

**A Novel Test for the Measurement of Skin Cholesterol,** Robert Zawdydiwski,<sup>1\*</sup> Dennis L. Sprecher,<sup>2</sup> Michael J. Eveleigh,<sup>1</sup> Peter Horsewood,<sup>1</sup> Carol Carte,<sup>1</sup> and Michelle Patterson<sup>1</sup> (<sup>1</sup> IMI International Medical Innovations Inc., 4211 Yonge St., Suite 300, Toronto, ON M2P 2A9, Canada; <sup>2</sup> The Cleveland Clinic Foundation, Preventive Cardiology Clinic, 9500 Euclid Ave., Cleveland, OH 44195; \* author for correspondence: fax 905-521-2613, e-mail rzawdydiwski@imin.ca)

Increased serum cholesterol is a risk factor for atherosclerosis and coronary artery disease (CAD) (1, 2). Reduction of serum cholesterol has, therefore, been targeted for the prevention and management of CAD with cholesterol screening and is the primary tool for initial risk assessment (3, 4). Cholesterol, however, can also be quantified in skin.

Skin reflects the vascular changes associated with age and aortic atherosclerosis. A simple, noninvasive procedure for the estimation of skin cholesterol, the "three-drop" test, has been proposed as an alternative screening method (5). The test, which uses three different concentrations of a digitonin-copolymer-horseradish peroxidase (HRP) conjugate and visual scoring, is capable of discriminating among healthy individuals, those at risk of developing atherosclerosis, and those with overt disease. Cholesterol 1,2,3<sup>TM</sup> is a refinement of this procedure. We describe here some properties of this test and report on our initial evaluation in a prospective clinical trial.

Cholesterol 1,2,3 uses quantitative interpretation and a single concentration of detector. Briefly, the detector and the positive control each are added to die-cut wells in a foam template affixed to the palm. After a 1-min incubation, the reagents are removed by blotting and the HRP substrate is added to these wells and to a third well, which serves as negative control. After an additional 2-min incubation, color development in the center test well is measured by reflectance with a hand-held instrument (MD22 Spectrophotometer; X-Rite, Inc.) interfaced with a computer, and the result is reported numerically as the hue. Assay validity is assessed by visual interpretation of control wells.

The specificities of digitonin conjugates have been demonstrated previously with several model systems [crystalline cholesterol, cholesterol-rich tissue sections, and antibody-immobilized LDL-cholesterol (LDL-C)], and the amount of skin-bound conjugate has been shown to correlate with epidermal cholesterol content (5). We extended the specificity studies to cholesterol analogs and skin lipids in a competition assay. Lipids and plant sterols were preincubated with the conjugate, and the activity of the uncomplexed conjugate was determined subsequently on cholesterol-coated microwells. Lipids were representative of the predominant classes found in the palm (6). Phytosterols were included because of their cholesterol-lowering activity and use in dietary management of CAD (7). The conjugate exhibited preferential affinity for sterols (Table 1), and although it showed negligible binding to a triglyceride [(TG); triolein] and a free fatty acid

**Table 1. Molecular specificity of the skin cholesterol test.<sup>a</sup>**

Lipid	I <sub>max</sub> <sup>b</sup> ± SD, %	I <sub>50</sub> <sup>c</sup> mg/L	Relative affinity <sup>d</sup>
β-Sitosterol	97 ± 2	14	1.14
Cholesterol	89 ± 3	16	1.00
Stigmastanol	82 ± 11	21	0.76
Cholesterol sulfate	92 ± 4	29	0.55
Ceramide 2	76 ± 7	53	0.30
Ceramide 5	76 ± 3	55	0.29
Cholesteryl oleate	52 ± 7	110	0.15
Triolein	41 ± 14	>170 <sup>e</sup>	<0.09
Stearic acid	9 ± 10	— <sup>f</sup>	—

<sup>a</sup> Affinity of conjugate for selected lipids (Sigma) was determined as described in the text.

<sup>b</sup> Inhibition of the conjugate binding to solid-phase cholesterol at the highest dose tested (125 mg/L).

<sup>c</sup> Concentration for 50% inhibition; estimated from the mean (n = 3) hyperbolic regression curve of data normalized to cholesterol as the internal reference (n = 17).

<sup>d</sup> Cholesterol I<sub>50</sub>/Lipid I<sub>50</sub>.

<sup>e</sup> Approximated by extrapolation.

<sup>f</sup> Inhibition too low for estimation.

(stearic acid), it demonstrated some affinity for the ceramides (30%, relative to cholesterol). Reactivity with β-sitosterol and stigmastanol is not surprising given their structural similarity with cholesterol. Although β-sitosterol can accumulate in surface skin, it constitutes only a minor fraction of epidermal lipids, even in individuals whose dietary intake of plant sterols is high (8, 9). Phytosterols are poorly absorbed (10–12); therefore, neither sterol is likely to contribute substantively to any quantification ascribed to skin cholesterol. The relatively high affinity of the conjugate for cholesterol sulfate is unexpected because digitonide formation is favored with sterols containing a free 3β-hydroxy group (13). However, several exceptions have been noted, including some 3β esters (14). The significance of the conjugate association with ceramides is unclear, although it is known that digitonin does not react exclusively with sterols (13). Whether these observations reflect specific interactions or artifacts of altered specificity of conjugated digitonin is open to speculation. Overall, these data indicate that Cholesterol 1,2,3 exhibits the desired molecular specificity.

We assessed the clinical performance of Cholesterol 1,2,3 in a study population of generally healthy patients, with no known CAD, who received the treadmill stress test at the Cleveland Clinic Foundation because of reported chest discomfort. The objectives were to determine the relationships between Cholesterol 1,2,3 and a positive stress test and between the skin test and serum lipids. The study protocol was approved by the Institutional Review Board, and informed consent was obtained. Patients 30 years of age and older were enrolled. Exclusion criteria included hepatitis, pregnancy within the previous 4 months, psoriasis or eczema on either hand, or recent use (within 24 h of Cholesterol 1,2,3 test) of topical medication, cream, or lotion. Patients using β-blockers, those

with evidence of left bundle-branch block, or those with uninterpretable S-T segments on the electrocardiogram were also excluded.

One hundred eleven patients were evaluated. The population (70% male) had a median age of 50 years (range, 34–77 years) and the following median (range) lipid concentrations: 5.30 (2.97–9.00) mmol/L [205 mg/dL (115–348 mg/dL)] total cholesterol; 1.11 (0.72–3.00) mmol/L [43 mg/dL (28–116 mg/dL)] HDL-cholesterol; 3.34 (1.40–7.03) mmol/L [129 (54–272 mg/dL)] LDL-C; and 1.59 (0.50–9.82) mmol/L [141 (44–869 mg/dL)] TGs. These patients were tested with an earlier version of Cholesterol 1,2,3, where skin cholesterol was estimated by absorbance after the transfer of a 25- $\mu$ L aliquot of the reaction product to microwells. Fifteen subjects had positive stress tests; their median skin cholesterol was 0.059 absorbance units [(AU); range, 0.031–0.166 AU] compared with 0.040 AU (range, 0.003–0.118 AU) for the 96 patients with negative stress tests. The two groups were statistically different (Mann–Whitney *U*-test,  $P = 0.006$ ). The proportion of patients with positive stress tests paralleled the increase in skin cholesterol (Fig. 1). Logistic regression analysis (MINITAB<sup>®</sup> 12; Minitab Inc.) indicated that the Cholesterol 1,2,3 results were significantly correlated with

a positive stress test [unadjusted odds ratio (OR) = 1.42 per 0.1 AU increment; 95% confidence interval (CI) = 1.13–1.79;  $P = 0.003$ ]. The predictive value of skin cholesterol did not diminish after the adjustment for serum lipids (total cholesterol, HDL-cholesterol, LDL-C, and TGs) and other risk factors, including age, gender, body mass index, hip-to-waist ratio, familial history of CAD, diabetes, hypertension, and smoking (OR = 1.81; 95% CI = 1.13–2.88;  $P = 0.013$ ). History of CAD in the family was the only covariate to achieve significance ( $P = 0.039$ ), whereas age approached significance ( $P = 0.056$ ). Univariate analysis of serum lipids as predictors of positive stress tests indicated that total cholesterol (OR = 1.19 per 100 mg/L increment; 95% CI = 1.04–1.36;  $P = 0.011$ ) and LDL-C (OR = 1.30; 95% CI = 1.10–1.54;  $P = 0.002$ ) were associated with significant risk. These effects were completely mitigated ( $P = 0.62$  and  $0.67$ , respectively) after the inclusion of HDL-cholesterol, TGs, and other traditional CAD risk factors into the model incorporating skin cholesterol. There was no evidence of correlations between skin and serum cholesterol or skin cholesterol and TGs. These observations suggest that skin cholesterol may be an independent risk factor. Alternatively, it is conceivable that a putative correlation between skin and serum cholesterol escaped detection because of the limited size of the study population.

The current test uses a portable reader to measure color directly on the palm. The attribute, hue, is an angular measurement that describes the position of the colors of the visible spectrum arranged in a circle. Conversion of the HRP substrate to the colored product involves a continuous transition in color that is recorded by the instrument as an increase in the hue angle ( $h^\circ$ ). The HRP substrate of Cholesterol 1,2,3 has a nominal span between  $50^\circ$  (colorless) and  $\sim 200^\circ$  (blue) when read against the background of palmar skin, and it is strongly correlated with absorbance over the range (0–0.25 AU) examined in the laboratory ( $h^\circ = 490 \times A_{450} + 57$ ;  $r^2 = 0.96$ ;  $P < 0.001$ ). Hue, therefore, exhibits sufficient scope and dose-dependence to serve as a quantitative measure of skin cholesterol. For the sake of simplicity, we have deleted the units when reporting skin hue or cholesterol, pending development of an algorithm relating hue to skin cholesterol mass. Because skin hue contributes more to the value obtained in the hue measurements of subjects with low skin cholesterol, as a result of little or no color development in the substrate, we compared skin tone in racially diverse individuals. There was no difference in the background hue of the palmar skin of African Americans and Caucasians. Although we have not formally examined the effect of skin thickness as a variable, we found no effect of subject handedness on measurement of skin cholesterol.

The analytical characteristics of Cholesterol 1,2,3, incorporating the color quantification procedure, were determined in apparently healthy volunteers, with no known history of CAD (although serum cholesterol was not measured, some may have had increased serum and/or skin cholesterol), recruited from laboratory and office personnel at McMaster University (Hamilton, ON, Cana-

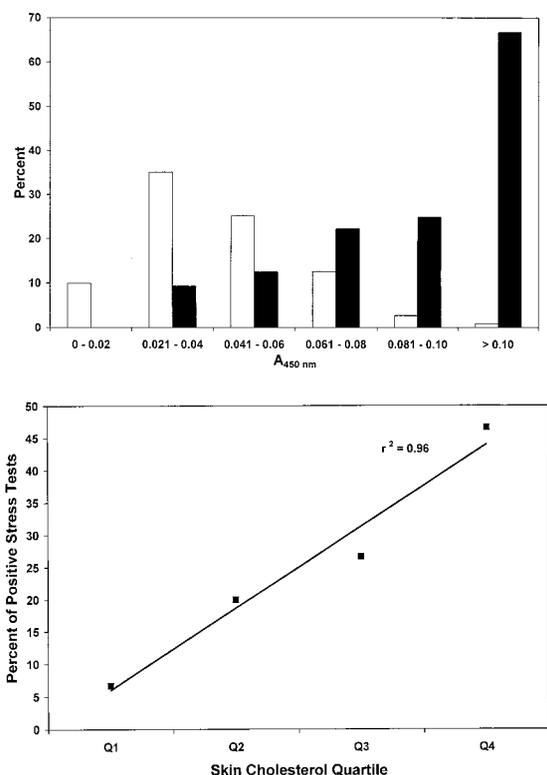


Fig. 1. Distribution and correlation of skin cholesterol with positive stress tests.

Top, skin cholesterol results in subjects with negative (□) and positive (■) treadmill stress tests shown as percentages of the total study population and population per absorbance range, respectively. Absorbance at 450 nm given as AU along the x-axis. Bottom, relationship between skin cholesterol and subjects with positive stress tests expressed as percentages of the total positive stress-test population.

da). The detection limit for skin cholesterol, defined as the value +2 SD from the mean background hue for a "zero" determination (conjugate omitted and replaced with diluent), was established in 20 individuals (9 males). The mean hue for the zero determinant was 65, and the SD was 8; therefore, the detection limit was 81.

The tentative reference interval for skin cholesterol incorporating the central 95% of observations, determined in an unselected population of adult volunteers (n = 115; >18 years of age; 62% female), was 61–134 (median, 93). There was no indication of gender difference. By comparison, skin cholesterol in a clinical population of 147 patients referred to the Cleveland Clinic Foundation for coronary angiography was 55–206 (median, 130).

Within-day precision was assessed in 6 tests (3 for each palm) performed in succession in 10 individuals. The mean subject skin cholesterol was 74–109; the CV was 5–19% (11% overall). Interassay variation was determined in five tests conducted over 5–9 days to minimize the potential influence of diet and/or life-style modification on skin cholesterol over prolonged periods. Day-to-day CV was 2–12% (7% overall; n = 10) in subjects with mean skin cholesterol of 86–111.

In review, we have described some of the characteristics and reported on a prospective evaluation of the Cholesterol 1,2,3, a simple, rapid, noninvasive procedure for quantification of skin cholesterol. The data revealed a positive correlation between skin cholesterol and abnormal treadmill stress-test results and suggested a possible role for the skin test in establishing risk for CAD. We have pursued the predictive potential of skin cholesterol in CAD. In a recent study of subjects referred for diagnostic catheterization, the presence and extent of vascular disease, as assessed by coronary angiography, was significantly associated with skin cholesterol (15). Determination of skin cholesterol values, therefore, could provide an independent, inexpensive screening tool for individuals at risk of disease and in need of more elaborate diagnostic follow-up.

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***Chlamydia pneumoniae* Seropositivity and Hyperhomocysteinemia Are Linked in Patients with Atherosclerosis**, Peter Wiesli,<sup>1\*</sup> Friedrich E. Maly,<sup>2\*</sup> Alfredo Meniconi,<sup>1</sup> Wolfgang Czerwenka,<sup>1</sup> Ulrich Hoffmann,<sup>3</sup> Wilhelm Vetter,<sup>1</sup> and Georg Schulthess<sup>1†</sup> (<sup>1</sup> Department of Internal Medicine, Medical Policlinic, <sup>2</sup> Institute of Clinical Chemistry, and <sup>3</sup> Department of Angiology, University Hospital, CH-8091 Zurich, Switzerland; \* these authors contributed equally to this work; † address correspondence to this author at: Department of Internal Medicine, University Hospital, CH-8091 Zurich, Switzerland; fax 0041-1-255 45 67, e-mail georg.schulthess@dim.usz.ch)

Atherosclerosis, with its different clinical manifestations, is the major cause of morbidity and mortality in the developed countries. *Chlamydia pneumoniae* infection and the ensuing chronic inflammation have been claimed to contribute to the atherosclerotic process (1). Concurrently, epidemiologic evidence suggests that hyperhomocysteinemia is associated with an increased atherosclerotic risk (2). Both *C. pneumoniae* infection and hyperhomocysteinemia have been assumed to increase the atherosclerotic risk independently of each other and independently of the classic risk factors.

In vitro, the growth of *C. pneumoniae* is enhanced in serum-free media and particularly by the depletion of lysine or methionine (3). In human metabolism, homocysteine is produced by demethylation of methionine, and defects in the recycling pathway (in which homocysteine is remethylated to methionine) are one cause of hyperhomocysteinemia (4). This constellation prompted us to investigate whether *C. pneumoniae* seropositivity and plasma homocysteine concentrations are related in patients with established atherosclerosis.