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Altered toxicological endpoints in humans from common quaternary ammonium compound disinfectant exposure

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ABSTRACT

Humans are frequently exposed to Quaternary Ammonium Compounds (QACs). QACs are ubiquitously used in medical settings, restaurants, and homes as cleaners and disinfectants. Despite their prevalence, nothing is known about the health effects associated with chronic low-level exposure. Chronic QAC toxicity, only recently identified in mice, resulted in developmental, reproductive, and immune dysfunction. Cell based studies indicate increased inflammation, decreased mitochondrial function, and disruption of cholesterol synthesis. If these findings translate to human toxicity, multiple physiological processes could be affected. This study tested whether QAC concentrations could be detected in the blood of 43 human volunteers, and whether QAC concentrations influenced markers of inflammation, mitochondrial function, and cholesterol synthesis. QAC concentrations were detected in 80 % of study participants. Blood QACs were associated with increase in inflammatory cytokines, decreased mitochondrial function, and disruption of cholesterol homeostasis in a dose dependent manner. This is the first study to measure QACs in human blood, and also the first to demonstrate statistically significant relationships between blood QAC and meaningful health related biomarkers. Additionally, the results are timely in light of the increased QAC disinfectant exposure occurring due to the SARS-CoV-2 pandemic.

Main Findings: This study found that 80 % of study participants contained QACs in their blood; and that markers of inflammation, mitochondrial function, and sterol homeostasis varied with blood QAC concentration.

1. Introduction

Humans are extensively exposed to quaternary ammonium compounds (QACs). QACs are disinfectants widely used in medical settings, restaurants, and food production facilities (US EPA 2006a). They are also ubiquitous in household cleaning products. Their widespread use has resulted in considerable environmental contamination. Despite their

prevalence, little is known about the extent of human exposure and the consequences of chronic low-level contact. Two common QACs are alkyl dimethyl benzyl ammonium chloride (ADBAC, also termed benzalkonium chloride or BAC) and didecyl dimethyl ammonium chloride (DDAC). Production volumes estimates in 2015 are 10–50 million pounds per year for each BAC and DDAC [51]. During production of BAC, a mixture of products is formed differing only in numbers of

Abbreviations: ADBAC, alkyldimethylbenzyl ammonium chloride; ANOVA, analysis of variance; BAC, benzalkonium chloride; CRP, C-reactive protein; DDAC, didecyldimethyl ammonium chloride; FCCP, trifluoromethoxy carbonyl cyanide phenylhydrazone; IL-6, interleukin 6; IL-10, interleukin 10; IL-12, interleukin 12; IRB, Institutional Review Board; LC, liquid chromatography; LOD, level of detection; LOQ, level of quantification; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa beta; NOEL, no effect level; OCR, oxygen consumption rate; OEL, occupational exposure limit; QAC, quaternary ammonium compounds; TNF α , tumor necrosis factor alpha; 7-DHC, 7-Dehydrocholesterol; 8-DHC, 8-Dehydrocholesterol; 7-DHD, 7-Dehydrodesmosterol.

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carbons units in the alkyl chain. Many commercial products also contain 2 QACs together such as BAC and DDAC. Mixtures can act synergistically or antagonistically to produce an effect that is different from the sum of the individual components and must be evaluated together when determining chemical exposure risk [1–3].

QAC disinfectants are historically viewed as having low toxicity; although exposure can cause asthma, contact dermatitis, ocular inflammation, and hypersensitivity [4–6]. Accidental overdoses from ingestion of the concentrate, while uncommon, have resulted in human deaths [7,8]. Risk assessments of BAC and of DDAC individually were conducted for regulatory purposes by the US EPA in 2006 and identified little risk to humans [9,10,53] Luz et al., 2020). However, mixtures of BAC and DDAC were not evaluated, and few toxicological endpoints other than changes in rodent body weight were tested. More importantly, no human studies have evaluated the extent, magnitude, and systemic response of exposure. This knowledge gap is particularly troubling in light of the increased use and exposure to QAC containing disinfectants due to the COVID-19 pandemic [11,12].

Chronic QAC toxicity has only recently been identified. Using *in-vitro* studies on both human and mouse cell lines, we found that QACs increase inflammation, disrupt mitochondrial function, alter estrogen signaling, and inhibit cholesterol synthesis [13–15]. In mice, we have shown that normal use of a disinfectant containing a mixture of BAC and DDAC in the vivarium inhibited reproduction, caused birth defects, and altered immune function [16–19]. With gestational exposure, BACs cross the placenta to alter cholesterol and lipid homeostasis in mouse neonatal brains [20]. Taken together, this suggests toxicity on both a cellular and organismal level. If these studies translate to humans, many basic physiological functions could be altered. There is, however, a complete data gap on the effects of chronic exposure in humans. The objective of this study was to determine the extent of human exposure and identify associated systemic responses.

2. Methods

2.1. Sample population characteristics

Blood samples were collected from a convenience sample of 43 participants recruited from a small college town. No demographic information was collected as per the IRB protocol; however, based on visual estimate of age, approximately two-thirds of the study participants were of college age, and one-third older adults. Inclusion criteria included: non pregnant individuals who were greater than 18 years of age with no history of chronic illness, no acute illness, and no previous blood draw in the last two weeks. All research was approved by the university's Institutional Review Board (IRB project number 1302366) in accordance with the Declaration of Helsinki, and informed consent was obtained from each participant.

2.2. Sample size calculation

We hypothesize that there is an association between measured toxicological endpoints and QAC concentration in blood. By estimating the correlation coefficient to be 0.5 and setting $\alpha = 0.05$ and power = 0.9, at least 31 blood samples were needed to determine associations between blood analytes and QAC concentrations.

2.3. Sample collection

Blood was collected into 3 separate heparinized tubes. One tube was used for mitochondrial analysis, a second tube was used for sterol and QAC analysis, while the third tube was used for cytokine analysis. Determination of each blood analyte was made blind to the participant's QAC concentration.

2.4. Mitochondrial function

Mitochondrial physiology was assessed with an XF24 Extracellular Flux Analyzer (Seahorse System, Agilent, Santa Clara, CA) which determines O₂ consumption under different test conditions. Briefly, 6 M white blood cells were aliquoted onto Seahorse XF24 plates at ~200,000 cells per well. Oxygen consumption rate (OCR) was determined at basal rate (ATP synthesis inhibited by oligomycin), maximal mitochondrial stimulation from addition of FCCP, and at full mitochondrial inhibition from addition of Antimycin A/rotenone (OCR from Proton Leak).

2.5. Sterol analysis

Analysis of Cholesterol and synthesis precursors: 7-Dehydrocholesterol (7-DHC), 8-Dehydrocholesterol (8-DHC), 7-Dehydrodesmosterol (7-DHD), Lanosterol, Zymosterol, Lathosterol, and Desmosterol, were determined by mass spectrometry. Lipids were extracted from the whole blood and the sterols analyzed by reverse phase LC-MS/MS assays as described previously [21].

2.6. Cytokine production

Cytokines were determined by ELISA. C-reactive protein (CRP) was determined in plasma (R&D Systems, Minneapolis, MN) and NF- κ B determined in whole blood (ABNOVA, Taipei, Taiwan). The remaining cytokines, IL-6, IL-10, IL-12, and TNF α , were determined in plasma (R&D Systems) under three conditions: unstimulated baseline; unstimulated whole blood culture baseline; and whole blood culture stimulated with 1 μ g/mL LPS. For the cultured conditions, heparinized whole blood was cultured for 12 h at 37 °C with 5 % CO₂, plasma was then collected. There was no difference in cytokine concentrations between the unstimulated baseline and the unstimulated cultured baseline; no further analysis was conducted on the cultured controls. IL-10 is generally considered an anti-inflammatory cytokine, but stimulation with LPS alone converts macrophages to the M1 pro-inflammatory phenotype and results in pro-inflammatory IL-10 production.

2.7. QAC determination

QAC levels in the blood were measured to accurately gauge exposure. Blood samples were spiked with known amounts of deuterium-labeled (*d*₇-benzyl) BACs as internal standards [15]. All samples were extracted by Folch solution (chloroform/methanol = 2/1). After extraction, the samples were re-constituted in the LC solvent and then analyzed by UPLC-MS/MS [22].

2.8. QAC nomenclature

QAC nomenclature is not consistent throughout the literature; for the purposes of this paper, QAC refers to both BAC and DDAC together; BAC, with no modifier refers to the various BAC alkyl chain lengths together; while the modifiers C10, C12, C14 and C16 BAC are used to designate specific BACs with the indicated alkyl chain length.

2.9. Statistical analysis

Analytes, in context of QAC concentration, were evaluated by means comparison, association (Statistix 10, Analytical Software, Tallahassee, FL), and effect size (SAS version 9.4, SAS Institute Inc., Cary, NC). Two-group means comparisons (no detectable QAC vs. any QAC) relied on two-sample t-tests or non-parametric Wilcoxon Rank-Sum tests, as appropriate. With the 4-group analysis, samples containing QACs were segregated into three evenly spaced QAC terciles. Comparisons were then made between those with no detectable QAC vs. low QAC vs. mid QAC vs. high QAC. Relationships between the groups were examined using analysis of variance (ANOVA) or non-parametric Kruskal-Wallis

tests, as appropriate, followed by Tukey’s HSD. Significance for 2 and 4-group means comparisons was set at $p \leq 0.05$. Two-group effect size was determined by Cohen’s d [23]. Four-group effect size was determined by eta-squared (η^2) [24]. Effect size was interpreted as small - d : 0.20; η^2 : 0.01, medium - d : 0.50; η^2 : 0.06, or large - d : 0.80; η^2 : 0.14. Only a few individuals contained C12 BAC; these were divided into 3 rather than 4 groups for analysis: no detectable C12, low C12, and high C12.

Bivariate associations between QAC concentrations and analyte values were determined by Spearman’s rank correlation (r_s). Because the no effect level (NOEL) threshold and nonmonotonic (non-linear) responses can interfere with linear associations across the entire sample population, individual correlations were determined over a range of sample numbers (N). The NOEL is a threshold concentration below which there is no measurable effect of the toxicant, and thus no correlation would be present at these lower concentrations. For each QAC species, blood samples were ranked high to low for a specific QAC. Associations between the QAC concentration and blood analyte were calculated across the ranked samples, increasing by 1 sample (N) in each calculation. For example, the 10 samples that contained C12 BAC were ranked from highest to lowest. Correlations were determined between analyte and C12 BAC for the five samples with the highest C12 BAC. A second correlation was then calculated for samples with the six highest C12 BAC concentrations. The process was repeated until correlations were determined for all 10 samples. Significance was taken at $r_s \geq 0.5$ with a $p \leq 0.05$ [25–27].

Nineteen of the twenty-seven analytes measured (including QACs) exhibited outliers (Statistix 10, Analytical Software, Tallahassee, FL). Typically, only a single outlier was seen per analyte; four analytes had two outliers. All outliers were greater than two standard deviations from the mean; 73 % of identified outliers were greater than three standard deviations from the mean. The identified outliers were not included in the analysis. Additionally, only one sample contained detectable C10 BAC, and no samples contained detectable concentrations of Baseline IL-6; no further analysis was conducted on these analytes.

Table 1

Concordance between significant means comparison p -values and large effect size for two, and four-group comparisons. Two-group comparisons were made between those without detectable QACs and those with any QAC using a t -test or non-parametric Wilcoxon Rank-sum test, and Cohen’s d for effect size. Four-group comparisons were made based on evenly spaced tertiles of QAC exposure (no detectable QAC vs. low QAC vs. mid QAC vs. high QAC) using analysis of variance (ANOVA) or non-parametric Kruskal-Wallis test, and eta-squared (η^2) for effect size. Significant means comparisons ($p \leq 0.05$) and large effect sizes ($d \geq 0.80$; $\eta^2 \geq 0.14$) are given. Means comparisons with less certainty ($p \leq 0.1$) and medium effect size ($d \geq 0.50$; $\eta^2 \geq 0.06$) are designated by = . All means, p -values, and effect sizes are given in Supplemental Tables 2-4.

Analyte	Two-Group Means Comparison and Effect Size									
	<i>t</i> -test <i>p</i> values					Effect size Cohen’s <i>d</i>				
	C12	C14	C16	DDAC	Total QAC	C12	C14	C16	DDAC	Total QAC
8-Dehydrocholesterol			0.016	0.048				0.80	==	
Maximal OCR from Basal			0.044			==		0.89		
Maximal OCR from Proton Leak			0.046							
Baseline TNF α				0.003	0.020	==			1.14	1.00
Stimulated IL-10	==					0.92	==			
Analyte	Four-Group Means Comparison and Effect Size									
	ANOVA <i>p</i> values					Effect size Eta ²				
	C12	C14	C16	DDAC	Total QAC	C12	C14	C16	DDAC	Total QAC
8-Dehydrocholesterol			0.016					0.237	==	==
Maximal OCR from Basal			0.028			==	==	0.252	==	0.159
Maximal OCR from Proton Leak						==		0.159		==
Baseline TNF α				0.010	0.029	==	==	==	0.219	0.230
Stimulated IL-6		0.037	0.008	==	==	==	0.157	==	==	0.152
Baseline IL-10				0.023		==			0.156	==
Stimulated IL-10	==		0.039			==	==	0.171	==	==
NF κ B										0.160

3. Results

3.1. Descriptive statistics

Descriptive statistics for blood analytes are summarized in Supplemental Table S1. QACs were detected in approximately 80 % of individuals, with half of these having Total QAC concentrations between 10–150 nM; a range shown to have physiological effects in cell culture models [14,15]. Of the individuals that contained QACs, median blood concentrations were 5, 2.4, 4.5, and 1.9 nM for DDAC, C16 BAC, C14 BAC and C12 BAC, respectively.

3.2. Means comparisons, effect size, and associations

Significant means comparisons and effect size are given in Table 1 while statistically significant associations are given in Table 2. Two-group and 4-group means comparisons for the entire dataset are given in Supplemental Tables S2; all 2 and 4-group effect sizes are given in Supplemental Tables S3 and S4; and all associations are provided in Supplemental Tables S5 - S9. While only large effect size is presented here, medium effect size, from at least one QAC species, was seen for all blood analytes except for Desmosterol (Table S4).

3.2.1. C12 BAC

Eleven individuals had C12 BAC in their blood; one outlier was identified (6.5 standard deviations from the mean) and was not included in the analysis. C12 BAC had a large effect on Stimulated IL-10, coupled with a means comparison that approached significance ($p = 0.0511$ by t -test; Table 1). Sequential associations, increasing 1 N each time, were calculated for all 10 samples containing C12 BAC (not including the outlier); only one association per analyte is shown in Fig. 1. Additional associations are given in Table 2 and Supplemental Table 5. The cholesterol pathway constituent Lanosterol demonstrated strong positive correlations (up to $r_s = 0.833$) for calculations which included 7 through 9 of the 10 individuals with C12 BAC, over a concentration range of 4.5 to 0.8 nM (Fig. 1A, Table 2). The increase in the precursor Lanosterol indicates a slowdown in a downstream step of the cholesterol synthesis pathway, or a general upregulation of cholesterol production.

Table 2

Significant correlation coefficients between QAC and blood analyte ($r_s \geq 0.5$ with a $p \leq 0.05$). Each QAC species was ranked high to low and Sequential associations, increasing 1 N each time, were calculated by QAC for all individuals containing that QAC (not including outliers) using the nonparametric Spearman's Rank Correlation test. Calculations for individuals with C12, C14, and C16 BAC in their blood numbered 10, 18, and 22 respectively indicated by † bars. Calculations for individuals with DDAC and Total QAC numbered 31 and 33 respectively; no significant correlations were found beyond an N = 26 (indicated by † bars). Significant correlation coefficients ($r_s \geq 0.5$, $p \leq 0.05$) are given. Correlation coefficients with less certainty ($r_s \geq 0.5$, $p \leq 0.1$) are designated by = = . All correlation coefficients and p -values are given in Supplemental Tables 5-9.

QAC	Analyte	Number of Samples Included in the Spearman's Rank Correlation																									
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27			
C12	Lanosterol			0.82	0.81	0.83	†																				
	Baseline IL-12			= =	0.83		†																				
C14	7-Dehydrodesmosterol					= =	-0.70	-0.77	-0.83	-0.69	-0.57				†												
	Baseline TNF α					= =	-0.70		-0.61		= =				†												
	Stimulated TNF α			= =	0.81	0.82	0.66	0.68			= =	0.62			†												
C16	Baseline IL-12									= =	= =	-0.59	-0.62														
	Stimulated TNF α								= =		0.54	0.58	0.53	0.60	0.65										†		
	Stimulated IL-6													0.57	0.64	0.57	0.57								†		
	Baseline IL-10										0.54	0.54	0.62	0.65	0.66										†		
DDAC	Stimulated IL-10		0.83	0.89	0.91	0.80	0.58	0.62	0.67	0.67	0.73	0.75	0.71	0.53	0.52	0.56									†		
	Cholesterol		0.83	0.89	0.86	0.80																					
	Zymosterol	= =	0.89	0.93	0.71	0.80	0.76		= =		0.75																
	Desmosterol	= =	0.89	0.93	0.71	0.77																					
	7-Dehydrocholesterol				= =	0.73	= =																				
	Lanosterol		0.83	0.89	0.79	0.85	0.73	0.64																			
	Max. OCR from Basal CRP				0.79	= =	-0.68	-0.73	-0.75																		
Total QAC	7-Dehydrodesmosterol	= =	0.89	0.89																							
	Max. OCR from Basal CRP				= =																						
	Stimulated TNF α				= =	0.79	0.75																				
	Stimulated IL-6		0.83																								
	Baseline IL-10				= =	= =	0.67					0.58	0.58														
Total QAC	Stimulated IL-10			0.79	0.86	0.90	0.89	0.92	0.76	0.65	0.57																

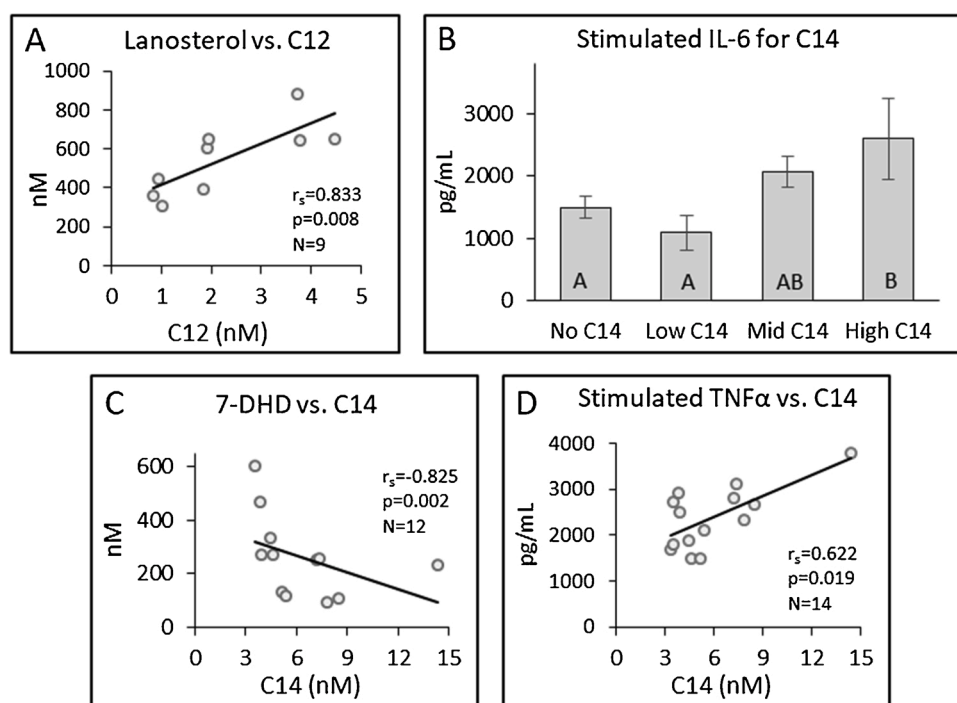


Fig. 1. Significant differences in selected analytes by C12 and C14 BAC concentration found in the blood. The 4-group bar graph depicts differences, as determined by ANOVA or the nonparametric Kruskal-Wallis test, between samples with no detectable C14 BAC ($N = 23$), and those with C14 BAC divided into three equally spaced tercile groups designated Low ($N = 6$), Mid ($N = 6$), and High ($N = 6$) concentration. Bars indicate (mean \pm SEM; those identified by different letters indicate a statistically significant difference ($p \leq 0.05$) between groups. Associations were based on Spearman's Rank Correlation Coefficients (r_s). Sequential associations, increasing 1 N each time, were calculated for all samples containing C12 or C14 BAC; only one association per analyte is shown here. Additional associations are given in Table 2 and Supplemental Tables 5 & 6.

3.2.2. C14 BAC

Twenty individuals contained C14 BAC; two outliers were identified (3.8 and 5.5 standard deviations from the mean) and were not included in the analysis. C14 BAC had a large effect on Stimulated IL-6, a common marker of inflammation, coupled with a dose response across the QAC tercile groups (Fig. 1B, Table 1). Sequential associations, increasing 1 N each time, were calculated for all 18 samples containing C14 BAC (not including the outliers); only one association per analyte is shown in Fig. 1. Additional associations are given in Table 2 and Supplemental Table 6. 7-DHD demonstrated strong negative associations (up to $r_s = -0.825$) for calculations from 10 individuals up to 14 out of the 18 individuals with C14 BAC, over a concentration range of 14.4 to 3.3 nM (Fig. 1C, Table 2). Stimulated TNF α was strongly associated (up to $r_s = 0.817$) with C14 BAC across much of the study population with significant associations beginning at 8 individuals and continuing across 14 individuals, over a concentration range of 14.4 to 3.3 nM (Fig. 1D, Table 2). Associations for both 7-DHD and Stimulated TNF α became weaker as samples with lower concentrations of C14 BAC were included in the calculations (Supplemental Table 6) and may be indicative of a threshold NOEL.

3.2.3. C16 BAC

Twenty-three individuals contained C16 BAC; one outlier was identified (6.3 standard deviations from the mean) and was not included in the analysis. C16 BAC affected sterol homeostasis, inflammation, and mitochondrial function (Fig. 2, Tables 1 and 2). When individuals without C16 BAC were compared to individuals with C16 BAC in the 2-group analysis, 8-DHC was significantly higher (Fig. 2B), while Maximum OCR from Proton Leak (Fig. 2C), and Maximum OCR from Basal (Fig. 2E) were significantly decreased with C16 BAC exposure. All were coupled with large effect sizes (Table 1). In the 4-group comparison, 8-DHC (Fig. 2A), Stimulated IL-6 (Fig. 2G), and Stimulated IL-10 (Fig. 2I) displayed nonmonotonic dose response across the four exposure groups. C16 BAC also had a large effect on Stimulated IL-10 (Table 1). Sequential associations, increasing 1 N each time, were calculated for all 22 individuals containing C16 BAC (not including the outlier); only one association per analyte is shown in Fig. 2. Additional associations are given in Table 2 and Supplemental Table 7. Positive

associations with C16 BAC were seen with Stimulated TNF α (up to $r_s = 0.651$, over 14–18 individuals, down to a concentration of 1.2 nM C16 BAC; Fig. 2F), Stimulated IL-6 (up to $r_s = 0.640$, over 16–19 individuals, down to 0.9 nM C16 BAC; Fig. 2H), and Baseline IL-10 ($r_s = 0.655$, over 14–18 individuals, down to 1.2 nM C16 BAC; Table 1). Stimulated IL-10 demonstrated a strong correlation (up to $r_s = 0.905$) over most of the study population, with significant positive associations beginning at 6 individuals and continuing to 19 individuals, over a concentration range of 6.4 to 0.5 nM (Fig. 2J, Table 1). The associations for Stimulated IL-10 became weaker as samples with lower concentrations of C14 BAC were included in the calculations (Supplemental Table 7) and may be indicative of a threshold NOEL.

3.2.4. DDAC

DDAC residues were seen in 32 of the 43 participants; one outlier was identified (5.9 standard deviations from the mean) and was not included in the analysis. DDAC affected cholesterol biosynthetic pathway intermediates, mitochondrial function, and inflammatory markers. In the 2-group comparison, 8-DHC was higher in individuals with DDAC exposure and was coupled with a medium effect size (Fig. 3A, Table 1). Baseline TNF α exhibited a greater than 10 fold increase in individuals containing DDAC residues over those without DDAC in their blood (Fig. 3H). This was accompanied by a large effect size (Table 1). In the 4-group comparison, Baseline IL-10 exhibited a J-shaped nonmonotonic dose response across the exposure groups (Fig. 3F). Baseline TNF α did not vary with the exposure concentration, and was significantly higher in all exposed individuals compared to those with undetectable DDAC in their blood (Fig. 3I). Sequential associations, increasing 1 N each time, were calculated for all 31 individuals containing DDAC (not including the outlier); only one association per analyte is shown in Fig. 3. Additional associations are given in Table 2 and Supplemental Table 8. Up-regulation of the cholesterol synthesis pathway was demonstrated by strong, positive correlations for Cholesterol, (up to $r_s = 0.893$, over 6–9 individuals, down to 7.4 nM DDAC; Table 2), Lanosterol (up to $r_s = 0.893$, over 6–11 individuals, down to 6.5 nM DDAC; Fig. 3B), Zymosterol (up to $r_s = 0.929$ over 6–13 individuals, down to 5.9 nM DDAC; Fig. 3C), and Desmosterol (up to $r_s = 0.929$, over 6–9 individuals, down to 7.4 nM DDAC; Fig. 3D). Maximal Mitochondrial OCR from Basal

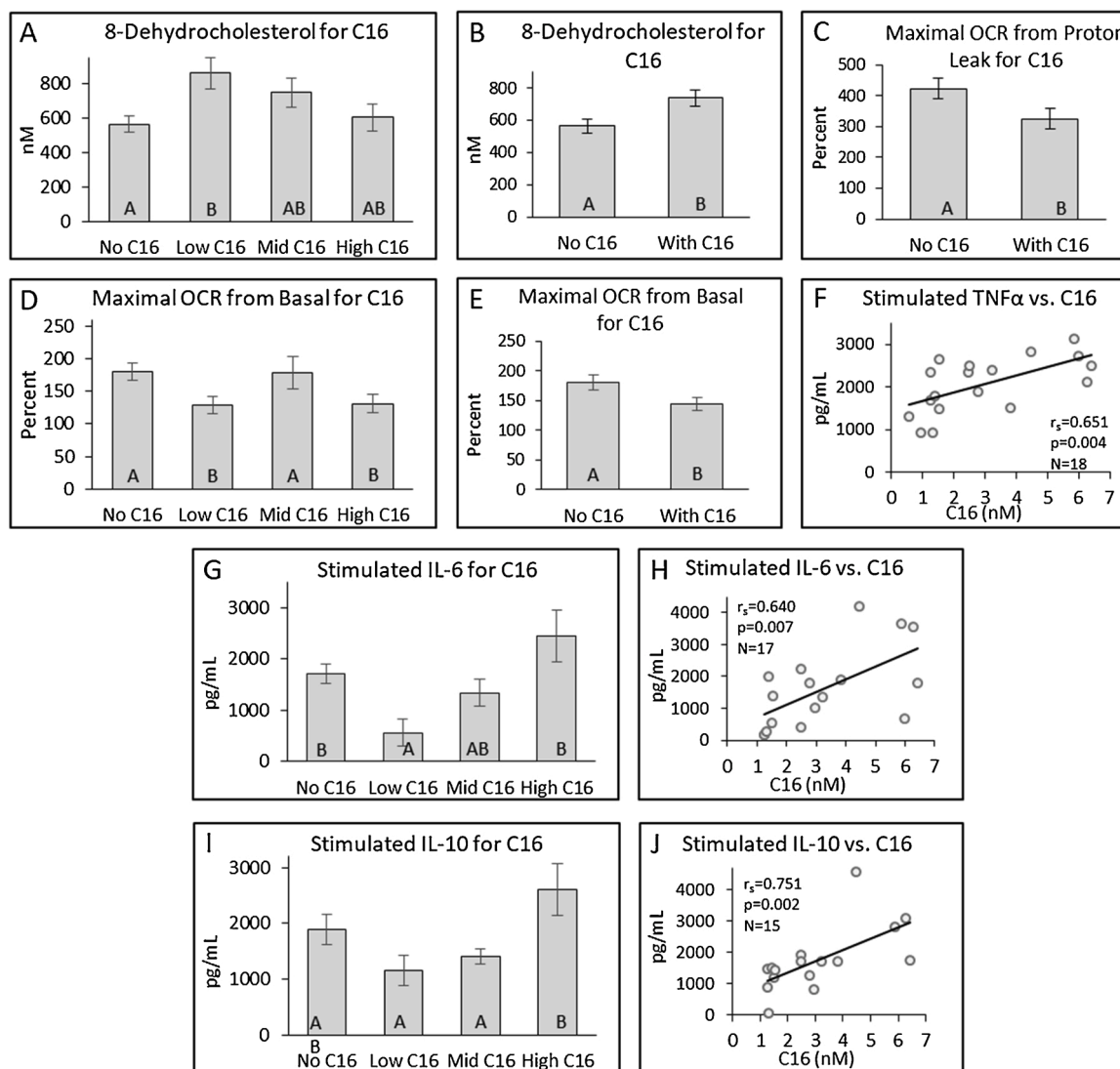


Fig. 2. Differences in selected analytes by C16 BAC concentration found in the blood. The 2-group bar graphs depict differences between those with no detectable C16 BAC ($N = 20$) and those with C16 BAC ($N = 22$) determined by either t -test or the nonparametric Wilcoxon Rank-sum Test. The 4-group bar graphs depict differences, as determined by ANOVA or the nonparametric Kruskal-Wallis test, between those with no detectable C16 BAC ($N = 20$), and those with C16 BAC divided into three equally spaced tercile groups designated Low ($N = 7$), Mid ($N = 8$), and High ($N = 7$). Bars indicate (mean \pm SEM; those identified by different letters indicate a statistically significant difference ($p \leq 0.05$) between groups. Associations were based on Spearman's Rank Correlation Coefficients (r_s). Sequential associations, increasing 1 N each time, were calculated for all samples containing C16 BAC; only one association per analyte is shown here. Additional associations are given in Table 2 and Supplemental Table 7.

showed strong negative correlations with DDAC (up to $r_s=-0.745$, over 9–11 individuals, down to 6.5 nM DDAC; Fig. 3E). Additionally, the inflammatory markers CRP (Fig. 3G), Stimulated TNF α (Table 2), and Baseline IL-10 (Table 2) were positively associated with DDAC concentration.

3.2.5. Total QACs

Thirty-five of the 43 participants contained one or more species of QAC which were summed to give the Total QAC concentration. Two outliers were identified (2.9 and 5.9 standard deviations from the mean) and were not included in the analysis. Analyte response to the Total QAC mixture can be enhanced if the responses to single QACs are additive, or potentiated. On the other hand, responses can be diminished if the response to a single QAC is diluted by minimal response to the other QACs, or if the responses of two different QACs are divergent. Most of the responses seen with individual QACs were blunted, as demonstrated by a smaller effect sizes with Total QAC, which likely indicates a dilution effect (Fig. 4, Tables 1 and 2, The significant increase in Baseline TNF α

with DDAC exposure, and the increase in Stimulated IL-6 from C14 and C16 BAC exposure carried over to the response seen in the Total QAC analysis with only slight blunting (Fig. 4D, 4E & 4 F) indicating these effects were not greatly influenced by the other QAC species. The decrease in Maximal OCR from Basal seen with DDAC carried over with the Total QAC associations (up to $r_s=-0.706$, over 10–16 individuals, and down to 12.1 nM Total QAC (Fig. 4A, Table 2). This association extended over a greater range than was seen with DDAC indicating a possible additive effect from other QAC species. The 2 and 4-group effect size analysis identified large effects from C16 BAC on Maximal OCR from Basal which could be contributing to the extended range in the Total QAC association. IL-10 was strongly correlated with Total QACs for both Baseline IL-10 (up to $r_s=-0.673$, over 10–18 individuals, and down to 7.9 nM Total QAC; Fig. 4B, Table 2) and Stimulated IL-10 (up to $r_s=-0.918$, over 7–26 individuals, down to 2.8 nM; Fig. C, Table 2). Of all the analytes, Stimulated IL-10 was most affected with the highest correlation coefficient, and the greatest range of concentrations over which an association was present. This response was most likely due to the

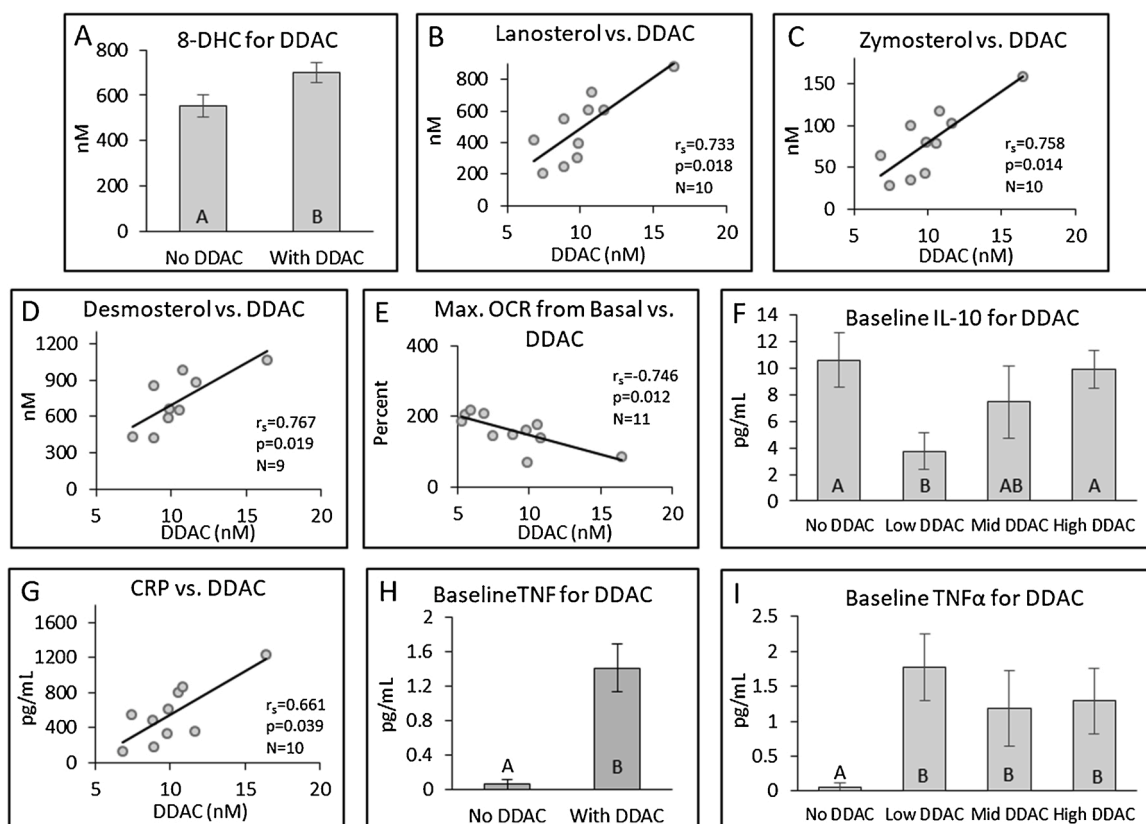


Fig. 3. Differences in selected analytes by DDAC concentration found in the blood. The 2-group bar graphs depict differences between those with no detectable DDAC (N = 11) and those with DDAC (N = 31) determined by either *t*-test or the nonparametric Wilcoxon Rank-sum Test. The 4-group bar graphs depict differences, as determined by ANOVA or the nonparametric Kruskal-Wallis test, between those with no detectable DDAC (N = 11), and those with DDAC divided into three equally spaced tercile groups designated Low (N = 10), Mid (N = 11), and High (N = 10). Bars indicate (mean ± SEM; those identified by different letters indicate a statistically significant difference ($p \leq 0.05$) between groups. Associations were based on Spearman's Rank Correlation Coefficients (r_s). Sequential associations, increasing 1 N each time, were calculated for all samples containing DDAC; only one association per analyte is shown here. Additional associations are given in [Table 2](#) and Supplemental Table 8.

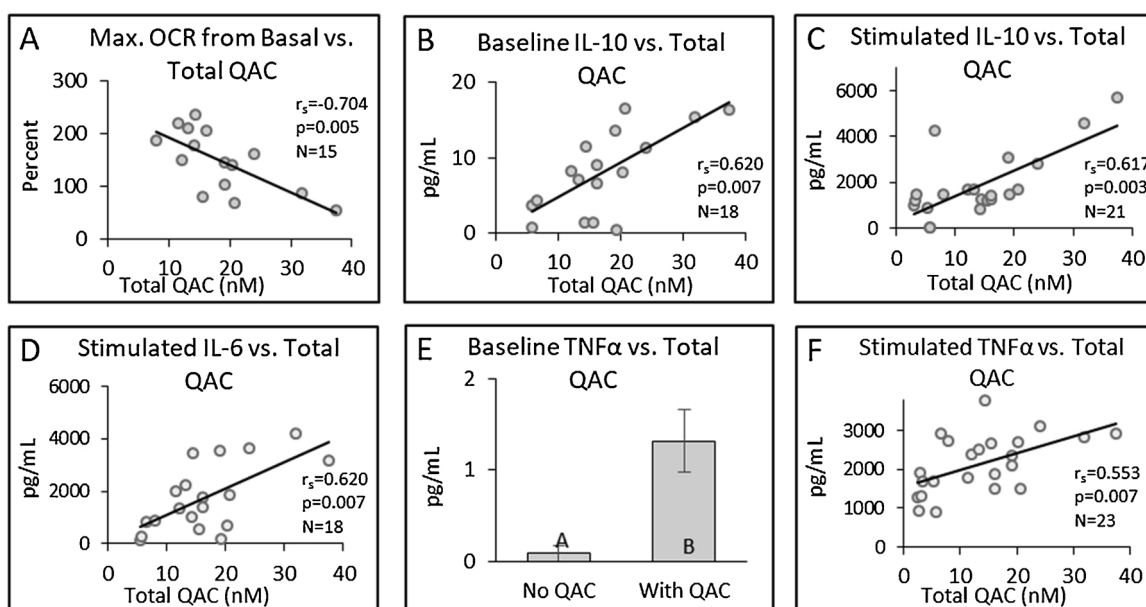


Fig. 4. Differences in selected analytes by Total QAC concentration found in the blood. The 2-group bar graph depicts differences between those with no detectable QAC (N = 8) and those with any QAC (N = 35) determined by either *t*-test or the nonparametric Wilcoxon Rank-sum Test. Bars indicate mean ± SEM; those identified by different letters indicate a statistically significant difference ($p \leq 0.05$) between groups. Associations were based on Spearman's Rank Correlation Coefficients (r_s). Sequential associations, increasing 1 N each time, were calculated for all samples containing any QAC; only one association per analyte is shown here. Additional associations are given in [Table 2](#) and Supplemental Table 9.

effect of C16 BAC as this QAC species showed strong associations and large effect sizes. However, the range of effect was greater in the Total QAC association than for C16 BAC, indicating a possible additive effect from other QAC species.

4. Discussion

Our studies, identifying chronic QAC toxicity in animal models, brings the issue of human toxicity to the forefront. The widespread use of QACs in household and commercial products has led to contamination of both the natural and built environment. It is estimated that about 75 % of the QACs utilized annually are released into water through wastewater treatment systems, and the rest is discharged directly into the environment [28]. This has long resulted in the presence of BAC and DDAC in urban wastewater, particularly downstream from point sources such as hospitals [29–31]. QACs are primarily removed from wastewater by sorption to the sludge [32]; however, the sludge is often then used as fertilizer. Plants take up the QACs resulting in residues in food products [33]. QACs discharged into the environment can be carried through storm water runoff into streams [31], and incorporate rapidly into Karst topography [34]. Karst aquifers are responsible for providing potable water for 40 % and 25 % of the US and world's population, respectively [35]. Thus people are potentially exposed through food and water sources, from direct contact with cleaning products, and from inhalation of aerosolized droplets produced by spray products. Surprisingly, sources of exposure, routes of exposure, and toxicokinetic properties of BAC and DDAC have not been investigated.

It is widely held that although exposure occurs, little if any accumulates systemically as BAC and DDAC pass through the intestine largely unabsorbed. This belief is based on unpublished rodent studies conducted for regulatory purposes [36,9,10]. For BAC, 90–98 % of an orally administered dose was eliminated in the feces, and only 0.03 to 0.5 % of the dose was retained in body tissues [9]. For DDAC, 89–99 % of an oral dose was eliminated in the feces, and only 0.003–0.675 % of the DDAC dose remained in the tissue [36]. However, these studies rely upon a single oral dose; tissue accumulation following chronic exposure, has not been assessed. Furthermore, substantial recovery of QAC in feces does not definitively demonstrate low absorption. Approximately 1/3rd of excreted BAC and 40 % of excreted DDAC were oxidized metabolites [9,37]. We have shown that BAC is converted to oxidized metabolites by hepatic cytochrome P450 enzymes expressed in the liver [22]. Others have also determined that the major excretory route of high molecular weight QACs, such as those examined in this study, is via bile into feces [38–40]. The extent of oxidized QAC metabolites in the feces could in fact reflect substantial intestinal absorption, followed by hepatic metabolism and biliary excretion back into feces.

The regulatory safety assessments were based on oral dosing. It is likely that this is not the primary route of exposure. Xue et al. [52] found much higher blood and tissue concentrations when BAC was given parenterally than when dosed orally. Similarly, mice that received only ambient exposure (most likely inhalant) from use of QAC disinfectants in the vivarium, showed similar degrees of reproductive and developmental impairment as mice receiving both oral and ambient exposures [17,18]. Inhalant exposure does not undergo hepatic first-pass metabolism as is seen with oral dosing, which implies that most of the observed toxicity was from the ambient exposure. Studies investigating systemic toxicity from inhalant QAC exposure are limited. A single study on inhalant toxicity of cetylpyridinium chloride, a common but structurally unrelated QAC, found that systemic toxicity in rats was greater from inhalant exposure over other routes [42]. While occupational exposure levels (OELs) for QACs have not been established, Dotson et al. [43] proposed a derived OEL of 0.1 mg/m³ for BAC and DDAC containing disinfectants. This would place QACs in OEL category 3 with moderate toxicity and high pharmacological activity. However, the estimated OEL was based on oral dosing extrapolated to inhalant exposure and may need to be adjusted as inhalant toxicity is better

understood. Due to the lack of information on routes of exposure and toxicokinetics, it is not known if the BAC and DDAC detected in our study population indicates prior exposure that day, or low level exposure over months.

Divergence in the outcomes of statistical tests can occur under some circumstances. A significantly different means comparison coupled with a small effect size would signal that the result could be simply due to chance. Conversely, a non-significant means comparison with a medium or large effect size likely indicates a nonmonotonic dose response, or a measurable response coupled with too small a sample size to resolve differences by *t*-test or ANOVA. Effect size analysis is independent of power or sample size and can overcome the limitation of a small data set. In our dataset, all the analytes showed concordance between statistically significant means comparisons and moderate to large effect sizes. This indicates the results are not likely due to chance. We did observe non-significant means comparison tests coupled with medium to large effect size which is likely due to the nonmonotonic dose responses observed and (or) the small sample size of this pilot study.

Divergence between means comparison tests and association tests can also occur. This can happen when the concentration of toxicant falls below the NOEL, as there is no longer a measurable effect of the contaminant on the determined end point. This may be why we observed sequential declines and eventual loss of significant correlation coefficients as more individuals, with lower blood QAC concentrations, were included in the correlation calculations. Alternatively, divergent test outcomes can be seen with nonmonotonic dose responses, which by definition are not linear across the concentration range. In this case, significant correlations may only be observed across a portion of the sample population, as was seen for a number of the analytes measured in this study.

The idea of a toxicant having a nonmonotonic dose response is not new and is reviewed in detail by Calabrese [44]. The nonmonotonic response demonstrates either an inverted U-shaped curve with decreased responses at low and high doses, or a J-shaped curve with enhanced responses at low and high doses. In an analysis of the published toxicological response literature, Calabrese and Baldwin [45] estimated that the overall frequency of nonmonotonic dose response across the spectrum of known toxicants is approximately 40 %. This includes responses to many well recognized toxicants such as DDT, mercuric chloride, lead, cadmium, chromium, toluene, and others [44]. With regards to QACs, an early study evaluating related histamine release from BAC exposed mast cells, identified a nonlinear dose response [46]. Additionally, BAC binds to the acetylcholine muscarinic receptor, and also the estrogen receptor [14,47]. Many receptor mediated signaling systems, including those involving the muscarinic and estrogen receptors, have a biphasic nonmonotonic dose response. Differential receptor binding by an agonist to two opposite acting receptors has been identified as a mechanism for these biphasic responses [44]. It has also been shown that low dose exposure to a toxicant induces a compensatory response to offset initial damage as the body tries to heal itself. This modest initial over-compensation to low dose exposure manifests as a nonmonotonic biphasic response [44]. The majority of participants in the study contained relatively low concentrations of QACs in their blood with median concentrations of 1.9, 4.5, 2.5 and 5 nM for C12 BAC, C14 BAC, C16 BAC, and DDAC respectively. At these lower exposures, we may be observing a compensatory response resulting in biphasic response curves.

Strong associations and dose dependent relationships in context of QAC concentration were clearly observed. The accepted significant correlation coefficient for evaluating environmental effects in human tissues is 0.5 [25–27]. We observed significant correlations from 0.5 to 0.9 for many blood analytes. Inflammatory cytokine production appears heavily impacted with increases in both baseline and stimulated responses at higher QAC concentrations. The inflammatory effects were particularly striking for TNF α , IL-6, and LPS-stimulated inflammatory IL-10. For example, DDAC exposure resulted in an order of magnitude

increase in baseline TNF α concentration. Mitochondrial function was also strongly and significantly inhibited in a dose dependent manner. These changes corroborate findings from our cell and mouse studies. Additionally, we found that cholesterol synthesis intermediaries were profoundly affected. Increasing concentrations of DDAC were significantly associated with increasing Zymosterol, 7-Dehydrocholesterol, Desmosterol, Lanosterol, and cholesterol while C12 BAC was positively associated with Lanosterol. Previously, we demonstrated *in-vitro* that BAC inhibition of the final step of cholesterol synthesis led to upstream accumulation of the synthesis precursors, particularly 7-DHC and 8-DHC [15]. The present study found an overall upregulation of cholesterol biosynthesis by DDAC suggesting that different types of QACs may have different effects on cholesterol biosynthesis.

C16 BAC affected more constituents than the other BAC species, despite the fact that C16 BAC was found at lower concentrations. DDAC also affected a large number of the blood analytes, and was the most abundant QAC species in the blood. The differing, and sometimes divergent, responses in analytes with the individual QAC species, coupled with the additive and ablative effects seen in the Total QAC analysis, clearly indicates that toxicity needs to be evaluated for individual QAC species, as well as for Total QAC mixtures. Some individuals in the study had a BAC or DDAC concentration that was over an order of magnitude higher than the next closest individual, indicating a wide range of exposures in the population. The extreme QAC concentrations were not attributable to a single individual, and were excluded as outliers in all calculations; thus the findings presented here may not be representative of outcomes in individuals with high QAC exposure. Additional studies are sorely needed to further evaluate the effects of BAC and DDAC particularly in individuals with high exposures.

It must be kept in mind that this is a small pilot study based on a convenience sample with a limited sample size. The lack of personal information and demographics collected on study participants prevents analysis of the sources and routes of exposure as well as correction for possible confounding variables such as age and sex. Even with this caveat, these data are provocative and indicate possible health ramifications from QAC exposure. If the changes in blood analytes observed in this study translate to the population at large, and are associated with pathophysiology, then exposure to QAC containing products could be involved in many and varied health outcomes. This is of particular concern with the increased use of QAC containing disinfectants due to the SARS-CoV-2 pandemic. The use of QAC disinfectants has increased with the pandemic [11] resulting in increased exposure to QACs in the home [12], and most likely in the workplace as well. In animal studies, we have demonstrated declines in male and female reproduction [16, 17], severe defects in neural development [18], and alterations in both neutrophil and T-cell phenotype and activity [19]. We also have found that chronic oral dosing during breeding and gestation resulted in maternal absorption and transfer of C12 BAC and C16 BAC across the placenta to the fetus, and disrupted lipid homeostasis in the neonatal brain [20]. BAC and DDAC also cross the blood-testis barrier to alter spermatogenesis [48]. QACs disrupt cell signaling through alterations in sterol and oxysterol formation [20], and through receptor mediated G protein Gi/Go signaling [49]. Taken together, there is a wealth of evidence suggesting that BAC and DDAC have profound effects on basic biochemical and physiological processes. The results of this pilot study indicates that QACs may affect human physiological responses as well.

5. Conclusions

This study is timely in light of the increased use of QAC containing disinfectants against SARS-CoV-2. We clearly demonstrate that QAC residues are present in human blood, and that inflammatory markers, mitochondrial function, and cholesterol synthesis intermediaries are altered in a dose dependent manner with QAC concentration. Further studies are critically needed to identify sources of exposure, toxicokinetic properties, and health effects associated with exposure. The

limitations of our pilot study, including small sample size and lack of demographic information to correct for possible confounding factors, prevent us from answering these important questions. However, the data clearly indicate that QAC exposure is extensive and there is significant potential for health ramifications from exposure to these compounds.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in Toxicology Reports.

Category 1

Conception and design of study: T.C.Hrubec, L. Xu, G.A Cortopassi, R.T. Williams; acquisition of data: R.P. Seguin, S. Datta, V.A. McDonald, C.A. Healy, T.C. Anderson, N.A. Musse; analysis and/or interpretation of data: T.C. Hrubec, R.P. Seguin, L. Xu, G.A Cortopassi, S. Datta, A.L. Hanlon, A.J. Lozano, V.A. McDonald, C.A. Healy, T.C. Anderson, N.A. Musse.

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Category 3

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

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