesis [18]. The other stage where RNS harmfully affect while RA is setting up; is during the fulminate stage; RNS enhance cytokines secretion (particularly IL-1 and 17A) and inundation of the synovial fluid, the cartilage is gradually become deprived of chondrocytes [18]. **Figure 1**. Summarizes the general pathways of RA pathogenesis and its relation with 3-NT.

Generally, RA oxidative/nitrosative stress candidate biomarkers are variable and dependent on targeting pathology' pathways and the reached stage. Lipid peroxidation occurs mainly in polyunsaturated fatty acids of cell membranes or in polyunsaturated fatty acids of low-density-lipoproteins (LDL) and leads to the formation of MDA[5]. The latter is a good marker of lipid peroxidation in this case [5]. LDL are the blood component most sensitive to oxidative stress and involved in the development of atherosclerosis [19]. DNA oxidation leads to 8-hydroxy-2'-deoxyguanosine (8-OHdG) fragments formation. DNA repair enzymes eliminate these fragments, but if these systems fail or are outdated, 8-OHdG will accumulate within the DNA and cause mutations. Therefore, its increased urinary levels reflect oxidative stress in the DNA [20]. C-reactive protein (CRP) has been recognized as an excellent biological marker of systemic inflammation, but it is also a predictor of cardiovascular accident risk [21] as shown in **Figure 2**.

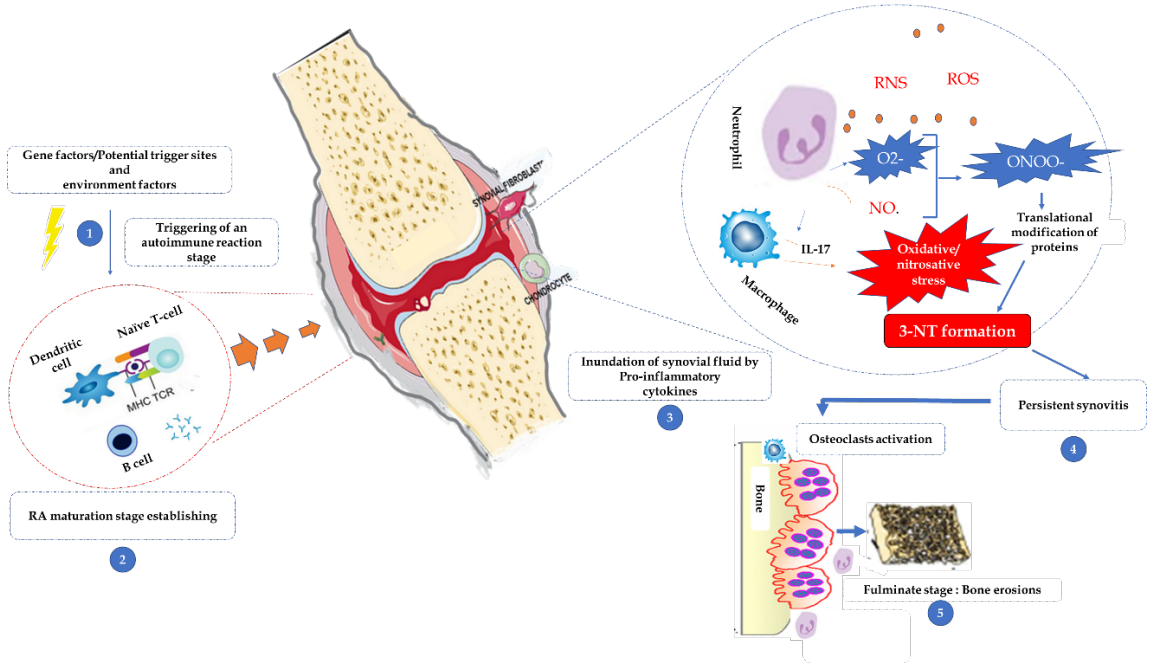

Figure 1: Summarized schematic for the general pathways of RA pathogenesis and its relation with 3-NT.

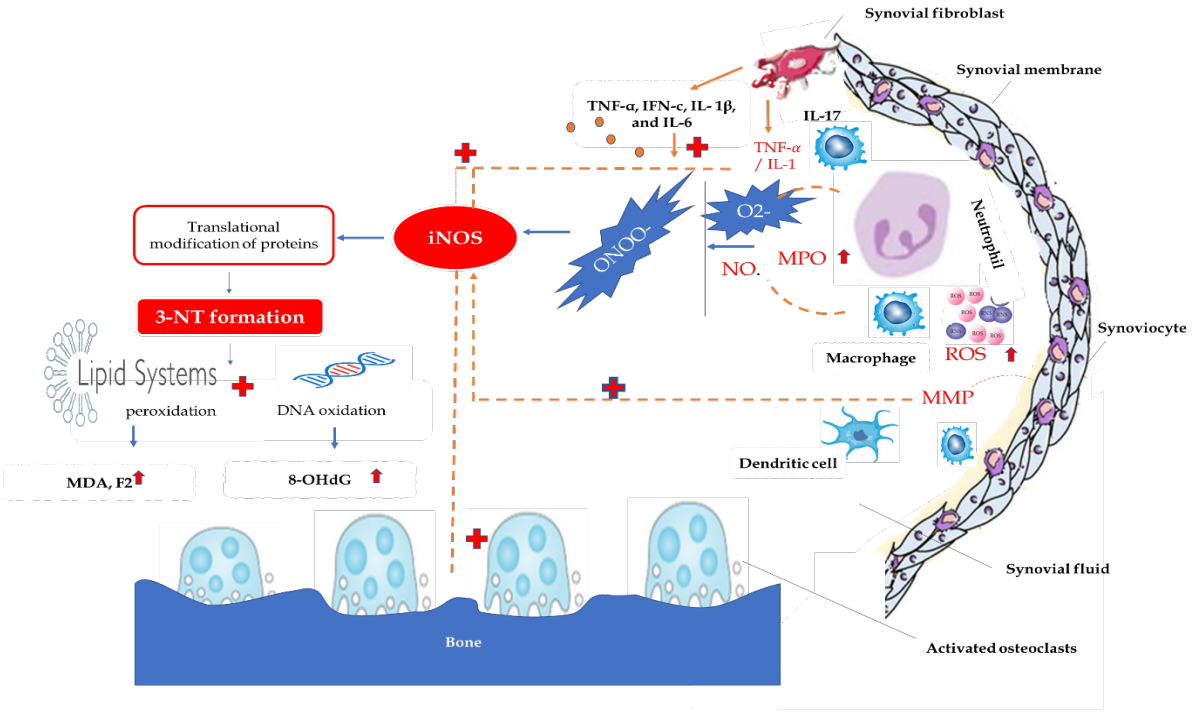

Figure 2: Summarized schematic for biomarkers and oxidative damage in joints and blood of RA patients.

Qualification and reliability of 3-NT as a biomarker of oxidative/nitrosative stress in RA

This brief literature-analysis will first assess the associations between serum, urine, and synovial fluid levels of 3-NT and RA evolution, then reveal the interest of 3-NT in those studies: Disease's prognostic, pathology evolution determination and/or therapy evaluation, and finally, evaluate and state the sensitivity and reliability of the used quantifying methods. This analysis was based on scrutinizing published scientific literature retrieved from all credible sources including PubMed, Elsevier, Research Gate, Web of Science, Google Scholar, Science Direct, Europe PMC, and Wiley Online library using keywords such as "3-Nitrotyrosine", "Biomarker", "Rheumatoid arthritis", "Oxidative/nitrosative stress", "Quantification" , "Mass spectrometry" or their combinations in the last three decades. More than 600 publications were retrieved after a cursory search of relevant scientific literature using the aforementioned keywords. Inclusion/exclusion criteria were set to narrow the scope and fulfill our review's objectives. To be included, articles needed to describe our primary factors, which are 3-NT, RA, and quantification methods. The experimental model used in the included studies were Clinical (11 studies) and pre-Clinical studies (8 studies). Some of these studies reported other biomarkers: five studies [22]- [23] associated MDA with 3-NT, Studies [22], [24] , [25], [26] adopted iNOS. Wadly *et al.*[27] and Ikonomidis *et al.*[28] utilized CRP. Misko *et al.*[13] reported nitrate and Nitrite, matrix metalloproteinase (MMP)-3, MMP-1, MMP-9, and more than 40 chemokines and cytokines. One study from China [29] used Monocyte chemo-attractant protein-1 as RA biomarker. Wadly *et al.* [27] to assess the impact of exercising on oxidative stress markers in RA, relied on protein carbonyls (PC) and lipid hydroperoxides. Although all these reported works show that these

biomarkers expressed a good response to disease activity, no one has comprehensively compared these biomarker' selectivity to the disease activity. **Table 1** summarized all these included studies. Different purposes and objectives were behind adopting the use of this molecule ; papers [13], [24], and [30] were exclusively and purely tracking the reliability and the relation of 3-NT with disease activity. Misko *et al*. [13], to qualify the clinical's rely on 3-NT as a biomarker in arthritis without and after treatment; compared the mean of this substance, and as aforementioned the other biomarkers MMP-3, MMP-1, MMP-9, and other chemokines and cytokines. They tested the response and the change in plasma NT concentration of anti-tumor necrosis factor (TNF) treatment. The decrease in NT synthesis after therapy of 6 months (not before this period) reinforced the concept of considering 3-NT a promising biomarker of nitrogen-free radical species [13]. Khan *et al*. [30], to investigate the prevalence of anti-3-NT antibodies in the joint synovial fluid of patients with RA, came up with proving the existence of these antibodies as a response of nitrated proteins. The level of 3-NT is correlated with disease severity, an enhanced level of nitration detected in patients with a higher level of RA disease activity. The results of this study came to prove that the measurement of protein nitration parameters may be an alternative marker of disease activity[30]. Significantly, nitrotyrosine is recognized as a biochemical marker of increased expression of iNOS at sites of inflammation[30]. Nemirovsky *et al*. [24], besides the interest of developing and

Table 1: Summary of some studies used 3-NT as biomarker of RA

Author Year	Country	Total Participants	Experimental model	Other RA Biomarkers	Potential and interest of use
Uesugi *et al.* [32] 2022	USA	Rabbits	Non-Clinical	NS	Evaluating the Inflammatory properties of IgG
Impellizzeri *et al*. [22] 2020	Italy	Mice (CIA model)	Clinical	iNOS poly-ADP-ribose MDA	Evaluating the effect of a new hyaluronic acid -carnosine conjugate on the modulation of the inflammatory response
Huang *et al*. [29] 2020	China	55 RA patients	Clinical	Monocyte chemo-attractant protein-1	Evaluation of the potential relation between monocyte chemoattractant protein-1 (MCP-1) and NT with the decrease/increase of inflammation and body mass during etanercept therapy
Steinz *et al.* [23] 2019	Sweden	30 RA Patients	Clinical	MDA	Proving that oxidation products promote skeletal muscle weakness
Moon *et al.* [36] 2017	Japan	Mice (CIA model)	Non-Clinical	IL-1β TNF-α MDA	Evaluating the effect of Leflunomide and its potentiality to induce heme oxygenase-1

Ahmed *et al.* [31] 2016	UK	225 RA Patients	Clinical	Oxidized, nitrated and glycated amino acids	Evaluating the early stage protein oxidation, nitration and glycation biomarkers in case of RA
Wadly *et al*. [27] 2014	UK	12 RA patients	Clinical	Protein carbonyls (PC) Lipid hydro peroxides Interleukin-8 CRP	Effect of exercise on markers of oxidative stress in RA, following acute exercise and a period of exercise training.
Moon *et al.* [35] 2014	Japan	Mice (CIA model)	Non-Clinical	Heme oxygenase 1 (HO-1)	Proving that Rebamipide Suppresses Collagen-Induced Arthritis Through Reciprocal Regulation of Th17/Treg Cell Differentiation and Heme Oxygenase 1 Induction
Ikonomidis *et al.* [28] 2014	Greece	80 RA Patients	Clinical	Interleukin-1 C-reactive protein MDA protein carbonyls	Investigated the effects of anakinra, an interleukin-1 receptor antagonist, on coronary and left ventricular function in coronary artery disease (CAD) patients with RA
Impellizzeri *et al*.[38] 2013	Italy	Mice (CIA model)	Non-Clinical	TNF-α, (IL)-6 and IL-1β MIP-1α and MIP-2 MDA	Assessing Palmitoylethanolamide and luteolin on CIA rats
Yamada *et al.* [25] 2013	Sweden	Mice (CIA model)	Non-Clinical	iNOS	Tracking Nitrosative modifications of Ca2+ release complex and actin underlie arthritis-induced muscle weakness
Misko *et al*. [13] 2012	USA	18 RA patients	Clinical	Nitrate and Nitrite MMP)-3, MMP-1, MMP-9 Chemokines cytokines.	Characterization of nitrotyrosine as a biomarker for arthritis and joint injury
Pham *et al.* [34] 2009	Germany	110 RA Patients	Clinical	Urinary nitrite	Proving that Nitrite correlates with 3-NT but not with the F2-isoprostane 15(S)-8-iso-PGF2a in urine of rheumatic patients

Nemirovskiy *et al.*[24] 2009	USA	Rats (CIA amd AIA model)	Clinical	iNOS	Reliability of 3-NT
Haruna *et al.* [37] 2007	Japan	Rats (AIA model)	Non-Clinical	4-hydroxy-2-nonenal (HNE)	Assessing the potentiality of Fluvastatin to reverses endothelial dysfunction and increased vascular oxidative stress in AIA
Griffiths *et al*. [39] 2006	UK	RA and OA patients	Clinical	Homocysteine - induced posttranslational modifications	Proving thar Hcy from endothelial cells promotes LDL nitration and scavenger receptor uptake
Khan *et al*. [30] 2006	INDIA	30 RA patients	Clinical	NR	Assessing the prevalence of anti-3-NT antibodies in in the joint synovial fluid of patients with RA
Cuzzocrea *et al.* [33] 2006	Italy	Mice (CIA model)	Non-Clinical	Poly (ADP-ribose) (PAR) iNOS Cyclooxygenase-2 (COX-2)	Investigating the effects of Glycogen synthase kinase-3ß inhibition on the degree of arthritis
Yonekura *et al*. [26] 2003	Japan	Rats	Non-Clinical	iNOS	Revealing the relation between the expression of iNOS by chondrocytes and its nitric oxide-generating activity.

improving a new sensitive and specific analysis method based on immuno-affinity 2-D LC-MS/MS, also assessed the role of iNOS in the disease pathology, as well as proof the pharmacology of iNOS inhibitors in an acute endotoxin challenge model, in models of RA such as rat adjuvant- and collagen-induced arthritis (AIA and CIA respectively). They have proved that plasma 3-NT is dependent on the severity of the inflammatory response; thus, a 20-fold increase was observed in the rat LPS model versus a 2.5-fold elevation in CIA[24]. Studies [12, 23,25,26,29,31–34] utilized this biomarker to better understand and enrich our findings about pathogenesis' mechanisms of RA. Shiojiri *et al.* [12] quantified nitrotyrosine in adjuvant-induced RA (AIA mice model) and investigated how the Peroxisome proliferator-activated receptor gamma (PPARγ) ligands could inhibit its formation. The overexpression of inflammatory mediators such as iNOS; suggested that PPARγ ligands reduce the inflammation of arthritis by suppressing the activated NF κB to regulate the transcription of various inflammatory mediators. All these works' objectives were summarized in **Table 1** as well. As for studies [22, 28,29, 35–39], monitoring and evaluating Hyaluronic acid -carnosine, Etanercept, Rebamipide, Anakinra, Leflunomide, Fluvastatin, Palmitoylethanolamide, and Homocysteine (respectively) effects and impacts on RA pathology, were the primary goal of these investigations. Widely *et al*.[27] also evaluated

the impact of three months of moderate-intensity exercise on the status of the generated oxidative stress of 12 patients suffering from RA by quantifying 3-NT, and some other plasma markers like protein carbonyls (PC), lipid hydroperoxides (LOOH), total antioxidant capacity (TAC) and catalase (CAT) activity, beside of some other inflammation markers. A decrease in disease activity was clinically observed. Biomarkers-wise, 3-NT concentration decreased alongside increases in aerobic fitness, unlike the other biomarkers that did not express any correlation with patients' activity, leading to considering 3-NT as a specific and reliable biomarker of RA activity compared to other biomarkers [27]. The employed analytical methods in these studies were variable; [28] and [30] have used ELISA, immunohistochemistry of joint tissues by [22, 26, 33, 35, 38], Western immunoblot by [23,25 ,37] and, HPLC with electrochemical detection by [27], Immunoaffinity two-dimensional (2D) LC-MS/MS by [13] and [24] . As shown in **Table 2,** most of these reported studies fail to fully validate these techniques before determining 3-NT in different biological fluids. This concludes that 3-NT is still in its early phases for comprehensive validation and clinical application. That is why a thorough exploration and evaluation of the current 3-NT quantification methods is necessary to expand 3-NT use in RA clinical practice.

3-NT quantification methods

Typically, quantification of NT imposes measuring either free NT or digested proteins and then applying various detection methods. It has been reported that the first and starting analysis methods applied were antibody-based techniques; immunohistochemistry or Western blot [40], drawbacks, and pitfalls like lower concentrations of this biomolecule (nM-range) in most biological fluids, difficulties to handle the samples which may lead to artifactual nitration of tyrosine by Nitrite and nitrate, besides of the unpredictable

Table 2: Summary of some methods used to detect 3-NT in case of RA

First Author	Biological Specimen	3-NT Detection Method	LOD	Detection range	Analysis Method specifications
Misko *et al.* [13]	Synovial fluid, plasma, and urine	Immunoaffinity two-dimensional (2D) LC MS/MS	50 pg	0-5000 pg /mL	Immuno-affinity two-dimensional (2D) liquid chromatography tandem mass spectrometry (LC-MS/MS) using an HP 1100 HPLC system.
Huang *et al.* [29]	Serum	Enzyme immuno-assays	NR	0-0.4 µg /mL	NR
Khan *et al.* [30]	Synovial Fluid and Serum	Direct ELISA	NR	NR	Poly L-tyrosine was exposed to nitrating species resulting in the formation of 3-NT, recognition of 3-NT by direct binding ELISA
Wadly *et al.* [27]	Plasma	HPLC with electrochemical detection	20 µM	0–100 µM	Vanadium (III) chloride (8 mg /mL) was added to fully reduce plasma nitrate

					to Nitrite. Sulphanilamide (50 µL, 2 %) and N-(1-naphthyl) ethylenediamine dihydrochloride (50 µL, 0.1 %) were then added as well.
Impellizzeri *et al*. [22]	Plasma	Immunohistochemistry	NR	NR	Anti-nitrotyrosine rabbit polyclonal antibodies (1:1000 in PBS, v/v) were used.
Ahmed *et al.* [31]	Plasma/serum and synovial fluid	LC-MS/MS	NR	NR	Ultrafiltrated, delipidated and hydrolysed enzymatically respectively.
Moon *et al.* [35]	Joint tissues	Immunohistochemistry	NR	NR	First incubated with primary antibodies against NT, then tissues were incubated for 1 hour with a biotinylated secondary antibody and a streptavidin–peroxidase complex. final colored product was developed using diaminobenzidine chromogen.
Ikonomidis *et al.* [28]	Joint tissues	ELISA	NR	2–430 nmol/L	NR
Moon *et al*. [36]	Seum	NR	NR	NR	NR
Haruna *et al*. [37]	Aortas tissue	Western immunoblot	NR	NR	NR
Impellizzeri *et al.* [38]	Joint tissues	Immunohistochemistry	NR	NR	Incubating with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.
Steinz *et al.* [23]	Muscles	Western immunoblot and Mass Spectrometry (MS)	NR	NR	NR
Uesugi *et al.* [32]	Serum/Synovial fluids	Radioimmunoassay	NR	NR	Monoclonal anti-nitrotyrosine antibodies were used
Yamada *et al.* [25]	Muscles	Western immunoblot	NR	NR	Protein content determined by using

					the Bradford assay. Total muscle homogenate was separated by electrophoresis and transferred onto membranes. Membranes were incubated with primary antibody. Immunoreactive bands were visualised using the Odyssey Infrared Imaging System.
Cuzzocrea *et al.* [33]	Joint tissues	Immunohistochemistry	NR	NR	Anti-nitrotyrosine rabbit polyclonal antibody (1:1000 in PBS, v/v) were used; Specific labeling was detected with a biotin conjugated goat anti-rabbit IgG.
Nemirovskiy *et al.* [24]	Plasma	2-D LC–MS/MS	2.5 pg /mL	5 to 200 pg /mL	Pronase first (5 mg /mL) and heating for 10 min at 100°C were applied to destroy endogenous enzymatic activity and denature proteins for more complete digestion with pronase.
Yonekura *et al.* [26]	Serum	Immunohistochemistry	NR	NR	Rabbit anti-nitrotyrosine polyclonal antibody, then incubated for 60min with peroxidase-labeled anti-rabbit IgG antibody (diluted 1 to 250in 10% serum) and then for 30 min in peroxidase substrate (0.03% H2O2 and 0.2 mg /mL 3-amino-9-ethyl carbazole)

NR: Not reported

reactions of generating an artifactual 3-NT after increasing temperature or while changing pH[41]. All these factors limited the utility of these techniques, affected the credibility, and changed their status of validity, but at the same time pushed ahead scientists to improve and seek out for more confidential and reliable analytical methods responding to previously mentioned criteria. A new tendency of using spectrum analytical

methodologies has been developed. Among these techniques: electrochemical detection (ECD) [42],enzyme-linked immunosorbent assay (ELISA)[43], high-performance liquid chromatography (HPLC) coupled with ultraviolet detection (UV) [44], and mass spectrometry (MS) based techniques [45]. **Figure 3** Summarizes the analytical approaches for the quantification of 3-NT in biological matrices.

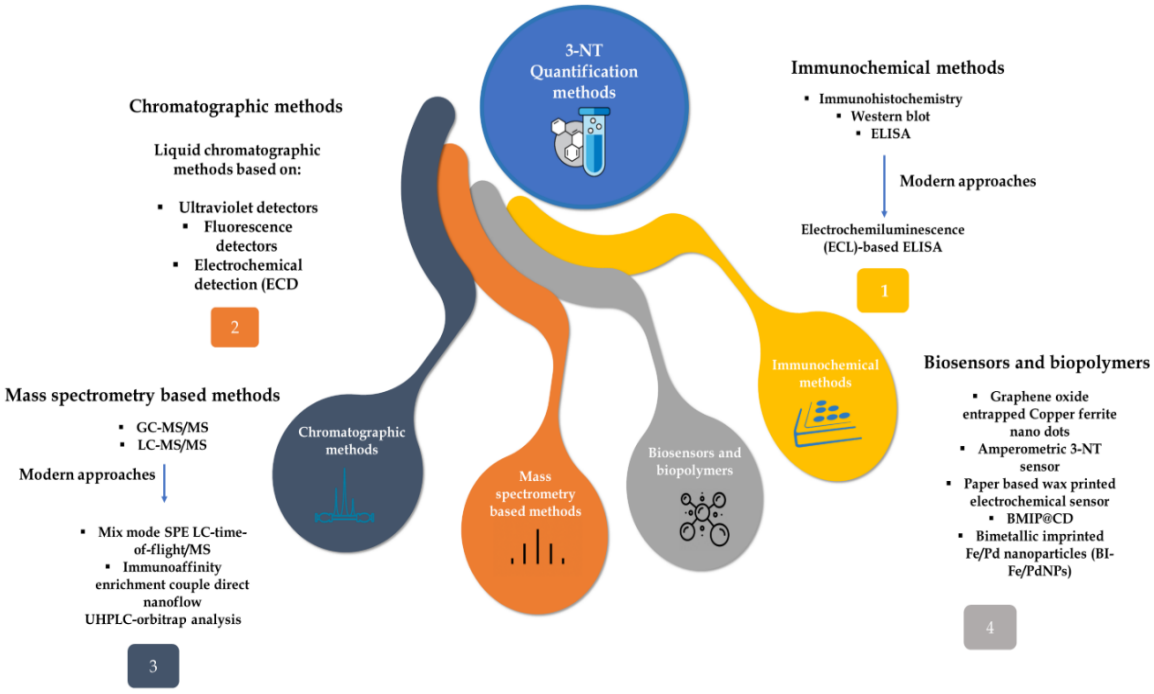

Figure 3: Analytical approaches for the quantification of 3-NT in biological matrices.

4.1. Mass spectrometry-based techniques

4.1.1. Liquid chromatography-mass spectrometry methods

The robustness and unambiguous identification of the mass spectrometry (MS) technique made the concept of combining it with the flexibility of liquid chromatography (LC) favorable to accurately quantify 3-NT in different samples and simultaneously covering up for some LC limitations[8]. Unlike gas chromatography (GC) based techniques, this method does not require a pre-derivatization which is an advantage in terms of circumventing the concern of occurrence of some pitfalls like the likelihood of artifacts formation and sample loss[46]. The scrutiny of scientific articles edited in this regard leads to state some factors and parameters along the way or set before this technique, for the sake of amending the selectivity and specificity of the analysis: linking triple-stage quadrupole instruments[47] and/or Hypercarb HPLC columns[48]; ESI before detection[49]; SPE[45], cut-off filter[50] and ion-trap instruments for a better target-analytes extraction[51].

The first reported study that used this method was by Althaus *et al.*[47] on an amino acid pool, generated from rat microvessels, aiming to improve the sensitivity and selectivity of their developed method based on LC-MS/MS, adopted with triple-stage quadrupole instruments, ESI, and SPE for pre-concentration phase. Noticeably their approach displayed an improved value of LOQ in the biological samples with 10 fmol

[47]. The same method applied to quantify free 3-NT in human plasma came up with a LOQ estimated 20 fmol [41]. Ion-trap instruments for sample extraction were also tested by Delatour *et al.*[51] on human plasma; displaying as well an ameliorated value of LOQ (3.2 fmol)[51]. Coming up with a new concept of antibodies immunoaffinity toward 3-NT, Radabaugh *et al.*[21] used an in-line antibody **(Figure 4)** column prior to MS/MS to increase the specificity of their method, attempting to measure free amino acid and protein 3-nitrotyrosine (digested after by utilizing pronase) in different biological fluids, including synovial fluid (SF). The estimated LOD and LOQ reported were 5 pg /mL (22.1 pM)[21]. extraction phase, usually an SPE base, either a simple[47] or mixed-mode[45], has constantly been proving a positive influence by improving the performance of the coming steps of the analysis[45,47]. A recently developed method (Gamon *et al.*[52]), with an outstanding outcome, coupled LC-MS with zwitterionic ion-exchange chromatography to quantify parent amino acids and their respective oxidation products after using methanesulfonic acid in the presence of tryptamine for proteins hydrolyze. The reported LOD and LOQ values of 3-NT were 0.423 and 1.41 pmol, respectively. Hydrolysis/derivatization free and non-invasive detection of urine 3-NT. Li, X *et al.*[53] developed an Ultra-Fast Liquid Chromatography (UFLC)-MS/MS based method, preceded by a step of extraction, represented by a Tailored Oasis MCX 96-well plate and MCX 1 Elution for SPE, where ammonium acetate (pH 9, 25 mM) has been selected as an elution buffer. The group came with a LLOQ estimated of 10 pg /mL and a total run time of 7 min, which is sharply distinguishable over previously reported methods[53]. **Table 3** summarizes some liquid chromatography-based fully validated methods used for the determination of 3-NT in biological samples.

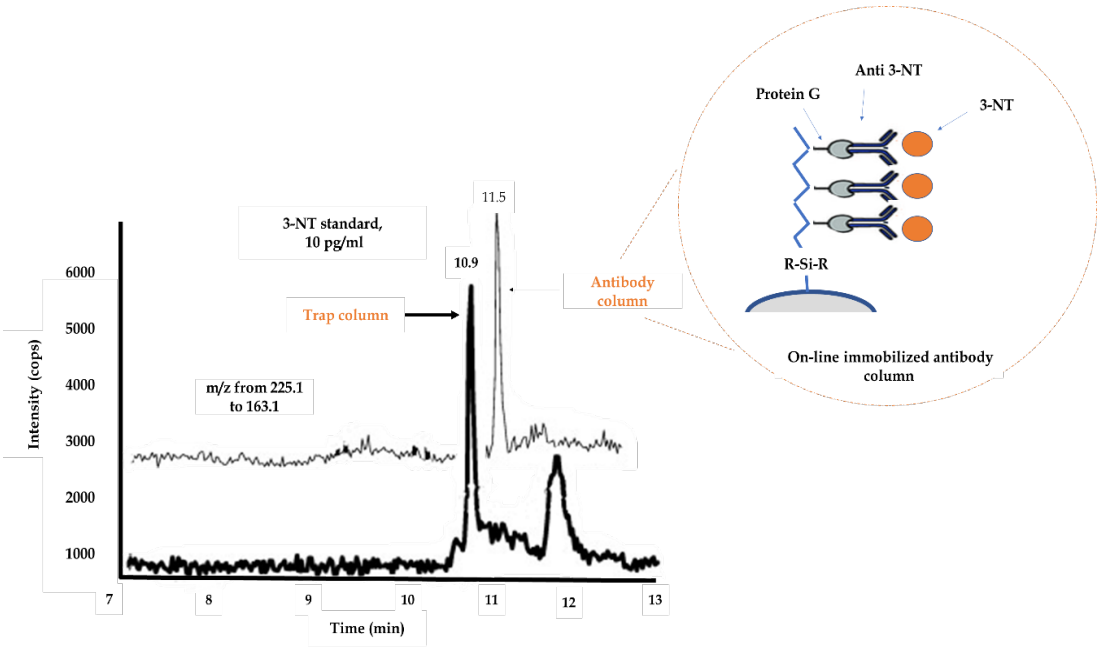

Figure 4: 3-NT detection using immunoaffinity LC-MS/MS.

Ion chromatograms for the multiple reaction monitoring (MRM) 225.1/163.1 for 3-NT with and without immunoaffinity column: in-line C18 trapping alone and with an in-line anti-nitrotyrosine antibody column coupled with a C18 trapping column as described [8].

Table 3: Summary of some reported and fully validated liquid chromatography-based methods for the determination of 3-NT in biological samples

Sample	Analytes	Proteins digestion way	Method specificity	Column	Eluents	Flow rate	Detection method	LOD LOQ	Ref
Rats microvessels	3-NT, Tyrosine	Enzymatic	Adopted with triple-stage quadrupole instruments and ESI Preceded by a SPE	Waters J'Sphere ODS-H80 (50 × 2 mm; 80 A˚) and a Phenomenex Luna C18 column (50 ×2 mm; 5 µm)	Mobile phase A: 99.9% water and 0.1% acetic acid Mobile phase B: 99.9% acetonitrile and 0.1% acetic acid	NS	LC-MS/MS	10 fmol NS	[47]
Human plasma	3-NT,3-Chlorotyrosine, 3-Bromotyrosine	NS	Adopted with ion trapping instrument Preceded by a SPE	Zorbax (5-mm resin, 1× 150 mm)	Mobile phase A: methanol/water/acetic acid (4/95/1, v/v/v, pH 3) Mobile phase B: methanol/water/acetic acid (95/4/1, v/v/v, pH 3.2)	1 mL/m	LC–MS/MS	3.2 fmol N.S.	[51]
Biological fluids	Free amino acid, protein 3-NT	Enzymatic (pronase)	An in-line antibody column	Immunoaffinity column: Targa (4.6 × 30 mm) C18 reversed-phase column: Betasil (2.1 ×100 mm; 5 µm)	Immunoaffinity column: 1% formic acid C18 reversed-phase column: 100% 10 mM ammonium acetate acetonitrile	Immunoaffinity column: 1 mL/min C18 reversed-phase column: 0.3 mL/min	Immunoaffinity LC/MS/MS	5 pg /mL 5 pg /mL (Lower LOQ)	[21]
Human plasma	Free 3-NT	NS	Mixed-mode of SPE before the analysis	Restek PFPP column (150mm×2.1mm,3 µm)	Mobile phase A: water with 0.1% formic acid Mobile phase B: 100% methanol	0.45 ml/min	LC–MS/MS	2 pg /mL 5pg /mL	[45]
Cell lysates and complex protein mixtures treated with hypochlorous acid	3-NT	Hydrolyzed with methanesulfonic acid in the presence of tryptamine and purified by strong cation exchange solid phase extraction	Prior derivatization Coupled with zwitterionic ion-exchange chromatography	Imtakt Intrada Amino Acid 100 × 3.0 mm	Mobile phase A: ACN/formic acid (100/0.3) Mobile phase B: ACN/100 mM ammonium formate (20/80)	0.8 ml/min	LC-MS	0.423 pmol 1.41 pmol	[52]
Plasma and urine	3-NT	Enzymatic	Two 5-µmHypercarbcolumns were used	Hypercarb™column	0.1% TFA with a linear gradient of 10-50%	0.2 mL/min	LC-MS/MS	0.022 pmol	[48]

	2.1 ×50 mm; 5 µm	acetonitrile	N.S.

NS: Not Stated

4.1.2. Gas chromatography-mass spectrometry methods

Analysis of 3-NT requires prior chemical derivatization and/or modification of some functional groups for the sake of increasing volatility and thermal stability[8]. Some other factors might play an enhancive role for clear analytes separation, like using proper conditions for ionization, suitable fluorine-containing derivatization agents, besides signal-to-noise ratio (S/N)[50]. Over the past three decades, the derivatization step usually called for using molecules like N heptafluorobutyryl derivative referred to as the schwedhelm derivative[54], N heptafluorobutyryl-O, O-di(t-butyldimethylsilyl) (TBDMS) derivative referred to as the Frost derivative[55], and di-O-methyl-di N-heptafluorobutyryl derivative referred to as the Soderling derivative[56]. No comparative study has been reported so far about the quality of the expected results of these three methods.

Concerning artifacts formation, many strategies have been highly developed aiming to avoid the occurrence of this problem, such as: reducing 3-NT to 3-amimotyrosine before derivatization[56]; capturing of nitrosated species with high concentrations of reactive aromatic compounds[56]; circumventing acidic conditions during derivatization using novel alkaline methods for the hydrolysis instead [55], incorporating of stable-isotope labeled of tyrosine to quantify artifactual 3-NT formed, and utilizing SPE (as mentioned above) or HPLC for 3-NT pre-isolation[56]. As an instance, Söderling *et al*.[56] developed an artifact-free derivatization method for quantifying the endogenous 3-NT content of biological samples by GC/negative chemical ionization MS/MS, based on reducing the nitro group of the molecule by dithionite, heptafluorobutyric acylation, and subsequent methyl derivatization; these processes of pre-treatment showed good selectivity, eliminating the problem of artifactual nitration besides of separating between free and protein-associated 3-NT[56].Another study performed by Tsikas *et al*.[57] in order to quantify and compare the basal plasma levels of 3-NT and 3-nitrotyrosinoalbumin relying on GC-MS and GC–tandem MS, a new method with interference-free and accurate quantitative determination in human plasma. Samples of albumin (ALB) and NT-ALB were proteolyzed directly by adding pronase in neutral pH and then ultrafiltered. Afterward, isolation of each compound by SPE (HR- cartridges), derivatization into their n-propyl ester-pentafluoropropionyl amide-tri-methylsilyl ether derivatives were all placed before quantifying the obtained proteins from proteolysis; tyrosine, and phenylalanine by HPLC system ultraviolet detection. Self-digested pronase adopted in this study contributed largely to minimize protein interference[57]. Employing the same technique, the group of Tsikas *et al*.[58] on human urine after oral intake of 3-nitro-l-tyrosine by healthy volunteers, realized the necessity of applying the new concept of sample purification; based on separating urinary 3-NT from Nitrite, nitrate, and l-tyrosine to avoid artifactual formation of 3-NT, by HPLC before launching the step of derivatization[58]. Hanff *et al*.[59] developed a simultaneous quantification method for MPO pathway 3-NT precursor (Nitrite) and MDA in the urine based on validated gas chromatography-electron capture negative-ion chemical ionization-mass spectrometry

(GC-ECNICI-MS). Pentafluorobenzyl bromide in acetone was used for derivatization (urine-acetone, 100:400 µL, 50 °C for 60 min) and ethyl acetate; dried over anhydrous sodium sulfate for extraction. The outcomes confirmed the assumption of a close relationship between MDA and Nitrite in urine[59]. **Table 4** summarizes some gas chromatography-based fully validated methods used for the determination of 3-NT in biological samples and **Figure 5** Summarizes and categorizes the different 3-NT chromatographic and mass spectrometry-based quantification methods depending on their selectivity.

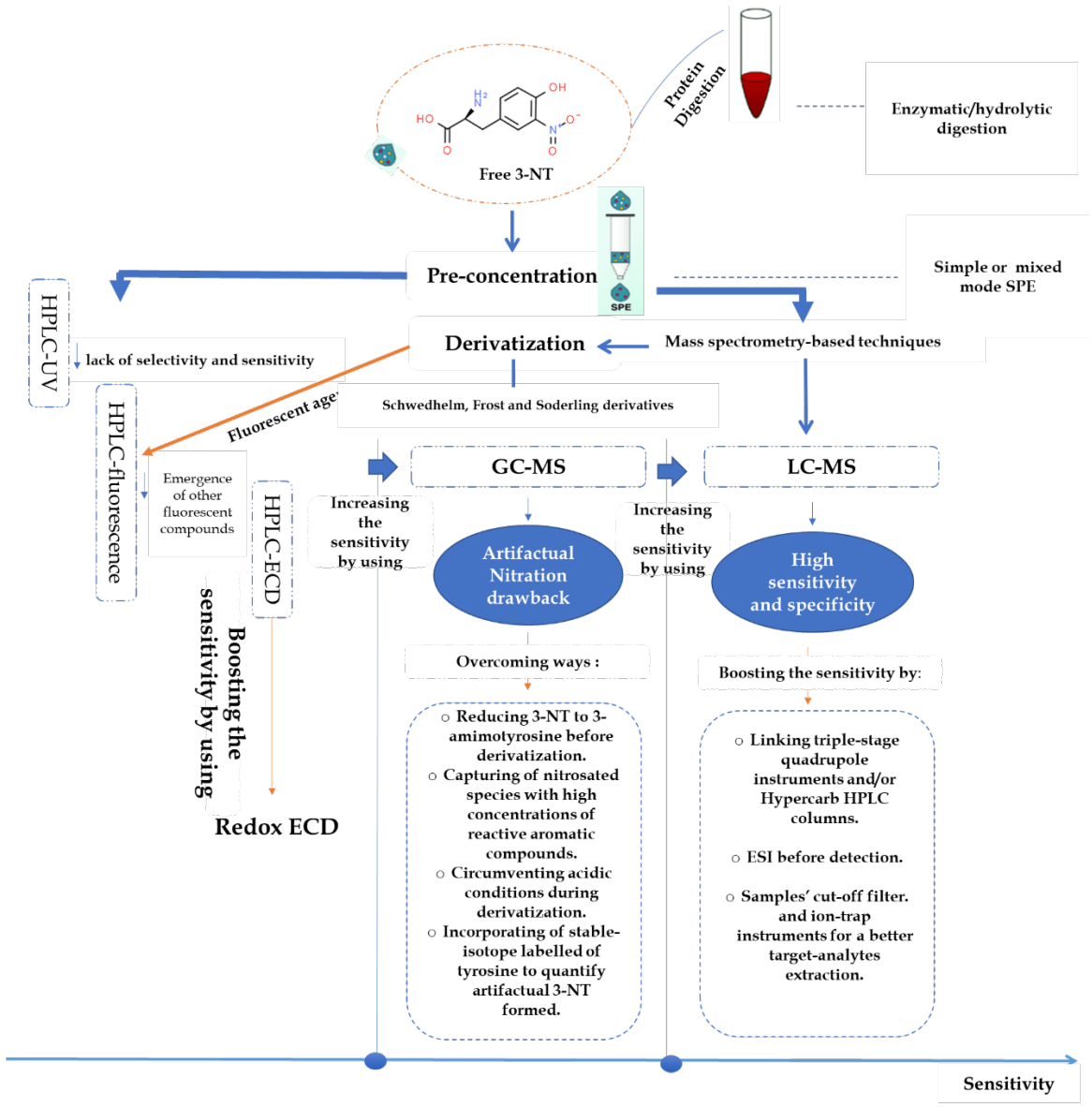

Figure 5: Summarized schematic for the different 3-NT chromatographic and mass spectrometry-based quantification methods depending on their sensitivity.

Table 4: Summary of some reported and fully validated gas chromatography-based methods for the determination of 3-NT in biological samples

Sample	Analytes	Derivatization method	Column	Internal standard	Eluents	Detection method	LOD LOQ	Ref
Human plasma	3-NT	Schwedhelm derivative	Optima (30× 0.25 mm; 0.25 µm)	3-nitro-L-[$^{2}H_3$] tyrosine	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa)	GC-MS/MS	4 amol 125 PM	54
Human plasma	3-NT	Frost derivative	capillary column (0.25 mm; 0.2 µm)	[$^{13}C_9$] nitrotyrosine	ammonia	GC–MS	NS	55
Human plasma	3-NT and 3-Nitrotyrosinoalbumin	Schwedhelm derivative	Optima 5-MS (30 × 0.25 mm; 0.25 µm)	d $_3$-Nitrotyrosin	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa)	GC–MS and GC-MS/MS	NS	57
Human plasma	3-NT	Soderling derivative	DB5-MS column (30 × 0.25 mm; 0.25 µm)	3-nitro[$^{13}C_6$] tyrosine	Helium (41 kPa); Methane (0.27e1.1 kPa)	GC-MS/MS	0.03 nM 0.3 nM	56
Human urine	3-NT	Schwedhelm derivative	Optima 5-MS (30 × 0.25 mm; 0.25 µm)	d 3-Nitrotyrosin	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa collision pressure)	GC-MS/MS	4 amol 125 pM	58

1.2. Biosensors and biopolymers

Electrochemical sensors and biosensors have experienced considerable development in recent decades due to their simplicity, reliability, speed, and selectivity[60]. They were the most attractive alternatives for conventional analytical methods in clinical biology[60]. A biosensor is an integrated device providing specific quantitative or semi-quantitative information through a biological origin recognition element in direct contact with a transduction element[61]. The biosensor concept includes any measuring device defined by a couple of selective biological ligands bound to a transducer that transforms a biochemical phenomenon into a measurable signal[60]. Group of Li *et al.*[62] to quantify PTM molecules more precisely (avoiding antibodies stereo hindrances), in other way but employing immunochemical based assays. They drew up an opinion on the grounds of PTM proteins' stoichiometry number (SN) of nitration on the one hand, and producing binding small-chemoselective label (aniline) linked with site-specific targeting agents in the modified protein, on the other[62].

Molecular imprinting polymers (MIPs) are networks of cross-linked polymers where cavities specific to a particular type of molecule are created. The affinity between polymers and their target molecules is similar to the observed

affinity between an antibody and its antigen[63]. Once the structure and Physico-chemical parameters of the target molecule have been determined, it is judicious to choose the adequate functional monomer. This choice is essential to determine in part the quality of the sites of recognition of MIPs[63].

3-NT quantification method first reported study based on this type of technology was about MIP, green-light emitting carbon dots (BMIP@CD) on sol-gel-sol imprinting. The concept of this technique is about recognizing the formed chemical bound of 3-NT in human serum samples with the added fluorescent agents. A prepared template of dopamine-containing 3-NT in a weak alkaline solution is required before extraction. The rest of the imprinted binding sites selectively recognize the target molecules, resulting in quenched fluorescence of BMIP@CDs measured using a fluorescence spectrophotometer (520 nm with an excitation wavelength of 425 nm). This technique displayed a 17 nM of LOD and 0.050–1.85 μM of linear concentration range[64]. Based on the same concept and accompanied by a sensing lab-on-a-chip technology for 3-NT detection, Martins *et al*. [65] manipulated a treated Wax-printed paper-based, where conductive bio-sensing inks spots were revealed by using silver and carbon, performing 49.2 nM of LOD [65]. **Table 5.** Summarizes some reported biosensors and biopolymers methods for the determination of 3-NT in biological samples.

Table 5: Summary of some reported biosensors and biopolymers methods for the determination of 3-NT in biological samples

Analyte	Sample	Biosensor / biopolymer	Concept of transduction and detection	Method sensibility	Ref.
3-NT	Urine and serum samples	Cadmium tungstate nano dots	Amperometric method: conversion of analyte-electrode binding into a measurable signal	Linearity range: 18.5–1.84 mM LOD of 3.24 nM.	[69]
3-NT	Human serum samples	Green-light emitting carbon dots (BMIP@CD)	Quenched fluorescence of the imprinted binding sites of BMIP@CDs measured by fluorescence spectrophotometer	Linearity range: 0.050–1.85 μM LOD of 17 nM	[64]
PTM	HEK-293 cells with different concentrations of peroxynitrite	Small-chemoselective labels (aniline)	Electrochemical detection and measurements (square Wave Voltammetry: SWV) of PTM proteins and their stoichiometry number (SN) of nitration measured by	NS	[62]
3-NT	Blood samples	Paper based wax printed electrochemical sensor (silver and carbon based as conductive inks)	Electrochemical detection: Lab-on-a-chip based technology, silver and carbon-based conductive inks to create a paper-based-screen printed bio-sensing electrode like a small ink spot	LOD of 49.2 nM	[65]
3-NT	Human serum and urine samples	Copper ferrite nano dots in porous reduced graphene oxide (RGO) nanosheets	Electrochemical detection: electro catalytic activity of the copper-graphene oxide composite was modified towards sensing 3-NT	LOD of 25.14pM	[70]
3-NT	Human serum and urine samples	MIP doped with gold nanoparticles (MIP-AuNP)	Electrochemical detection: produced electrical signals by charge transfer from AuNP enhance the cyclic	LOD of 50.0 nM	[71]

			voltammetry (CV) responses		
3-NT	Human serum and phosphate buffer (PBS)	Nickel-doped graphene localized surface plasmon resonance (NDG-LSPR)	Emission Spectrum	Linearity range: 0.5 pg /mL –1 ng /mL LOD of 0.13 pg /mL in PBS	[72]
3-NT	Human blood serum, plasma, and urine samples	Bimetallic imprinted Fe/Pd nanoparticles (BI-Fe/Pd NPs) imprinted using acrylamide monomer and N-N'-methylene bisacrylamide	Electrochemical detection: square wave and differential pulse voltammetry SWV	Linearity range: 4.90–867.57 µg/L LOD of 1.20 µg	[73]

Validation of 3-NT quantification methods: Behind the scenes

3-NT analyzing defects may be briefly summarized into choosing the reliable analytical way to reach the required sensitivity and accuracy, circumventing the artificial formation of 3- NT during the step of sampling, extraction, and derivatization; and finally, establishing a concentration range in the selected biological matrix basing on a reliable validating process[66]. Scientists in their publications have reported concentrations for 3-NT and 3-NT-Proteins in different biological samples with several orders of magnitude (pM-range to µM-range), indicating indirectly the occurrence of analytical pitfalls and shortcomings rather than evidence of significant biological variation[67].

So far, Groupe of Li *et al.*[68] thorough validated LC-MS/MS method reported the lowest 3-NT concentrations in plasma of 54 apparently healthy human subjects, with a reported lower limit of quantification of 5 ng/L and concentration range of 8.8–177 pM (mean 49 pM) in plasma. One issue could be pointed out in this method is the use of an instable-isotope labeled analogs at concentrations not close enough to the expected concentrations of the endogenous compounds in biological samples. The step-by-step validation process is very recommended in 3-NT measurement due to the reasons mentioned earlier, taking into consideration the quality of the column used (routine washing) and the appropriate maintenance of instruments[50].

Conclusion and perspectives

The coalesced knowledge about analysis and pathophysiology of 3-NT offered by GC–MS/MS, LC-MS/MS, and the new applied biosensors and biopolymers methods represent a gleam of hope for scientists to the fully clinical rely on this biomolecule as a biomarker of oxidative/nitrosative stress in RA. Methods based on mass spectrometry hold exceptional promise for research, discovery, and clinical use. In the near future, it will be necessary to develop practical analytical methods circumventing the pre-analytical, analytical shortcomings and pitfalls.

Declaration of interest

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