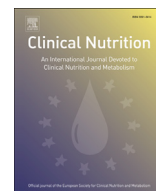




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## Original article

# The effect of 2 mMol glutamine supplementation on HSP70 and TNF- $\alpha$ release by LPS stimulated blood from healthy children

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## SUMMARY

**Objective:** Glutamine has been shown to promote heat shock protein 70 (HSP70) release both within experimental *in vitro* models of sepsis (2–10 mM) and in adults post trauma (0.5 g/kg), although the efficacy varies and is dependent on the model used. The effect of glutamine supplementation on HSP70 release in children is less clear. Therefore, the aim of this study was to investigate the effect of 2 mM glutamine added to incubation media on HSP70 and inflammatory mediator release in an *in vitro* model of paediatric sepsis using whole blood from healthy paediatric volunteers.

**Methods:** An *in vitro* whole blood endotoxin stimulation model using 1  $\mu$ g/ml lipopolysaccharide (LPS) over a 24 h time period was used to investigate the effects of 2 mM glutamine on HSP70 and inflammatory mediator release in healthy children.

**Results:** The addition of 2 mM glutamine to the incubation media significantly increased HSP70 release over time ( $p < 0.05$ ). This was associated with an early pro-inflammatory effect on TNF- $\alpha$  release at 4 h ( $p < 0.005$ ) which was not seen at 24 h. There was a non significant trend towards higher levels of IL-6 and IL-10 following the addition of 2 mM glutamine, which appears to differ from the response reported in adult and animal models.

**Conclusion:** Glutamine supplementation of incubation media promotes HSP70 and early TNF- $\alpha$  release in an *in vitro* model using blood samples from healthy children.

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### What is known:

- The addition of glutamine to incubation media promotes HSP70 release in *in vitro* experimental models of sepsis.
- During times of infection, HSP70 release signals via cell surface ligands TLR2 and 4 activating inflammatory pathway of NF- $\kappa$ B promoting the release of inflammatory mediators.

### What this study adds:

- The addition of 2 mM glutamine to incubation media in a paediatric *in vitro* model of sepsis significantly increased HSP70 levels over time.
- The addition of 2 mM glutamine to incubation media appeared to promote an early pro-inflammatory response in a paediatric model of sepsis.

## 1. Introduction

HSP70 forms part of the cellular response to stress [1]. Levels of extracellular HSP70 are increased, in response to infection, forming a network of molecules discharged by stressed or damaged cells [2,3]. HSP70 has powerful immunoregulatory effects [4–7], providing cellular protection [8] preventing apoptosis and cell death [9–13]. Under normal circumstances HSP70 is detectable in

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plasma of healthy individuals (who have no evidence of inflammation), suggesting that during times of homeostasis, HSP70 does not promote an inflammatory response and its immunoregulatory/inflammatory functions are tightly controlled [3]. However, during times of stress HSP70 is able to interact with antigen presenting cells (APC's) and activate both innate as well as adaptive immunity. The type of response elicited depends on whether HSP70 is within or external to the cell, the particular cell surface receptor sites it binds to [14] and the type of T cells stimulated. In experimental model of sepsis, *in vitro* cell culture models HSP70 has been shown to signal via the Toll like receptors (TLR) 2/4-MyD88-nuclear factor kappa B (NF- $\kappa$ B) pathway promoting the release of inflammatory mediators [15] influencing the mix of cytokines released [3]. As the stress response resolves, HSP70 acts to dampen down the inflammatory and immunoregulatory response, restoring cellular homeostasis [16,17].

As such the effects of HSP70 appear to be dependent of the model used (e.g. cell culture, animal, human). A recent analysis considering the effect of HSP70 in animal models compared to humans indicate that whilst HSP70 confers an almost entirely protective effect in animals (97.1%) the same may not be true in humans, with only a 50% protective effect demonstrated [1]. Therefore the clinical benefit of HSP70 upregulation during times of stress remains unclear, especially as both low and high levels in children and adults are associated with increased risk in children and adults mortality and infection risk [1,18]. In adults following trauma HSP70 levels  $\leq 15$  ng/ml is associated with increased mortality [19], conversely, levels  $\geq 60$  ng/ml is associated with increased mortality in traumatic brain injury [20] and severe sepsis in adults [21] and children [22]. Our own work in children with acute meningococcal disease, found significantly higher levels of HSP70 in the acute phase of illness, although this were not associated with increased mortality [23].

Glutamine, as a modulator of the heat shock response [24] is well described in experimental models of sepsis [25] and in adults following trauma [26]. Glutamine exerts a complex regulatory activity with respect to the activation of intracellular signalling pathways associated with inflammation and can influence the milieu of inflammatory mediator release in response to stress independently of HSP70 [27,28].

We have previously shown that glutamine depletion occurs in critically ill children and is correlated with length of stay and illness severity scores [23]. There is, however, limited information regarding the effect of glutamine supplementation on the HSP70 and inflammatory mediator release in children [29]. As such we sought to investigate the *in vitro* effects of endotoxin and glutamine on HSP70 release and its association with markers of inflammatory response, using an *in vitro* whole blood model in healthy paediatric volunteers. As HSP70 is produced by a large variety of cells including granulocytes and peripheral mononuclear cells [3] we chose to use an *in vitro* whole blood endotoxin stimulation model. Endotoxin has been used extensively to understand the pathophysiological response to sepsis/stress as well as for the study of cytokine [30] and HSP70 release [31], providing further insight into the host response [32].

The aim of this study was to investigate the effect of the addition of 2 mM glutamine to incubation media on HSP70 and inflammatory mediator release in an *in vitro* model of paediatric sepsis.

## 2. Materials and methods

This study was approved by the St Mary's Hospital Imperial College Foundation Trust (reference number EC3262). After obtaining informed parental consent, 25 healthy children were prospectively enrolled from a general paediatric outpatient clinic.

Heparinised whole blood was diluted 1:1 with glutamine free RPMI (Sigma Aldrich), stimulated with  $\pm$  LPS (1  $\mu$ g/ml) in duplicate, and incubated at 37 °C, 5% CO<sub>2</sub>. After 2 h of culture, 2 mM glutamine was added to half the conditions and incubated for another 2 h and for a further 22 h. At each time point (4 h and 24 h) supernatant was removed, centrifuged for 10 min at 1200 g and immediately stored at  $-80$  °C. HSP70 and inflammatory mediators were measured as per manufacturer's instructions using High Sensitivity HSP70 (HSP72) ELISA (Enzo Life Sciences CA; USA) and MSD Human pro-inflammatory – 3 II ultra sensitive ELISA (Meso Scale; MY; USA) measuring inflammatory mediators IL-6, TNF- $\alpha$  and IL-10. As plasma from whole blood *in vitro* model was used, we were not able to discriminate between the source (e.g. granulocytes, lymphocytes or peripheral mononuclear cells) and subsequent role of the HSP70 released from the *in vitro* cell mix.

Statistical analysis of clinical variables, HSP70 and cytokine data was completed using statistical analysis package Graphpad Prism 4.0 for Windows (Graphpad Software, San Diego, CA) and Statistical Package for Social Sciences 19.0 (SPSS: An IBM Company, Chicago, IL).

## 3. Results

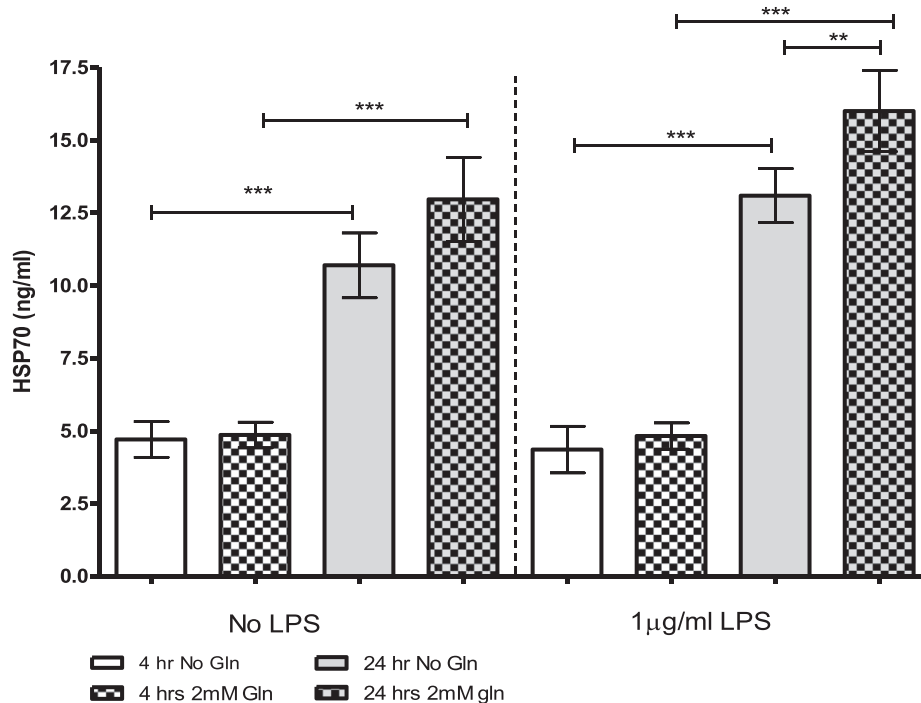
### 3.1. Effect of 2 mM glutamine on HSP70 release in endotoxin stimulated whole blood from healthy paediatric volunteers

In children, the addition of 2 mM glutamine to incubation media significantly upregulated HSP70 release at 24 h in endotoxin stimulated whole blood ( $p < 0.005$ ) in comparison to conditions with no glutamine (Fig. 1). In unstimulated blood, the release of HSP70 was significantly upregulated at 24 h in conditions with/without glutamine compared to those at 4 h ( $p < 0.005$ ) (Fig. 1).

The addition of 2 mM glutamine to incubation media with endotoxin significantly stimulated the release of TNF- $\alpha$  at 4 h ( $p < 0.05$ ) but not at 24 h (Fig. 2). A similar effect was seen with IL-6, although this was not significant (Fig. 3). 2 mM glutamine appeared to have no effect on the release of IL-10 (Fig. 4). There was a positive correlations between HSP70 and IL-6 ( $n = 25$ ,  $r = 0.61$ ,  $p = 0.004$ ) and TNF- $\alpha$  ( $n = 25$ ,  $r = 0.61$ ,  $p = 0.005$ ) at 4 h following the addition of 2 mM glutamine to incubation media (Fig. 5a and b) but there was no relationship between HSP70 and IL-10 ( $n = 25$ ,  $r = 0.191$ ,  $p = 0.382$ ).

## 4. Discussion

In an *in vitro* model of paediatric sepsis the addition of 2 mM glutamine to incubation media significantly increased HSP70 release at 24 h, concurring with other models of experimental sepsis [25,33] and critically ill adults [26]. However, in addition, to this we also found there was a significant release of HSP70 in unstimulated whole blood control wells with and without glutamine. The significant increase in HSP70 levels appears to be a physiological response to the experimental conditions, and has previously been described *in vitro* and *in vivo* studies [25,34–36] and is unlikely to be due to endotoxin contamination given that inflammatory mediator levels were low at each time point in control wells. The addition of 2 mM glutamine to incubation media in endotoxin stimulated blood from paediatric volunteers significantly increased the release of TNF- $\alpha$  at 4 h, although at 24 h the release of TNF- $\alpha$  in endotoxin stimulated blood was not significantly different. There was a similar but non significant trend for IL-6 release after the addition of 2 mM glutamine to incubation media. However, The addition of 2 mM glutamine had no discernible effect on IL-10 at 4 or 24 h following endotoxin stimulation though it may be that dosage greater than 2 mM glutamine is required to significantly influence the release of inflammatory mediators [37,38].

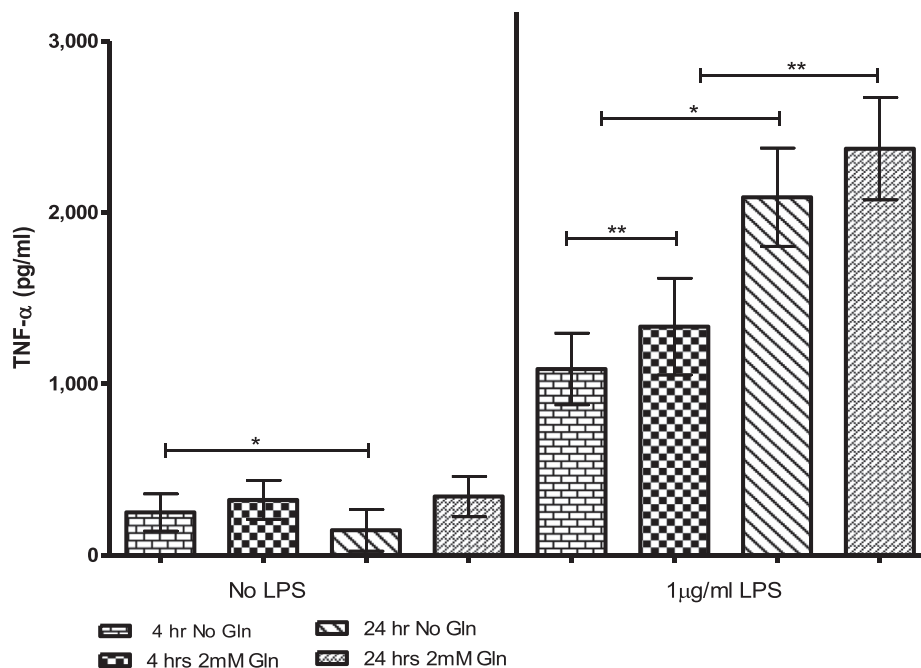


**Fig. 1.** The effect of 2 mM glutamine supplementation on LPS stimulated HSP70 release at 4 h and 24 h in whole blood from healthy paediatric volunteers ( $n = 25$ ). Results are expressed as mean;  $\pm$  SEM. Glutamine = glutamine, LPS = endotoxin \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ .

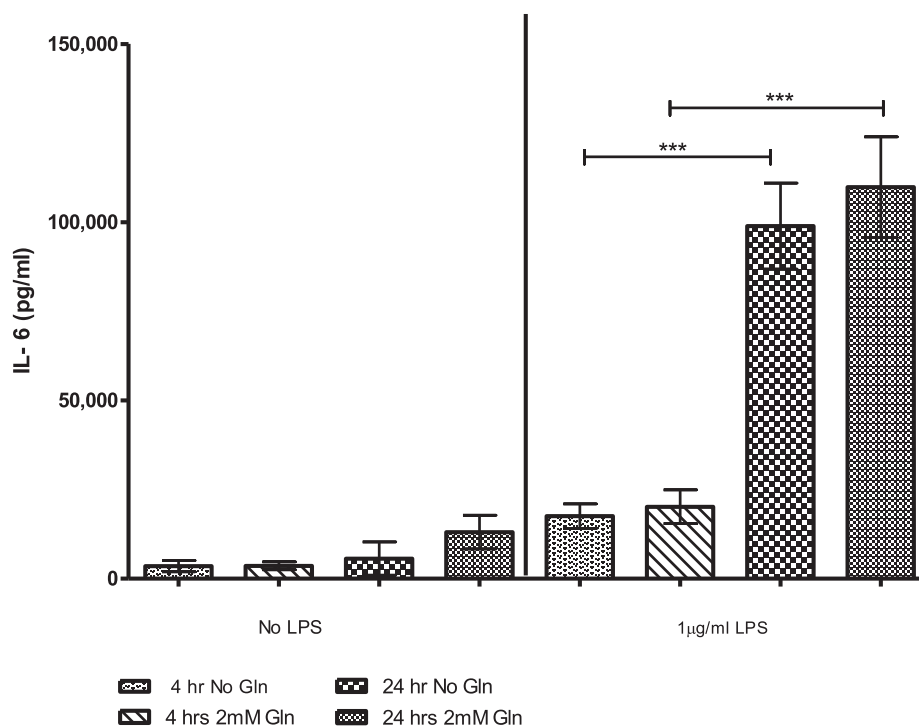
The putative role of extracellular HSP70 during times of stress in children has yet to be clearly defined, making it challenging to interpret our results. It has been postulated that constitutive levels of extracellular HSP70 *in vivo* help maintain immune homeostasis through T regulatory cell control. During times of stress high levels of HSP70 are released at sites of infection or tissue damage resulting in a loss of T regulatory cell control contributing

to a pro-inflammatory response, which reflects the response seen in our *in vitro* model. As the stress response resolves and HSP70 levels decline T regulatory cell function is restored with a resolution of the inflammatory response and return to homeostasis [39,40].

HSP70 also appears to influence the mix of cytokines released, which is dependent on the model used, for example an *in vitro*



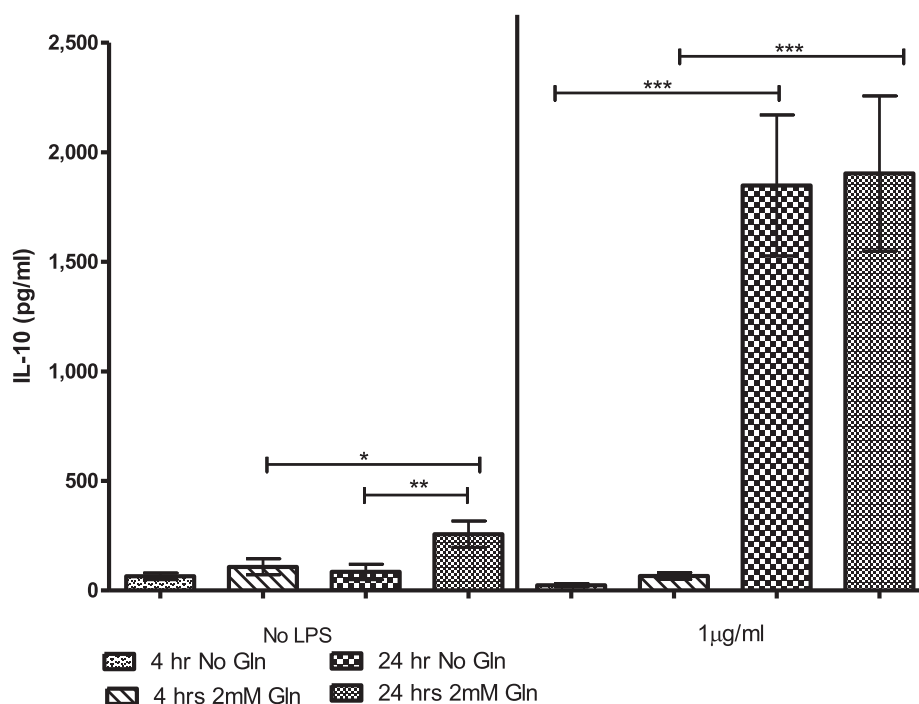
**Fig. 2.** The effect of glutamine supplementation on endotoxin stimulated release of TNF- $\alpha$  in whole blood from healthy paediatric volunteers at 4 h and 24 h ( $n = 25$ ). Results are expressed as mean;  $\pm$  SEM. Glutamine = glutamine, LPS = endotoxin \* $p < 0.05$ , \*\* $p < 0.005$ .



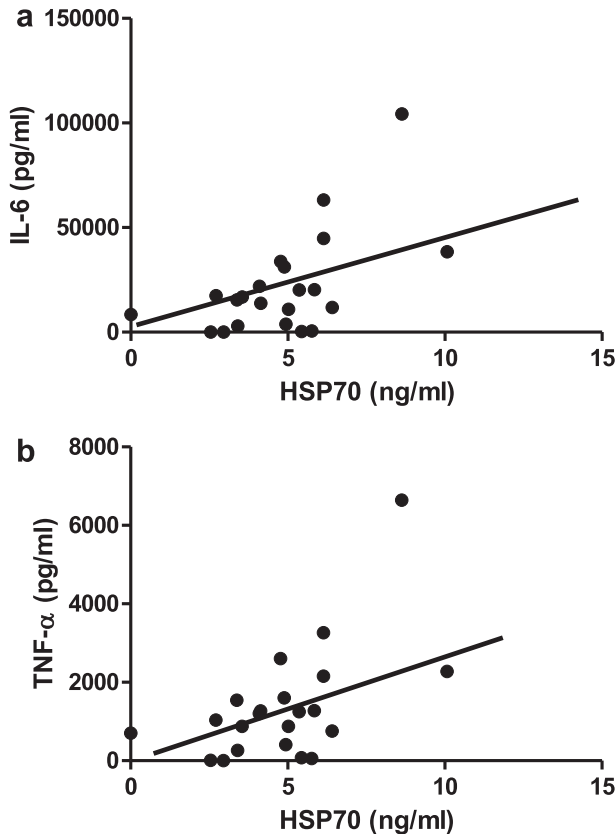
**Fig. 3.** The effect of glutamine supplementation on endotoxin stimulated release of IL-6 in whole blood from healthy paediatric volunteers at 4 h and 24 h (n = 25). Results are expressed as mean;  $\pm$  SEM. Glutamine = glutamine, LPS = endotoxin \*\*\*p < 0.0005.

model of sepsis model using, either lipoteichoic acid or LPS, shows that pre-treatment of human neutrophils with recombinant HSP70 prior to the administration of endotoxin attenuates the release of TNF- $\alpha$  [41]. This is corroborated in other *in vitro* animal models where the use of recombinant HSP70 provides a similar anti-

inflammatory mode of action by inhibiting C/EBP $\beta$  and C/EBP $\delta$  transcription factors from signalling via TLR2-ERK-STAT2-1L10 pathway, attenuating the release of pro-inflammatory mediators [42]. Converse to this, when healthy subjects were inoculated with endotoxin, levels of HSP70 and proinflammatory mediators



**Fig. 4.** The effect of glutamine supplementation on endotoxin stimulated release of IL-10 in whole blood from healthy paediatric volunteers at 4 h and 24 h (n = 25). Results are expressed as mean;  $\pm$  SEM. Glutamine = glutamine, LPS = endotoxin. \*p = 0.05, \*\*p < 0.005, \*\*\*p < 0.0005.



**Fig. 5.** a) Graphical representation (scatter plot) correlations between the effect of glutamine supplementation on endotoxin stimulated release of IL-6 in whole blood from healthy paediatric volunteers at 4 h ( $n = 25$ ,  $r = 0.61$ ,  $p = 0.004$ ). b) Graphical representation (scatter plot) correlations between the effect of glutamine supplementation on endotoxin stimulated release of TNF- $\alpha$  in whole blood from healthy paediatric volunteers at 4 h ( $n = 25$ ,  $r = 0.61$ ,  $p = 0.005$ ).

subsequently increased although the causality regarding the pleiotropic effect of HSP70 was not established [31].

In our study the addition of 2 mM glutamine to incubation media appeared to promote the release of pro-inflammatory mediators [43], which could be reflective of experimental conditions, levels of extracellular HSP70 release or glutamine levels within the *in vitro* model. The cellular response to infection is a complex process co-ordinated by a wide range of intra- and extracellular mediators. Within this dynamic, glutamine and HSP70 influence and are influenced by, the mix of inflammatory mediators, activating protective cellular defence mechanisms [44], and a challenge for the future is to elucidate whether there is any clinical relevance of this in critically ill children.

Glutamine supplementation in children has yielded conflicting results. In studies of glutamine supplementation in pre-term infants [45–50], infants with gastrointestinal disease and surgery [51,52], children with burns [53,54] and malnutrition [55–57], little or no benefit on survival, late onset sepsis or duration of hospital stay have been described [18]. In addition recent evidence indicates that either hypo-glutamine  $<430 \mu\text{mol/L}$  [58] or hyper-glutamine levels  $>930 \mu\text{mol/L}$  are associated with increased mortality [59,60]. The mechanism for the effect of supra physiological glutamine levels on HSP70 release and inflammatory mediators in critical illness is not known. However, an *in vitro* model of human skeletal muscle linked supraphysiological levels of glutamine to respiratory uncoupling and energy wasting within the mitochondria which was associated with cell death [61]. In addition, Krajcova

et al. have suggested the hypoglutaminaemia seen during critical illness may be a protective mechanism. They show that supra physiological levels of glutamine decrease the efficiency of the mitochondrial respiratory chain contributing to the bioenergetics failure of the mitochondria (a feature of critical illness), where engorged mitochondria are not able to meet adenosine tri phosphate demands of tissues, resulting in cell death [61]. As such the hypoglutaminaemia described by us [23] and others during critical illness [62], may be a protective mechanism decreasing mitochondrial size allowing for restoration and repair during the recovery phase [61].

Furthermore, it may also be the increased mortality seen in these studies may be as a result of the metabolic decompensation associated with sepsis/septic shock and multiorgan failure rather than the effect of glutamine per se [59,60]. The effective modulation and management of the inflammatory response to infection remains elusive, as complete abrogation of inflammation and/or over stimulation results in increased mortality [63].

Our results suggest that the addition of 2 mM glutamine to incubation media using an *in vitro* endotoxin model from whole blood of healthy children, may promote increased levels of TNF- $\alpha$ , and mediated via HSP70 release, although the clinical significance of this is not known. Since the results from Heyland and Dhaliwal [64] describing the increased risk of mortality following the use of glutamine in sepsis, supplementation is no longer recommended [65]. Furthermore, the effect of glutamine supplementation has not been elucidated in critically ill children and so its use cannot be recommended in immune competent children [18]. Further work is required to understand what the role of glutamine is in critically ill children.

The conclusions from our work should be regarded with some caution owing to certain limitations of the study, which include the investigation of only one dose of glutamine (2 mM) that significantly upregulated HSP70 release in paediatric endotoxin stimulation models. The effect of 2 mM glutamine on the release of inflammatory mediators was less clear. It may be that a large dose of glutamine ( $\geq 2 \text{ mM}$ ) is required in the paediatric models to better understand the effect of glutamine mediated HSP70 release and subsequent milieu of inflammatory mediators. Although a range of pro- and anti-inflammatory mediators were selected for study based on previous work in meningococcal disease, it was not possible to examine all those of interest within a single validated multiplex plate as we were limited on blood volume available from paediatric samples. Another limitation of our *in vitro* models was the lack of discrimination between live and dead cells, which prevented the source of HSP70 release (e.g. passive release or as a result of necrosis) from being determined.

## 5. Conclusion

These results would suggest that the addition of 2 mM glutamine to incubation media promotes HSP70 release in an *in vitro* endotoxin stimulation model in children. The results from this work highlight the need to explore the relationships of glutamine, HSP70 and inflammatory mediators further, particularly given the conflicting reports of benefit of glutamine during critical illness. Given the current paucity of evidence regarding the efficacy of glutamine supplementation during paediatric critical illness and sepsis it should not be given until the effects of supplementation on the inflammatory response are better understood.

## Conflict of interest

The authors declare no conflict of interest.

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