Contrast-Enhanced Ultrasound and Protein Shakes Are No Alternatives for Inulin Clearance and Meat to Assess Renal Functional Reserve in Humans

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Renal functional reserve · Ultrasound · Perfusion index · Protein load

Abstract

Introduction: The measurement of renal functional reserve (RFR) can unmask glomerular hyperfiltration in residual nephrons, but its determination is time-consuming. In this study, we assessed whether contrast-enhanced ultrasound (CEUS) is a valuable alternative to the gold standard inulin clearance and whether L-arginine or protein shakes lead to similar changes in glomerular filtration rate (GFR) as animal proteins in men and women. Methods: Changes in GFR and renal microperfusion were studied in 25 healthy subjects (8 men, 17 women) by simultaneously performing inulin clearance and CEUS (perfusion index, PI) before and 1 and 2 h after different protein loads (L-arginine, protein shake or meat). The Doppler parameters – renal resistive index (RRI) and pulsatility index (PuI) – were also measured. Results: None of the oral protein loads induced significant changes in CEUS-assessed PI. Only meat increased inulin clearance (from 111.2 ± 16.0 to 149.8 ± 27.2 mL/min, p < 0.05). Protein shakes had a neutral effect. There were no correlations between changes in inulin clearance and PI. At Doppler, RRI and PuI increased after meat intake (from 0.647 ± 0.029 to 0.694 ± 0.050 a.u., p < 0.05 and from 1.130 ± 0.087 to 1.318 ± 0.163 a.u., p < 0.05, respectively), but their changes also did not correlate with changes in inulin clearance. Results were similar in both sexes. Conclusions: CEUS is not a valuable alternative for inulin clearance to measure RFR. Meat ingestion leads to modest changes in renal Doppler parameters and to glomerular hyperfiltration in both women and men, while protein shakes and L-arginine do not.

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Introduction

Chronic kidney disease (CKD) is defined as evidence of structural or functional kidney abnormalities that persist for at least 3 months or a glomerular filtration rate (GFR) < 60 mL/min/1.73 m², with or without (micro)albuminuria, irrespective of its cause [1]. Large interindividual differences have been described in CKD progression toward end-stage kidney disease (ESKD) [2]. This
may be partly linked to the imprecision of markers used to measure or estimate GFR and hence detect early loss of nephrons, the hallmark of CKD. The ability to develop compensatory mechanisms such as glomerular hyperfiltration that limit the loss of GFR also contributes to the variability of the GFR decline in individuals. Indeed, according to Brenner’s hypothesis, a nephron loss will lead to a compensatory hyperfiltration in the remaining ones [3]. This compensatory hyperfiltration explains why GFR may remain remarkably stable over time despite the ongoing loss of nephrons. The renal functional reserve (RFR) actually describes the capacity of the kidney to augment its level of function under certain stimuli. Hence, it is an elegant way to unmask compensatory hyperfiltration. A reduction in RFR is classically observed in patients with hypertension, diabetes, and CKD. It has recently been associated with some hypertensive phenotypes [4].

In theory, RFR represents a sensitive tool to identify early nephron loss, but easily applicable, rapid tests to measure RFR are lacking in clinical practice [5]. The determination of RFR is usually based on the administration of a substance leading to an acute increase in GFR. The most frequently used stimulus is a high animal protein meal with meat, with reported increases in GFR of on average 34 mL/min [6–9].

L-Arginine has been used as an alternative to a protein-rich meal, as it can be easily administered orally or intravenously. L-Arginine leads to intrarenal vasodilation and thus to an increase in GFR, with larger increases reported after oral forms compared to intravenous L-arginine [10, 11]. However, studies have not shown uniform results [12]. Ingestion of vegetable or milk-based proteins has also been proposed as an alternative to animal proteins, but again with mixed results [6, 9, 13, 14].

Regarding the measurement of the changes in GFR, most studies have used either inulin or iothalamate, DTPA, or EDTA as exogenous filtration markers. These methods are reliable but cumbersome and time-consuming [14–17]. Recently, contrast-enhanced renal ultrasound (CEUS) has been proposed as an alternative for measuring RFR [18].

CEUS associates conventional ultrasonography with the IV administration of microbubbles-based contrast agents. The microbubbles remain intravascular; therefore, CEUS can depict renal vessels as small as 40 μm, and quantify renal microcirculation, without nephrotoxic side effects [19]. The increases in GFR during RFR testing are largely explained by proportional increases in renal blood flow (RBF) [5]. CEUS may be an interesting alternative to inulin clearance. Recently, Kalantarinia et al. [18] performed CEUS before and 2 h after the administration of a protein-rich meal in 19 healthy volunteers (10 women), and reported, indeed, a 43% increase in renal microperfusion. Unfortunately, results were not reported by sex, and a direct head-to-head comparison to the gold-standard of RFR, i.e., inulin clearance, was not performed.

The purposes of this study were (1) to assess whether CEUS is a valuable alternative for inulin clearance to measure RFR, (2) to assess whether different sources of oral protein lead to similar degrees of glomerular hyperfiltration and CEUS-assessed hyperperfusion, and (3) to determine whether a similar degree of glomerular hyperfiltration occurs in women after a protein load as compared to men.

Materials and Methods

Study Design and Participants

The study population consisted of 25 healthy subjects (8 men, 17 women) and was part of the GenderBOLD study (clinical trial gov NCT04085094, PI Dr. M. Pruijm). Subjects were categorized into three groups according to the type of protein load they received. The first 11 participants received oral L-arginine (Dynamisan forte®), the next 7 a protein cocktail (whey protein isolate), and the last 7 meat in the form of a cooked steak. GFR was measured by inulin clearance before and 60 and 120 min after the consumption of the different sources of protein. Renal hemodynamic responses were assessed simultaneously by using Doppler ultrasound and CEUS before and 120 min after protein intake (shown in Fig. 1).

All participants were recruited by advertisement and all visits were performed in the outpatient clinic of the Service of Nephrology and Hypertension of University Hospital in Lausanne (CHUV). Subjects were normotensive, untreated healthy individuals without a history of kidney disease, arterial hypertension, or any other concomitant disease. Informed written consent was obtained from all the participants. Other inclusion criteria were age >18 years and the ability to understand the study protocol and to sign informed consent. Subjects were excluded if they had a known allergy to inulin, Dynamisan forte, protein shakes, or contrast products. Vegetarians were also excluded.

Renal Functional Reserve Measurements

Inulin Clearance and Creatinine Clearance

The study visit started at 8:00 a.m. On the day preceding the study visit, 24-h urines were collected to measure the daily excretion of Na, K, creatinine, protein, and albumin. Dietary protein intake was estimated from 24-h urinary urea excretion according to Maroni’s et al. [20] formula. All subjects had to abstain from alcohol and from consuming meat on the day before the study visit. An ultrasound exam confirmed that the urine bladder was empty before starting the study and after each urine collection at specific time points.

Inulin clearance was performed under standardized hydration conditions by perfusing inulin at a continuous rate (5 mg/min) for at least 2 h until a steady state was reached, as previously reported.
[21]. The method is technically validated in our lab by using internal quality control at different levels. The measured intra-assay coefficient values of variation at 3 levels of sinistrin concentrations, i.e., 3 mg%, 25 mg%, and 100 mg% are 2.0, 3.3, and 3.3%, respectively, and for the inter-assay values of 9.4, 3.5, and 3.4%, respectively. The limit of quantification is 0.5 mg%.

Thereafter, participants took either L-Dynamisan (Dynamisan forte®, containing a mixture of L-aspartate and L-arginine) at a standard oral dose of 1 g/kg, a protein shake (whey protein isolate) at a dose of 2 g protein/kg, or a steak at a dose of 1–1.2 g protein/kg (i.e., 4–4.8 g of steak/kg). Blood and urine samples were collected before, at 60 min and 120 min after protein load. Urinary and plasma sodium and potassium concentrations were measured by flame photometry (IL-943; Instrumentation Laboratory, Milan, Italy). Creatinine levels were determined using the Jaffe Kinetic method (Cobas-Mira; Roche, Basel, Switzerland), while plasma and urinary inulin were determined by adaptation of a diphenylalanine procedure on an Autoanalyzer II – Technicon (Bran and Luebbe, Norderstedt, Germany). Changes in inulin clearance were used to calculate the RFR. Finally, plasma and urinary creatinine levels and urine volumes measured at baseline, 60 and 120 min were used in order to calculate baseline and stressed GFR after each protein load by calculating the endogenous creatinine clearance (CrCl) that was corrected for the body surface area according to the following formula: CrCl = (urinary creatinine × urinary volume)/(collection time × serum creatinine)/body surface area × 1.73 [22, 23].

Renal Doppler Ultrasound
Renal grayscale and color duplex ultrasounds were performed at baseline in the fasting state and 120 min after the protein load, according to a standardized procedure by the same experienced operator as described previously [24]. Briefly, the renal resistive index (RRI) and the pulsatility index (Pul) were measured in 3 segmental arteries (superior, middle, and inferior) of each kidney. The values were then averaged to obtain a mean value for each participant (shown in online suppl. Fig. 1a; for all online suppl. material, see www.karger.com/doi/10.1159/000527313).

Contrast-Enhanced Ultrasound
CEUS sessions at low mechanical index (MI = 0.08) were performed in each participant just before protein consumption and 120 min after protein load in order to assess whether changes in the CEUS-assessed perfusion index (PI) corresponded to changes in GFR. The most accessible kidney (usually the right) was chosen for image acquisition in a sagittal plane and the probe was oriented so that renal length was highest in order to assure the largest cortical surface area.

Sonovue® was selected as the contrast medium and administrated at a perfusion dose of 0.014 mL/kg/min in order to saturate the systemic circulation and the kidneys with microbubbles. Once saturation was reached after 1–2 min, the destruction-replenishment technique was used to quantify intrarenal perfusion, as published previously [25, 26]. Briefly, the rate at which microbubbles replenish renal tissue after destruction by pulses at high MI is proportional to the local blood flow and is expressed as time-intensity curves (TICs). Division of the intensity of the signal (expressed as regional blood volume, RBV) by the mean transit time (mTT) allows the calculation of the so-called PI as a proxy of renal microcirculation [19] (shown in online suppl. Fig. 1b). A minimum of four consecutive destruction–reperfusion (DR) sequences were performed at each time point. Special attention was paid to obtain after each flash zero luminance (no visible microbubbles). All DR sequences were performed in breath hold in order to avoid movement artefacts.

Statistical Analysis
Clinical data were analyzed using STATA 16.0 (StataCorp, College Station, TX, USA). Quantitative variables were expressed as mean ± standard deviation (SD). Qualitative variables were expressed as the number of patients and percentage. Paired Student’s t tests were used to compare within-group changes and one-way
analysis of variance (ANOVA) to study between-group differences. RRI was calculated as the ratio of the difference between the peak systolic velocity (PSV) and the end diastolic velocity (EDV) divided by the PSV value: $RRI = \frac{\text{PSV} - \text{EDV}}{\text{PSV}}$. The PuI value was obtained by dividing the difference between PSV and EDV by the mean velocity (MV): $PI = \frac{\text{PSV} - \text{EDV}}{\text{MV}}$. Concerning CEUS, the PI was calculated as the mean transit time/regional blood volume. Protein-induced changes in PI (called “Delta PI”) were compared with protein-induced changes in GFR (called “Delta GFR”) with Spearman’s rank correlation.

In order to assess whether significant changes in PI were detected after a protein load, and based on an expected change in PI value of 40% (±15) as reported in a previous study [18] and an α of 0.05 with a two-sided significance level, 7 subjects had to be included per group to have a power of 80%. Statistical significance was considered for a two-sided $p$ value < 0.05.

### Results

#### Participants and Baseline Characteristics

The study population consisted of 25 healthy subjects (8 men and 17 women) who were divided into three different groups based on the type of the administrated protein load (Dynamisan $n = 11$, protein shake $n = 7$, meat $n = 7$). Subjects did not differ significantly in terms of age, body mass index (BMI), or baseline office systolic and diastolic blood pressures. Baseline serum creatinine and estimated GFR by the CKD-EPI formula did not differ significantly among groups. A summary of baseline characteristics is shown in Table 1.

|                                | Total participants ($n = 25$) | Dynamisan ($n = 11$) | Protein shake ($n = 7$) | Steak ($n = 7$) | $p$ value |
|--------------------------------|-------------------------------|-----------------------|-------------------------|-----------------|-----------|
| Females, $n$ (%)                | 17 (68)                       | 6 (54.5)              | 6 (85.7)                | 5 (83.3)        | 0.68      |
| Age, years                      | 30.72±9.7                     | 31.1±6.9              | 32.7±15.0               | 28.1±7.6        | 0.58      |
| BMI, kg/m²                      | 23.8±3.9                      | 23.1±4.2              | 25.2±3.9                | 23.6±3.8        | 0.58      |
| Serum creatinine, μmol/L        | 71.5±10.4                     | 73.6±8.3              | 71.6±13.8               | 68.1±10.3       | 0.57      |
| eGFR-EPI, mL/min/1.73 m²        | 108.7±15.1                    | 108.8±15.7            | 102.1±16.0              | 115.1±12.4      | 0.28      |
| Serum urea, mmol/L              | 4.5±1.0                       | 4.9±1.0               | 4.1±1.1                 | 4.2±0.8         | 0.17      |
| Serum sodium, mmol/L            | 140.1±1.8                     | 139.7±1.7             | 140.3±1.9               | 140.6±1.8       | 0.60      |
| Serum potassium, mmol/L         | 3.9±0.2                       | 3.9±0.3               | 4.0±0.1                 | 3.8±0.3         | 0.37      |
| Serum uric acid, μmol/L         | 270.4±82.7                    | 246.9±44.9            | 289.9±102.6             | 288.0±107.9     | 0.46      |
| 24-h urine sodium, mmol/day      | 136.2±62.1                    | 154.1±72.3            | 139.9±62.5              | 104.4±32.1      | 0.25      |
| Daily protein intake, g/day     | 332.4±87.6                    | 291.4±79.9            | 383.3±83.0              | 345.9±83.0      | 0.08      |
| Baseline SBP, mm Hg             | 114.3±13.3                    | 114.6±18.0            | 111.9±9.2               | 116.8±4.4       | 0.82      |
| Baseline DBP, mm Hg             | 70.6±8.1                      | 70.2±7.6              | 71.1±9.9                | 70.7±8.6        | 0.96      |
| Baseline heart rate, beats/min   | 66.4±10.8                     | 64.0±12.2             | 65.5±7.2                | 72.4±12.1       | 0.37      |

Values are expressed as mean±standard deviation or percentage, as appropriate. SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HR, heart rate.

### GFR Derived from Inulin Clearance before and after the Protein Load

Inulin clearance increased significantly 2 h after the steak ingestion (from 111.2 ± 16.0 to 149.8 ± 27.2 mL/min, $p < 0.05$) while inulin clearance decreased after L-arginine (Dynamisan) intake (from 106.7 ± 45.3 to 86.3 ± 42.6 mL/min, $p < 0.05$) and remained unchanged after protein shakes (shown in Fig. 2). The estimated GFR values between the two 1-h clearances of inulin varied on average by 7.0% ± 4.3% (Min: 0.32%, Max: 16.6). When examining the time-course (0 min, 60 min, and 120 min) of the absolute values of mGFR (measured GFR) by inulin clearance, a significant increase occurred only in the meat group at 120 min, not earlier. The change of mGFR (inulin clearance) over time is graphically presented in online supplementary Figure 3.

In a separate analysis by sex (see Table 2), inulin clearance decreased significantly in women but not in men who received L-arginine. However, there was also a lowering trend in men. Protein shakes did not alter the inulin clearance, neither in men nor in women, but there was only one man included in this group. Finally, inulin clearance increased significantly after intake of steak in women but not in men. The changes in mGFR (inulin clearance) induced by each type of protein load are graphically shown per sex in online supplementary Figure 2. There were no significant changes in blood pressure and heart rate after the protein load (online suppl. Table 1).
Creatinine Clearance and eGFR derived from CKD-EPI formula did not change significantly after each protein load (online suppl. Table 2).

Ultrasound-Assessed Changes in Renal Perfusion before and after Protein Loads

At Doppler ultrasound, no changes occurred in RRI, except for the group who ate the steak, in which RRI increased from 0.64 ± 0.03 to 0.69 ± 0.05 a.u. (p < 0.05). In this group, the PuI also increased from 1.130 ± 0.087 to 1.318 ± 0.163 a.u. (p < 0.05). As for the CEUS-assessed PI, there was no significant change after the different protein loads (see Table 3).

Table 2. Changes in inulin clearance by protein load, overall and by sex

| Protein load | Total population | p value | Women | p value | Men | p value |
|--------------|------------------|---------|-------|---------|-----|---------|
|              | inulin Cl baseline, mL/min |         | inulin Cl baseline, mL/min |         | inulin Cl baseline, mL/min |         |
| Dynamisan (n = 11) | 106.7±45.3 | 0.01 | 92.2±18.4 | 0.01 | 124.0±63.4 | 0.34 |
| Protein shake (n = 7) | 101.7±33.7 | 0.79 | 97.9±35.2 | 0.73 | 124.6±0 | a |
| Steak (n = 7) | 111.2±16.0 | 0.003 | 118±13.1 | 0.001 | 94.4±7.7 | 0.46 |

Values are expressed as mean ± standard deviation, as appropriate. Inulin Cl baseline, baseline inulin clearance; Inulin Cl post, inulin clearance after protein. a One man only in this subgroup.

Fig. 2. Measured GFR by inulin clearance before and after protein load.

Relationship between Protein-Induced Changes in GFR and Renal Ultrasound Parameters

There was no correlation between the protein-induced change in mGFR (Δ GFR) and the change in CEUS-assessed PI (Δ PI) (shown in Fig. 3a). While there was a trend toward a proportional increase in inulin clearance and RRI after protein load (shown in Fig. 3b), no statistically significant correlation was found between Δ GFR and Δ RRI (spearman’s rho 0.27, p = 0.20). The same was observed for the relationship of Δ GFR and Δ PuI (spearman’s rho 0.35, p = 0.35). We also did not find any significant association between Δ GFR and Δ RRI or Δ PuI when analyzed separately by the type of protein load.
Table 3. Ultrasound parameters before and after protein load

| Protein load     | RRI pre (a.u.) | RRI post (a.u.) | p value | PuI pre (a.u.) | PuI post (a.u.) | p value | PI pre (a.u.) | PI post (a.u.) | p value |
|------------------|----------------|-----------------|---------|----------------|-----------------|---------|---------------|---------------|---------|
| Dynamisan (n=11) | 0.63±0.08      | 0.63±0.07       | 0.99    | 1.140±0.302    | 1.110±0.246     | 0.70    | 3,371.4±3,352.9 | 3,910.2±3,039.1 | 0.50    |
| Protein shake (n=7) | 0.65±0.06     | 0.62±0.03       | 0.12    | 1.164±0.179    | 1.073±0.088     | 0.11    | 2,856.0±2,025.2 | 2,975.1±2,193.2 | 0.92    |
| Steak (n=7)      | 0.64±0.03      | 0.69±0.05       | 0.02    | 1.135±0.087    | 1.318±0.163     | 0.01    | 4,771.1±2,618.4 | 6,018.8±5,289.7 | 0.53    |

Values are expressed as mean±standard deviation, as appropriate. RRI, renal resistive index; PuI, pulsatility index; PI, perfusion index.
Discussion

The main findings of our study were that CEUS-assessed renal microperfusion did not capture any changes in renal perfusion induced by oral protein loads, and that there was no correlation between the protein-induced changes in GFR and CEUS-assessed PIs. Hence, according to our data, CEUS cannot be recommended as an alternative to inulin clearance to measure RFR in humans. Second, among the different protein loads, only meat animal proteins increased inulin clearance and mobilized amino acids as a consequence of oral protein loads, whereas whey protein shakes had a neutral effect. These results are in contrast with previous studies and suggest that oral L-arginine is no alternative to red meat to assess RFR. Finally, we observed significant increases in the RRI and in the pulsatility index after the ingestion of meat, whereas no changes in these Doppler parameters occurred after the intake of L-arginine or protein shakes. The changes in RRI and pulsatility index correlated weakly but not significantly with the changes in inulin clearance. As there were large interindividual variations in the association between ΔGFR and ΔPI, it seems unlikely that renal Doppler ultrasound offers an alternative to inulin clearance to assess RFR.

We are not the first to report that different protein sources exert different effects on GFR. It is well known that animal proteins induce stronger changes in GFR than vegetal proteins [27]. Nonessential amino acids in meat are usually held responsible for this effect, as they are able to release glucagon from the alpha-cells of the pancreas, which leads on its terms to vasodilation of preglomerular arterioles and thus hyperfiltration [28, 29]. In line with this hypothesis, dogs and humans who have undergone pancreatectomy do not exhibit RFR. Besides, the use of somatostatin, an inhibitor of glucagon excretion, also blocks RFR [30, 31].

Why protein shakes did not induce changes in GFR in our study is less clear and goes against the common belief that these shakes are bad for the kidneys. Although previous studies demonstrated that protein shakes can lead to an important rise in glucagon [32], it remains unclear why this does not lead to a uniform increase in GFR [33, 34]. This may be because of their different amino acid composition or a different rate of gastrointestinal absorption [35]. In this context, whey protein is a “fast” protein due to its rapid digestion and amino acid absorption, which results in a rapid increase in plasma amino acid concentrations. Hence, this could potentially lead to a different time-dependent renal hemodynamic response that is not captured by classic protocols that typically measure GFR 120 min after a protein load. However, the changes of mGFR induced by meat and by Dynamisan occurred at 120 min after the intake and not earlier, which is in line with previous studies supporting that measurement of RFR should be performed no earlier than 2 h after the protein load [7, 36].

As for L-arginine, it has been reported that an enhanced GFR response to oral and iv arginine is associated with increases in glucagon concentrations [11]. However, a recent study by Jeppsson et al. [37] minimized the importance of arginine in the recruitment of RFR. In our study, L-arginine also failed to increase GFR and, surprisingly, even led to a reduction in GFR. This was true for both men and women. The reason for this paradoxical response is unclear. The decrease in GFR cannot be explained by changes in renal perfusion or blood pressure, as both remained constant. The selected dose of 1 g/kg of L-arginine might have been insufficient to increase intrarenal NO availability or insulin and glucagon levels. However, the dose was higher than the dose used in previous studies [11]. Whatever the reason, our study does not favor the use of oral L-arginine to assess RFR.

Another interesting explanation for the lack of effect of protein shakes and L-arginine on GFR may be that in contrast to cooked meat—they lack abundance in advanced glycation end products. In two recent studies, advanced glycation end product and not the protein-content were found to be responsible for the renal hemodynamic effects of meat [38, 39].

Importantly, the aforementioned protein-induced changes in GFR measured by inulin clearance could not be captured by using the creatinine clearance formula. Interestingly, in the group who received Dynamisan (L-arginine), the creatinine clearance slightly increased, which contrasted with the decrease in inulin clearance observed after Dynamisan intake. While alterations in serum creatinine values can occur due to changes in glomerular filtration, they can also change due to changes in its production by the muscles, physical activity, or diet. The urinary secretion of creatinine is also influenced by tubular secretion, which may differ among subjects and may be affected by the type of proteins given to test RFR. In line with the latter, in a study by Bello and Caramelo, the intravenous infusion of L-arginine induced an increase in the tubular secretion of creatinine [40]. Therefore, creatinine clearance is not a good parameter to capture changes in GFR when amino acids such as L-arginine are given to assess RFR.

Interestingly, despite the proven roles of RRI and RFR in the prognosis of renal disease progression in
Contrast-Enhanced Ultrasound and Protein Loads for Assessing RFR

To the best of our knowledge, our study is the first to perform CEUS, Doppler renal ultrasound, and inulin clearance simultaneously. We found a simultaneous increase in RRI and inulin clearance 2 h after meat ingestion but no significant change in the PI. The latter finding is in contrast with the study of Kalantarinia and others, who reported a 40% increase in CEUS-assessed renal microperfusion, versus 26% in our study [18]. The smaller amount of meat consumed in our study (1 g/kg vs. 1.5 g/kg in the study by Kalantarinia) may be partly responsible for the observed differences. Besides, it is well known that changes in renal blood flow after red meat intake vary largely between individuals [36]. As both studies included only a limited number of subjects, the difference might also be due to chance. We have no explanation for the increase in RRI that occurred after meat intake. RRI is an incompletely understood parameter that refers not only to the intrarenal circulation but is also influenced by systemic factors such as heart rate, blood pressure, and pulse pressure [46], which in our study did not differ significantly before and after each protein load. Besides, some drugs are known to increase (e.g., calcineurin inhibitors) or decrease RRI (RAS-blockers), but no drugs were given during our study. Animal studies suggest that ureteral pressure may also alter intrarenal Doppler flow patterns and RRI [47]. However, the bladder was emptied at regular intervals in our study, and water intake was modest (150 mL/h); besides, no dilatation of the pyelon was noted when ultrasounds were repeated after 120 min.

Thus, we can only hypothesize on why RRI increased after meat intake in our study. The PuI and PI both increased, suggesting an increase in renal perfusion, although the increase in PI was not significant. It may be that the steak has different effects on pre- and post-glomerular vascular tonus or that changes in the ureteral pressure occurred, but this clearly needs more detailed studies. These studies should include the gold standard method for measuring RBF, i.e., para-aminohippuric acid (PAH) in order to calculate filtration fractions and appreciate possible changes in the pre- and post-glomerular arteriolar resistance. As for the pulsatility index, an indirect index of the degree of vasoconstriction, this parameter has received much less attention in the medical literature. Our study shows that a high protein load increases the pulsatility index, which is in accordance with the changes in RRI.

Our study has several limitations. First, we had a limited number of participants in each group and an unequal proportion of men in the group who received meat and protein shake as protein stimuli. Therefore, sex-differences could not be explored in detail. Second, we did not measure PAH-clearance as the gold standard of RBF due to the lack of this substance on the market when we performed our study. Third, we did not measure RRI and CEUS at earlier time points (e.g., 60 min) that might have given more information on different time-dependent renal hemodynamic responses. Additionally, the lack of hormonal measurements such as glucagon, insulin, catecholamines, or renin, which appear important in the pathophysiology of RFR and the regulation of renal hemodynamics, especially in the group who received the steak, is another limitation of our study. Finally, the study was not powered to detect changes in PI <40%.

Taken together, considering the three objectives of our study, one could conclude that CEUS is not a valuable alternative to inulin clearance to measure RFR. Some interesting changes in Doppler-assessed parameters are observed with CEUS after meat intake, but additional studies are needed to understand their significance. Our data also support the hypothesis that the intake of supranormal quantities of proteins other than meat proteins does not induce glomerular hyperfiltration and is therefore unlikely to be harmful for glomerular filtration. At last, the GFR response to a high protein load does not appear to be reduced in women when compared to men.

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Statement of Ethics
This study protocol was reviewed and approved by the local Ethical Committee (the “Commission cantonale d’Ethique de la Recherche sur l’être humain”), under number 2016-01971. Written informed consent was obtained from participants.

Conflict of Interest Statement
None declared.
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**Author Contributions**

Menno Pruijn, Aikaterini Damianaki, and Michel Burnier: contributed to the conception and study design, analysis of data, and drafted of the article; provided intellectual content; and approved the final version submitted. Wendy Brito, Jonas Garessus, and Marc Maillard: contributed to data collection and analysis, drafted the article, provided intellectual content, and approved the final version. Antoine Schneider: contributed to the conception and study design, drafted the article, and approved the final version.

**Data Availability Statement**

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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