Monitoring of warfarin therapy: preliminary results from a longitudinal pilot study

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Highlights

- Methods for the analysis of warfarin and its metabolites in oral fluid.
- Patients undergoing warfarin therapy were longitudinally monitored over time.
- Reduced variability of warfarin response if single patients are followed over time.

Abstract

The aim of this study was to investigate the relationship between warfarin dosage, international normalized ratio, plasma and oral fluid concentrations of warfarin, RR/SS- and RS/SR-warfarin alcohols. Nine patients on long-term warfarin therapy (4 with stable and 5 with unstable international normalized ratio values) were longitudinally monitored for over two months by recording warfarin dosage and measuring international normalized ratio, warfarin and warfarin alcohols concentrations in oral fluid and in plasma.

At equivalent dose (24 *vs* 22 mgweek^{-1}) and international normalized ratio (2.5 *vs* 2.2), stable patients showed nearly double plasma warfarin (4200 *vs* 2000 nM, p <1E–4), unbound plasma warfarin (35 *vs* 16 nM, p <1E–4), and nearly triple oral fluid warfarin (18 *vs* 7 nM, p <1E–4) concentrations compared to unstable patients. Correlations between warfarin dosage and total plasma concentration of warfarin (r = 0.65, p <0.01) or RS/SR-warfarin alcohols (r = 0.66, p <0.01), as well as between stimulated oral fluid and total plasma concentrations of warfarin (r = 0.72, p <0.01) and RS/SR-warfarin alcohols (r = 0.95, p <0.01) suggest that the relative changes of the oral fluid concentrations of these species may provide clinically useful information for monitoring individual patients. Follow-up data revealed that even in the absence of changes of warfarin dose, the oscillations of plasma and oral fluid of RS/SR-warfarin alcohols parallel oscillations of international normalized ratio. Due to the long delay of its biological action, monitoring the plasma concentration of warfarin might help to predict variations of international normalized ratio and prevent the risk of thrombotic or haemorrhagic events. The information collected suggests that non-invasive monitoring of warfarin in oral fluid might represent a suitable tool for this purpose.

Keywords: Warfarin, Warfarin alcohols, international normalized ratio, oral fluid, plasma

1. Introduction

Vitamin K is necessary to convert inactive precursors of vitamin k-dependent coagulation factors to active zymogens. The vitamin K antagonists, such as warfarin (WAR), are the most widely diffused oral anticoagulant drugs [1]. Warfarin is thought to interfere with clotting factors by inhibiting the C1 subunit of the vitamin K epoxide reductases (VKORC1) enzyme complex, thereby reducing the regeneration of vitamin K1 epoxide. In blood, WAR is highly bound to albumin (≈99%) with a high affinity (K_d = 3.4 ± 0.7 μM). It is mainly metabolized in the liver by cytochrome (CYP) P450 to inactive hydroxylated metabolites (OH-WAR) and also by ketone reductases to warfarin alcohols (RR/SS- and RS/SR-warfarin alcohols) [2]. These latter metabolites show an anticoagulant activity six-times lower than WAR [3], but their possible role in the anticoagulation process has received little attention up to now.

Despite its effectiveness, the WAR treatment has several shortcomings related to many factors that may interfere with the anticoagulant therapy by impairing metabolic pathways or increasing the pharmacologically active fraction due to the displacement of WAR from serum albumin [4–8]. The large inter- and intra-individual variability in patients' responses makes WAR therapy difficult to control, and this increases the risk of bleeding or thrombosis [9]. Such events occur in approximately 12% of patients, with a higher probability during the first two weeks of therapy than during maintenance [9]. A complex and expensive service has been set-up in most countries to monitor WAR effects by measuring the prothrombin time (PT) in blood expressed in terms of international normalized ratio (INR) and limit risks for the patients [10].

Warfarin shortcomings have prompted the development of direct oral anticoagulants (DOACs), which target key coagulation factors (e.g. factors Xa and IIa) instead of vitamin K [11]. Compared to WAR, DOACs have a more rapid onset of action (time to peak concentration: 2–4 hours *vs* 72–96 hours), shorter half-lives (5–17 hours *vs* 40 hours), are administered at fixed doses, and do not require a routine coagulation monitoring [12–15]. Nevertheless, the new anticoagulant drugs also have drawbacks: a) they are more expensive compared to the traditional vitamin K antagonists, b) patients quickly lose the anticoagulant effect and are unprotected from thrombosis if a single DOACs dose is missed, c) specific antidote (i.e. Idarucizumab) is currently available only for Dabigatran and d) DOACs are unsuitable for treating patients with mechanical heart valves.

For these reasons, WAR is not going to disappear from the scene. Several pilot studies have been conducted to verify if the safety and effectiveness of the WAR therapy may be improved by the knowledge of the plasma concentrations of WAR [16–19]. All these cross-sectional studies failed to find a significant correlation between the measured plasma concentration of WAR and INR. Such result is not surprising in view of the large inter-individual variability in the dose-effect

ratio [4–8]. However, our group hypothesized that a correlation between unbound plasma WAR and INR might be found based on measurements of WAR concentrations in oral fluid (OF) [20].

Based on this background, we performed a longitudinal pilot study enrolling nine patients undergoing a long-term WAR therapy. Our purpose was to get preliminary experimental evidences about the relationship between WAR dosage, INR, plasma and OF concentration of WAR, RR/SS- and RS/SR-warfarin alcohols.

2. Materials and Methods

2.1 Statement of ethics and data collection

Nine patients (5 males, 4 females) on long-term WAR therapy were monitored for over two months. During this period the drug has been regularly taken. Clinical variables such as age, sex, INR target range and duration of WAR therapy were collected at the enrolment by a patient interview. Possible patient-specific factors (e.g. new drugs administered) affecting the anticoagulant effect of WAR were also recorded at each sampling time.

A patient was considered stable when his INR values had remained within the target range (2.0–3.0) over (at least) three consecutive months before the first medical examination.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The pilot study was conducted at the Azienda Ospedaliero-Universitaria Pisana (Pisa, Italy) upon approval by the Ethical Committee of the Pisa Hospital. Informed consent was obtained from all individual participants included in the study.

2.2 Blood sample collection

Two venous blood samples were consecutively collected into vacuum tubes (Vacutest Kima, Padua, Italy) containing 109 mM (3.2%) sodium citrate at least 12 hours after the administration of the last WAR dose. Blood samples were immediately centrifuged at room temperature for 10 minutes at 3000 rpm to obtain platelet-poor plasma. Plasma samples were divided in two aliquots and stored in polypropylene tubes at –80°C until use. An aliquot of sample was used to measure the INR value, whereas the other was used to measure concentrations (both total and unbound fraction) of WAR, RR/SS- and RS/SR-warfarin alcohols.

2.3 Oral fluid sample collection

After blood collection, stimulated OF samples were collected in a quiet room between 7 and 10 AM by asking the patient to roll a Salivette polyester swab (Sarstedt, Nümbrecht, Germany) in mouth for 2 minutes. Patients were asked not to take any food or beverages within 1 hour prior to OF collection. After sampling, OF pH was measured by two independent observers using a Pehanon narrow range (6.0<pH<8.1) pH paper strips (Macherey Nagel, Düren, Germany) with a resolution of 0.3 pH units. The OF flow rate (grams per minute) was calculated from the ratio between the weight difference of the sampling device before and after sampling and the collection time, considering the density of OF equal of 1 gmL^{-1}. The sampling procedure and pH measurements were always completed in less than 3 minutes. The OF was recovered by centrifugation of the swabs at 3000 rpm for 5 minutes at room temperature and then stored in a polypropylene tube at –80°C until use.

2.4 Determination of international normalized ratio

Spectrophotometric INR measurements were carried out at a wavelength of 671 nm by an ACL TOP700 automatic system (Instrumentation Laboratory, Oragenburg, USA) equipped with an auto-sampler. The quality control procedure for INR measurements consisted in analysing three reference samples (normal, low and high INR levels) provided by the Instrumentation Laboratory at least once every eight hours. A RSD <1% for measurements performed on the same day (n = 6) and <3% for measurements performed on different days (n = 6) demonstrated the good inter- and intra-day precision of the method.

2.5 Determination of warfarin and warfarin alcohols in oral fluid and plasma

Warfarin and its metabolites (i.e. RR/SS- and RS/SR-warfarin alcohols) concentrations in OF and plasma samples were determined by a high-performance liquid chromatography (HPLC) (Jasco Europe, Lecco, Italy) equipped with an autosampler (AS 2055), a quaternary low-pressure gradient pump (PU 2089) and a fluorescence detector (FP 2020). The full details of the analytical procedures are reported elsewhere [21]. Briefly, an aliquot of OF (1 mL) or plasma (0.5 mL) was acidified with H_2SO_4 (2 mL, 0.5 M) and extracted with 4 mL of dichloromethane/hexane 1:5, (v/v). The resulting mixture was vortex-mixed for 30 s and then centrifuged at 5000 rpm for 5 minutes at room temperature. The supernatant was recovered, evaporated under nitrogen and reconstituted in 0.25 mL (OF) or 1 mL (plasma) of phosphate buffer solution (PBS) 25 mM at pH = 7. An aliquot (25 µL) of the reconstituted sample was finally injected into the HPLC system. Warfarin, RR/SS- and

RS/SR-warfarin alcohols were separated with a Poroshell EC-C-18 reversed-phase column (100 × 4.6 mm, 2.7 µm) (Agilent, Santa Clara, USA) connected to a TC-C-18 guard column (12.5 x 4.6 mm, 5 µm) (Agilent, Santa Clara, USA) using isocratic elution with 30% methanol and 70% phosphate buffer 25 mM at pH = 7 at a flow rate of 0.7 mL/min. Fluorescence detection was performed at excitation and emission wavelengths of 310 and 390 nm, respectively. The HPLC run time was 25 minutes. Calibration curves of 3–9740 nM were evaluated by the Deming regression analysis. Inter- and intra-day precision and recovery were determined for spiked OF sample at 6, 32 and 65 nM and for spiked plasma sample at 650, 3250 and 4500 nM. The recoveries of RR/SS-warfarin alcohols, WAR and RS/SR-warfarin alcohols in OF were 85% (RSD = 4%), 80% (RSD = 3%) and 85% (RSD = 3%) respectively, whereas the recoveries of RR/SS-warfarin alcohols, WAR and RS/SR-warfarin alcohols in plasma were 60% (RSD = 5%), 90% (RSD = 4%) and 70% (RSD = 3%), respectively. For both OF and plasma, the inter- and intra-day precisions (%RSD) for WAR and its metabolites were always lower than 5%.

Unbound WAR, RR/SS- and RS/SR-warfarin alcohols concentrations in plasma were measured by ultrafiltration [21]. In short, an aliquot of plasma sample (1 mL) was centrifuged at 5000 rpm for 1 hour at 25°C by an Amicon Ultra-4 tube (Millipore, Massachusetts, USA) with a molecular weight cut-off of 3 KDa. The filtrate sample was then injected (25 µL) into the HPLC system without any further treatment, and analysed according to the chromatographic method described above. Calibration curves in the range 3–97 nM were evaluated by the Deming regression analysis. Inter- and intra-day precision was determined for spiked plasma samples at 650, 3250 and 4500 nM, whereas the recovery was determined for standard working solutions at 6, 32 and 65 nM.

The inter- and intra-day precisions (%RSD) at a concentration level of 32 nM were 6% and 4% for RR/SS-warfarin alcohols, 8% and 5% for WAR, and 8% and 6% for RS/SR-warfarin alcohols, respectively. The recovery of RR/SS- and RS/SR-warfarin alcohols and WAR was 90% (RSD = 8%) and 70% (RSD = 8%), respectively.

2.6 Statistical analysis

Continuous variables were reported as means ± standard deviation and ranges. The distribution of variables was tested for normality by the Shapiro-Wilk test, whereas the gender difference was analysed by the Mann-Witney test. The association between the OF concentrations and INR as well as between plasma concentrations and INR were evaluated by the Spearman correlation test. A two-tailed *p*-value of less than 0.05 was considered statistically significant for all analyses. Statistical analysis and principal component analysis (PCA) was performed using GraphPad Prism v.6.0 (GraphPad, La Jolla, USA) and XLSTAT v.2015.4.01 (Addinsoft, New

177 York, USA), respectively.

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180 **3. Results**

181 The patient profiles, including clinical conditions, dosage and concentrations of WAR and its
182 metabolites (i.e. RR/SS- and RS/SR-warfarin alcohols) in OF and plasma samples (both total and
183 unbound fraction) are summarized in Table 1.

184

185 **Table 1**

186 Characteristics of patients enrolled (n = 9) at first point collected. Data are shown as mean ±
187 standard deviation (range).

Demographics	
Age, yr	67 ± 14 (38–83)
Gender, male:female	5:4
Warfarin therapy	
Warfarin dose, mgweek^{-1}	30 ± 20 (1.25–48.75)
INR	2.4 ± 0.6 (1.8–3.7)
Oral fluid parameters	
Stimulated pH	7.6 ± 0.3 (6.9–8.1)
Stimulated flow rate, mL/min	0.9 ± 0.1 (0.5–1.1)
Warfarin concentration, nM	20 ± 10 (3–45)
RR/SS-warfarin alcohols concentration, nM	<LOD*
RS/SR-warfarin alcohols concentrations, nM	10 ± 6 (1–20)
Unbound plasma fraction concentration	
Warfarin, nM	30 ± 10 (10–55)
RR/SS-warfarin alcohols concentration, nM	1 ± 1 (1–3)
RS/SR-warfarin alcohols concentrations, nM	15 ± 10 (2–30)
Plasma concentration	
Warfarin, nM	4200 ± 1600 (1300–6500)
RR/SS-warfarin alcohols concentration, nM	100 ± 70 (40–260)
RS/SR-warfarin alcohols concentrations, nM	1950 ± 1100 (400–3600)

Abbreviation: INR, international normalized ratio.

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189 Patients' average age and WAR dose were 67 ± 14 years (range, 38–87 years) and 30 ± 20

mgweek^{-1} (range, 1.25–48.75 mgweek^{-1}), respectively. International normalized ratio values ranged from 1.8 to 3.7, with an average value of 2.6 ± 0.6. The Mann-Witney test did not reveal statistically significant gender differences (p <0.05) for any of the above parameters.

A therapeutic range of WAR of 970–5840 nM was calculated, corresponding to the 2.5th to 97.5th percentile of the plasma WAR concentration for patients showing INR within 2–3, which is coherent with previously reported range [16].

To get an overall view of the internal structure of the data, a PCA was performed on the entire dataset consisting of all measurements from all patients (n = 66, Fig. 1).

In the score plot, measurements belonging to a same patient appear to form relatively coherent clusters, whereas clusters corresponding to patients with unstable INR values are scattered along the vertical axis. Interestingly, data of all stable patients (square dots) are in the right part of the plot, corresponding to higher doses and WAR concentration values, whereas data of patients with unstable INR values (circle dots) are in the left part. This observation is confirmed by an independent descriptive statistical analysis, which reveals that despite similar average INR and WAR dose, stable patients have higher values of plasma and OF concentrations of WAR and WAR metabolites compared to patients with unstable INR values.

The loadings plot (Fig. 1A) shows no correlation between WAR dose and INR and regression analysis confirms such result (r = –0.14, p = 0.24). On the contrary, there is a good correlation between WAR dosage and total plasma concentration of WAR (r = 0.65, p <0.01), RS/SR-warfarin alcohols (r = 0.43, p <0.01) and RR/SS-warfarin alcohols (r = 0.68, p <0.01).

The position of plasma and OF concentrations of WAR and RS/SR-warfarin alcohols in the loadings plot suggests a poor correlation with INR, confirming the results obtained from other authors [16–19].

The significance of PCA, in particular as concerns the coherence of OF and plasma concentrations of WAR and its metabolites, is supported by linear regression between unbound and total plasma fraction as well as between stimulated OF and unbound plasma fraction of both WAR and RS/SR-warfarin alcohols (Fig. 2).

In fact, linear regression reveals statistically significant correlations between: a) unbound plasma fraction and total plasma concentration of WAR (Fig. 2A, r = 0.79, p <0.01); b) stimulated OF and unbound plasma WAR (Fig. 2B, r = 0.80, p <0.01); c) unbound plasma fraction and total plasma concentration of RS/SR-warfarin alcohols (Fig. 2C, r = 0.93, p <0.01); stimulated OF and unbound plasma RS/SR-warfarin alcohols (Fig. 2D, r = 0.95, p <0.01).

Fig. 2 shows a good correlation between OF and plasma data from all patients enrolled in our longitudinal study. The average ratio between the concentration in OF and the unbound fraction in plasma, assessed from the slope of the regression line, is about 0.5 for both of WAR and RS/SR-warfarin alcohols, and this confirms the dilution effect occurring during sample collection reported by Lomonaco et al [22].

Fig. 3 shows changes over time of dosage, unbound plasma WAR and RS/SR-warfarin alcohols concentrations and INR in two representative patients, one with a stable (P3) and the other with unstable INR values (P5). Figs. S1 and S5 in the supplementary information reports the corresponding data for the other patients.

Fig. 4 reports INR values versus the OF concentration of WAR for patients P3 and P5.

4. Discussion

Results suggest that each patient under WAR therapy has his own dose to effect ratio, i.e. he attains the desired anticoagulant effect with a personal plasmatic WAR concentration that is largely independent on the assumed dose. Despite this, our main finding in this study is that the oscillations of INR during therapy parallel those of the concentrations of WAR and its metabolites in plasma, and that OF WAR concentrations mirror plasma WAR concentrations. If one considers the long delay in the biological action of vitamin K antagonists, it seems reasonable to think that monitoring concentration changes in plasma (and perhaps OF concentration of WAR and its metabolites) may help to prevent INR oscillations out of the therapeutic range and to reduce the risk of thrombotic and haemorrhagic events.

The present study also shows that patients with higher concentrations of WAR and its metabolites in plasma and OF are likely to obtain a more stable therapeutic effect. If this result is confirmed in a larger set of patients, WAR plasma concentrations during therapy may be used to stratify patients according to their risk of going out of the therapeutic range.

The perspective of a minimally invasive monitoring of WAR therapy is particularly appealing because large meta-analysis studies have shown that DOACs are not safer than WAR in terms of haemorrhagic or thrombotic incidents [23–25]. As a consequence, vitamin K inhibitors will continue to be used in future due to the limited indications of DOACs and inherent restrictions such as poor renal function, and for this reason any improvement of WAR therapy would reduce risks and costs of the anticoagulant therapy in a large number of patients.

Despite the numerous factors potentially affecting WAR metabolism, we found a strict association between changes of the plasmatic concentration of RS/SR-warfarin alcohols and INR, and this made us suspect that the pharmacological action of these metabolites, although less potent than WAR's, may contribute to the overall therapeutic effect more than expected.

The use of PCA to analyse effects of WAR therapy provided specific advantages for the visualization of multivariate data. The basic assumption with this technique is that variance has an informative content, whereas variables with poor variations have not. In addition, multivariate data often contain redundant information related to the correlation of variables. At the same time, they are very difficult to visualize, as we are used to interpret bi- or three-dimensional plots and cannot really figure out a higher dimensional space. To get rid of redundancy, PCA defines a new set of uncorrelated variables (principal components) that are linear combinations of the "old" variables and are oriented (in the multidimensional space) along the directions where the maximum variance is observed. Data and previous variables are then plotted versus the principal components. From a geometrical point of view, this corresponds to project multidimensional points onto the plane where the maximum variance can be shown, so that data can be looked at from the best possible perspective.

In practice, PCA produces two plots in which similar items are located close to one another: the score plot shows the relationships between the objects (in our case the array of measurements of INR, dose and concentration values obtained from a specific patient at each observation time), and the loadings plot shows the correlation between variables. If two variables are close one another in the loadings plot then they are highly correlated, if they are symmetrical compared to the origin of axes then they are negatively correlated, whereas in case the lines connecting the origin of axes with the two variables are orthogonal it means that variables are not correlated. If the score and loadings plots are superimposed, a so-called bi-plot is obtained in which objects characterized by a high value of a specific variable will be located close to the variable.

In our case, it is evident that the first principal component is strictly related to the concentrations of WAR and RS/SR-warfarin alcohols and to a minor extent to the WAR dosage, whereas the second principal component mainly depends on INR. The explained variance is nearly 80%, and this means that most of the original information contained in the data is retained in the plot.

It is noteworthy that dots corresponding to a same patient cluster in the score plot. This suggests that most variability in the WAR dose to response ratio depends on inter-individual differences, whereas each patient has his own sensitivity to the drug possibly related to individual genetic factors. Clusters corresponding to patients with unstable INR values show a vertical spread,

a consequence of the large INR variability in a restricted range of dosage. They are located in the left part of the score plot whereas stable patients are in the right part, exactly where WAR and RS/SR-warfarin alcohols concentrations can be found in the loadings plot. In fact, stable patients show higher plasma and OF concentrations of WAR and metabolites compared to patients with unstable INR, suggesting that they are less sensitive to WAR and require higher plasma concentrations to obtain a similar anticoagulant action. Thus, it seems that patients with higher plasma and OF concentrations of WAR are more liable to attain a stable dose–effect ratio.

The loadings plot shows that INR and WAR dosage are not correlated. Due to the many factors interfering with the therapy, this result is not surprising [4–8]. In fact, there is consensus in literature about the lack of a univocal and clear relationship between WAR dose and therapeutic effects, and this is why the monitoring of patients undergoing WAR therapy is needed.

Loadings plot and linear regression show that higher dosages result in higher plasmatic concentrations of both WAR and RS/SR-warfarin alcohols. There are correlations between the unbound and total plasma concentrations of WAR and its metabolite, as well as between their OF and unbound plasma concentrations. The degree of correlation is evident in Fig. 2, where WAR data appear somewhat more scattered that RS/SR-warfarin alcohols data. This phenomenon might be related to the longer half-life of these metabolites compared to WAR (20 days *vs* 40 hrs), so that short term fluctuations of the concentration average over a longer length of time. A further explanation might lie in the lower pKa of WAR (5.15 ± 0.04 at 25 °C, [26]) which makes it more prone to the dilution effect related to the sampling of stimulated OF.

Our findings are different from those reported from previous authors who had found much poorer correlations among these variables in cross-sectional studies [17–19]. The large variability in the individual response to WAR can probably explain such difference, which, is reduced if single patients are followed over time, as in the present study.

We hypothesize the existence of individual concentration-effect curves, which may allow the use of stimulated OF analysis to monitor the unbound plasma concentrations of WAR and its metabolites and possibly INR.

The data presented in Figs. 3 and 4 and supplementary Figs. S1 and S5 seem to confirm that this might actually be possible in some case, but in about 30% of our patients one or more confounding factors puzzle the scene and do not allow to find a clear relationship between OF concentrations and INR. Fig. 3 reports the trends over time of WAR dosage, WAR and RS/SR-warfarin alcohols concentrations in plasma (unbound) and OF, and INR of the illustrative patients P3 and P5 (the corresponding data relevant to the other patients are reported in Figs. S1 and S5 of supplementary information). Patient P3 had a stable therapy and INR values within the target range

(2.0–3.0); the slight fluctuations of INR values were perfectly mirrored from OF WAR and to a lesser extent from OF RS/SR-warfarin alcohols. The correlation between OF WAR and INR is more evident in Fig. 4, where linear regression allows to predict INR value once the OF WAR concentration is known. Also for patient P5, which shows a remarkable fluctuation of INR values probably due to the large number of other drugs needed to manage concomitant comorbidities, a good correlation between OF WAR and INR was found.

5. Conclusion

On the whole, the above findings suggest that in each patient there is a different but constant dose-effect ratio of WAR, and that each patient attains the desired anticoagulant effect with different plasma concentrations of WAR, in part independent from the dose assumed; indeed, we found that the oscillations of INR may reflect the oscillations of the concentrations of WAR, and in particular of its RS/SR metabolite, the one showing the strongest association with INR in PCA.

In consideration of the delayed pharmacological action of WAR, the measurement of WAR plasma concentration may be helpful in managing patients by anticipating the risk of bleeding or thrombotic events. A simple instrument for measuring OF WAR concentration would allow to monitor WAR therapy, to reduce the need on frequent INR monitoring, and to prevent the occurrence of out of range INR values, thus possibly reducing the risk of thrombotic or haemorrhagic events in patient's counter indicated for DOACs therapy.

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List of figure captions

Fig. 1. Principal component analysis of the complete patients' dataset (*n* = 66). Legend: warfarin dosage (DOSE); stimulated oral fluid concentration of warfarin (SOF WAR) and RS/SR-warfarin alcohols (SOF RS/SR-WAR); unbound plasma concentration of warfarin (UP WAR) and RS/SR-warfarin alcohols (UP RS/SR-WAR); total plasma concentration of warfarin (TP WAR); RS/SR-warfarin alcohols (TP RS/SR-WAR) and RR/SS-warfarin alcohols (TP RR/SS-WAR); international normalized ratio (INR).

Fig. 2. Relationship between unbound and total plasma of warfarin (A) and RS/SR-warfarin alcohols (C) and between stimulated oral fluid and unbound plasma fraction of warfarin (B) and RS/SR-warfarin alcohols (D) for the enrolled patients (*n* = 66) undergoing warfarin therapy.

Fig. 3. Warfarin dosage, unbound plasma warfarin and RS/SR-warfarin alcohols concentrations and international normalized ratio trends over time in two representative patients undergoing warfarin therapy: a stable patient (P3, A) and a patient with unstable international normalized ratio values (P5, B).

Fig. 4. Warfarin concentrations in oral fluid *vs* international normalized ratio in two representative patients undergoing warfarin therapy: a stable patient (P3, A) and a patient with unstable international normalized ratio values (P5, B).

Supporting information

Fig. S1. Warfarin dosage, oral fluid, total and unbound plasma warfarin and RS/SR-warfarin alcohols concentrations and international normalized ratio trends over time in two patients undergoing warfarin therapy: an unstable patient (P1, A) and a patient with stable international normalized ratio values (P2, B).

Fig. S2. Warfarin dosage, oral fluid, total and unbound plasma warfarin and RS/SR-warfarin alcohols concentrations and international normalized ratio trends over time in two patients undergoing warfarin therapy: a stable patient (P3, A) and a patient with unstable international normalized ratio values (P4, B).

Fig. S3. Warfarin dosage, oral fluid, total and unbound plasma warfarin and RS/SR-warfarin alcohols concentrations and international normalized ratio trends over time in two patients undergoing warfarin therapy: an unstable patient (P5, A) and a patient with stable international normalized ratio values (P6, B).

Fig. S4. Warfarin dosage, oral fluid, total and unbound plasma warfarin and RS/SR-warfarin alcohols concentrations and international normalized ratio trends over time in two patients undergoing warfarin therapy: a stable patient (P7, A) and a patient with unstable international normalized ratio values (P8, B).

Fig. S5. Warfarin dosage, oral fluid, total and unbound plasma warfarin and RS/SR-warfarin alcohols concentrations and international normalized ratio trends over time in an unstable patient (P9, A) undergoing warfarin therapy.

Fig. 1

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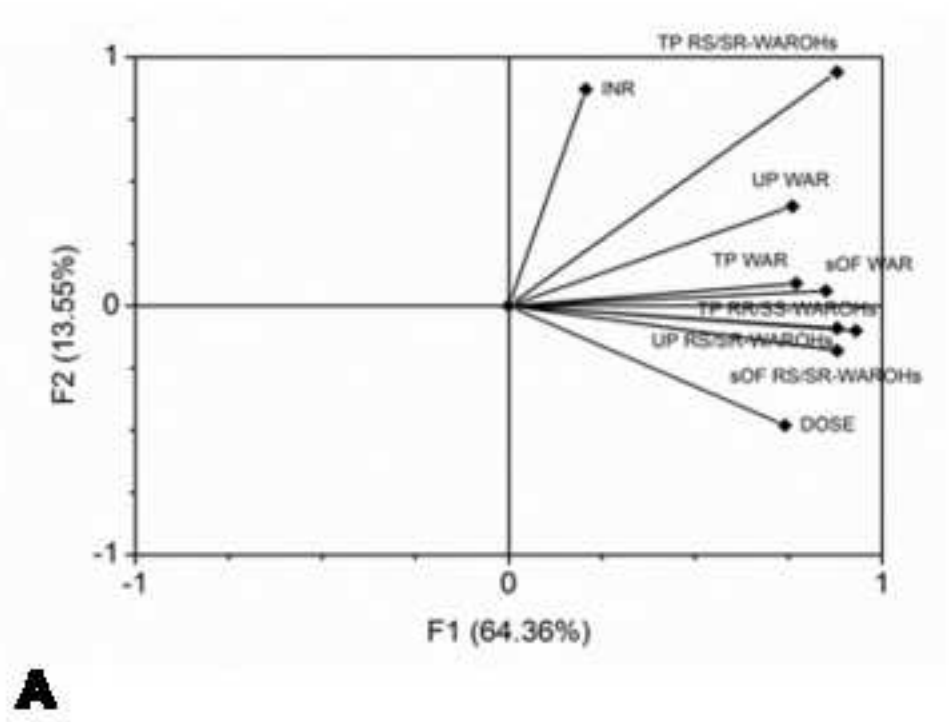
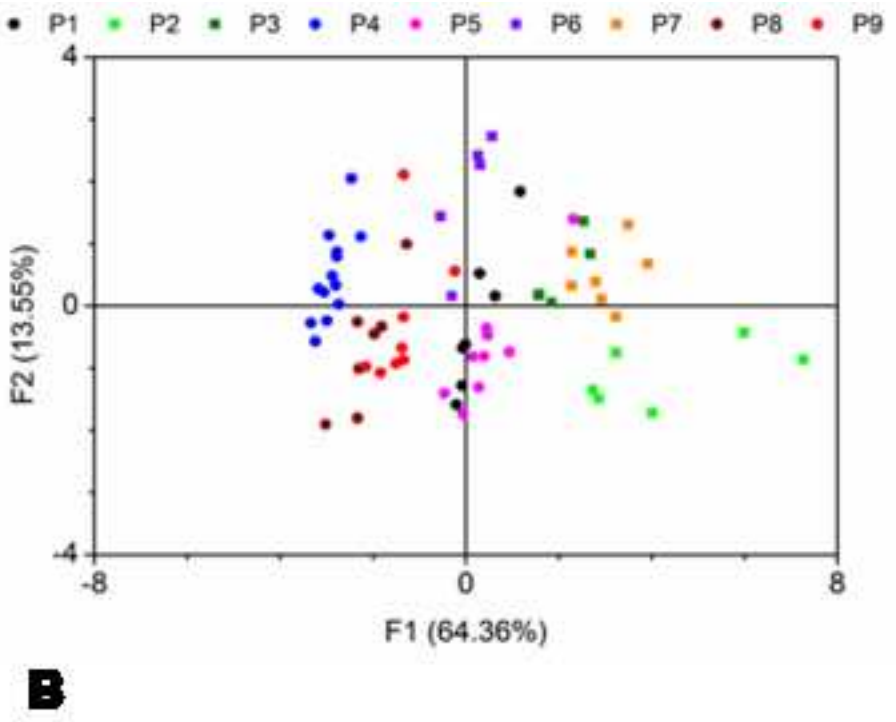


A



B

Fig. 2

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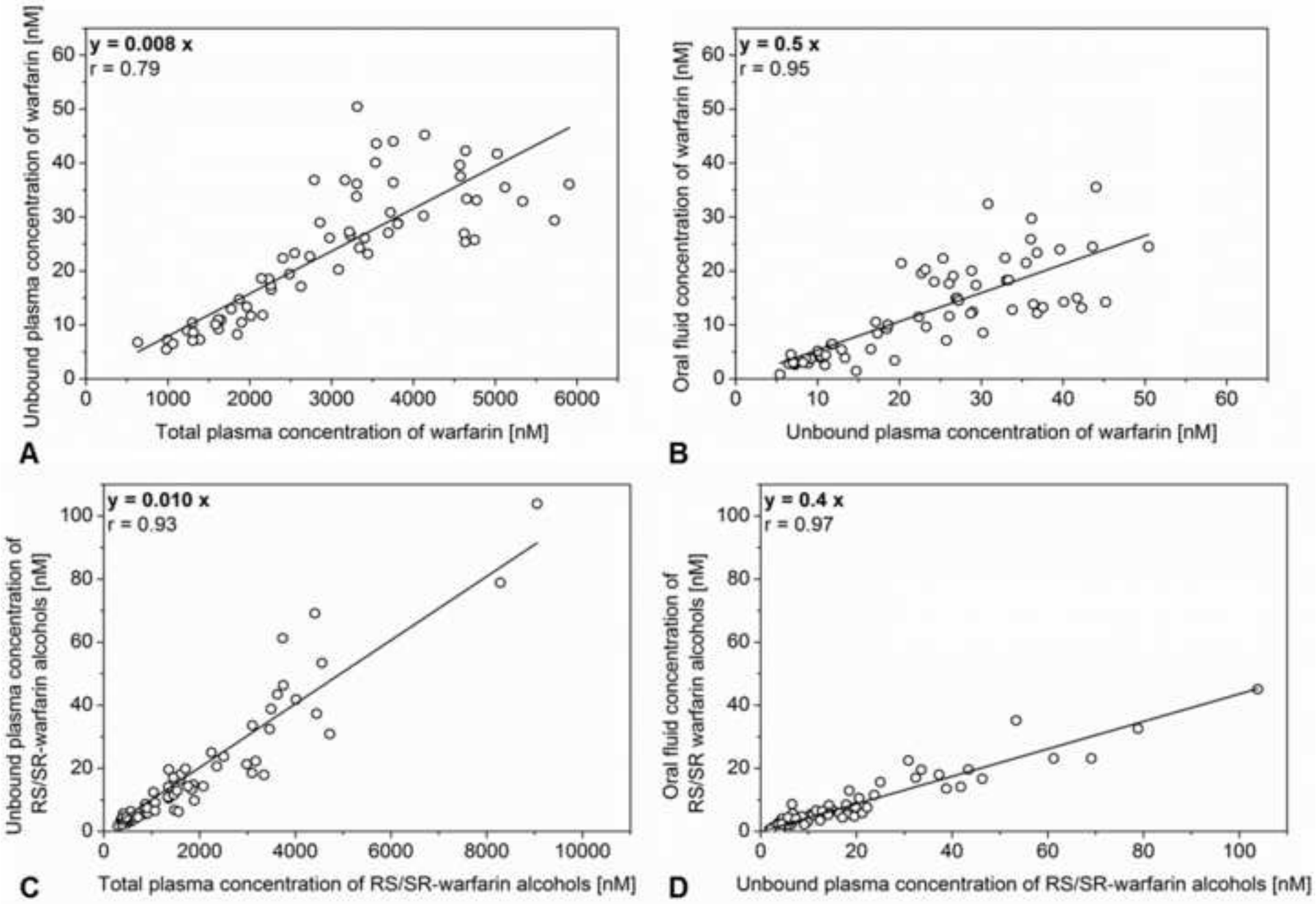

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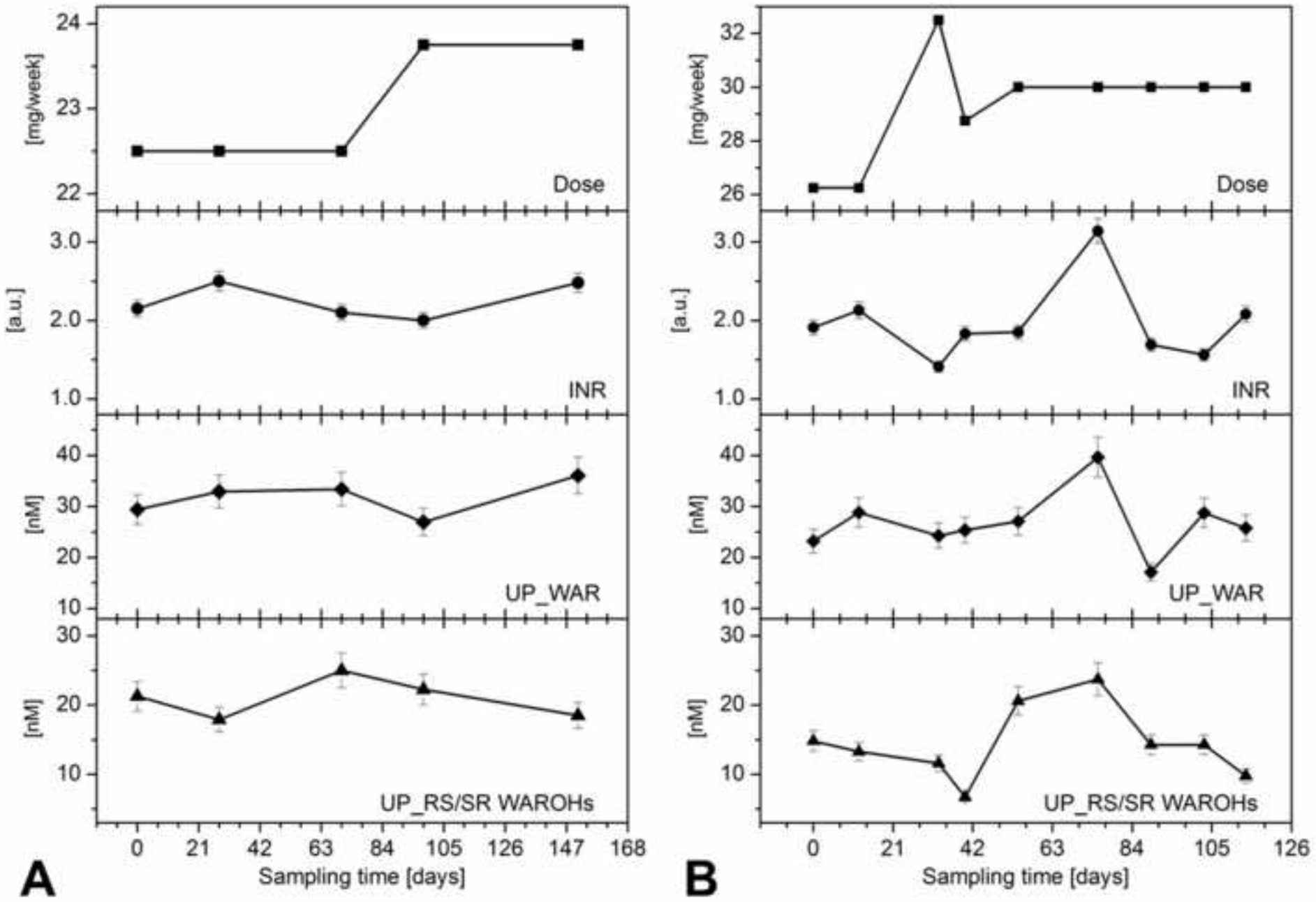

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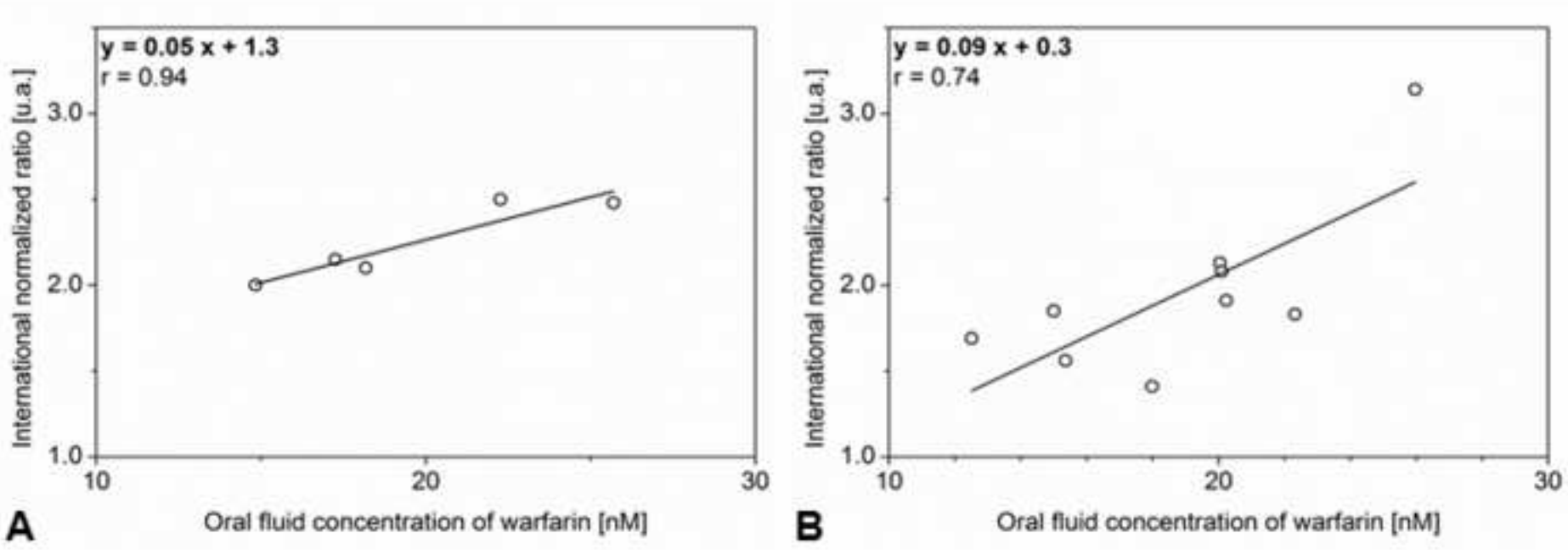

Fig. S1

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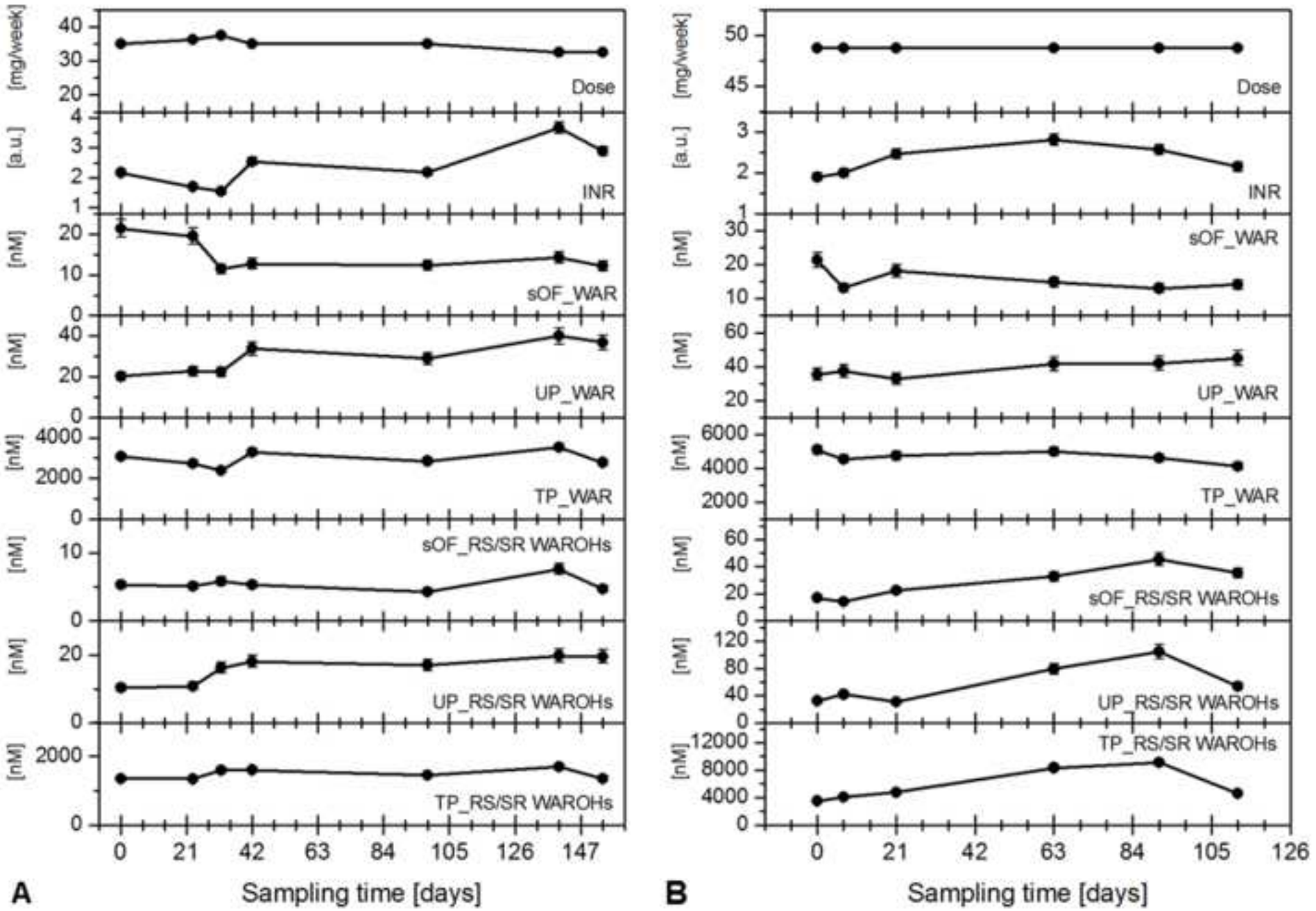

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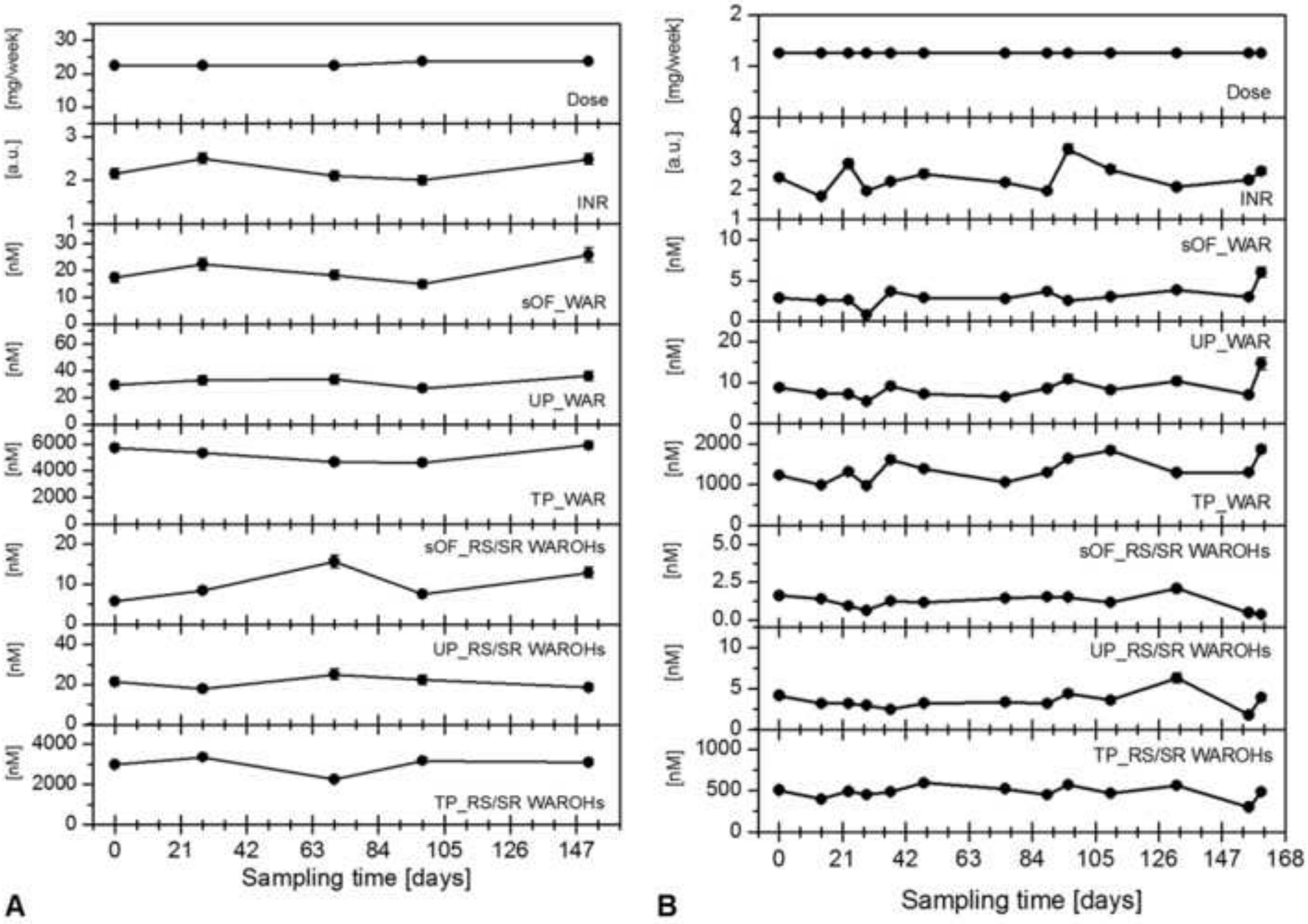

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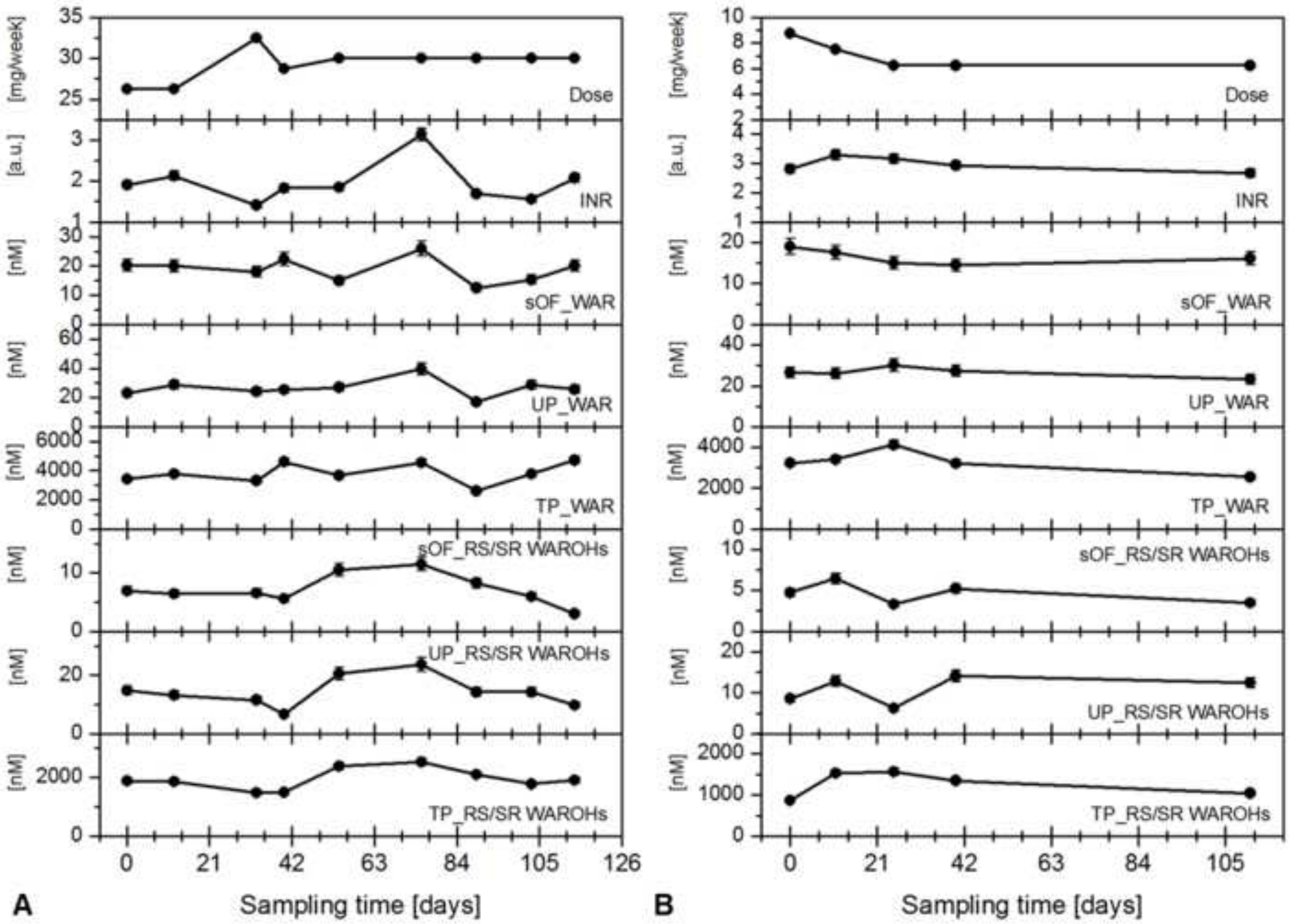

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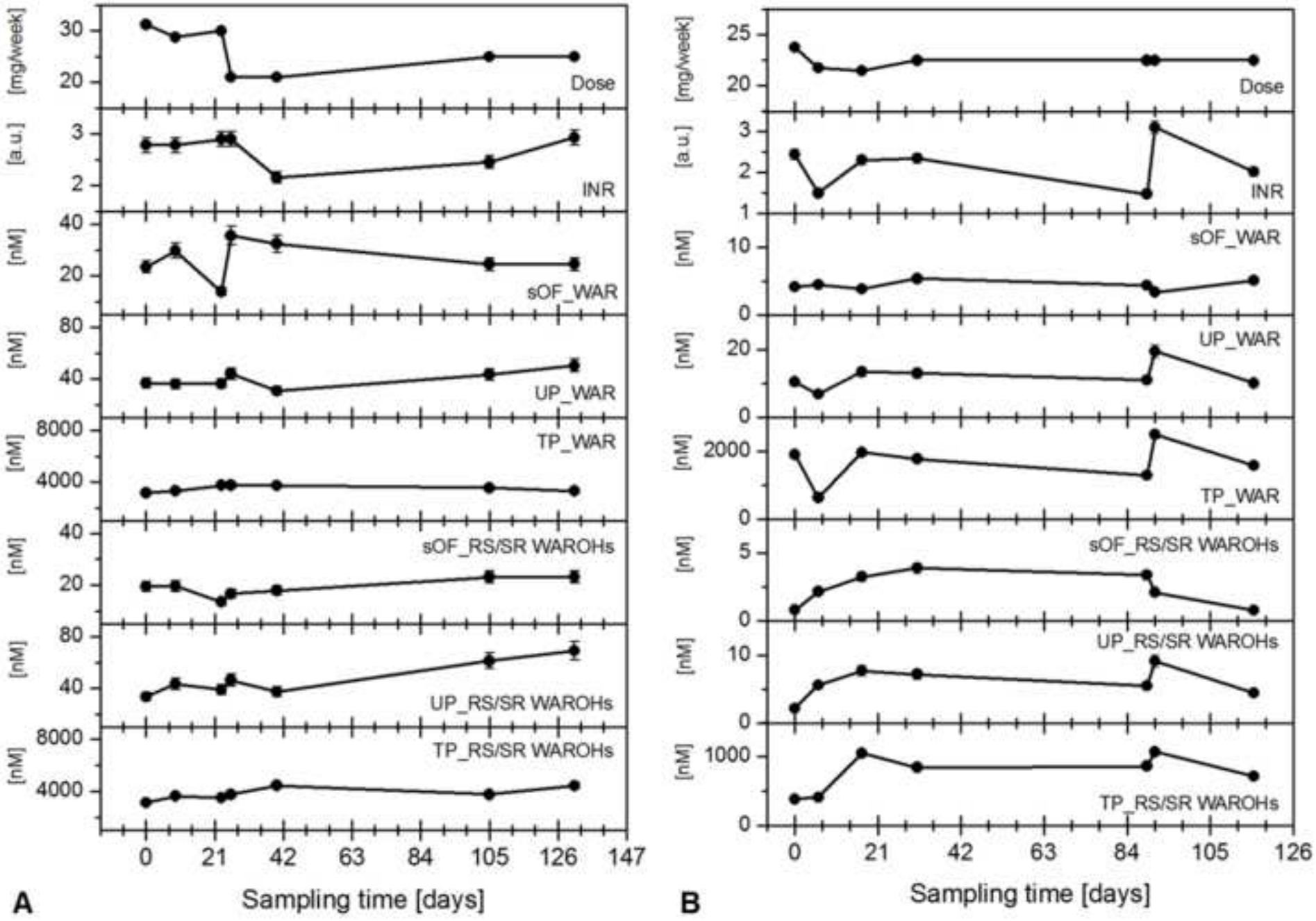

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