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Authors

Aschbacher, Kirstin

Milush, Jeffrey M

Gilbert, Amanda

et al.

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Chronic Stress is Associated with Reduced Circulating Hematopoietic Progenitor Cell Number: A Maternal Caregiving Model

Kirstin Aschbacher[1,2], **Jeffrey M. Milush**[3], **Amanda Gilbert**[1], **Carlos Almeida**[1], **Elizabeth Sinclair**[3], **Lorrie Epling**[3], **S. Marlene Grenon**[4,5,6], **Elysa J. Marco**[7], **Eli Puterman**[8], and **Elissa Epel**[1]

[1] Department of Psychiatry, University of California San Francisco, San Francisco, CA

[2] The Institute for Integrative Health, Baltimore, MD

[3] Core Immunology Laboratory, Division of Experimental Medicine, University of California San Francisco, San Francisco, CA

[4] Department of Surgery, University of California San Francisco, San Francisco, California; CA

[5] Department of Surgery, Veterans Affairs Medical Center, San Francisco, CA

[6] Viperx Lab, San Francisco

[7] Department of Neurology, University of California San Francisco, San Francisco, CA

[8] School of Kinesiology, University of British Columbia, Canada

Abstract

Background—Chronic psychological stress is a risk factor for cardiovascular disease and mortality. Circulating hematopoietic progenitor cells (CPCs) maintain vascular homeostasis, correlate with preclinical atherosclerosis, and prospectively predict cardiovascular events. We hypothesize that 1) chronic caregiving stress is related to reduced CPC number, and 2) this may be explained in part by negative interactions within the family.

Methods—We investigated levels of stress and CPCs in 68 healthy mothers - 31 of these had children with an autism spectrum disorder (M-ASD) and 37 had neurotypical children (M-NT). Participants provided fasting blood samples, and $CD45^+CD34^+KDR^+$ and $CD45^+CD133^+KDR^+$ CPCs were assayed by flow cytometry. We averaged the blom-transformed scores of both CPCs to create one index. Participants completed the perceived stress scale (PSS), the inventory for

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depressive symptoms (IDS), and reported on daily interactions with their children and partners, averaged over 7 nights.

Results—M-ASD exhibited lower CPCs than M-NT (Cohen's *d*=.83; *p* .01), controlling for age, BMI, and physical activity. Across the whole sample, positive interactions were related to higher CPCs, and negative interactions to lower CPCs (*all p's*<.05). The adverse effects of group on CPCs were significantly mediated through negative interactions with the child (indirect *ß*=−.24, *p* .01). In the full model, greater age (*ß*=−.19, *p*=.04), BMI (*ß*=−.18, *p*=.04), and negative interactions with the child (*ß*=−.33, *p*<.01) were independently associated with lower CPCs. M-ASD had a less healthy lipid profile (total cholesterol/HDL), which in turn, was associated with lower CPCs.

Conclusions—Chronic stress adversely impacts CPC number, an early-stage biomarker that predicts subclinical atherosclerosis and future CVD events, independent of traditional cardiovascular risk factors and inflammatory factors. Among maternal caregivers, child-related interpersonal stress appears to be a key psychological predictor of stress-related CVD risk.

Keywords

endothelial progenitor cells (EPCs); circulating angiogenic cells (CACs); monocytes; cardiovascular risk; preclinical atherosclerosis; cholesterol; chronic stress; maternal caregiving; family interactions; autism

Introduction

Psychological stress is associated with a heightened risk of cardiovascular disease (CVD) (1). This risk may arise not only because markers of damage (e.g., inflammation) are elevated, but also because the body's endogenous mechanisms for repair are impaired. Hematopoietic progenitor cells, which are derived from bone marrow, promote tissue repair and regeneration (2, 3). Circulating hematopoietic progenitor cells (CPCs) can be mobilized into circulation and identified by combinations of cell surface markers: $CD45^+CD34^+$ KDR^+ and $CD45^+CD133^+$ KDR^+. CPCs (previously termed endothelial progenitor cells, or EPCs) play a role in vascular repair, vascular aging (4), and axonal or white matter protection (5), placing them at the intersection of neurovascular health.

Caring for an ill family member is one of the best-established human models of chronic stress. Caregiving is associated with higher risk of endothelial dysfunction (6), pro-coagulant and pro-inflammatory activity (7, 8), metabolic dysregulation (9), and cardiovascular disease (10, 11). One reason that caregiving is a potent stressor may be because it encapsulates the experience of having one's closest interpersonal attachments disrupted for years upon end. However, most self-report measures quantify stress as a quality of an *individual*, not of a family system – i.e., as the daily stressful interactions with family members.

The current study's model of chronic stress contrasts mothers of children with an autism spectrum disorder (M-ASD) with demographically similar mothers of healthy, neurotypical children (M-NT). Other studies show M-ASD endorse significantly higher stress levels and poorer mental health than M-NT (12). While most parents experience parenting stressors on a daily basis, there are differences in the types and severity of the stressors for children with

developmental disorders. Children with autism can engage in unpredictable aggression, self-injury, oppositional behavior, and unresponsiveness. In some cases, autistic children also express less affection, contributing to fewer positive interactions. We assessed maternal reports of daily positive and negative interactions with their children and spouses over the course of a week, to place caregiving stress in the context of daily family life.

CPCs may constitute a valuable early CVD risk marker, a potential mechanism, and a protective factor. A meta-analysis of over 1000 patients at high CVD risk found that $CD34^+KDR^+$ cells were prospectively associated with an increased risk of cardiovascular morbidity and mortality, independent of inflammatory and traditional CVD risk factors (13). Thousands of studies have investigated CPCs (in fresh blood) and early EPCs (in culture) in relation to disease and regenerative cell-therapy. However, only a few have examined associations with psychological factors (14-17). None of these published studies used an *objectively defined exposure* to chronic stress (such as caregiving).

The immunologic definition of CPCs is still evolving and has suffered some confusion in the literature. We, and others, refer to the $CD45^+CD34^+KDR^+$ and $CD45^+CD133^+KDR^+$ cell populations derived from circulating blood as CPCs. We use the term CPCs to distinguish these rare cells found in fresh blood from their counterparts derived from cell culture models. Historically, $CD34^+KDR^+$ and $CD133^+KDR^+$ cells were termed endothelial progenitor cells (EPCs). EPCs were measured by a combination of surface markers (to specify phenotypes) and cell culture models (to investigate function). Subsequently, it was established that blood-derived "EPCs" that emerge early in cell culture (7 days) are not true endothelial cells and do not form new blood vessels (18). Hence, these cultures of "early EPCs" are increasingly renamed circulating angiogenic cells (CACs) (19-21). We intentionally use the term CPCs, because cell culture models like CACs contain several different types of immune cells, and their phenotypes are influenced by cell culture media and conditions. Moreover, less than 1% of CACs in culture models express the stem cell markers, $CD34^+$ and $CD133^+$, while the majority express hematopoietic and monocytic markers ($CD45^+$ and $CD14^+$) (22). In sum, we use the term CPCs to refer to $CD45^+CD34^+KDR^+$ and $CD45^+CD133^+KDR^+$ cells.

In animal models, chronic social stress accelerates the development of hematopoietic stem cell pools in the bone marrow. In turn, this leads to an increase in pro-inflammatory monocytes and promotes their infiltration into atherosclerotic lesions (23). Hematopoietic progenitor cells have the capacity develop into the major types of immune cells (including $CD14^+$ monocytes) dependent on their microenvironment and cytokine milieu (24). As an exploratory hypothesis, we investigated whether chronic stress would be associated with alterations in $CD14^+$ monocytes or with CPCs co-expressing $CD14^+$. Secondarily, we also investigated the associations of CPCs with traditional cardiovascular risk factors.

We hypothesized that mothers of children with autism spectrum disorders would have significantly greater levels of psychological distress and fewer $CD45^+CD34^+KDR^+$ and $CD45^+CD133^+KDR^+$ CPCs than mothers of healthy, neurotypical children. Furthermore, we tested whether differences in CPCs could be explained by pinpointing the most central characteristic of maternal caregiver stress – daily negative mother-child interactions. We

contrasted these interactions with other sources of psychological distress, such as marital interactions, perceived stress, and depressive symptoms.

Methods

Participants

The current study was conducted as part of a larger study on chronic caregiving stress and cellular aging. Participants were 68 mothers living in the San Francisco Bay area, recruited through local schools, parenting publications, social media, mailings, child development centers, and through the University of California, San Francisco Sensory Neurodevelopment and Autism Program. Eligible mothers were non-smokers between 20 and 50 years of age, with at least one child between 2 and 16 years of age. Thirty-eight percent (n=26) had one child, 47% (n=32) had two, 10% (n=7) had three, and 4% (n=3) had four children. Inclusion criteria for mothers in the higher stress, caregiver group were caring for a child diagnosed with an autism spectrum disorder (including labels such as autism, Asperger syndrome, or pervasive developmental disorder not otherwise specified) and having a minimum perceived stress score (PSS) of 13 upon the initial phone screen. Mothers were eligible for the lower-stress, control group if they were caring for a neurotypical child without other chronic disease and reported PSS 19 during the phone screen. Overlap in PSS scores was permitted so that perceived stress could be better disentangled from the objective characteristic of caring for a child with an autism spectrum disorder. We then reassessed the PSS at the baseline visit to align our psychological and biological measures in time. Because depression is common in states of chronic stress, depression was allowed in the caregiving group. Thus, at recruitment, mothers were excluded from the control group, but not the chronic stress group, if they met criteria for current major depressive disorder or were taking antidepressants. Two controls who later started taking antidepressants were not excluded from the study as a whole, and subanalyses were included to test that their exclusion did not change the significance of the results. Exclusion criteria included major chronic diseases (e.g., diabetes, cardiovascular, autoimmune, history of stroke, brain injury, cancer, endocrine disorders), and regular use of steroid prescription medications. Participants meeting criteria for current posttraumatic stress, bipolar, or eating disorders were also excluded. This study was approved by the Committee for Human Research at the University of California, San Francisco, and all participants gave written consent.

Perceived Stress Scale (PSS)

The Perceived Stress Scale-10 (25) is a standard 10-item questionnaire that assesses subjective perceptions of stress over the previous month. The scale has been normed in several large national surveys, and the average PSS scores among women was roughly 16 (26). Response options form a 5-point Likert scale ranging from 0=never to 4=very often. Cronbach's alpha was .88 in the current sample. PSS scores were missing for two participants.

Inventory for Depressive Symptomatology (IDS)

The Inventory for Depressive Symptomatology (27) is a 30-item self-report scale that measures signs and symptoms of depression. All items are equally weighted and use scores

on a 4-point Likert scale ranging from 0 to 3. The Cronbach's alpha in this sample was .87. IDS scores were missing for 3 participants.

Daily Maternal-Child Interactions

Participants were asked to complete a nightly diary, over a 7-day period (blood was drawn on day 4), and answer online questions about the quality of their interactions with their child and spouse. If participants had multiple children, they chose a target child – i.e., either their child with ASD, or for controls, their most difficult child. All items were scored on a continuous line scale from "Not at all" to "A lot", which was translated to a score between 0 and 100. Negative interactions with one's child (NIC) were assessed using four items tapping the extent to which mothers reported experiencing difficult interactions, felt overwhelmed, blamed themselves for difficult interactions, or felt ashamed of their child's behavior (full scale provided in the supplement). Positive interactions with child (PIC) were assessed by two items asking mothers to rate the extent to which they thought the interactions with their child that day were positive, and whether they were able to pay full attention to their children during interactions. The final scores for negative and positive interactions were quantified as the average over the week. The Cronbach's alphas in this sample for NIC and PIC were satisfactory, .82 and .74 respectively, suggesting some consistency across days.

Daily Spousal Interactions

Fifty-eight mothers also rated the quality of their spousal interactions. Two high stress and three control mothers did not have spouses, and five participants did not provide data. Six items assessed negative interactions with one's partner (NIP), related to the experience of tension, criticism, disappointment, ignored, and self-blame (see supplementary documents). Three items assessed positive interactions with one's partner (PIP), related to feeling satisfied, respected, and giving one's full attention to one's partner. The Cronbach's alphas for NIP and PIP spousal interactions were .87 and .70 respectively, suggesting consistency across days.

Flow Cytometry Assays of CPC Number

On the fourth day of the daily questionnaire, women came into the Clinical Research Center for a fasting blood draw, between 7am and 10am. During this same clinic visit, the PSS and IDS were also completed, and blood pressure was taken by trained research nurses. Peripheral blood mononuclear cells (PBMC) were isolated from 10 mL of whole blood using Ficoll Histopaque®-1077 (Sigma-Aldrich). Samples were layered on Ficoll and centrifuged at 25°C for 30 minutes at $400 \times g$ without a brake. The PBMC layer was recovered, washed twice with phosphate buffered saline (PBS), and then treated with ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Lonza Walkersville, Inc) to remove red blood cell contamination. Four million PBMCs were stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, blocked with 1mg/ml Human IgG (BioDesign International, A08400H), then stained on ice for 30 minutes with the following fluorophore-conjugated antibodies: Alexa Fluor®700-conjugated anti-CD45 (HI30) (ThermoFisher Scientific), Phycoerythrin (PE-conjugated anti-CD133 (AC133 and 293C3) (Miltenyl Biotec), Alexa 647-conjugated anti-KDR (89106) (BD Pharmigen), BV605-conjugated anti-CD14 (M5E2) (BioLegend), and FITC-conjugated anti-CD34 (8G12) (BD Biosciences). Stained PBMCs

were washed once with FACS buffer (PBS containing 0.5% bovine serum albumin and 1mM Ethylenediarninetetraacetic Acid), fixed in 0.5% paraformaldehyde (Electron Microscopy Sciences) in PBS, and processed on a BD LSR II Flow cytometer (BD Biosciences), collecting the entire sample for each subject. CS&T beads (BD Biosciences) were used for instrument set up for each run, and Rainbow bead (Spherotec) standardized instrument settings between runs. FMO controls were also prepared on each sample to check that gates were set consistently between runs. Data were compensated and analyzed in FlowJo V9.8.1 (TreeStar). Cells were gated by standard singlet inclusion, dead cell exclusion, and CD45 gating, and CPC subsets were defined as $CD45^+CD133^+KDR^+$ and $CD45^+CD34^+KDR^+$ (Supplementary Figure 1 illustrates the gating strategy).

Assessment of Physical Activity

Each night for one week, participants were asked to describe the physical activities they had engaged in that day, by selecting any (or all) of the following intensity ratings: "very little", "some light", "some moderate", and/or "some vigorous activity". Participants were provided examples of activities within each intensity category, and asked to report the number of minutes for each activity. Descriptions and definitions for each category, adapted for daily use, were based on previously published work (28) and the Center for Disease Control and Prevention descriptions[i]. Activities were converted to metabolic equivalent of task scores (METS)[ii], and each activity's METS was multiplied by the number of minutes participants reported engaging in that activity that day. The seven days were added to obtain the total weekly minutes of moderate and vigorous activity, in METS. Because the distribution was bimodal, this variable was recoded into any physical activity (1) versus none (0).

Cardiovascular Risk Factors, Menstrual Cycle, & Medications

Blood was assayed by Quest Diagnostics for a Comprehensive Metabolic Panel, from which markers of cardiovascular risk are derived (e.g., triglycerides and cholesterol), and data was missing for one participant. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol were assessed continuously and also using clinically relevant cut-offs validated for the prediction of cardiovascular disease in women (29, 30). The following numbers of participants were taking NSAIDs/analgesics (n=6), progestin-only contraceptives (n=12), estrogen/progestin contraceptives (n=4), antidepressants (n=8), or antihypertensives (n=2). These numbers did not significantly differ between the groups per chi-square analyses. No participants were taking any statin, anticoagulant, antiarrhythmic, antiplatelet, ACE inhibitors, angiotensin II receptor blockers, or vasodilator drugs. Participants provided data on approximately how many weeks ago their previous period occurred. Ten participants had difficulty recalling the exact timing or reported that their period had not come during the previous cycle, and were classified as longer than five weeks.

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[i]http://www.cdc.gov/physicalactivity/basics/adults/
[ii]https://community.plu.edu/~chasega/met.html

Data Analysis

All physiological outcomes and proposed mediators were examined for deviations from normality both visually and using the Kolmogorov-Smirnov test. All cell counts were blom-transformed to improve the normality of the distribution (mean=0, std=1). A total CPC factor was formed by averaging the blom-transformed scores for $CD34^+KDR^+$ and $CD133^+KDR^+$. Group differences between mothers of children with autism spectrum disorders (M-ASD) and mothers of neurotypical children (M-NT) were tested using analysis of covariance (ANCOVA), controlling for exercise, age, and body mass index (BMI). Note that ANCOVA results and Figures 1 and 3 use blom-transformed dependent variables, which have normal distributions. However, we report untransformed means in the text for interpretability. Because CPCs are very rare cells, and constitute a very small percentage of total PBMCs, we use the notation $M \pm SE * 10^{-3}$ to indicate that the mean and standard error must be multiplied by 10^{-3}.

To determine which psychological factors were potential mediators of the group-CPC relationship, we conducted regression analyses in the whole sample testing each psychological factor (PSS, IDS, or daily interactions) as an independent predictor of total CPCs (blom transformed), not including group, but controlling for exercise, age, and BMI. Those factors bearing significant relationships to CPCs were subsequently tested as potential mediators. Mediation models were bootstrapped with 10,000 iterations and a critical alpha of 0.05, using the mediation macro within SPSS 23.0 by developed by Andrew Hayes (31). Psychological factors, age, and BMI were standardized prior to entry into the mediation analysis. Because of sample size limitations on power, separate regression and mediation models were conducted for each potential mediator/psychological factor. Small differences in the degrees of freedom occur across the models due to missing data.

Results

Group Characteristics

The groups, M-ASD and M-NT, did not significantly differ on age, BMI, Caucasian race or education (all p's>.13; Table 1). M-ASD reported no significant differences from M-NT in the use of medications (all p's>.05), although there was a non-significant trend that more M-ASD than M-NT were taking antidepressants. As expected, M-ASD reported significantly greater levels of psychological distress than M-NT, as indexed by higher scores on the PSS (p=0.013), IDS (p=0.001), NIC (p=0.001) and NIP (p=0.005), and lower scores on PIC (p=0.002) and PIP (p=0.015), in unadjusted t-test comparisons (Table 1). M-ASD were significantly more likely to meet clinically relevant criteria for LDL and HDL levels associated with cardiovascular risk (29, 30) than M-NT (Table 1). No group differences in blood pressure or triglycerides were found.

Group Comparisons on CPCs

M-ASD had lower overall CPCs than M-NT ($F(1,62)=8.079$, p=0.006; Figure 1). The magnitude of this effect, quantified using Cohen's d was 0.83, a large effect[iii]. Examining each CPC subset separately, M-ASD ($M \pm SE * 10^{-3}$: 0.568±0.100) had significantly lower $CD133^+KDR^+$ cells ($M \pm SE * 10^{-3}$: 1.052±0.131) than M-NT ($M \pm SE * 10^{-3}$: 1.851±0.196;

$F(1,62)=8.583$, $p=0.005$) and a trend toward lower CD34+KDR+ cells than M-NT (M $\pm$SE*10^{-3}: 0.811 ± 0.118; $F(1,62)=3.399$, $p=0.070$). Group also remained a significant predictor when additionally controlling for time since last menstrual cycle ($p=.019$), education, and HDL, LDL or the cholesterol/HDL ratio (all $p's<.05$) in sequential analyses. The addition of variables coding for medications, including antidepressants and oral contraceptives, also did not change the significance of these findings. Furthermore, we confirmed that when *excluding* all participants taking antidepressants, group remained a significant predictor of CPCs ($p=.025$, Cohen's d: .73). Also, when excluding participants taking oral contraceptives, group remained a significant predictor of CPCs ($p=.035$, Cohen's d: 0.81).

As expected, in these analyses greater age was a significant predictor of lower CPCs and $CD133^+KDR^+$ cells ($p's<.05$). Greater BMI was a significant predictor of lower CPCs and $CD34^+KDR^+$ cells ($p's<.05$). Activity was a significant independent predictor of greater $CD133^+KDR^+$ ($p=.044$), but not $CD34^+KDR^+$ or overall CPC counts.

Psychological Distress & CPCs

We hypothesized that daily negative maternal-child interactions constitute the primary psychological mechanism to explain group differences in CPCs. To provide the initial foundation for mediation analyses, we conducted regression analyses for each psychological factor predicting CPCs, without including group, but while controlling for physical activity, centered age, and centered BMI. Factors significantly related to CPCs (i.e., interactions with children and spouses) were subsequently examined in mediation analyses. NIC ($\beta(62)=-0.452$, $p<0.001$) , PIC ($\beta(62)=0.296$, $p=0.012$), NIP ($\beta(53)=-0.268$, $p=0.038$) and PIP ($\beta(53)=0.268$, $p=0.039$) were all significant predictors of CPCs, while not including group in the model. PSS ($\beta(60)=-0.230$, $p=0.054$) and IDS ($\beta(62)=-0.225$, $p=0.064$) showed non-significant trends. Figure 2 illustrates the correlations of CPCs with negative and positive maternal-child interactions. Adding family size as a covariate did not change the pattern of significance, though it showed a borderline independent relationship with lower CPCs, independent of age. Time since child's diagnosis, as a measure of chronicity, was not significantly related to CPCs independent of age. We tested group by psychological factor interaction terms, and none were significant.

Full Mediation Model

As the final mediation model, we tested each psychological factor separately. NIC significantly mediated the relationship between group and total CPCs, while controlling for physical activity, age, and BMI. This model accounted for 33% of the variance in CPCs, per the adjusted R^2. The adverse effects of group on CPCs were significantly mediated via NIC in path analysis (indirect β(SE)$=-0.242(0.107)$, LB=-0.480, UB=-0.067, p 0.05). When the indirect path via NIC was included, the direct effect of group became non-significant (β(SE)$=-0.308(0.192)$, $p=0.114$), indicating there was no longer a significant effect of group, independent of NIC. Furthermore, in this full model, higher maternal age (β(SE)$=$

[iii]Cohen's d is quantified as the difference in means divided by the pooled standard error: (0.297- (−.350))/.777, using estimates derived from the blom-transformed total CPC factor, unadjusted for covariates.

−0.193(0.091), p=0.038), BMI (ß(SE)=−0.181(0.088), p=0.043), and NIC (ß(SE)= −0.326(0.095), p=0.001) all exerted significant, direct effects on CPCs, with trending benefits for physical activity ß(SE)=0.343(0.196), p=0.084). In contrast, PIC, NIP and PIP were not significant mediators of group effects on CPCs, although there was a trending indirect effect of group on CPCs via PIC within a 90% confidence interval (indirect ß(SE)= −0.117(.081), LB=−0.264, UB=−0.006, p 0.10) and a trending direct effect of PIC on CPCs (ß(SE)=0.180(0.099), p=0.075).

Group Comparisons on CD14+ Monoctye subsets

Analysis of CPC-containing cell culture models suggests that the molecular phenotype of CPCs may resemble CD14+ monocytes (32), which play a key role in atherosclerosis (33). However, few studies have examined the overlap of CPC and CD14+ markers in fresh blood, as opposed to after 7-days of culture, during which time, the culture media exerts a strong influence on the phenotype. Hence, as a secondary question, we explored group differences in CD14+ monocytes and their co-expression with CPC markers. M-ASD (M±SE: 12.349±0.771) had higher percentages of CD14+ monocytes than M-NT (M±SE: 10.333±0.695; F(1,62)=5.280, p=0.025; Figure 3). However, the subsets of CD14+ monocytes positive for CPC markers were all significantly lower in M-ASD compared to M-NT. Specifically, M-ASD had fewer CD14+KDR+ (M±SE*10^{-3}: 5.081±.0.750) monocytes than M-NT (M±SE*10^{-3}: 30.675±11.039; F(1,62)=12.169, p=0.001). Moreover, when expressing CPCs as a percentage of CD14+ cells, group differences became even more pronounced, such that M-ASD had fewer CD14+CD34+KDR+ (M±SE*10^{-3}: 1.039±0.171) and CD14+CD133+KDR+ (M±SE*10^{-3}: 2.042±0.351) monocytes than M-NT (respectively: M±SE*10^{-3}: 63.796±30.005, F(1,62)=16.776, p<0.001; M±SE*10^{-3}: 10.392±1.988, F(1,62)=18.451, p<0.001).

Associations of Major Immune Cell Subsets with Cardiovascular Risk Factors

Given relations between CPCs and CVD risk factors (34), in secondary analyses, we examined CVD risk factors in this sample. Table 2 demonstrates that lower CPCs were significantly associated with lower HDL levels, while higher CD14+ counts were significantly associated with higher LDL levels. CPCs and overall CD14+ counts were not significantly related, underscoring that these two metrics generally represent distinct cell populations in fresh blood, unless flow gates are specifically designed to isolate the relatively infrequent cell subsets co-expressing CD14+ and CPC markers.

Discussion

Chronically stressed mothers caring for a child with an autism spectrum disorder exhibit alterations in immune and cholesterol biomarkers that reflect increased cardiovascular risk. Chronically stressed mothers have significant reductions in CD45+CD34+KDR+ or CD45+CD133+KDR+ circulating hematopoietic progenitor cell populations, compared to mothers of healthy, neurotypical children. The effect size of this association was .83, which is considered large by statistical standards. Low CPCs are a prospective risk factor for cardiovascular morbidity and mortality in meta-analytic research (13), independent of

inflammatory and traditional CVD risk markers. These data raise the possibility that reduced CPCs may constitute a novel pathway linking chronic psychological stress with CVD.

This study defines chronic stress in humans using the objectively defined exposure of caring for a child with an autism spectrum disorder. This stress exposure is not general, but *role-specific*. Maternal-child negative interactions were the primary psychological mechanism, which significantly explained (or statistically mediated) group differences in CPCs. Daily partner interactions correlated with CPC's, but did not explain group differences. One-time, self-report measures of an individual's stress and depressive symptoms showed only trend relationships with CPCs.

When it comes to the question, "what makes stress stressful?" these data suggest that *daily stressful experiences* have implications for cardiovascular risk. They may be more sensitive measures than the more commonly used scales for general perceived stress. Specifically, *interpersonally-based* chronic stressors may be particularly potent. Clinical interventions might target daily maternal coping skills with parenting stressors, as well as family based emotion regulation and communication skills. Family centered interventions could have the potential to thereby alter long-term trajectories of cardiovascular risk.

This study's data are broadly consistent with findings from animal models of chronic social stress. In animal studies, chronic stress increases sympathetic noradrenergic inputs to the bone marrow, leading to hematopoietic progenitor cell proliferation, which, in turn, increases the number of inflammatory monocytes and accelerates the development of atherosclerotic plaques (23). Animal models of caregiver stress specifically (i.e., cohabitation with a sick partner) also identify heightened sympathetic and immune responses (35).

Atherosclerosis is driven by a synergy of factors. Impaired endothelial integrity is a critical initial stimulus for monocyte recruitment into the vasculature (36). CPCs may play a protective role by maintaining endothelial integrity (37). Both CPCs and monocytes traffic from the bone marrow into blood vessel linings, where they alter the local milieu by secreting cytokines and growth factors. This milieu, combined with factors like oxidized LDL, stimulates monocytes to differentiate into lipid-laden "foam" cell macrophages, which promote atherosclerotic plaque development (38). In this study, significantly more chronically stressed women had LDL levels above the clinically relevant threshold of 130 mg/dL (30), and HDL levels below the clinically relevant threshold of 56 mg/dL (29). Higher LDL was significantly related to higher $CD14^+$ counts, while lower HDL was related to fewer CPCs.

This study found a *greater* percentages of cells expressing $CD14^+$ among the chronically stressed group, but significantly *fewer* $CD14^+$ monocytes co-expressing $CD34^+KDR^+$ and $CD133^+KDR^+$. (39). $CD14^+$ monocytes play a key role in cardiovascular disease (38). In one study, $CD14^+$ counts were a better independent predictor of carotid plaque formation than pro-inflammatory factors like interleukin-6 and C-reactive protein (40). Of the three monocytes subsets, the "inflammatory/classical" and "intermediate" subsets are associated with CVD events, while the infrequent "non-classical" subset is not (41-43). This study's

CD14$^+$ gating is consistent with expression levels found in classical or intermediate subsets (41). However, additional cell surface markers (CD16, CCR2, CD86 or HLA-DR) would be needed for precise classification (41). These data suggest the possibility that chronic stress is associated with a pro-atherogenic skewing of monocyte phenotypes, though deeper surface phenotyping is needed.

The phenotypic and functional overlap between CPCs and monocytes is a matter of ongoing debate (32, 44). In these data, the majority of CPCs do not coexpress CD14. KDR co-expression is associated with "intermediate" monocytes (38, 45). However, CD14$^+$KDR$^+$ cells are also associated with beneficial, pro-angiogenic processes (44). We cannot determine whether CD14$^+$ CPC counts are lower because of changes in the lineage trajectory or due to redistribution – e.g., cells could have migrated out of circulation into tissue compartments like the vasculature. Hence, the significance of finding lower CD14$^+$ CPC counts in chronically stressed women remains unclear.

Atherosclerotic plaque development is fueled not only by inflammation, but also by impairments in repair and resolution (38). Low CD34$^+$KDR$^+$ number correlates with lower subclinical atherosclerotic plaque build up as indexed by carotid intima-media thickness among healthy middle-aged adults, independent of classic CVD and inflammatory risk markers (37). Low CPC counts also prospectively predict the progression of atherosclerosis and cardiovascular events, independent of traditional CVD risk factors (46). Atherosclerosis begins decades before its clinical manifestations. Traditional CVD risk indices can be poor predictors of subclinical atherosclerosis, especially among women (47). CPCs constitute an early marker of endothelial integrity, which can be used to measure the efficacy of lifestyle interventions to reverse preclinical atherosclerosis in high risk populations (48).

An important future step in this line of research will be to assess whether stress-associated reductions in CPC number result in clinical impairments in vascular repair *in vivo*. A previously published study by our lab found that psychological stress was associated with reduced migratory and paracrine function of CACs (a cell culture model containing CPCs) *in vitro* (19). *In vitro* CAC function is highly correlated with vascular repair capacity *in vivo* (49). The effects of stress on cell-mediated vascular repair *in vivo* could be tested by isolating human CACs or CD34$^+$ cells from high versus low-stress healthy adults, and transplanting them into an animal model of vascular injury (49).

There are several pathways by which stress could potentially impact CPC number, including autonomic (23), neuroendocrine (1), oxidative/nitrative (49), and lipid (50) mediators. We previously demonstrated that cortisol, a stress-responsive hormone, can inhibit CAC function *in vitro* (19). Prior research also suggests that low HDL may decrease CPC counts (50).

One limitation is that different HDL and LDL cut-offs have been used across the literature. We selected the lower, literature-supported cut-offs because our population does not have frank cardiovascular disease. Another limitation of the current study was that it focused on women, although previous studies have also found relationships between self-reported distress and CPC number among men (15). It is possible that family stress could exhibit

different relationships with CPCs in mothers versus fathers. The use of a multicolor panel, which specifically gated out dead cells, was an important strength of the current study's methods, given the rarity of CPCs. Despite the large effect size, because CPCs are rare cells with higher measurement variability, it will be important to replicate these results in a larger study of both men and women.

In a maternal caregiving model, we show that chronic stress is associated with reduced CPC number and an increased cardiovascular risk profile. In these data, general self-reports of stress and depression were not as predictive of cardiovascular risk markers as daily stressful family interactions. CPC number is an early-stage biomarker of endothelial repair that prospectively predicts CVD events. It is detectable in healthy individuals prior to clinical signs of CVD, and has been used as a primary outcome to assess the efficacy of short-term behavioral interventions (48). Future stress-reduction interventions might seek not only to mitigate markers of damage, but also to enhance the endogenous capacity for repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Highlights

- Circulating Hematopoietic Progenitor cells (CPCs) are CD34$^+$KDR$^+$ and CD133$^+$KDR$^+$.

- Low CPCs prospectively predict cardiovascular events in meta-analyses.

- Chronic stress of maternal caregiving is associated with reduced CPCs.

- Negative daily maternal-child interactions explain this stress-immune relationship.

- As expected, low CPCs are associated with a poorer lipid profile in this sample.
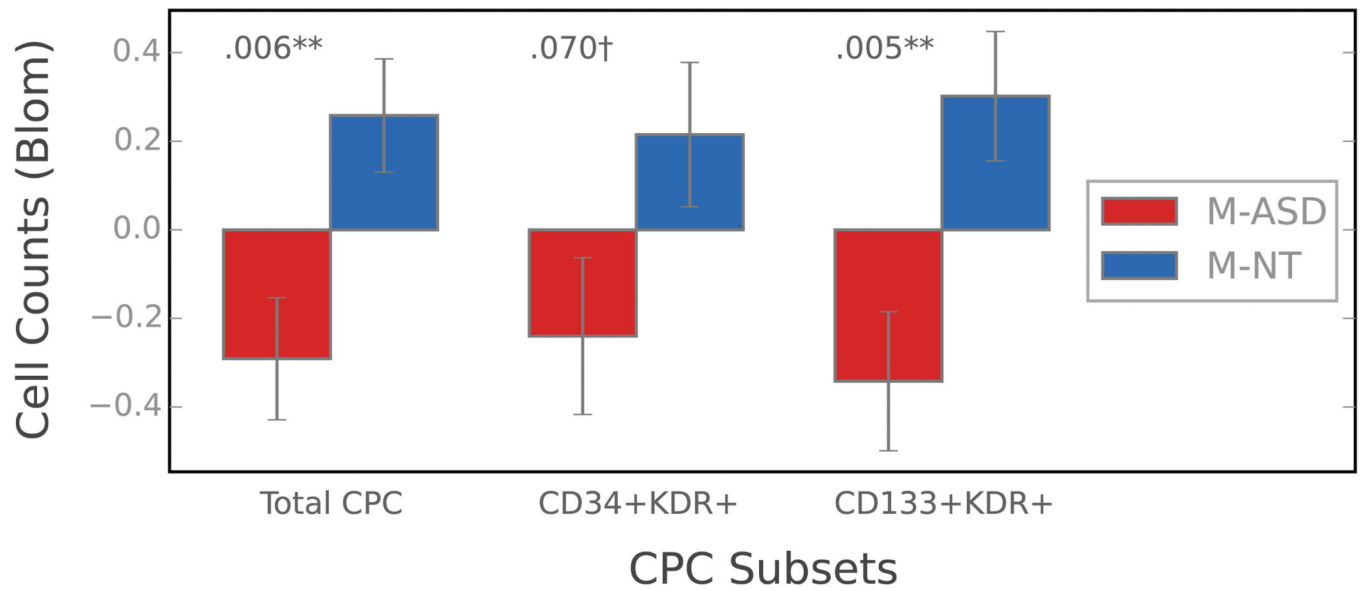
Figure 1.
Group Differences in Total CD45$^+$CD34$^+$KDR$^+$ and CD45$^+$CD133$^+$KDR$^+$ CPCs
Note: **p .01, *p .05, †p .10. CPC = Circulating Hematopoietic Progenitor Cell. M-ASD = Mothers of children with autism spectrum disorders. M-NT = Mothers of neurotypical children. The y-axis is the averaged blom-transformed scores for the CD45$^+$CD34$^+$KDR$^+$ and CD45$^+$CD133$^+$KDR$^+$ subsets.
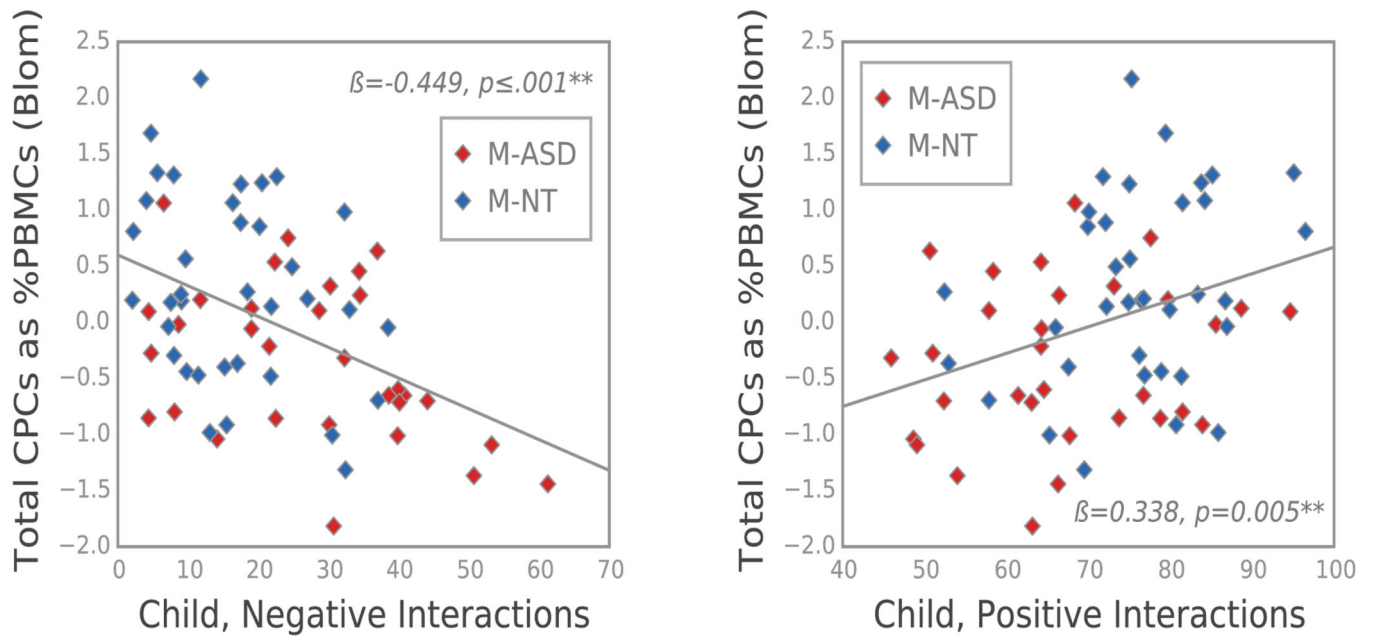
Figure 2.
Mother-Child Interactions are Associated with Total CPCs

Note: **p .01, *p .05, †*p* .10. The standardized regression coefficient and p-value are given above. CPCs = Circulating Hematopoietic Progenitor Cells. M-ASD = Mothers of children with autism spectrum disorders. M-NT = Mothers of neurotypical children.

Figure 3.
Group Differences in CD14^{+} Monocytes and their Co-Expression of CPC Markers
Note: **p .01, *p .05, †*p* .10. CPC = Circulating Hematopoietic Progenitor Cell. M-ASD = Mothers of children with autism 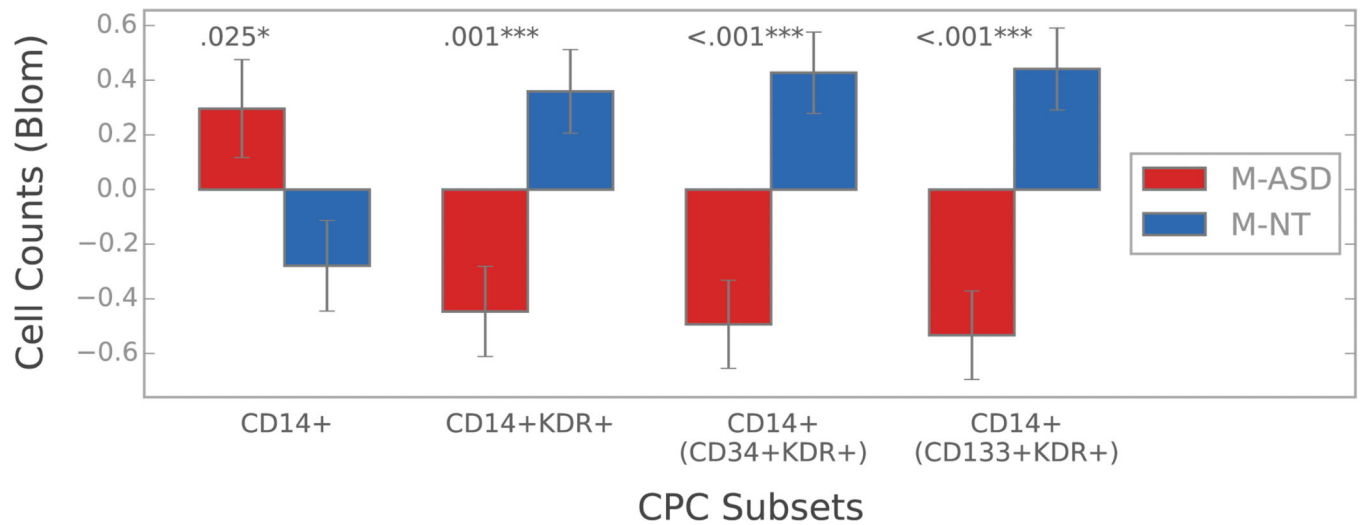spectrum disorders. M-NT = Mothers of neurotypical children. CD14[+](CD34^{+}KDR^{+}) and CD14[+](CD133^{+}KDR^{+}) represent the percentage of CD14+ monocytes that co-express the CPC markers CD45^{+}CD34^{+}KDR^{+} or CD45^{+}CD133^{+}KDR^{+}.

Table 1

Group Comparisons of Demographic, Psychological and Health Factors

N=68	M-ASD	M-NT
Demographic Characteristics	**(n=31)**	**(n=37)**
Age, years[a]	43.45 (.87)	41.86 (.77)
Caucasian race[b]	24 (77%)	27 (73%)
Advanced education[b]	11 (36%)	20 (54%)
Psychological Factors		
Perceived Stress Scale[a]	19.80 (.1.03)[*]	16.31 (5.43)
Depressive Symptoms[a]	17.55 (1.60)[**]	11.22 (1.11)
Negative Child Interactions[a]	27.68 (2.75)[**]	16.77 (1.69)
Positive Child Interactions[a]	66.96 (2.30)	76.03 (1.64)[*]
Negative Partner Interactions[a]	25.20 (14.00)[*]	16.35 (9.09)
Positive Partner Interactions[a]	60.47 (15.67)	69.75 (12.43)[*]
Cardiovascular Health		
Body Mass Index[a]	24.26 (0.71)	25.02 (0.69)
Exercise (None/Any)[a]	19 (61%)	28 (78%)
Systolic Blood Pressure[a]	112.74 (1.71)	111.11 (2.17)
Diastolic Blood Pressure[a]	68.35 (1.51)	66.76 (1.78)
Triglycerides[a]	82.03 (6.99)	73.14 (4.59)
LDL[a]	113.00 (5.08)[†]	100.35 (4.06)
High LDL 130 mg/dL[a]	9 (30%)[**]	3 (8%)
HDL[a]	57.80 (2.26)	67.32 (2.30)[**]
Low HDL 56 mg/dL[a]	15 (50%)[**]	9 (24%)
Total Cholesterol/ HDL[a]	3.35 (0.15)[**]	2.81 (0.11)

Note:

P-values indicate significant group differences at a critical alpha of .05, and are placed next to the group with the higher mean.

FET = Fisher's exact test, 2-sided. LDL and HDL comparisons use continuous values and also clinically relevant cut-offs based on recent literature (see methods for references). No participants had diabetes or cardiovascular disease, and smoking was an exclusion criteria.

[**]
p .01

[*]
p .05

[†]
p .10.

[a]Mean (SEM)

b
n(%).

Table 2

Pearson Correlations among CPCs, CD14+ Monocytes, and Cardiovascular Risk Factors

	All CPCs	CD34+KDR+	CD133+KDR+	CD14+	SBP	DBP	TG	LDL	HDL	Chol/HDL
All CPCs										
CD34+KDR+	.853 **	---								
CD133+KDR+	.856 **	.460 **	---							
CD14+	−.058	.011	−.110	---						
SBP	−.165	−.107	−.174	.262 *	---					
DBP	−.082	−.071	−.069	.210 †	.701 **	---				
TG	−.191	−.167	−.158	−.065	.221 †	.155	---			
LDL	−.193	−.139	−.191	.327 **	.281 *	.293 *	.354 **	---		
HDL	.259 *	.282 *	.161	−.186	−.116	−.008	−.422 **	−.206 †	---	
Chol/HDL	−.286 *	−.240 †	−.249 *	.291 *	.236 †	.167	.655 **	.724 **	−.748 **	---

Note:

***
p .001

CPCs = Circulating Hematopoietic Progenitor Cells; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; TG = Triglycerides; LDL = Low-Density Lipoprotein; HDL = High-Density Lipoprotein; Chol/HDL = Total Cholesterol to HDL Ratio. N=68 for correlations among CPC subsets and n=67 for correlations of cells with cardiovascular risk factors.

**
p .01

*
p .05

†
p .10, critical alpha = .05.