Received: 2 September 2019 | Revised: 18 January 2020 | Accepted: 20 February 2020

DOI: 10.1111/odi.13313

ORIGINAL ARTICLE

ORAL DISEASES WILEY

Inflammatory mediators in saliva and gingival fluid of children with congenital heart defect

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Funding information
Universitetet i Bergen; University of Science and Technology; Ahmed Gasim Cardiac Centre

Abstract

Objectives: (a) To compare levels of pro- and anti-inflammatory mediators in saliva and gingival crevicular fluid (GCF) in children with and without congenital heart defects (CHD cases and controls) and to test whether a systemic component exists in CHD cases by controlling for gingivitis and plaque scores. (b) To correlate the levels of pro- and anti-inflammatory mediators in GCF and saliva with plaque bacterial composition among CHD cases and controls.

Materials and Methods: Whole un-stimulated saliva and GCF samples were collected (60 CHD cases, 60 controls [Sudan]) and were analysed for levels of prostaglandin E2 (PGE2), interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), interleukin-1ra (IL-1ra) and interleukin-10 (IL-10) levels. These levels were correlated with the previously reported levels of four red complex bacteria.

Results: Significantly elevated levels of PGE2 and IL-1β in GCF and IL-1β and TNF-α in saliva were detected among CHD cases compared with controls. General linear model (GLM) analyses revealed that PGE2 and IL-1β levels remained significantly higher in GCF and saliva samples, respectively, among CHD cases after controlling for gingivitis and plaque score, whereas TNF-α and IL-10 levels were significantly lower in their GCF samples. Additionally, IL-1β level was significantly positively correlated to the counts of the four red complex species in their GCF.

Conclusion: In addition to higher levels of some pro-inflammatory mediators in saliva and GCF corresponding to more gingivitis in CHD cases, also a systemic inflammatory component exists and is reflected in these two oral fluids.

KEYWORDS

children, congenital heart defects, gingival crevicular fluid, gingivitis, inflammatory mediators, saliva

1 | INTRODUCTION

Congenital heart defects (CHD) affect approximately 8–10 per 1,000 child births (van der Linde et al., 2011). Besides their cardiac issues, it has been shown that children with CHD also suffer from a variety of other systemic health problems (Radford, Thong, Beard, & Ferrante, 1990). Several studies have shown the increased susceptibility of these children to different infections, for example respiratory tract infections, endocarditis and brain abscesses

(Canpolat et al., 2015; Ishiwada et al., 2005; Shu-yuan, 1989). These infections are often severe, prolonged, recurrent and challenging to treat (Radford & Thong, 1988). An earlier study suggested that this susceptibility is not only a consequence of their impaired cardiac function but rather due to some degree of immune deficiencies that is commonly associated with CHD (Radford & Thong, 1988). Furthermore, the concurrent occurrence of CHD with several immunodeficiency syndromes led to further studies to explore the immunological profiles among children with CHD (Khalil, Trehan, Tiwari, Malik, & Arora, 1994; Radford, Lachman, & Thong, 1986; Radford et al., 1990). Serum sample analyses have been conducted to compare the level of several immunological components (immunoglobulin, complement factors, B cells, T helper and T suppressor cells) between children with CHD and their healthy counterparts and have revealed significant differences (Khalil et al., 1994; Radford et al., 1990; Dorothy Jane Radford et al., 1986). Those studies concluded that children with CHD have a higher predisposition to infections and this could be partially explained by possible underlying immunological disturbances (Khalil et al., 1994; Radford et al., 1990).

With regard to the oral health of the children with CHD, studies have also shown that they are more prone to prevalent and long-lasting gingival inflammation compared to their healthy counterparts (Nosrati et al., 2013; Steelman et al., 2000) and that they harbour higher counts of several bacterial species that are known to be involved in initiation and progression of caries and gingival inflammation (Steelman et al., 2000; Steelman, Rosen, Nelson, & Kenamond, 2003). We recently demonstrated that a group of Sudanese children with CHD were found to harbour altered bacterial profiles which were correlated with higher cariogenicity and perio-pathogenicity (Ali et al., 2017). However, the mechanism behind the severe and prolonged gingivitis particularly among children with CHD is still unclear. Bacteria have long been known as powerful immune-stimulatory elements (including several virulence factors and lipopolysaccharide antigens [LPS]), which initiate local inflammatory and immune responses (Birkedal-Hansen, 1993). Mediators are released mainly include interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor α (TNF-α) and the arachidonic acid derivative prostaglandin E_2 (PGE_2) (Birkedal-Hansen, 1993; Faizuddin, Bharathi, & Rohini, 2003; Ulker, Tulunoglu, Ozmeric, Can, & Demirtas, 2008). Following the inflammatory process, different molecules such as interleukin-10 (IL-10), interleukin-4 (IL-4), interleukin-11 (IL-11) are produced to enhance resolution and return tissues to normal homeostasis (Hasturk, Kantarci, & Van Dyke, 2012; Kinane & Attström, 2005; Opal & DePalo, 2000; Van Dyke & Serhan, 2003). The disturbance of the balance between pro-inflammatory and anti-inflammatory processes has also been associated with the delayed resolution of inflammation and tissue destruction (Hasturk et al., 2012; Kinane & Attström, 2005; Van Dyke & Serhan, 2003).

These immune products are present in varying amounts within the oral fluids, including gingival crevicular fluid (GCF) and saliva (Bostanci et al., 2013; Chiappin, Antonelli, Gatti, Palo, & Elio, 2007; Delima & Van Dyke, 2003; Grant et al., 2010; Taylor & Preshaw, 2016). Both saliva and GCF have been investigated for the measurement of inflammatory processes in the periodontal tissues and were considered superior to serum analysis (Jaedicke, Preshaw, & Taylor, 2016; Taylor & Preshaw, 2016). In addition, saliva has long been proposed as a diagnostic tool with proven effectiveness for investigations of several other systemic conditions (Khaitan, Kabiraj, Bhattacharya, Ginjupally, & Jha, 2015; Lima et al., 2010; Matczuk, Zendzian-Piotrowska, Maciejczyk, & Kurek, 2017; Pappa, Kousvelari, & Vastardis, 2018).

On the other hand, studies have shown that the development of atherosclerosis begins earlier during childhood in children with CHD and they are at higher risk of developing vascular diseases and their complications upon reaching adulthood (Bresolin et al., 2013; Giannakoulas et al., 2009; Nosrati et al., 2013; Pemberton et al., 2010). This increased risk among children with CHD has also been linked to the enhanced levels of systemic inflammatory mediators following prolonged gingival inflammation, together with other risk factors, which might contribute to future development of acquired heart diseases and atherosclerosis (Dyke & Winkelhoff, 2013). A study from Brazil demonstrated that improved oral hygiene and periodontal treatment provided to children with CHD resulted in marked reduction of blood lipid parameters which are known as risk factors for future atherosclerosis (Bresolin et al., 2013).

Most of the previously reported findings have focused on systemically derived inflammatory mediators but no study has been reported comparing the levels of a panel of inflammatory mediators in GCF and saliva of children with CHD. Given the possible underlying immune insufficiency found in some of the children with CHD, we hypothesize that the more severe gingival inflammation observed will be reflected as increased inflammatory mediators and reduced amounts of anti-inflammatory molecules in their saliva and GCF compared to healthy children. Therefore, the aims of the study were as follows:

1. To measure the levels of selected pro- and anti-inflammatory mediators in saliva and GCF samples in children with CHD (CHD cases) compared with levels in their healthy counterparts (controls) and to test if a systemic component exists in CHD cases by controlling for gingivitis and plaque scores.
2. To study the expression of pro- and anti-inflammatory mediators in GCF and saliva samples and to correlate them with plaque bacterial composition among CHD cases and controls.

2 | MATERIALS AND METHODS

2.1 | Study subjects

A total number of 120 children from Khartoum city were enrolled in this study: 60 with CHD (CHD cases) and 60 controls (Ahmed Gasim cardiac Centre for CHD cases and schools and kindergartens for the

controls). Details regarding the setting, sampling and study design were previously reported (Ali et al., 2016). Children with confirmed diagnosis with a CHD in the age group 3–12 years were included, but critically ill children and those using medications other than for CHDs were excluded (Ali et al., 2016). Data collection was done in 2011 and in 2014 (Ali et al., 2016). In the current study, analyses were performed in children examined in 2014 when saliva and GCF samples were included (Ali et al., 2016).

Congenital heart defects cases and controls were matched for age, gender and use of antibiotics (frequency or group matching). Children underwent oral clinical examination, with plaque and gingivitis measured using simplified forms of the Gingival Index (GI) and the Plaque index (PI) (Ainamo & Bay, 1975) including measurements from six sextant teeth (55/16, 51/11, 65/26, 75/36, 71/31 and 85/46). Dichotomous scoring for gingivitis was used, where visual inflammation and a tendency to spontaneous bleeding was scored as 1, while the absence of these signs was scored as 0. The same scoring system was used for measuring plaque, where the presence of visible plaque on at least one surface was given the score of 1 and the absence of plaque 0.

2.2 | Saliva sample collections

Whole saliva samples (un-stimulated) were collected from all children (60 CHD cases, 60 controls). They were instructed to allow saliva to collect in their mouths before gently emptying into a sterile 2 ml tube. The un-stimulated whole saliva (UWS) samples were processed by centrifuging for 10 min at 4°C at 10,000 *g* in a micro-centrifuge (Beckman Coulter Microfuge 22 R Centrifuge). Each supernatant was dispensed into two tubes and stored in liquid nitrogen for further analysis. Saliva protein contents were measured using NanoDrop UV-Vis spectrophotometer at 260 and 280 nm.

2.3 | Collection of GCF samples

Perio-paper (PERIOPAPER® Gingival Fluid Collection Strips; Oraflow Inc) was used for collection of GCF from the mesio-buccal site of the four posterior teeth (55/16, 65/26, 75/36, and 85/46). Prior to the GCF collection, the area was isolated with cotton rolls, and thereafter, a perio-paper was placed in the sub-gingival sulcus for 15 s. The perio-papers were stored in an empty tube at −80°C.

2.3.1 | Protein measurement in GCF

Gingival crevicular fluid perio-papers were embedded in 230 ml Tris buffer (12 mM [pH 7.6]). Then, GCF samples were centrifuged at 4°C at 10,000 × g in a micro-centrifuge for 5 min and the supernatants were stored at −80°C. BCA™ Protein Assay Kit (PIERCE Thermo Fisher Scientific Inc.) was used for the protein measurement following manufacturer's instructions.

2.3.2 | Analyses of PGE_2

Following sample preparation, GCF and saliva samples were analysed using ELISA kits (NEOGEN CORPORATION) for the detection of prostaglandin E_2 mediator following the instructions from the manufacturer. Plates precoated with monoclonal antibodies against PGE_2 were used. Samples were diluted in the assay buffer of the ELISA kit with a dilution factor of 1:10. The plate was read at 450 nm, and concentrations were measured by FLUO star OPTIMA in ng/ml for the samples.

2.3.3 | Multiplex fluorescent bead-based immunoassay

Bead-based multiplex immunoassay was performed using a custom-made assay for the detection of four cytokines from the Bio-plex Pro Human Cytokine-plex assay Group 1 (Bio-Rad Laboratories). Two pro-inflammatory cytokines were selected for detection (IL-1β and TNF-α) as well as two anti-inflammatory cytokines (IL-1ra and IL-10) following the manufacturer's instructions. The plates were then analysed using the Luminex platform (Luminex), also known as xMAP, for the processing of the Bio-Plex® 200 systems. Concentrations measured are presented as pg/ml for saliva and GCF samples.

2.4 | Plaque bacterial composition (members of the red and orange complexes)

The amount of the bacterial members of the red complex (*Tannerella forsythia (T. forsythia)* , *Porphyromonas gingivalis (P. gingivalis)*, *Eubacterium nodatum (E. nodatum)* and Terponema *denticola (T. denticola*) has previously been reported in pooled plaque samples taken from the four posterior teeth of each child in the two groups and described in detail (Ali et al., 2017). Briefly, the collected plaque samples were kept in sterile tubes and stored at −80°C. DNA extraction and purification were done using Master-Pure TM DNA Purification kits (EPICENTRE Biotechnologies) and the DNA–DNA hybridization (Checkerboard) technique. We followed the procedures described by Socransky et al. where DNA extracted from known numbers of bacteria was used for the creation of standard curves used for estimation of counts (Socransky et al., 2004). The mean counts of red bacterial species in the plaque samples from 60 CHD cases and 60 controls were previously published (Ali et al., 2017).

2.5 | Ethical approvals

For the CHD cases, ethical approvals were obtained from Ahmed Gasim Hospital, Federal Ministry of Health Sudan, Research Ethical

Committee at the University of Science and Technology, and ethical approval was also obtained from the Regional Committee for Medical Research Ethics Western Norway (No. 2265) Biobank (No.2355). For the controls, ethical approvals were obtained from the State Ministry of Education (Khartoum), the State Ministry of Primary and Pre-school Education in the three localities of the capital Khartoum. Confidentiality was ensured, and a translated consent form was filled out and signed by the participants' guardians upon agreement to participate (both cases and controls).

2.6 | Statistical analysis

Data were entered and analysed using Statistical Package for the Social Science (SPSS) version 22. Data are presented as medians of the readings (continuous variables) and the Mann–Whitney test was used for the comparisons. General linear models were used for controlling the potential effect of the differences in levels of gingivitis and plaque among the two groups on the analysed inflammatory mediators. A *p* value of .05 was used to evaluate the statistical significance. Spearman's rho test was used to determine the correlation between the level of each of the investigated mediators and the level of each of the red complex bacterial species (*T. forsythia*, *P. gingivalis*, *E. nodatum* and *T. denticola*).

3 | RESULTS

There were no significant differences in distribution of age (CHD = 7.09 ± 2.68 years, controls = 6.93 ± 2.56 p = .750), gender (CHD males = 50%, controls males = 48.3% p = .853) or antibiotic use (CHD cases = 54.9%, controls = 45.1%) between CHD cases and controls (p = .307). The mean number of sites with gingivitis for CHD cases was significantly higher compared to controls (GI = 4.3 ± 1.9 vs. 1.9 ± 1.9) as well as the mean number of sites with plaque (PI = 5.1 ± 1.5 vs. 3.9 ± 2.1, with (p = .001) and (p = .002), respectively.

Saliva samples were analysed for PGE_2, and the results showed that it was detected among 85% of CHD cases compared to 65% of controls (p < .05). PGE_2 level was considerably higher among CHD cases compared with controls in both saliva and GCF, but reached the statistical significance only in the latter (Table 1).

Additional analyses using both saliva and GCF samples were carried out to determine the levels of the pro-inflammatory cytokines IL-1β and TNF-α, as well as the anti-inflammatory cytokines IL-10 and IL-1ra. IL-1β was found in significantly enhanced levels in both saliva and GCF samples of the CHD cases compared to controls (Table 1). While on the other hand, TNF-α level in the GCF samples was significantly lower among the CHD cases but in contrast was significantly higher in saliva samples compared to controls (Table 1). Comparisons also revealed that IL-10 was found in significantly higher levels in saliva samples, while IL-1ra was significantly higher in both fluids among CHD cases compared with controls (Table 2).

General linear regression models (GLMs) were used to control for the effect of the level of gingivitis and plaque upon all mediators analysed. In the saliva samples, GLM regression revealed that IL-1β was the only inflammatory mediator that persisted in significantly higher level among CHD cases compared with controls (Table 3). For the GCF samples, GLM regression revealed that the pro-inflammatory mediator PGE2 remained significantly higher among the CHD cases while TNF-α level remained significantly lower among

TABLE 1 Levels of the lipid mediator PGE_2 and the pro-inflammatory cytokines in GCF and saliva samples

	GCF			Saliva		
	CHD Cases (*n* = 57)	**Controls (*n* = 57)**	***p* value**	**CHD cases (*n* = 54)**	**Controls (*n* = 52)**	***p* value**
PGE2						
Median	5.98 ng/15 s	5.09 ng/15 s	.009**	0.56 ng/ml	0.28 ng/ml	.152
(Min–Max)	2.95–9.72	3.15–13.50		0.00–1.97	0.00–2.25	
	CHD Cases (*n* = 58)	**Controls (*n* = 58)**		**CHD Cases (*n* = 34)**	**Controls (*n* = 44)**	
IL-1β						
Median	413.58 pg/15 s	316.45 pg/15 s	.033*	29.91 pg/ml	9.05 pg/ml	.001**
(Min–Max)	69.84–1,674.03	42.09–1,458.63		0.00–174.32	0.00–131.12	
	CHD Cases (*n* = 58)	**Controls (*n* = 58)**		**CHD Cases (*N* = 34)**	**Controls (*N* = 44)**	
TNF-α						
Median	10.89 pg/15 s	15.69 pg/15 s	.001**	22.70 pg/ml	8.72 pg/ml	.019*
(Min-Max)	0.39–36.96	1.32–39.27		0.00–83.76	0.00–69.90	

Note: Comparisons between CHD cases and controls for levels of prostaglandin E_2, interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in gingival crevicular fluid (GCF) and saliva samples using Mann–Whitney test.

Abbreviation: *n*, number of children.

*p < .05

**p < .01.

TABLE 2 Levels of anti-inflammatory cytokines in GCF and saliva samples

	GCF			Saliva		
	CHD Cases (*n* = 58)	Controls (*n* = 57)	*p* value	CHD Cases (*n* = 54)	Controls (*n* = 55)	*p* value
IL-10						
Median	7.62 pg/15 s	8.64 pg/15 s	.110	2.54 pg/ml	1.84 pg/ml	.013*
(Min–Max)	1.56–24.63	1.56–31.68		0.78–22.22	0.33–94.60	
	CHD Cases (*n* = 34)	Controls (*n* = 44)		CHD Cases (*N* = 34)	Controls (*N* = 44)	
IL-1ra						
Median	145,617.42 pg/15 s	119,072.13 pg/15 s	.022*	11,289.18 pg/ml	6,378.03 pg/ml	.015*
(Min–Max)	34,735.26–490,660.08	12,462.06–449,637.51		0.00–64,087.88	0.00–323,615.36	

Note: GCF, gingival crevicular fluid; interleukin-10 (IL-10) and interleukin-1ra (IL-1ra), Mann–Whitney test was used for comparisons. *n* = number of children.

*$p < .05$; Level of significance is .05.

TABLE 3 Pro- and anti-inflammatory mediator levels in saliva of CHD cases and controls

Inflammatory mediator	CHD cases (*n* = 34) Mean ± *SD*	Controls (*n* = 44) Mean ± *SD*	*p* Value
PGE2 (ng/ml)	0.63 ± 0.46	0.51 ± 0.64	.603
IL-1β (pg)/ml	52.56 ± 54.35	19.98 ± 32.11	.013*
TNF-α (pg/ml)	25.00 ± 22.04	15.74 ± 19.21	.220
IL-10 (pg/ml)	3.67 ± 3.76	4.64 ± 14.35	.805
IL-1ra (pg/ml)	15,611.28 ± 15,837.91	15,228.89 ± 49,496.96	.973

Note: Comparison of the levels of pro- and anti-inflammatory mediators in saliva samples between CHDs cases and controls using the general linear models while controlling for gingivitis and plaque scores. *N* = number of children.

*$p < .05$.

the CHD cases. The anti-inflammatory IL-10 turned out to be significantly lower in the GCF among CHD cases compared with the controls (Table 4). Further correlation analyses revealed that IL-1β detected in GCF was the only pro-inflammatory mediator that had a significant positive correlation with the levels of the all four bacterial species of the red complex (Table 5), while none of the pro- and anti-inflammatory mediators in saliva showed significant correlations with any of the red complex bacterial species (data not shown).

4 | DISCUSSION

The current study is the first to investigate the levels of pro- and anti-inflammatory mediators in both saliva and GCF samples from CHD cases and to correlate them to plaque bacterial status. Findings revealed distinct differences in the levels of both pro and anti-inflammatory mediators between CHD cases and controls in saliva and GCF samples. The pro-inflammatory response was in general more dominant in the CHD cases in both fluids. Since the CHD cases had higher scores of gingivitis and plaque, we sought to investigate whether the above-mentioned differences were due to different levels of gingivitis and plaque between the two groups of children. GLM were used to control for the differences and revealed that IL-1β was the only factor that maintained significant higher levels in saliva samples among the CHD cases, and demonstrate a systemic contribution to the level of this cytokine in CHD cases.

In GCF, PGE_2 maintained a significant higher level and IL-10 at a lower level in CHD cases even after controlling for gingivitis and plaque score, the findings indicate that an inflammatory component is reflected in GCF. The pro-inflammatory cytokine IL-1β was significantly higher in CHD cases in GCF, but after controlling for GI and PI the difference was no longer significant and indicates that the cytokine is mainly produced locally in gingiva due to higher GI in CHD cases compared to controls.

The systemic component of saliva is known to be more dominant than in the GCF. By comparing the two fluids, we hoped to identify mediators most likely to be altered due to a systemic effect and to a local oral effect in CHD cases. GCF best reflects local periodontal responses since the fluid is derived from the gingival extracellular fluid (Greabu et al., 2009). Fortunately, this quantification has recently been made more efficient by advances in the technologies for detecting small concentrations in low fluid volumes which had been the main challenges in analysing oral GCF (Matczuk et al., 2017; Pappa et al., 2018). Our findings demonstrate that parallel analyses

TABLE 4 Pro- and anti-inflammatory mediator levels in GCF samples of CHD cases and controls

Inflammatory mediator	CHD cases Mean ± *SD*	Controls Mean ± *SD*	*p* Value
PGE2 (ng/15 s)	6.15 ± 1.62	5.256 ± 1.45	.013*
IL-1β (pg/15 s)	487.25 ± 422.79	455.965 ± 315.38	.616
TNF-α (pg/15 s)	11.37 ± 7.79	17.311 ± 8.27	.002**
IL-10 (pg/15 s)	7.87 ± 4.64	10.661 ± 5.95	.023*
IL-1ra (pg/15 s)	164,556.61 ± 86,835.80	132,331.88 ± 85,491.16	.117

Note: Comparisons of levels of pro- and anti-inflammatory mediators in GCF samples between CHDs cases and controls using the general linear models while controlling for gingival index and plaque index scores. CHD cases are 34 and controls are 44 children.

*$p < .05$.

**$p < .01$

TABLE 5 Pro- and anti-inflammatory mediators in GCF in relation to the bacteria levels of the red complex

	CHD cases			Controls	
Inflammatory mediator	Bacterial species	Correlation coefficient	*p* Value	Correlation coefficient	*p* Value
PGE2	*T. forsythia*	.235	.055	.060	.772
	P. gingivalis	−.002	.985	−.075	.714
	E. nodatum	.131	.289	.004	.984
	T. denticola	.170	.168	.006	.976
IL-1β	*T. forsythia*	.582	.0001**	−.058	.722
	P. gingivalis	.383	.018*	.179	.270
	E. nodatum	.524	.001**	.027	.869
	T. denticola	.437	.006**	.105	.518
TNF-α	*T. forsythia*	−.128	.431	−.113	.518
	P. gingivalis	−.292	.071	−.147	.398
	E. nodatum	−.198	.226	−.167	.338
	T. denticola	−.281	.083	−.235	.174
IL-10	*T. forsythia*	−.138	.397	−.010	.950
	P. gingivalis	−.102	.536	.056	.740
	E. nodatum	−.151	.360	.093	.577
	T. denticola	−.115	.486	−.104	.535
IL-1ra	*T. forsythia*	.180	.280	.220	.179
	P. gingivalis	.070	.683	.013	.939
	E. nodatum	.093	.584	.136	.410
	T. denticola	.051	.764	−.007	.965

Note: Each mediator was correlated to each bacterial specious of the red complex bacteria. Spearman's rho test was used for the correlation analyses. CHD cases ($n = 57$) and controls ($n = 58$). GCF, gingival crevicular fluid.

*$p < .05$.

**$p < .01$.

of both fluids give important additional information as also observed in previous studies (Matczuk et al., 2017; Pappa et al., 2018).

In the present study, PGE_2 was measured in saliva and GCF samples because it is one of the immune products known to indicate a local chronic ongoing inflammatory process and is a robust marker for measuring tissue inflammatory condition (Kalinski, 2012). PGE_2 is a member of the prostanoid family and a product of arachidonic acid derivatives produced by the sequential actions of the cyclo-oxygenase-2 (PTGS2/COX2) enzymes (Yao et al., 2009). It is found abundantly at inflammation sites and mediates tissue damage by suppressing acute inflammatory mediators while promoting immune responses associated with chronic inflammation (Kalinski, 2012; Yao et al., 2009). The present findings show that significantly more CHD cases compared to controls had detectable levels of PGE_2 in saliva and higher mean levels of PGE_2 in both saliva and GCF samples than controls. This increased PGE_2 can be explained by

the higher red complex bacterial counts, and the latter is previously published (Ali et al., 2017). We suggested in the previous study that these higher counts of red complex bacteria may be attributed to changes in the oral microenvironment which favour the growth of these bacteria and/or to poor oral hygiene, and/or to the intake of more cariogenic nutrition in children with CHD.

IL-1β is a key pro-inflammatory cytokine that plays an important role in the cellular response to microbial infection (Schenk et al., 2014). It is involved in a variety of cellular activities including immune activation, cytokine release and differentiation of monocytes to dendritic cells (Schenk et al., 2014). Locally, IL-1β plays a fundamental role in periodontal tissue destruction following exposure to various pathogens such as the red complex bacteria (Sanchez, Miozza, Delgado, & Busch, 2013). This relationship was also supported by correlation analyses, where the level of IL-1β was significantly positively correlated to the levels of the four members of the red complex, in particular; *T. forsythia* and *E. nodatum*. This relationship was also demonstrated by the fact that red complex bacterial species (*P. gingivalis*, *T. forsythia* and *E. nodatum*) were all shown to be linked to gingival and periodontal diseases because of their potential virulence factors, and their presence in > 70% of cases of periodontal disease cases (How, Song, & Chan, 2016). *Porphyromonas gingivalis* being the mostly studied species since it's known to possess several virulence factors, such as cysteine proteinases (gingipain), lipopolysaccharide, capsule and fimbriae. *T. forsythia*, in addition, is thought to exacerbate periodontal disease via symbiosis with the highly pathogenic *P. gingivalis* and *T. denticola* (Falcao & Bullón, 2019).

Moreover, the significant lower TNF-α level in the GCF samples of CHD cases might also be associated with the suppressive actions of PGE_2 in gingiva that has been reported to reduce the production of several pro-inflammatory cytokines, including TNF-α, which promote a shift towards chronic inflammation (Kalinski, 2012).

Periodontal tissue destruction has also been shown to be promoted by the delayed clearance of leucocytes at the site of inflammation (Deo & Bhongade, 2010). This clearance is mediated by several anti-inflammatory mediators, including IL-10, IL-1ra and several other interleukins (Deo & Bhongade, 2010). IL-1ra acts by antagonizing the action and production of IL-1 for the purpose of dampening the pro-inflammatory IL-1 activity (Gabay, Lamacchia, & Palmer, 2010). It was demonstrated, however, that IL-1ra concentration needs to exceed the IL-1 concentration by 100- or 1,000-folds in order to reach an inhibitory effect (Slotwinska, 2013). In the current study, significant differences in saliva and GCF levels of IL-1ra between CHD cases and controls were seen, but when controlling for GI and PI in GLM, no such differences between the groups were found. These findings support an oral contribution of this cytokine in the two fluids, and not a systemic one.

With respect to methodology, saliva and GCF samples are both considered superior to serum analysis with respect to reflection of oral inflammatory processes (Greabu et al., 2009). The GCF volume measurement was not applicable in the present study due to the fieldwork settings, and cytokine levels are therefore presented as amounts/15 s, which is a limitation of this study. The use of ELISA to measure the inflammatory mediators is considered the gold standard, as was used in conjunction with the multiplex immunoassay, which is reported to have good sensitivity and specificity (Tighe, Ryder, Todd, & Fairclough, 2015). The use of dichotomous scoring of gingivitis in the current study did not reveal the degree of severity of gingival inflammation. However, we chose to the dichotomous GI due to the expected low cooperation of young children and to avoid long-lasting dental examinations. In addition, the consulting cardiologist advised us to not to provoke any unnecessary bleeding, and therefore, the least invasive index for the scoring of gingivitis was chosen.

We therefore conclude from the present findings that the levels of IL-1β and PGE_2 in both saliva and GCF samples were significantly elevated among CHD cases compared with controls, respectively. In addition, the levels IL-1β in GCF were associated with higher levels of red complex bacterial counts. We also observed a systemic inflammatory component in both GCF and saliva since PGE2 and IL-1β remained significantly elevated, respectively, in CHD cases after controlling for gingivitis and plaque scores. Our study confirms therefore that CHD cases have a systemic inflammation that is reflected in oral fluids and can possibly make them more vulnerable to develop more severe periodontal diseases later in their lifespan. It is particularly important to prevent development of periodontal diseases in CHD cases by focusing on improved oral hygiene.

ACKNOWLEDGEMENTS

This work was supported by the Faculty of Medicine and Dentistry, Department of Clinical Dentistry, University of Bergen, University of Science and Technology, Omdurman, Sudan and Ahmed Gasim Cardiac Centre, Khartoum, Sudan. The contributions of cardiologist, Siham Hasabalrasol, and the study participants are gratefully acknowledged. The authors would like to thank Hasaan G. Mohamed, and we also acknowledge the contributions of Hager R. Zein Elabdeen and Samih Salah.

CONFLICT OF INTEREST

This work was funded by the University of Bergen and no conflict of interest to be declared by any of the co-authors.

AUTHOR CONTRIBUTIONS

Dr. Hiba, Dr. Manal and Prof. Ellen have contributed in the designing, data collection and statistical analyses and drafting the paper. While Dr. Salwa, Dr.Osama and Dr. Raouf have contributed in the data collection and analysis of samples as well as the drafting of the paper.

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How to cite this article: Mohamed Ali H, Mustafa M, Suliman S, Elshazali OH, Ali RW, Berggreen E. Inflammatory mediators in saliva and gingival fluid of children with congenital heart defect. *Oral Dis*. 2020;26:1053–1061. https://doi.org/10.1111/odi.13313