Antibody-free LC-MS/MS protein analysis of TRAIL

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Chapter 5

Quantitative antibody-free LC-MS/MS analysis of sTRAIL in sputum and saliva at the sub-ng/mL level

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Abstract

Soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) induces apoptosis via the extrinsic death receptor pathway and may be a biomarker in the pathogenesis of a broad range of diseases. To investigate the role of sTRAIL in asthma, we developed a quantitative LC-MS/MS method with a lower limit of quantitation (LLOQ) of ≈ 3 pM in induced sputum (174 pg/mL) and saliva (198 pg/mL) without the use of antibodies. sTRAIL was enriched by immobilized metal affinity chromatography (IMAC) solid-phase extraction (SPE) followed by tryptic digestion and subsequent enrichment of a signature peptide by strong cation exchange (SCX) SPE. The method was validated with respect to stability, accuracy and precision using the standard addition approach and a fully metabolically ^{15}N-labelled hrTRAIL as internal standard. Our results indicate that it is possible to quantify cytokines like sTRAIL at the pM level by LC-MS/MS without the use of antibodies, which has, to our knowledge, never been shown before.

Introduction

Research on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has mainly focused on the use of a recombinant form (hrTRAIL) as a drug in cancer therapy until now, because of its high selectivity to induce apoptosis in tumor cells without affecting normal cells.[1–3] However, in its endogenously occurring form, TRAIL has been shown to be also involved in the pathophysiology of other diseases having an inflammatory and/or autoimmune component.[4–10] This rather pleiotropic activity profile may be related to the essential role of apoptosis in the immune system and the variety of cells on which membrane-bound TRAIL is expressed in the innate and adaptive immune system.[11] Asthma is characterized by inflammation of the airways and a T_H2-mediated immune response.[12] TRAIL is also found in a soluble form following proteolytic cleavage from the cell surface (sTRAIL; extracellular domain, amino acids 114-281 of sequence P50591).[13] A link between sTRAIL and promoting a T_H2-mediated immune response in asthma was demonstrated by Weckmann *et al.*[14] Increased levels of sTRAIL in the airway epithelium, bronchoalveolar lavage fluid and induced sputum of asthmatic patients were associated with eosinophilic survival, eosinophils being the most important effector cell of asthmatic airway inflammation.[15]

It is thus of interest to quantify sTRAIL in induced sputum or saliva and investigate whether levels may be related to asthma. Secondly, a procedure relying not on antibodies provides advantages like low cost, variability and more overall robustness.[16] To this end, we developed an LC-MS/MS approach based on enzymatic digestion of the protein analyte and quantitation of a signature peptide, with two steps of solid-phase extraction (SPE); one at the protein and one at the signature peptide level. The LC-MS/MS method was applied to induced sputum and saliva samples. Saliva and the more commonly used induced sputum were both selected as matrices instead of bronchoalveolar lavage fluid, because of the relatively easy and noninvasive way of collection.[17] Recent reviews of proteomics in saliva for biomarker discovery showed its potential in clinical diagnostics.[18–20] Although being less protein-rich than serum or plasma, saliva and induced sputum are both challenging matrices for targeted

low-abundant protein quantification by LC-MS/MS, since they have total protein concentrations of ≈ 3 and ≈ 0.7 mg/mL and contain a wide diversity of approximately 2000 and 250 proteins, respectively.[21–24] Protein concentrations in saliva and sputum cover a wide dynamic range from the high-abundant amylase and mucus proteins to low abundant proteins like sTRAIL.

Metabolically ^{15}N-labeled hrTRAIL was used as internal standard to account for variation and losses during the entire sample preparation procedure including protein/peptide enrichment and tryptic digestion.[25,26] Endogenous sTRAIL was quantified with a standard addition method, since various surrogate matrices did not sufficiently correct for the variable matrix effects of saliva and induced sputum.[27,28] The method was validated with respect to stability, accuracy and precision and used to measure sTRAIL levels in saliva and induced sputum from asthma patients and healthy controls.

Materials and methods

Chemicals and protein standards

A Milli-Q® Advantage A10® System (conductivity 18.2MΩ/cm, Millipore, Billerica, Massachusetts, USA) was used for preparing HPLC-grade water. Acetonitrile and methanol were obtained from Avantor Performance Materials (J.T.Baker®, LC-MS grade, Center Valley, Pennsylvania, USA). Disodium hydrogen phosphate, sodium dihydrogen phosphate, glycerol, Tween® 20, formic acid, ammonia, sodium chloride, imidazole, hydrogen peroxide, iodoacetamide, dithiothreitol and trypsin from porcine pancreas (Type IX-S) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Sputolysin® Reagent (dithiothreitol concentrate in phosphate buffer pH 6.5-7.5) was purchased from Millipore. Human recombinant TRAIL (hrTRAIL) and metabolically labeled ^{15}N-rhTRAIL were both expressed in *E. coli* and purified as described by Reis *et al.*[29] and Wilffert *et al.*[30] ^{15}N-rhTRAIL was metabolically labeled by growing *E. coli* in a medium containing ^{15}N-labelled ammonium acetate as the sole nitrogen source. Both standards were stored in aliquots at -80˚C.

Induced sputum and saliva sample collection

Sputum induction was done according to a standard protocol of the University Medical Centre Groningen. Volunteers inhaled 4.5% hypertonic saline vapor for 5 min via an ultrasonic nebulizer (Ultraneb, DeVillbiss, Somerset, PA, USA) from which the output was calibrated at 1.5 mL/min. Afterwards, they were encouraged to cough and spit sputum. Induced sputum samples, which showed a high viscosity, were processed according to the method of Fahy *et al.*[31] Briefly, induced sputum was reduced by addition of 1:1 (w/w) dithiothreitol (Sputolysin® Reagent) for 15 min at 37˚C and filtered through a nylon (48µm) gauze followed by 10 min centrifugation at 450g at 20˚C. The supernatant was aliquoted and stored at -80˚C.

Saliva was collected using the passive drool technique. Volunteers were first asked to rinse their mouths with water and then instructed to generate saliva. After 1 min, the volunteers were asked to drool the saliva into a tube (50 mL).

After collection, the saliva was centrifuged for 10 min at 450g at room temperature and the supernatant was aliquoted and stored at -80˚C.

Enrichment with immobilized metal affinity chromatography (IMAC) at the protein level and strong cation exchange (SCX) at the peptide level

For quantification according to the standard addition method, each saliva or induced sputum sample was divided into two aliquots of 50 µL each: one aliquot with addition of 50 µL of ^{15}N-rhTRAIL internal standard at 4 ng/mL and one aliquot with 50 µL addition containing both 8 ng/mL of rhTRAIL and 4 ng/mL of ^{15}N-rhTRAIL.

IMAC enrichment was performed with 100 µL Ni^{2+} resin (Ni Sepharose™ 6 fast flow, slurry in 20% (v/v) ethanol in water, 6% cross-linked agarose, particle size ≈ 90 µm, GE Healthcare, Freiburg, Germany) which was transferred to a 1.5 mL tube. Five-hundred µL of water was added to the slurry and vortexed, followed by centrifugation at 2000g for 3 min. The water was removed and replaced by 500 µL IMAC buffer pH 8 (20 mM phosphate buffer, 10% glycerol, 0.005% Tween® 20) containing 15 mM imidazole to equilibrate the IMAC resin. After vortexing the resin was sedimented by centrifugation at 1500g for 3 min. The buffer was removed and 800 µL of new IMAC buffer pH 8 containing 15 mM imidazole was added. One hundred µL of prepared saliva or induced sputum were added to the IMAC resin (in IMAC buffer pH 8 containing 15 mM imidazole) and mixed on a rotating wheel for 10 min at 20 rpm. Afterwards, the slurry was transferred to an empty SPE cartridge and the IMAC resin was washed with 500 µL IMAC buffer pH 8.0 containing 100 mM NaCl and 15 mM imidazole and eluted with 200 µL IMAC buffer pH 8.0 containing 100 mM NaCl, 150 mM imidazole and 20% acetonitrile. For each step, positive air pressure was applied until the last drop was removed from the resin. For each sample, fresh Ni^{2+}-resin and new empty SPE cartridges were used.

Ten µL of 200 µg/mL trypsin were added to 200 µL of the enriched IMAC sample resulting in an approximate enzyme-to-substrate ratio (w/w) of 1:10. Proteins were digested by incubation at 37 °C with vortex-mixing at 450 rpm for 2 hours in a Thermomixer comfort (Eppendorf, Hamburg, Germany).

Digestion was stopped by adding 10 µL of 22% formic acid. SCX enrichment of the signature peptide was subsequently performed with SPE columns containing 100 µL SCX-resin (TSKgel SP-3PW slurry in 20% (v/v) ethanol, particle size ≈ 30 µm, 250 Å, TOSOH Bioscience, Tokyo, Japan) that was conditioned with 1 mL methanol and 1 mL of 1% formic acid. The digest was diluted two times with 1% formic acid before being loaded on the SPE column and washed with 1 mL 200 mM sodium chloride containing 10% acetonitrile and 1% formic acid followed by 1 mL acetonitrile. Peptides were eluted with 400 µL 1% ammonia containing 40% acetonitrile. Finally, the eluent was evaporated until dryness in approximately 30 min at 45˚C under nitrogen and reconstituted in 50 µL of 3% acetonitrile and 0.1% formic acid. Twenty µL of the final 50 µL were injected into the LC-MS/MS system.

Micro Liquid Chromatography – Mass Spectrometry (LC-MS/MS)

Quantification of the signature peptide of sTRAIL was performed with an Acquity M-Class nanoUPLC system coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with an IonKey source (Waters, Milford, MA, USA). The mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). After trapping peptides on a Trap Symmetry C_{18} column (Waters, 50 x 0.3 mm, 5 µm, 100Å) at a flow rate of 20 µL/min for 2.5 min at 3% B, the IonKey was switched online and separation was performed on a microfluidics IonKey UPLC CSH C_{18} column (Waters, 100 x 0.15 mm, 1.7 µm, 130Å) at 40˚C at a flow rate of 3 µL/min. Gradient elution was started at 3% B linearly increasing at 1.5% B/min for 17 min. The column was cleaned for 2 min at 90% B and equilibrated. This resulted in a total run time of 24 min per sample. Positive electrospray ionization (ESI^+) was used for mass spectrometry detection and the following source parameters were set: source temperature 150 ˚C, capillary voltage 3.5 kV, cone voltage 50 V, cone gas 20 L/h, collision gas (argon) 0.18 mL/min and nebulizer gas pressure 7.0 bar.

Three SRM transitions were monitored for the signature peptide SGHSFLSNLHLR of sTRAIL, one SRM transition for quantification and two SRM transitions for confirmation. For quantification purposes the SRM

transition 456.6 m/z ($[M+3H]^{3+}$) > 739.4 m/z (y_6^+) was monitored, which corresponds to the triply charged parent ion fragmenting to a singly charged y_6 daughter ion. Confirmation was performed by monitoring the SRM transitions: 456.6 m/z ($[M+3H]^{3+}$) > 652.4 m/z (y_5^+) and 456.6 m/z ($[M+3H]^{3+}$) > 852.5 m/z (y_7^+). The ^{15}N-labeled internal standard was monitored at the SRM transitions 463.2 m/z ($[^{15}N\text{-}M+3H]^{3+}$) > 751.4 m/z ($^{15}N\text{-}y_6^+$) (quantifier) and 463.2 m/z ($[^{15}N\text{-}M+3H]^{3+}$) > 663.4 m/z ($^{15}N\text{-}y_5^+$), and 463.2 m/z ($[^{15}N\text{-}M+3H]^{3+}$) > 865.5 m/z ($^{15}N\text{-}y_7^+$) (qualifiers). A collision energy of 18 V was applied for all SRM transitions. Results were accepted if the ratios of the peak areas between, the two confirmatory SRM transitions divided by the quantifier SRM transition were within ±25% of the mean ratio.

Statistical analysis

The mean sTRAIL levels between the asthma patient and the control groups and the influence of age, gender, food intake and allergies were evaluated by statistical analysis with GraphPad PRISM (Version 5.0, GraphPad Software Inc., La Jolla, CA, USA) for Windows. To uncover significant differences ($p < 0.05$), a Mann-Whitney's test was selected, because a Gaussian distribution was not observed.

Results and discussion

A fully validated, quantitative LC-MS/MS method for human recombinant TRAIL (hrTRAIL) in human and murine serum was adapted to the enrichment of endogenous soluble TRAIL (sTRAIL) from induced sputum or saliva.[30] Immobilized metal affinity chromatography (IMAC) SPE on Ni^{2+}-Sepharose proved applicable without major modifications due to the fact that sTRAIL and hrTRAIL share the same pattern of surface-exposed histidine residues, albeit that induced sputum had to be reduced with dithiothreitol, because of the high viscosity. Performance of IMAC enrichment was not affected, since sTRAIL does not contain any disulfide bridges and thus does not change in structure upon reduction.

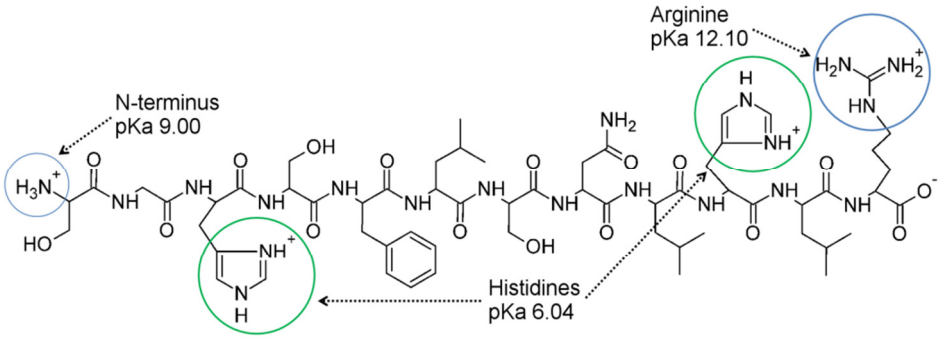

Figure 1. *Primary structure of the selected sTRAIL-specific signature peptide SGHSFLSNLHLR. The peptide contains four positive charges at pH ≈ 2.2, as used for strong cation exchange (SCX) SPE, unlike most other commonly observed tryptic peptides that contain only two positive charges at the N-terminus and the C-terminal Lys or Arg residues.*

SCX peptide enrichment

In order to reach the necessary concentration sensitivity to quantify sTRAIL in induced sputum or saliva, an additional enrichment step was needed at the peptide level after trypsin digestion. A strong cation exchange (SCX) SPE step was added, since the selected signature peptide SGHSFLSNLHLR contains four positive charges at pH ≈ 2.2 (1% formic acid) (**Figure 1**) ensuring strong interactions with the negatively charged sulphonic acid groups on the SCX resin. Most tryptic peptides from possible contaminating matrix proteins contain

only two positive charges due to the N-terminus (pK_a 9.00) and an arginine (pK_a 12.10) or lysine (pK_a 10.67) residue at the C-terminus. We therefore anticipated that the two additional histidines would discriminate this signature peptide from the majority of the other tryptic peptides in the mixture with respect to binding to an SCX resin at low pH. Applying a high ionic strength wash step with 200 mM sodium chloride removed a large part of interfering peptides while still retaining the signature peptide. Peptides that were bound to the SCX resin by possible hydrophobic interactions due to the polymeric nature of the resin, were removed by a second wash step with acetonitrile. Final elution was performed by increasing the pH to ≈ 11.4 (1% ammonia) thus neutralizing the positive charges on the histidine residues and the N-terminus of the signature peptide. Combining enrichment at the protein level by IMAC with enrichment at the peptide level by SCX allowed us to reach concentration sensitivities (LLOQs) of 174 pg/mL in induced sputum and 198 pg/mL in saliva (**Figure 2**).

Quantitation and method validation

Since sTRAIL is an endogenous protein and analyte-free biological matrix does not exist, calibration samples have to be prepared in a suitable surrogate matrix to allow quantitation at the relevant physiological levels. We observed a large variability in sensitivity (slopes of the calibration curves) between samples obtained from different volunteers making selection of a single surrogate matrix impossible. This might have been caused by differences in the composition of saliva and sputum from different individuals, for example due to the concentration of mucus proteins which influences viscosity. As a result, the method of standard addition was selected for quantitation. Induced sputum and saliva samples were analyzed twice, with and without addition of 8 ng/mL hrTRAIL, using the most intense SRM transition for quantification and two additional transitions as qualifiers (**Figure 2**). The ratios of intensities between the quantifier transition and the qualifiers were within the ±20% limit down to a concentration of 320 pg/mL below which the qualifiers were no longer detectable.

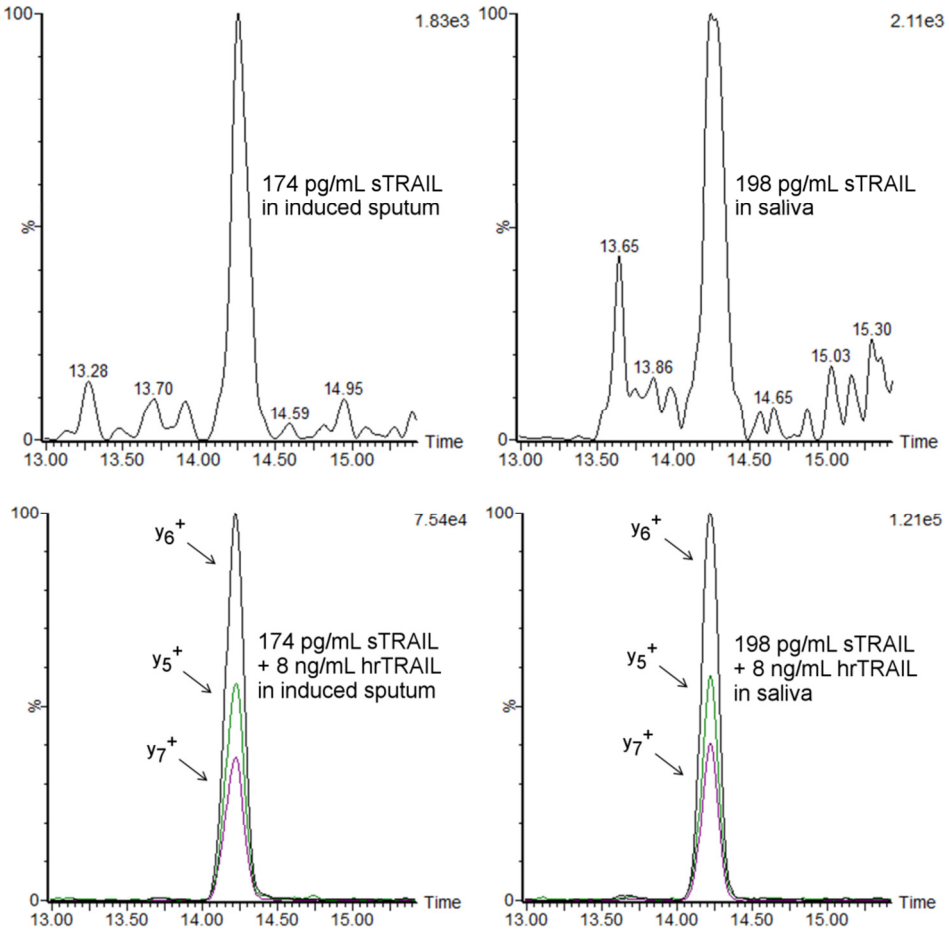

Figure 2. *LC-MS/MS chromatograms of sTRAIL at the lower limit of quantitation (LLOQ) of 174 pg/mL in (unspiked) induced sputum and 198 pg/mL in (unspiked) saliva, respectively, and after standard addition of 8000 pg/mL rhTRAIL. Three SRM transitions of the signature peptide SGHSFLSNLHLR were selected from which the most intense one (y_6^+) was used for quantification and the other two (y_5^+ and y_7^+) for confirmation (qualifiers).*

Table 1 provides an overview over the quantitative performance of the method in induced sputum at the endogenous levels of 174 pg/mL and saliva at 198 pg/mL sTRAIL (LLOQ), respectively, and at the standard addition level of 8000 pg/mL. All the results were within ±20% at the LLOQ and within ±15% at the standard addition level with respect to both accuracy (bias) and precision (CV). Bias at the LLOQ of sTRAIL was determined by comparing results after a single addition of 8000 pg/mL hrTRAIL and after the addition of different amounts to reach the following concentrations of hrTRAIL (200, 400, 800, 2000, 4000, 8000, 20000, 40000 pg/mL). Linearity over the entire concentration range was excellent with a correlation coefficient (R^2) of >0.997. Due to sample volume limitations, within-run precision was performed in both matrices while between-run accuracy and precision was only determined in induced sputum. Stability studies in table 1 addressing the following conditions; autosampler stability for 72 hours at 10˚C, bench-top storage for 24 hours and 5 complete freeze thaw cycles, showed that sTRAIL stability was acceptable with all biases and CV's within ±20% at the LLOQ and within ±15% at the standard addition level.

<u>sTRAIL analysis in induced sputum and saliva from asthma patients and healthy controls</u>

The LC-MS/MS method was applied to saliva and induced sputum samples from a group of asthma patients and healthy controls. Due to the noninvasive nature of collecting saliva, we extended the group of healthy controls to 45 volunteers. Within this group gender, age, food intake (within 1 hour of sampling) and allergies were evaluated and showed to have no significant influence ($p > 0.05$) on mean sTRAIL levels (supporting information, Figure S1). In a preliminary, small-scale study between asthma patients (510 ± 174 pg/mL, n=12) and a healthy control group (627 ± 118 pg/mL, n=45) there was no significant difference in the saliva concentrations of sTRAIL ($p = 0.236$) (**Figure 3A**). However, there was a tendency of sTRAIL being decreased in asthma patients in contrast to the potential "increase" in induced sputum that was observed in our small-scale study, with a mean concentration of 1193 ± 1107 pg/mL (n=3) sTRAIL in asthma patients and 857 ± 370 pg/mL (n=4) in controls ($p = 0.886$) (**Figure 3B**).

Table 1. *Validation of the quantification of sTRAIL in induced sputum and saliva at the lower limit of quantitation (LLOQ) and at the standard addition level. Accuracy is expressed in terms of bias from the nominal concentration and precision as the coefficient of variation (CV).*

		Induced Sputum		**Saliva**	
	[TRAIL] pg/mL	Bias %	CV %	Bias %	CV %
Accuracy and Precision					
Within-run	LLOQ	15.6*	19.4*	-8.1	17.3
(n=6)	+ 8000	-6.5*	12.6*	3.1	2.0
Between-run	LLOQ	8.5	18.3		
(3 days, n=6)	+ 8000	2.2	14.4		
Stability					
Autosampler	LLOQ	-2.8	17.6	-18.7	12.7
(n=3, 72h at 10˚C)	+ 8000	-3.6	10.0	2.1	0.7
Bench-top	LLOQ	-9.6	8.9	-19.8	12.1
(n=3, 24h)	+ 8000	5.1	4.8	1.3	2.5
Freeze / thaw	LLOQ	8.6	12.0	-16.5	12.0
(n=3, 5 cycles)	+ 8000	11.5	2.8	5.0	2.1

LLOQ: lower limit of quantitation of 174 pg/mL in induced sputum and 198 pg/mL in saliva
**Maximally observed values for within-run accuracy and precision are presented (see supporting information, Table S1 for more details)*

This is in line with the results of Weckmann *et al.*[14], who also reported an increase in the mean concentration of sTRAIL in asthma patients. These results show that the antibody-free LC-MS/MS method is suitable to support further studies to validate sTRAIL as an asthma biomarker in saliva and notably in induced sputum.

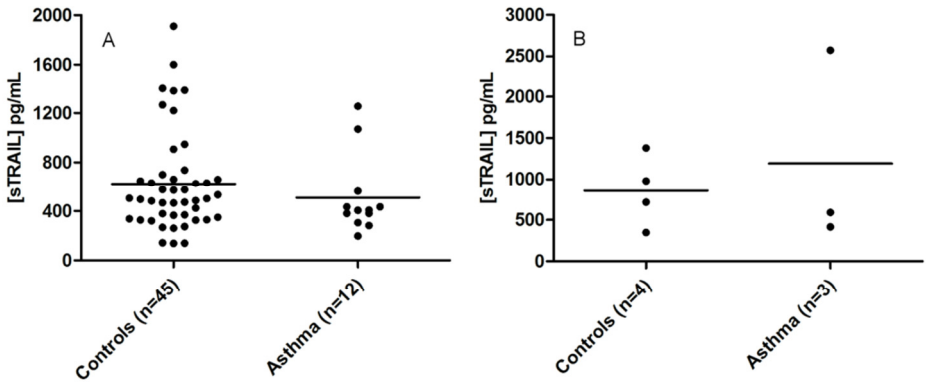

Figure 3. *Concentrations of sTRAIL in saliva (A) and induced sputum (B) of healthy volunteers and asthma patients by LC-MS/MS analysis. No significant difference was found in the mean concentration of sTRAIL in saliva (p = 0.236).*

Conclusion

We developed an antibody-free LC-MS/MS method to quantify endogenous levels of sTRAIL in induced sputum and saliva down to a level of ≈ 3 pM. A small scale study in saliva and induced sputum of asthma patients compared to healthy controls demonstrated that the method is 'fit-for-purpose'. Enrichment of sTRAIL was performed at the protein level with immobilized metal affinity chromatography (IMAC) followed by digestion and further enrichment at the peptide level with strong cation exchange (SCX) SPE. Absolute quantification was based on a standard addition approach with a metabolically labeled ^{15}N hrTRAIL as internal standard. The method complied with all current guidelines for the bioanalysis of small molecules concerning accuracy, precision and stability. We are currently investigating whether sTRAIL may serve as biomarker for other diseases with a strong inflammatory and/or autoimmune component as suggested in the literature.[4–10]

Supporting Information

This material is available online via the following link:

Supporting information chapter 5 (password: DWilffert102015)

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