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A semi-automated mass spectrometric immunoassay coupled to selected reaction monitoring (MSIA–SRM) reveals novel relationships between circulating PCSK9 and metabolic phenotypes in patient cohorts

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a key regulator of circulating low density lipoprotein cholesterol (LDL-C) levels. Besides its full-length mature form, multiple variants of PCSK9 have been reported such as forms that are truncated, mutated and/or with posttranslational modifications (PTMs). Previous studies have demonstrated that most of these variants affect PCSK9's function and thereby LDL-C levels. Commercial ELISA kits are available for quantification of PCSK9, but do not allow discrimination between the various forms and PTMs of the protein. To address this issue and given the complexity and wide dynamic range of the plasma proteome, we have developed a mass spectrometric immunoassay coupled to selected reaction monitoring (MSIA–SRM) for the multiplexed quantification of several forms of circulating PCSK9 in human plasma. Our MSIA–SRM assay quantifies peptides spanning the various protein domains and the S688 phosphorylation site. The assay was applied in two distinct cohorts of obese patients and healthy pregnant women stratified by their circulating LDL-C levels. Seven PCSK9 peptides were monitored in plasma samples: one in the prodomain prior to the autocleavage site at Q152, one in the catalytic domain prior to the furin cleavage site at R218, two in the catalytic domain following R218, one in the cysteine and histidine rich domain (CHRD) and the C-terminal peptide phosphorylated at S688 and unmodified. The latter was not detectable in sufficient amounts to be quantified in human plasma. All peptides were measured with high reproducibility and with LLOQ and LOD below the clinical range. The abundance of 5 of the 6 detectable PCSK9 peptides was higher in obese patients stratified with high circulating LDL-C levels as compared to those with low LDL-C ($p < 0.05$). The same 5 peptides showed good and statistically significant correlations with LDL-C levels ($0.55 < r < 0.65$; $0.0002 \leqslant p \leqslant 0.002$), but not the S688 phosphorylated peptide. However, this phosphopeptide was significantly correlated with insulin resistance ($r = 0.48$; $p = 0.04$). In the pregnant women cohort, none of the peptides were associated to LDL-C levels. However, the 6 detectable PCSK9 peptides, but not PCSK9 measured by ELISA, were significantly correlated with serum triglyceride levels in this cohort. Our results also suggest that PCSK9 circulates with S688 phosphorylated at high stoichiometry. In summary, we have developed and applied a robust and sensitive MSIA–SRM assay for the absolute quantification of all PCSK9 domains and a PTM in human plasma. This assay revealed novel relationships between PCSK9 and metabolic phenotypes, as compared to classical ELISA assays.

Abbreviations: PCSK9, proprotein convertase subtilisin/kexin type 9; LDL-C, low density lipoprotein cholesterol; PTM, posttranslational modification; MSIA–SRM, mass spectrometric immunoassay coupled to selected reaction monitoring; LLOQ, lower limit of quantification; LOD, limit of detection; CHRD, cysteine and histidine rich domain; LDLR, low density lipoprotein receptor; ApoB, apolipoprotein B; BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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1. Introduction

The secretory proprotein convertase subtilisin/kexin type 9 (PCSK9) [1] is a key regulator of circulating low density lipoprotein cholesterol (LDL-C) levels. Its gene represents the third locus associated with familial hypercholesterolemia, following the low density lipoprotein receptor (LDLR) and apolipoprotein B (ApoB) [2]. Numerous loss and gain of function mutations have been described in the gene encoding PCSK9 (reviewed in [3]). The reduced cardiovascular risk in patients with loss-of-function alleles [4] has prompted the development of monoclonal antibodies for the treatment of hypercholesterolemia that are currently in clinical trials, some of them as far as in phase 3 (reviewed in [3,5]).

PCSK9 affects levels of circulating LDL particles by decreasing the level of the LDLR protein at the plasma membrane [6,7]. It does so through an extracellular pathway, where circulating PCSK9 binds the EGF-A domain of the LDLR protein leading to internalization and degradation of the complex, thereby rendering LDLR unavailable for the cellular uptake of LDL-C [8]. Intracellular pathways have also been proposed, where PCSK9 can enhance LDLR degradation prior to secretion [9] or promotes its exit from the endoplasmic reticulum [10].

A positive association between circulating PCSK9 and LDL-C has been described in various patients cohorts, with correlation coefficients ranging from 0.24 to 0.54 [11–18]. These studies report PCSK9 abundance values obtained by ELISA assays, some of them being commercially available. There is, however, a lack of standardization amongst measurements obtained from different ELISA kits. Moreover, this assay intrinsically yields only one value per sample, so it lacks the discriminative power to resolve various protein forms and monitor posttranslational modifications (PTMs). Yet several PCSK9 protein species have been detected in plasma [19]. The shorter, Furin-cleavage product of PCSK9 is believed to be inactive [19–22], or to show reduced activity [23]. PTMs of PCSK9 have also been observed such as sulfation at tyrosine 38 [19], phosphorylation at serines 47 and 688 [24] and N-glycosylation at asparagine 533 [1]. Depending upon the antibody's specificity, an ELISA will report on either the mature, or both the mature and other protein species (including the shorter, furin-cleaved species), without discrimination or information on PTMs.

We describe herein a mass spectrometric immunoassay for the monitoring of PCSK9 abundance including one of its post-translational modifications. Immuno-capture of the protein prior to mass spectrometric analysis addresses the issue of the low abundance of the protein (low ng/ml) compounded with the wide dynamic range of plasma proteins, a non-negligible challenge for current mass spectrometers. The specificity and multiplexing capability of SRM provides a means to assess distinct domains and PTMs. We have previously briefly described the development of a PCSK9 MSIA–SRM assay [25]. Herein we have extended our work with the inclusion of a phosphopeptide and have applied this assay to two distinct patient cohorts to study the relationship between PCSK9 abundance, blood lipid profile and insulin resistance.

2. Materials and methods

2.1. Human subjects

2.1.1. Obese patient cohort

Twenty-nine obese patients enrolled between 2012 and 2014 in the ETAPP cohort were studied. The goal of the ETAPP cohort is to study factors involved in the complications associated with obesity before and after bariatric surgery. Only pre-surgical samples were included in the present analyses. Patients were enrolled if they were (1) obese with 35–55 kg/m^2 BMI, (2) 18–60 years old, (3) sedentary (<3 h weekly physical activity). Patients were excluded from study participation if they had (1) type 2 diabetes for more than 10 years or type 1 diabetes, (2) an acute cardiovascular event in the last 6 months, (3) an infection in the last month, (4) chronic or inflammatory diseases or cancer, (5) a known coagulation disorder, (6) uncontrolled thyroid or pituitary disorders, (7) been treated with insulin or medications known to interfere with metabolism except stable hypothyroidism replacement therapy, (8) history of alcohol or drug abuse. This study conformed to the principles outlined in the Declaration of Helsinki as revised in 2000 and was approved by the IRCM and Hôpital du Sacré-Coeur de Montréal Ethics Committees. All subjects gave prior written informed consent.

In a first analysis, participants were stratified as having high or low LDL-C levels according to the median value of the group. Accordingly, patients with LDL-C levels <3.06 mM were classified as low LDL-C group (n = 14; 2 males, 12 females) whereas patients having LDL-C levels $\geqslant$3.06 mM were classified as high LDL-C group (n = 15; 1 male, 14 females). In a second analysis, we studied the relationship between PCSK9 peptides abundance and insulin resistance as assessed by HOMA-IR. For the purpose of this second analysis, obese patients who had type 2 diabetes were excluded (n = 11) in order to avoid the confounding effects of their diabetes treatment on insulin resistance.

All anthropometric and blood measurements were obtained after an overnight fast. Blood was collected in EDTA tubes, centrifuged at a relative centrifugal force of 850 at 4 °C for 10 min and plasma was then aliquoted and stored at −80 °C until analysis. The anthropometric and lipid profiles of each group are summarized in Supplementary Table 1.

2.1.2. Pregnant women cohort

The clinical samples were from an ongoing study aimed at determining the reference ranges of novel biomarkers during pregnancy in healthy women. This study was approved by the ethics committee of the McGill University Health Center. Written informed consent was obtained and the samples were provided to the proteomic platform after anonymization. Healthy women at 12–13 weeks of pregnancy (n = 88) were stratified according to their LDL-C levels and for the purpose of the present study, the 12 women with the highest LDL-C levels (>2.8 mmol/L) were classified as high LDL-C group whereas the 12 women with the lowest LDL-C values (<1.75 mmol/L) were classified as low LDL-C group. Blood was collected in EDTA tubes after an overnight fast, centrifuged at a relative centrifugal force of 850g at 4 °C for 10 min and plasma was then aliquoted and stored at −80 °C until analysis. High density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and triglyceride (TG) levels were measured with the use of standard automated assays, and LDL cholesterol was calculated with the Friedewald equation. PCSK9 was measured by ELISA assay as described in [15]. The lipid profiles of each group are summarized in Supplementary Table 2.

2.2. Chemicals and reagents

Recombinant human PCSK9 was purchased from R&D Systems, resuspended according to the manufacturer's recommendations, aliquoted for single use and stored at −80 °C until analysis. Custom MSIA DARTs were covalently coupled to PCSK9 antibody AF3888 from R&D Systems by Thermo Fisher Scientific as previously described [25]. This PCSK9 antibody AF3888 is polyclonal and was raised against the mature full-length form of PCSK9 (Gln31Gln692). It was previously shown that this antibody recognizes PCSK9's pro-domain, full-length form, furin-cleaved form as well as the N-terminal segment of the furin-cleaved PCSK9 [22].