

Usefulness of Increased Skin Cholesterol to Identify Individuals at Increased Cardiovascular Risk (from the Predictor of Advanced Subclinical Atherosclerosis Study)

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In patients with symptomatic coronary heart disease, skin cholesterol (SC) content is associated with the presence and extent of coronary artery disease; however, its relation to subclinical arterial disease in asymptomatic patients is unknown. The purpose of this study was to determine the relations between SC and carotid intima-media thickness (CIMT) in asymptomatic subjects across a wide range of cardiovascular risk. SC was measured using a noninvasive assay. CIMT and carotid plaque presence were determined by high-resolution B-mode ultrasound. Associations among SC, CIMT, carotid plaque presence, and cardiovascular risk factors were evaluated by multivariable logistic regression analyses. SC and CIMT were measured in 565 asymptomatic subjects (57 ± 10 years of age, 38% women) from 6 sites in North America. The mean Framingham 10-year cardiovascular risk was $8.4 \pm 7.2\%$. A 10-U increase in SC was associated with a 12% increase in the odds of having increased CIMT ($p = 0.006$) and a 15% increase in carotid plaque presence ($p = 0.002$). Odds ratios (95% confidence intervals) associated with SC >110 U were 2.19 (1.25 to 3.85, $p = 0.006$) for increased CIMT and 2.89 (1.61 to 5.19, $p < 0.001$) for carotid plaque presence. In conclusion, SC identified the presence of advanced subclinical atherosclerosis. The relations among increasing SC, increasing CIMT, and carotid plaque presence were consistent across all levels of cardiovascular risk and were independent of cardiovascular risk factors. SC may be a useful test for cardiovascular risk prediction. © 2008 Elsevier Inc. All rights reserved. (Am J Cardiol 2008;101:986–991)

Approximately 11% of the body's total cholesterol content is in the skin.¹ Because skin cholesterol (SC) content is associated with deposition of cholesterol in the coronary arteries and aorta, it has been suggested that quantifying SC content may be a useful technique for identifying patients with atherosclerotic arterial disease.^{2,3} Using a noninvasive assay that measures cholesterol content within the stratum corneum, SC has been associated with the presence and extent of angiographic coronary artery disease^{4,5} and the presence of myocardial ischemia³ in patients with positive stress test results. In asymptomatic patients, there is an association between SC and coronary artery calcium⁶ and

circulating inflammatory markers⁷ and with carotid intima-media thickness (CIMT).⁸ These findings suggest that SC may be a marker of subclinical atherosclerosis. Because SC can be measured noninvasively using a point-of-care test, SC content could become a useful office tool for assessing cardiovascular risk, if it is shown to identify the presence of subclinical atherosclerosis. The purpose of this study was to determine the relation between SC content and common carotid artery CIMT in asymptomatic subjects across a wide range of cardiovascular risk.

Methods

This was a multicenter study performed at 6 centers in the United States. This study was approved by the institutional human subjects review board at each participating institution. All subjects provided informed consent before enrollment. Subjects were 40 to 80 years old. Exclusion criteria included known coronary artery disease, peripheral arterial disease, cerebrovascular disease, current use of cholesterol-lowering medications, hepatitis, pregnancy, or skin disease on either hand. Enrollment was stratified by site with a goal of recruiting $\geq 33\%$ women and $\geq 25\%$ African-Americans. Subject entry also was stratified by predefined tertiles of low ($<5\%$), intermediate (5% to 20%), or high ($>20\%$) risk of cardiovascular disease.⁹ Patients with type 2 diabetes mellitus were considered to be at high risk.⁹

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SC was measured noninvasively using Prevu*POC (PreMD, Inc., Toronto, Ontario, Canada).¹⁰ After cleaning with alcohol, a foam well template was affixed to the hypothenar eminence of the right palm, and a solution containing a synthetic digitonin–copolymer–horseradish peroxidase conjugate was applied. After a 1-minute incubation, the area was blotted and an indicator solution containing a horseradish peroxidase substrate was applied in the well and to positive and negative controls. SC levels were quantified based on color change as measured by a hand-held spectrophotometer (MD22 Spectrophotometer, X-Rite, Inc., Grand Rapids, Michigan). The resulting change in hue was reported in units. The test uses a detector reagent (digitonin/horseradish peroxidase–conjugated copolymer) to bind cholesterol. Each operator participated in a live demonstration of the Prevu*POC test and reviewed the training and certification procedures. For certification, each operator had to measure SC on the right and left palms of 3 volunteers, and the results were sent to the sponsor for verification. Operators were certified if the coefficient of variation between results of the left and right palms for each volunteer was <20% and the average of all 6 results was <120 U. Coefficients of variation for SC assays by a single operator making 6 measurements on the palms of 20 volunteers with normal (n = 10, mean SC 96 U) and high SC levels simulated with high concentration detector (n = 10, mean SC 166 U) were 11% and 7%, respectively. The coefficient of variation for repeat testing with 3 different kit lots in 10 volunteers with normal SC values was 10%. Mean SC values for the left and right hands of volunteers were not significantly different (p = 0.92) and had a coefficient of variation of 9%.¹⁰

All sonographers were trained and certified to perform the imaging protocol by a central core laboratory (University of Wisconsin Atherosclerosis Imaging Research Program, Madison, Wisconsin). The protocol for evaluating CIMT was modified from the Atherosclerosis Risk In Communities (ARIC) study.^{11,12} Digital images of the far walls of the distal 1 cm of the common carotid artery from 3 angles of interrogation and of the bifurcation were obtained using high-resolution B-mode ultrasound. Ultrasound images were shipped to the core laboratory on digital media for central analysis. The core laboratory was blinded to SC and all laboratory measurements. Two experienced readers measured the mean far wall CIMT of the common carotid artery in triplicate from each angle for a total of 6 segments (18 measurements) per subject (Access Point 2004, Freeland Systems, Westfield, Indiana). Mean CIMT was considered to be increased if it was >75th percentile for age, gender, and race in the ARIC study, based on published nomograms and regression modeling.^{12,13} Carotid plaque was defined as a focal area of echogenic carotid wall thickening that encroached on the arterial lumen, with a minimal intimal plus medial thickness >1.2 mm.¹⁴ Approximately 10% of subjects from each site had repeat carotid ultrasonography performed \geq 1 day apart. Sixty pairs of duplicate scans were measured by readers who were blinded to their previous measurements. Mean \pm SD absolute differences in CIMT between scans for readers 1 and 2 were 0.011 ± 0.012 and 0.041 ± 0.031 mm, respectively. Their

Table 1
Subject characteristics (n = 565)

Age (yrs)	57 \pm 10
Women	215 (38%)
White	429 (66%)
Hypertension	260 (46%)
Diabetes mellitus	98 (17%)
Current smoker	145 (26%)
Framingham cardiovascular risk (%)	8.4 \pm 7.2
Total cholesterol (mg/dl)	200 \pm 41
Low-density lipoprotein cholesterol (mg/dl)	127 \pm 36
High-density lipoprotein cholesterol (mg/dl)	53 \pm 16
Triglycerides (mg/dl)	134 \pm 99
Systolic blood pressure (mm Hg)	131 \pm 18
Diastolic blood pressure (mm Hg)	78 \pm 11
High-sensitivity C-reactive protein (mg/L)	2.7 (1.7–4.6)
Lipoprotein-associated phospholipase A ₂ (ng/ml)	148 \pm 48
SC (U)	90.6 \pm 21.6
Mean CIMT (mm)	0.758 \pm 0.154
CIMT >75th percentile	201 (36%)
Carotid plaque presence	300 (53%)

Continuous values are presented as mean \pm SD, except high-sensitivity C-reactive protein, which is presented as median (interquartile range). Categorical values are presented as number of patients (percentage).

coefficients of variation were 1.0% and 3.5%. Assessment of plaque presence was 97% reproducible for the 2 readers.

Blood samples were obtained by venipuncture after subjects had fasted for \geq 12 hours. All laboratory tests were performed at Orion Laboratory (Seymour, Connecticut). Fasting plasma glucose, serum total cholesterol, triglycerides, and high-density lipoprotein cholesterol were measured using standard enzymatic assays and low-density lipoprotein cholesterol was calculated using the Friedewald equation.¹⁵ C-reactive protein was measured using a high-sensitivity assay (high-sensitivity C-reactive protein, extended range assay, Dade Behring, Deerfield, Illinois), and lipoprotein-associated phospholipase A₂ was measured using the PLAC test (Diadexus, Inc., South San Francisco, California).

The primary analysis was to determine if SC levels could identify patients with CIMT >75th percentile for age, gender, and race based on projections from the ARIC study.^{12,13} Unadjusted logistic regression models were created and then adjusted for traditional cardiovascular disease risk factors, the Framingham risk score (FRS), and emerging risk factors (high-sensitivity C-reactive protein, lipoprotein-associated phospholipase A₂). Response variables were CIMT >75th percentile and carotid plaque presence. First, SC was treated as a continuous variable with an odds ratio (OR) and 95% confidence intervals (CIs) calculated for each 10-U increase in SC. Second, the relation was assessed with adjustment for the FRS and cardiovascular risk factors. Forward stepwise regression models (entry criterion of p < 0.05) were used to select the set of variables (Table 1) that best characterized CIMT >75th percentile. Models then were stratified by cardiovascular risk (low <5%, intermediate 5% to 20%, and high >20% or type 2 diabetes mellitus). Areas under the receiver operating characteristic curves (with 95% CIs) for the prediction of increased CIMT were calculated for each risk factor. Similar analyses were performed for the outcome variable of carotid plaque presence.

Based on previous research,⁵ SC also was analyzed as a

Table 2
Associations with carotid intima–media thickness for skin cholesterol, lipids, and blood pressure, overall and by cardiovascular risk status

Variable	Overall		Low Risk (n = 186)		Intermediate Risk (n = 245)		High Risk (n = 134)		AUC for CIMT >75th Percentile (95% CI)
	r Value	p Value	r Value	p Value	r Value	p Value	r Value	p Value	
	SC	0.03	0.47	−0.03	0.70	0.12	0.06	0.12	
Total cholesterol	0.07	0.11	0.16	0.03	0.13	0.04	0.03	0.72	0.505 (0.467–0.551)
Low-density lipoprotein cholesterol	0.12	0.004	0.15	0.05	0.15	0.02	0.08	0.35	0.525 (0.483–0.567)
High-density lipoprotein cholesterol	−0.16	<0.001	0.05	0.52	0.03	0.67	−0.13	0.15	0.561 (0.519–0.603)
Systolic blood pressure	0.27	<0.001	0.12	0.11	0.07	0.28	0.18	0.04	0.572 (0.530–0.613)
Diastolic blood pressure	0.08	0.07	0.01	0.92	−0.11	0.08	0.04	0.66	0.559 (0.517–0.600)

AUC = area under the receiver operating characteristic curve.

Table 3
Associations among skin cholesterol, increased carotid intima–media thickness, and carotid plaque presence*

	CIMT >75th Percentile	p Value	Carotid Plaque Presence	p Value
SC/10 U	1.12 (1.03–1.21)	0.006	1.15 (1.06–1.26)	0.002
Intermediate vs low SC	1.45 (0.97–2.16)	0.07	1.08 (0.74–1.57)	0.70
High vs low SC	2.19 (1.30–3.72)	0.004	2.53 (1.46–4.40)	0.001

* ORs and 95% CIs adjusted for cardiovascular risk (low <5%, intermediate 5% to 20%, and high >20% or type 2 diabetes mellitus).

categorical variable with low (<80 U), intermediate (80 to 110 U), and high (>110 U) levels. SC <80 U is considered low because 80 U is the theoretical lower limit of detection of the test.¹⁰ This was based on measurements of substrate alone on different palms and adding 2 SDs to the mean value obtained. The upper cutpoint (>110 U) identifies approximately the top quartile of healthy subjects based on previous studies.⁵ All analyses were performed with SAS 9.1 (SAS Institute, Cary, North Carolina) using an alpha value equal to 0.05 to establish statistical significance. The projected sample of 600 subjects was estimated based on the ability to detect an OR for CIMT >75th percentile of ≥1.85 for subjects with high SC with 80% power.

Results

In total 650 subjects were recruited. Twelve subjects were excluded before data analysis (6 did not have blood drawn, 1 had missing lipid values, 2 were on cholesterol-lowering medications, 3 did not have complete CIMT studies). Also, data from 73 subjects were excluded because of operator error in measuring SC (Appendix 1). Therefore, SC, CIMT, and laboratory data were analyzed in 565 subjects (Table 1). Mean FRS was 8.4 ± 7.2% (37% low risk, 39% intermediate risk, 24% high risk). Mean SC was 90.6 ± 21.6 U. There were 196 subjects (35%) with SC <80 U, 282 (50%) with SC 80 to 110 U, and 87 (15%) with SC >110 U. There was no significant association between SC and age (r = 0.03, p = 0.46) or gender (men vs women 90 ± 22 vs 92 ± 22 U, p = 0.18). Neither high-sensitivity C-reactive protein (r = −0.06, p = 0.17) nor lipoprotein-associated phospholipase A₂ (r = 0.07, p = 0.10) was strongly associated with SC. In univariate analysis, SC was not correlated with

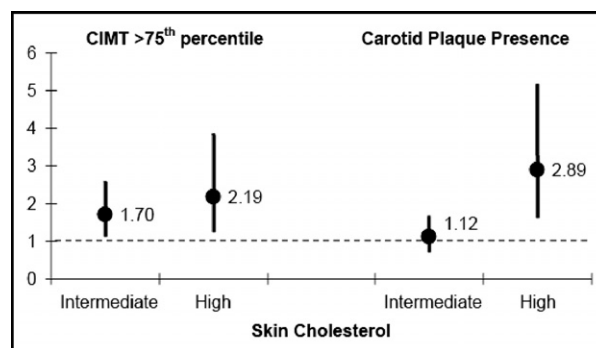


Figure 1. Adjusted ORs (adjusted for age, gender, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, systolic and diastolic blood pressures, glucose, smoking status) with 95% CIs for prediction of CIMT >75th percentile and carotid plaque presence by SC levels (intermediate 80 to 110 U and high >110 U vs low <80 U).

CIMT (r = 0.03, p = 0.47); however, a weak correlation was seen in participants at intermediate risk (r = 0.12, p = 0.06; Table 2). In this group of participants, the relation between SC and increased CIMT was stronger than for high-density lipoprotein cholesterol (r = 0.03, p = 0.67) and systolic blood pressure (r = 0.07, p = 0.28) and similar to that of diastolic blood pressure (r = −0.11, p = 0.08). The area under the receiver operating characteristic curve for the ability of SC to identify patients with increased CIMT (0.560) was higher than for total cholesterol (0.505) and similar to low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, and diastolic blood pressure (Table 2).

Multivariate models were more informative. For each 10-U increase in SC, the unadjusted OR for CIMT >75th percentile was 1.12 (95% CI 1.03 to 1.21, p = 0.006), which was not changed after adjustment for the FRS (OR 1.12, 95% CI 1.03 to 1.21, p = 0.007). For each 10-U increase in SC, the OR for carotid plaque presence was 1.13 (95% CI 1.04 to 1.22, p = 0.004). Adjustment for the FRS did not change this relation appreciably (OR 1.15, 95% CI 1.06 to 1.26, p <0.001; Table 3). Relations between SC and the 2 markers of subclinical atherosclerosis were similar in subjects at low, intermediate, and high cardiovascular risk (data not shown). In models that considered SC as a categorical variable and adjusted for cardiovascular risk factors, intermediate (p = 0.02) and high (p = 0.006) levels of SC were significant predictors of CIMT >75th percentile, and high

Table 4
Stepwise logistic regression models of predictors of increased carotid intima-media thickness and carotid plaque presence

	CIMT >75th Percentile	p Value	Carotid Plaque Presence	p Value
Age/10 yrs	0.54 (0.43–0.67)	<0.001	2.05 (1.66–2.53)	<0.001
Diabetes mellitus	1.93 (1.19–3.13)	0.008	1.68 (1.02–2.77)	0.04
Female gender	2.25 (1.49–3.39)	<0.001	0.68 (0.46–0.99)	0.04
High-density lipoprotein cholesterol	0.98 (0.97–1.00)	0.006		NS
Low-density lipoprotein cholesterol/10 mg/dl		NS	1.07 (1.02–1.13)	0.009
SC/10 U	1.14 (1.05–1.24)	0.003	1.17 (1.07–1.28)	<0.001
Smoker		NS	2.19 (1.41–3.41)	<0.001
Systolic blood pressure/10 mm Hg	1.24 (1.11–1.38)	<0.001	1.16 (1.04–1.30)	0.008

Candidate variables for inclusion in each model were age, gender, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, diastolic blood pressure, diabetes mellitus, smoking, high-sensitivity C-reactive protein, and lipoprotein-associated phospholipase A₂.

($p < 0.001$) SC was a significant predictor of carotid plaque presence (Figure 1 and Table 3). Similar results were seen when the relations between SC and the 2 markers of subclinical atherosclerosis were adjusted for cardiovascular risk category (Table 3).

Results of stepwise logistic regression modeling are presented in Table 4. In the model for CIMT >75th percentile, only age, systolic blood pressure, and gender were better predictors than SC; however, SC remained an independent predictor of increased CIMT after adjustment for these parameters. The same relations were observed in the model for predicting carotid plaque presence, with SC also selected as the third best explanatory variable after age and systolic blood pressure. Directions of the ORs for age and gender were divergent between models because the CIMT percentiles were based on age and gender. To evaluate how SC compared with other novel risk markers, logistic regression models were created with high-sensitivity C-reactive protein and lipoprotein-associated phospholipase A₂ as predictor variables, with adjustment for the FRS. For each 1.0-mg/L increase in high-sensitivity C-reactive protein, the ORs for CIMT >75th percentile and carotid plaque presence were 1.03 (95% CI 1.00 to 1.06, $p = 0.03$) and 0.99 (95% CI 0.96 to 1.01, $p = 0.25$), respectively. For each 10-mg/dl increase in lipoprotein-associated phospholipase A₂, the ORs for CIMT >75th percentile and carotid plaque presence were 1.02 (95% CI 0.98 to 1.05, $p = 0.42$) and 1.03 (95% CI 0.99 to 1.07, $p = 0.13$), respectively.

Discussion

These data demonstrate that SC, as measured by a noninvasive assay, identified the presence of advanced subclinical atherosclerosis. A high level of SC (>110 U) was associated with nearly a 2.2-fold increase in the odds of having CIMT >75th percentile and an approximately 2.5-fold increase in the odds of having carotid plaque. Associations between SC and advanced subclinical atherosclerosis were strong and independent of traditional and emerging risk factors. Relations between SC and subclinical atherosclerosis were robust, because neither the manner in which SC was modeled (i.e., continuous or categorical) nor the manner in which adjustments for traditional cardiovascular risk were performed (i.e., individual risk factors, FRS, cardiovascular risk category) affected the conclusions. The area

under the receiver operating characteristic curve for SC was higher than for total cholesterol and similar to other recognized coronary heart disease risk factors. These analyses emphasize that SC contributes to the explanation of variability in the outcome of CIMT >75th percentile to an extent similar to traditional cardiovascular risk factors; however, none of the markers can adequately predict risk by themselves. The magnitude and independence of these findings suggest that measuring SC may be a useful test to identify increased cardiovascular risk.

The need for simple, noninvasive tests to assist health care professionals with cardiovascular risk stratification has been recognized.^{9,16,17} However, most tests that have been recommended are relatively complicated and require expensive instrumentation or highly trained technicians to be performed correctly. These alternative risk prediction methods are not widely available and/or may involve harmful exposures such as to ionizing radiation. A noninvasive test that could be performed in a clinical practice setting by office personnel after a brief training program, such as measurement of SC, could be a useful clinical tool. SC levels reflect changes that occur with aging and atherosclerosis.³ Cholesterol selectively accumulates in the skin of mice with defects in cholesterol regulation, and in a recent study of mice deficient in low-density lipoprotein receptors and apolipoprotein A1, accumulation of epidermal cholesterol was associated with macrophage infiltration and inflammatory changes.¹⁸ Similar processes occur in the arterial intima during the development of atherosclerosis, thus explaining the relation among SC, CIMT, and cardiovascular disease risk.

Although this study identified independent associations between SC levels and 2 markers of advanced subclinical atherosclerosis, it was not designed to evaluate the ability of SC levels to predict incident cardiovascular events. A longitudinal study investigating the predictive power of SC levels is in progress. Like all techniques, measurement of SC is subject to operator error. Despite standardized training, SC values from 1 operator were excluded because of operator error (Appendix 1). When this operator's data were included, statistically significant ($p < 0.05$), but weaker, associations between SC and the markers of advanced subclinical atherosclerosis still were observed. A critical step in the SC test process that is subject to operator variability is

blotting, which requires the operator to remove an unbound detector from the palm before adding the indicator. Excess residual indicator solution can result in falsely increased SC levels. A simplified and standardized blotting procedure with the objective of eliminating the kind of operator variability seen in this study has been developed by the manufacturer. As with all laboratory tests, operator training remains critical for obtaining accurate and reproducible results.

The reliability of the findings in this study is supported by the strength of associations observed between SC and 2 markers of advanced subclinical atherosclerosis (increased CIMT and carotid plaque presence)^{14,16} and the consistency of the associations between SC and these markers across a wide range of cardiovascular risks. Increased SC was associated with increased risk of carotid plaque and, to a lesser extent, CIMT, even in subjects at low and intermediate risk according to traditional risk assessment. Furthermore, the reproducibility of the carotid ultrasound outcomes used in this study were as good as or better than that reported in the literature.^{11,19,20} In summary, SC can noninvasively identify patients with CIMT >75th percentile and with carotid plaque, markers of advanced subclinical atherosclerosis that predict incident cardiovascular events.

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Appendix 1

During analysis of the data from the entire cohort of 650 subjects, the effect of SC on CIMT >75th percentile and on plaque presence was found to be significantly modified by site. Further investigation revealed that the SC values from 1 site where 96 subjects were recruited were higher than the other participating sites. A single operator's SC values accounted for the variance. This operator measured SC in 73 subjects (76%) at this site. The mean SC for this operator was 123 ± 30 U compared with 104 ± 26 U for the other operator at this site and 90 ± 21 U for all other subjects. An unadjusted general linear model showed that this operator had significantly higher SC values than the other operator at this site and all other operators ($p < 0.001$ for the 2 analyses). Subjects for whom SC was measured by this operator were similar to the other operator at this site and the rest of the study participants for all characteristics listed in Table 1, except that they had slightly lower low-density lipoprotein cholesterol ($p = 0.048$) and lipoprotein-associated phospholipase A₂ ($p < 0.001$) values. The difference in SC levels persisted even after adjusting for all cardiovascular risk factors listed in Table 1 ($p < 0.001$). These results support the conclusion of operator error. Upon investigation, it was discovered that after training and certification this operator had not measured SC in a subject for 8 weeks, whereas other operators measured SC within 4 weeks of training. A longer interval between training and research testing may have resulted in aberrant SC results from this operator. All data from this operator were excluded from the primary analysis.

Appendix 2

The core laboratory for this study was the University of Wisconsin Atherosclerosis Imaging Research Program, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin (James H. Stein, MD, principal investigator; Claudia E. Korcarz, DVM, RDCS; Susan E. Aeschlimann, RDMS, RVT; Carol K.C. Mitchell, RDMS, RDCS, RVT; Mary Jane Washburn, RRT). The following institutions (personnel) participated in this study: University of Chicago Pritzker School of Medicine, Chicago, Illinois (Jeanne M. DeCara, MD, site principal investigator; Georgeanne Lammertin, RDCS; Anne Ryan, RN); University of Minnesota School of Public Health, Minneapolis, Minnesota (Alan T. Hirsch, MD, site principal investigator; Faye Imker-Witte; Kristi Jacobson; Kathy Knauth); University of Pennsylvania Medical School, Philadelphia, Pennsylvania (Emile R. Mohler, MD, site principal investigator; Wendy S. Tzou, MD, Elizabeth Medenilla; Natalia Zisman); Johns Hopkins Bayview Medical Center, Baltimore, Maryland (Pamela Ouyang, MD, site principal investigator; Sandra Lima; Jeanne Wingo; Melanie Herr); Radiant Research, Inc., Chicago, Illinois (Michael H. Davidson, MD, site principal investigator; Linda Arnold; Paul Espinosa).

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