Circulating Proprotein Convertase Subtilisin Kexin Type 9 Has a Diurnal Rhythm Synchronous With Cholesterol Synthesis and Is Reduced by Fasting in Humans

Lena Persson, Guoqing Cao, Lars Ståhle, Beatrice G. Sjöberg, Jason S. Troutt, Robert J. Konrad, Cecilia Gälman, Håkan Wällén, Mats Eriksson, Ingiöld Hafström, Suzanne Lind, Maria Dahlin, Per Åmark, Bo Angelin, Mats Rudling

Objective—To gain insight into the function of proprotein convertase subtilisin kexin type 9 (PCSK9) in humans by establishing whether circulating levels are influenced by diurnal, dietary, and hormonal changes.

Methods and Results—We monitored circulating PCSK9 in a set of dynamic human experiments and could show that serum PCSK9 levels display a diurnal rhythm that closely parallels that of cholesterol synthesis, measured as serum lathosterol. In contrast to these marked diurnal changes in cholesterol metabolism, serum low-density lipoprotein (LDL) cholesterol levels remained stable during the diurnal cycle. Depletion of liver cholesterol by treatment with the bile acid–binding resin, cholestyramine, abolished the diurnal rhythms of both PCSK9 and lathosterol. Fasting (>18 hours) strongly reduced circulating PCSK9 and lathosterol levels, whereas serum LDL levels remained unchanged. Growth hormone, known to be increased during fasting in humans, reduced circulating PCSK9 in parallel to LDL cholesterol levels.

Conclusion—Throughout the day, and in response to fasting and cholesterol depletion, circulating PCSK9 displays marked variation, presumably related to oscillations in hepatic cholesterol that modify its activity in parallel with cholesterol synthesis. In addition to this sterol-mediated regulation, additional effects on LDL receptors may be mediated by hormones directly influencing PCSK9.

Key Words: circulating PCSK9 ■ cholesterol synthesis ■ LDL cholesterol ■ diurnal rhythm ■ growth hormone ■ cholesterol-lowering drugs ■ lipoproteins

Genetic variants of proprotein convertase subtilisin kexin type 9 (PCSK9) influence plasma low-density lipoprotein (LDL) cholesterol in humans, accounting for both hypercholesterolemia and hypcholesterolemia and altered coronary risk.1,2 PCSK9 modulates the number of LDL receptors (LDLRs) by triggering the degradation of LDLRs.3 Gain-of-function mutations in the PCSK9 gene produce a phenotype of familial hypercholesterolemia,1 whereas loss-of-function mutations reduce plasma LDL cholesterol levels.2 Circulating PCSK9 is largely derived from the liver,4,5 and plasma levels relate to the hepatic expression of PCSK9.6–9 It is still unclear to what extent PCSK9 is physiologically regulated and if such regulation may influence plasma LDL cholesterol levels in humans. Novel therapies aiming at lowering serum LDL cholesterol by interfering with PCSK9 activity are under development.10 Fasting plasma levels of PCSK9 correlate positively with LDL cholesterol levels in healthy and diabetic patients7,11–14; however, in the most extensive study,7 with >3000 subjects, PCSK9 levels only predicted 7% of the variation in LDL cholesterol. In animals, the hepatic gene expression of PCSK9 is partly under hormonal control: treatment with glucagon or high-dose estrogen reduces PCSK9 mRNA and increases the number of LDLRs,15 whereas insulin15,16 and growth hormone (GH)17 both increase PCSK9 mRNA levels in rat liver.

The gene expressions of PCSK9, the LDLR and 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase (the rate-limiting step in cholesterol biosynthesis), are all regulated by the transcription factor steroid regulatory binding protein (SREBP)-2 that is activated by a reduction of membrane...
cholesterol. A positive correlation between mRNA levels for HMG-CoA reductase, LDLR, PCSK9, and SREBP-2 has also been shown in human liver. Hepatic cholesterol synthesis exhibits a diurnal rhythm, peaking at late night, which can be monitored by measuring the serum marker lathosterol. In this study our aims were as follows: (1) to explore whether circulating PCSK9 has a diurnal rhythm driven by the level of hepatic cholesterol, (2) to evaluate in detail if PCSK9 in serum is altered by food deprivation or a ketogenic diet, and (3) to determine if treatment with GH alters serum PCSK9. Therefore, we analyzed a large set of previous and new experiments in humans designed to study metabolic changes during (1) the diurnal phases and the interference of such by cholesterol; (2) short (up to 18 hours) and long (more than 18 hours) fast, including starvation (7 days); and (3) GH treatment. An experiment in which subjects were treated with atorvastatin was used as a reference. Our data show that the level of circulating PCSK9 is under more dynamic control than previously conceived and that such a regulation may contribute to the relatively stable LDL cholesterol levels generally observed in healthy humans.

Methods

Subjects and Study Design

Nine different experiments, with 90 patients or healthy subjects, were analyzed.
arranged by the Swedish Survival Guild. The study was designed to evaluate survival strategy at authentic outdoor conditions. Twelve healthy participants (5 women and 7 men) were divided into 2 groups. Up to 66 hours of fasting was compared with up to 50 hours of sleep deprivation in a crossover design, with baseline measurements on days 1 and 8. Group 1 (Figure 4A and 4B) started with a 3-day fast; samples were drawn after 18, 42, and 66 hours. After sampling on day 4, 500 kcal were consumed (divided into 4 meals over 24 hours), followed by unrestricted caloric intake on days 5 to 8. From day 5 to day 7, subjects were also sleep deprived. Group 2 (Figure 4C and 4D) followed the same scheme but in the reverse order. Further details of this experiment are shown in the supplemental data.

The effects of GH treatment (Figure 5A–5C) were evaluated by analyses of samples from 15 healthy men treated with increasing doses of GH for 1 week per dose; the last week, 0.1 IU/kg per day was taken. Atorvastatin, 80 mg/d, was given to 19 subjects (14 women and 5 men) for 4 weeks in a double-blind crossover design with a 4-week washout; fasting morning samples were drawn (Figure 5D–5F).

All subjects or their parents gave informed consent to participate in the studies, which were all approved by the Ethics Committee of Karolinska Institute, Stockholm, Sweden. Samples were stored at −80°C.

Serum Analyses

PCSK9 levels were measured using a PCSK9 dual–monoclonal antibody sandwich ELISA, with minor modifications, as previously described. Unesterified lathosterol was determined by isotope dilution mass spectrometry after the addition of deuterium-labeled internal standard, as previously described. Lathosterol levels were corrected for...
total cholesterol level, as previously outlined,30 because lathosterol is transported with cholesterol-rich lipoproteins.

Statistics
Differences between groups were tested by repeated measurements using 1-way ANOVA followed by the Dunnett multiple comparison test or, when appropriate, by 2-tailed paired Student t test using computer software (GraphPad Prisim). Diurnal rhythms were evaluated by fitting sinus curves with a 24-hour period by nonlinear regression.

Results
Circulating PCSK9 Has a Diurnal Rhythm Synchronous With Cholesterol Synthesis
When circulating PCSK9 was observed during the day in healthy subjects, the levels showed a diurnal rhythm, with a nadir between 3 and 9 PM and a peak at 4:30 AM (Figure 1A). These distinct diurnal changes were similar to those for serum lathosterol, a marker for cholesterol synthesis, as previously reported (Figure 1A).21 Both PCSK9 and lathosterol levels correlated strongly to 24-hour sinus functions ($r^2=0.81$ [Figure 1D] and $r^2=0.62$ [Figure 1E], respectively). Interestingly, despite these pronounced changes in PCSK9 and lathosterol levels, serum cholesterol levels were stable (Figure 1B). To evaluate if food intake was related to the reduction of PCSK9 that occurred between 9 AM and 4 PM, we measured its concentration in a repeated study in which the initial overnight fast was prolonged until 4PM (Figure 1A versus Figure 1C).
Depletion of Hepatic Cholesterol Increases PCSK9 Levels and Cholesterol Synthesis

Next, we evaluated if short-term depletion of hepatic cholesterol would influence serum PCSK9 levels. Treatment with cholestyramine during the first 12 hours of a 4-day experiment abolished the diurnal rhythm of PCSK9 the following day (Figure 2). Moreover, PCSK9 levels remained elevated 2 to 3 days after the cessation of treatment. A similar pattern of response was seen for lathosterol, indicating that both PCSK9 and HMG-CoA reductase expressions were induced rapidly after the reduction of liver microsomal cholesterol.

Fasting, But Not a Ketogenic Diet, Suppresses PCSK9 Serum Levels

We analyzed samples from 2 experiments in which subjects had fasted for 2 or 7 days, respectively;23,24; and found that PCSK9 levels were reduced by 70% to 80% (Figure 3A and 3D). In parallel, serum lathosterol levels were reduced by 50% to 60% (Figure 3B and 3E), again showing a parallel regulation with PCSK9. The serum total cholesterol level was unchanged and only tended to increase in these situations (Figure 3C and 3F), whereas serum ketone bodies were strongly increased from 0.08±0.07 (mean±SD) to 3.2±0.8 mmoles/L, and from 0.09±0.1 (mean±SD) to 6.4±1.4 mmoles/L, in response to 2 and 7 days of fasting, respectively, as previously described.26 To exclude that ketosis per se may reduce PCSK9 levels, we studied the effect of a ketogenic diet.25 Although this fat- and protein-rich diet reduces glucose in a similar way as fasting,26 it did not reduce circulating PCSK9; instead, lathosterol and total cholesterol levels increased by 24% and 37%, respectively (Figure 3G–3I).

Parallel temporal changes of circulating PCSK9 and cholesterol synthesis over 3 days of fasting were determined from an experiment in which 2 groups (n = 6 for both) fasted for up to 66 hours and were sleep deprived for up to 50 hours, using a crossover design (Figure 4). The supplemental Figure provides a detailed description. After 18 hours of fasting, initiated at 4 PM, PCSK9 levels were reduced by 35% and 38% in the 2 groups, respectively (Figure 4A and 4C). Continued fasting reduced serum levels of PCSK9 even further; and after 66 hours, they were lowered by 64% and 97%, respectively. After the restricted diet, PCSK9 levels increased somewhat. Lathosterol levels closely followed those of PCSK9 in both groups (Figure 4A and 4C); and when relating all individual PCSK9 and lathosterol values of this experiment to each other, a strong correlation was evident (Figure 4E). Furthermore, although serum triglycerides were reduced by fasting as expected (Figure 4B and 4D), serum total and LDL cholesterol levels were stable in both groups during the whole period (Figure 4B and 4D).

Opposing Changes in PCSK9 Levels During Stimulation of LDLRs by GH Treatment or Statin Therapy

We assayed PCSK9 levels in 15 subjects treated with GH28 (Figure 5A–5C). GH treatment lowered LDL cholesterol levels by 16% in this experiment, whereas PCSK9 levels were reduced by 17% (Figure 5A and 5C). Interestingly, statin therapy in humans increases liver LDLRs to a similar extent as does GH treatment.32 However, atorvastatin treatment, which reduced serum LDL cholesterol by 50%, increased serum PCSK9 levels by 33%, in agreement with previous observations.6,33 (Figure 5D–5F). This finding is in concert with the proposed parallel regulation of HMG-CoA reductase and PCSK9 by SREBP-2 in this situation33 and indicates that GH treatment stimulates LDLRs through mechanisms other than statin therapy.

Discussion

The present studies demonstrate that, in several experimental situations, fasting strongly reduces circulating PCSK9 in healthy humans. This occurs concomitantly with suppressed cholesterol synthesis, as monitored by lathosterol concentrations. Despite these pronounced dynamic changes, LDL cholesterol levels were not reduced. In fasting mice, a reduced expression of PCSK9 protects against hypercholesterolemia and postprandial hypertriglyceridemia.34,35 The strong correlations between PCSK9 and lathosterol observed in our studies make it reasonable to postulate that hepatic PCSK9 and HMG-CoA reductase are regulated by a common mechanism in these situations. SREBP-2 is an established coregulator of the gene expression of PCSK9, HMG-CoA reductase, and the LDLR18,19; and data indicate that this concept is also valid in humans.36 SREBP-2 is mainly driven by the sterol content in the cell membrane,18,19 and it is known that fasting increases liver, but not plasma, cholesterol in pigs.36 The lack of any reduction of serum LDL cholesterol levels in response to fasting, despite a strong decrease in serum PCSK9, would support the concept that the gene expression of the LDLR, in addition to that of PCSK9 and HMG-CoA reductase, is suppressed during fasting. Such a situation would be in agreement with what has been observed in mice.16 An important question to be answered is whether the fasting-induced response with an 80% reduction of plasma PCSK9 is important for the adaptation to fasting/starvation.

The dynamic regulation of PCSK9 demonstrated in fasting humans also seems to operate during the diurnal phases in humans. Serum PCSK9 had the same diurnal variation as cholesterol synthesis.21,22 Notably, these diurnal changes in PCSK9 occurred while LDL cholesterol levels were stable. One explanation for this may be that dynamic changes of the regulatory pool of hepatic membrane cholesterol occur during the day. At late night, when cholesterol synthesis and serum PCSK9 reach their peaks, presumably because of a nadir in liver cholesterol, the LDLR transcriptional activity should also peak. The fact that serum LDL cholesterol remained stable suggests that the transcriptional responses of PCSK9 and LDLR outbalance each other, resulting in constant LDLR numbers and, consequently, stable serum LDL levels. The findings of a diurnal variation of PCSK9 concomitant with stable serum LDL levels should partly explain why serum PCSK9 levels relate poorly to plasma LDL cholesterol. This weak correlation between serum PCSK9 levels and LDL cholesterol has been interpreted to indicate that circulating PCSK9 is a poor measure of PCSK9 activity.7 In contrast, our present findings instead suggest that the circulating PCSK9...
level may actually well reflect PCSK9 activity. The SREBP pathway in mice has recently been shown to be governed by both nutritional status and an independent circadian clock function exerted by REV-ERB α. Short-term fasting (18 hours) until 4 PM did not influence the 9 AM to 4 PM pattern of diurnal rhythms of either PCSK9 or lathosterol level. However, whether prolonged fasting (for 1 to 3 days) eliminates these rhythms is still unclear. The fact that prolonging the overnight fast from 9 AM to 4 PM (total fast of 18 hours) did not result in serum PCSK9 levels that could be distinguished from those during the normal diurnal rhythm of PCSK9 highlights the importance of always having appropriate controls when studying PCSK9 serum levels.

Our finding that a short-term depletion of hepatic cholesterol by cholestyramine treatment resulted in a prolonged increase of serum PCSK9 and of cholesterol synthesis was unexpected because it indicates that short-term perturbations of hepatic cholesterol metabolism may induce long-lasting effects on the cholesterol balance. Clearly, further studies on how regulation of PCSK9 may influence the dynamics of hepatic cholesterol metabolism in humans will be important. In addition to sterol-mediated regulation of PCSK9, other mechanisms, such as those exerted by various hormones, may be involved. In humans, but not in rodents, an increase in GH secretion is part of the normal response to fasting, contributing to the maintenance of a normal blood glucose level. The pleiotropic effects of GH on cholesterol metabolism include stimulation of hepatic LDLRs and LDL clearance, leading to reduced LDL cholesterol. We could show that PCSK9 is reduced after GH treatment, which could partly explain the reduction of plasma LDL cholesterol in this situation. This response is opposite to that observed in rats after GH treatment. Indeed, there are several species differences between rodents and humans regarding the effects of GH on cholesterol metabolism. Although we cannot exclude that GH may exert some or all of its effect on PCSK9 through SREBP-2 because of changes in microsomal cholesterol biosynthesis, the potential relevance in humans of other fasting-related hormones that regulate the expression of hepatic PCSK9 in rodents, such as glucagon and insulin, needs further exploration. A progressive relative lack of GH may also underlie the known reduction in LDL clearance that occurs with ageing.

The present studies provide novel information on the regulation of circulating PCSK9 during basal physiological conditions. From our data, the hypothesis emerges that during the normal diurnal rhythms of either PCSK9 or lathosterol level, the potential relevance in humans of other fasting-related hormones may be involved. In humans, but not in rodents, an increase in GH secretion is part of the normal response to fasting, contributing to the maintenance of a normal blood glucose level. The pleiotropic effects of GH on cholesterol metabolism include stimulation of hepatic LDLRs and LDL clearance, leading to reduced LDL cholesterol. We could show that PCSK9 is reduced after GH treatment, which could partly explain the reduction of plasma LDL cholesterol in this situation. This response is opposite to that observed in rats after GH treatment. Indeed, there are several species differences between rodents and humans regarding the effects of GH on cholesterol metabolism. Although we cannot exclude that GH may exert some or all of its effect on PCSK9 through SREBP-2 because of changes in microsomal cholesterol biosynthesis, the potential relevance in humans of other fasting-related hormones that regulate the expression of hepatic PCSK9 in rodents, such as glucagon and insulin, needs further exploration. A progressive relative lack of GH may also underlie the known reduction in LDL clearance that occurs with ageing.

The proposed use of current and novel treatment modalities aiming at interacting with PCSK9.

Acknowledgments

We thank Ingela Arvidsson, BS, for expert technical assistance; and Catharina Sjöberg, RN, Katarina Hertel, RN, and Sabine Söllow-Barin, RN, for valuable assistance in blood samplings.

Sources of Funding

This study was supported by the Swedish Research Council; the Stockholm City Council (ALF); the Swedish Heart-Lung and Diabetes Foundations; the Foundation of Old Female Servants; the Swedish Rheumatism Association; and the Cardiovascular Program, Karolinska Institute/Stockholm City Council.

Disclosures

Drs G.C., J.S.T., and R.J.K. are employees of Eli Lilly and Company.

References


Circulating Proprotein Convertase Subtilisin Kexin Type 9 Has a Diurnal Rhythm Synchronous With Cholesterol Synthesis and Is Reduced by Fasting in Humans
Lena Persson, Guoqing Cao, Lars Stähle, Beatrice G. Sjöberg, Jason S. Troutt, Robert J. Konrad, Cecilia Gälman, Håkan Wallén, Mats Eriksson, Ingiäld Hafström, Suzanne Lind, Maria Dahlin, Per Åmark, Bo Angelin and Mats Rudling

Arterioscler Thromb Vasc Biol. 2010;30:2666-2672; originally published online September 30, 2010;
doi: 10.1161/ATVBAHA.110.214130
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/12/2666

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/09/30/ATVBAHA.110.214130.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplement Material.

Detailed description of the experiment shown in Figure 4 where the dynamic effects of 1-3 days of fasting or sleep deprivation were evaluated in 12 healthy volunteers (5 women and 7 men) divided into two groups (6/group). Up to 66h of fasting was compared with up to 50h of sleep deprivation in a crossover design with base line measurements on day 1 and day 8.

Meals were taken at fixed times, and blood samples were drawn once daily at 10 AM, 2h after breakfast. Group 1, started with 3 days of fast; samples drawn after 18, 42 and 66h of fast. After sampling day 4, 500 kcal were consumed (divided into four meals over 24h), followed by unrestricted caloric intake on days 5 to 8. From day 5 to day 7 subjects were also sleep deprived. Group 2 followed the same scheme but in the reverse order.

**Supplemental Table I**

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Day</th>
<th>Time</th>
<th>Blood drawn</th>
<th>Situation at time of blood sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 AM</td>
<td>Yes</td>
<td>None = Basal level</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4 PM</td>
<td>No</td>
<td>Start of fast</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10 AM</td>
<td>Yes</td>
<td>18h of fast</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10 AM</td>
<td>Yes</td>
<td>42h of fast</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10 AM</td>
<td>Yes</td>
<td>66h of fast, end of fast.</td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>24hrs</td>
<td>No</td>
<td>125kcal is digested per meal in four meals (=500kcal)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10 AM</td>
<td>Yes</td>
<td>End of restricted calorie intake</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10 AM</td>
<td>Yes</td>
<td>26h since last sleep</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10 AM</td>
<td>Yes</td>
<td>50h since last sleep, end of sleep deprivation</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10 AM</td>
<td>Yes</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group2</th>
<th>Day</th>
<th>Time</th>
<th>Blood drawn</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 AM</td>
<td>Yes</td>
<td>None = Basal level</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10 AM</td>
<td>Yes</td>
<td>26h since last sleep</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10 AM</td>
<td>Yes</td>
<td>50h since last sleep, end of sleep deprivation</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4 PM</td>
<td>No</td>
<td>Start of fast</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10 AM</td>
<td>Yes</td>
<td>18h of fast</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10 AM</td>
<td>Yes</td>
<td>42h of fast</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10 AM</td>
<td>Yes</td>
<td>66h of fast, end of fast.</td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td>24hrs</td>
<td>No</td>
<td>125kcal is digested per meal in four meals (=500kcal)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10 AM</td>
<td>Yes</td>
<td>End of restricted calorie intake</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>10 AM</td>
<td>Yes</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>
Ketogenic diet

The administration of the Ketogenic Diet (KD) followed a standard protocol for the classic KD, which is a slightly modified version of the protocol of the Johns Hopkins Hospital (Freeman et al., 1998).

Initially, children were hospitalized during a 4–5-day stay. Before starting the diet, the children fasted 24-36 hours. The diet was introduced gradually by increasing the amount of calories reaching a full diet within 3 days. The vast majority of children started the diet on a 4:1 ratio of fat to protein and carbohydrates. The calculations of the total amount of calories were based on a 2-day diary kept by the parents before admission. The caloric needs of the child were modified to the recommended amount of calorie according to age as well as to the expected level of physical activity and rate of metabolism. The target of the total daily calorie intake was based on approximately 75% of the recommended daily allowance of calories for the child’s desirable weight for height (the 50th percentile of weight for height). As for the protein content, a minimum of 1g/kg body weight per day was given. From the start of the diet, all children were supplemented with multivitamins and minerals (including calcium, magnesium, zinc, and selenium), and all were provided with 100 mg/kg/day of carnitine. After one month on the diet, children were usually supplemented with 4 g/day of omega-3 fatty acids. The total calorie level and the composition of individual meal plans and supplements were calculated by a dietitian.