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## New combined treatments avoided transplantation in a child with severe pulmonary hypertension

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## CARDIOVASCULAR MEDICINE

## Increased serum concentrations of soluble CD95/Fas and caspase 1/ICE in patients with acute angina

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**Objectives:** To investigate the expression of death inducing receptors in the sera of patients with stable and unstable angina.**Design:** 80 consecutive patients with stable (n = 40) or unstable (n = 40) angina pectoris were studied. Serum concentrations of soluble CD95 (sCD95), soluble CD95 ligand (sCD95L; CD178), tumour necrosis factor (TNF)  $\alpha$ , soluble TNF $\alpha$  receptor type 1 (sTNFR1), and interleukin 1 $\beta$  converting enzyme (ICE; caspase 1) were measured by enzyme linked immunosorbent assay (ELISA).**Results:** Significant increases in the concentrations of sCD95 and ICE ( $p < 0.001$  and  $p < 0.023$ , respectively) were found in the serum from patients with unstable angina relative to those with stable angina. There were no significant differences in the concentrations of sCD95L, TNF  $\alpha$ , and sTNFR1 between the groups.**Conclusions:** These data provide the first evidence that sCD95 and ICE are important serological markers that may help to discriminate between stable and unstable angina. This observation may warrant further clinical study to elucidate the clinical impact of sCD95 and ICE in acute coronary syndromes.

Coronary artery disease is associated with systemic immune responses and signs of chronic inflammation.<sup>1,2</sup> Apart from the well known traditional risk factors, seroepidemiological studies have suggested a link between atherosclerosis and microbial infections.<sup>3,4</sup> The atherosclerotic lesions contain large numbers of immune cells, particularly monocytes, macrophages, and T cells. Most T cells are CD3<sup>+</sup>, CD4<sup>+</sup>, and T cell receptor  $\alpha\beta$ <sup>+</sup>.<sup>2</sup> They are largely T helper cell 1 subtype, which secrete interferon  $\gamma$ , interleukin 2, and tumour necrosis factor (TNF)  $\alpha$ .<sup>5</sup> These proinflammatory cytokines, as well as interleukin 1, interleukin 6, interleukin 8, and C reactive protein, cause activation of T cells, macrophages, and vascular endothelial cells. They also augment expression of adhesion molecules, chemoattractants, and other cell surface markers.<sup>5,6</sup>

The maintenance of T cell homeostasis and peripheral tolerance to self antigens following repeated cell stimulations is regulated mainly by apoptosis. Apoptosis, or programmed cell death, is initiated by death-inducing receptors. These are transmembrane receptors that include the TNF receptors and a variety of immune cell receptors (CD95, CD40, CD30, and CD27) and their corresponding ligands.<sup>7,8</sup> It has been previously shown that interaction between CD95 (Fas; apolipoprotein 1) and CD95 ligand (CD95L; Fas ligand, CD178) induces apoptosis in human plaque derived vascular smooth muscle cells.<sup>9</sup> Activated cytotoxic CD4<sup>+</sup>/CD161<sup>+</sup> T cells can lyse capillary endothelial cells in the presence of C reactive protein at concentrations frequently found in patients at risk for coronary heart disease.<sup>10</sup> In addition, patients with unstable angina have increased concentrations of shed membrane microparticles, originating from activated CD3<sup>+</sup> cells, endothelial cells, and macrophages, hence indicating increased intravascular peripheral apoptotic turnover.<sup>11</sup>

It is not well known whether patients with acute coronary syndrome have increased concentrations of soluble CD95 (sCD95) and sCD95L, soluble TNF receptor type 1 (sTNFR1), and interleukin 1 $\beta$  converting enzyme (ICE; caspase 1). Therefore, we investigated comparatively the concentration of these molecules, which are involved in the apoptotic

pathways, in the serum from patients with stable and unstable angina.

## METHODS

## Study group

Eighty consecutive patients admitted to our institution for the assessment of angina chest pain were studied and had undergone coronary angiography. Table 1 summarises demographic and some baseline clinical characteristics. Stable angina (n = 40) was defined by typical exertional chest pain angina relieved by rest, glyceryl trinitrate administration, or both with positive responses to exercise ECG stress testing and  $\geq 50\%$  diameter stenosis in  $\geq 1$  coronary arteries at catheterisation. Unstable angina (n = 40) was defined according to Braunwald.<sup>12</sup> All patients with unstable angina class IIIB had diagnostic ST segment changes, T wave inversion, or both, as well as negative cardiac enzymes. No patient included in the study had evidence of ongoing systemic or cardiac inflammatory processes as defined by clinical history. Blood samples were taken at the time of admission into hospital. The investigation conformed to the principles outlined in the Declaration of Helsinki.

## Angiographic analysis

Coronary angiography was carried out according to the Judkins technique. Images of the coronary tree were obtained in routine, standardised projections with standard equipment (Siemens BICORTOP, Erlangen, Germany). To assess the extent of atherosclerotic disease the coronary artery tree was

**Abbreviations:** CD95L, CD95 ligand; ELISA, enzyme linked immunosorbent assay; FADD, Fas associated death domain; ICE, interleukin 1 $\beta$  converting enzyme; sCD95, soluble CD95; sCD95L, soluble CD95 ligand; sTNFR1, soluble tumour necrosis factor receptor type 1; TNF, tumour necrosis factor; TNFR1, tumour necrosis factor receptor type 1

**Table 1** Demographic and clinical data of patients with stable and unstable angina pectoris

	Stable angina (n = 40)	Unstable angina (n = 40)
Men/women	29/11	29/11
Mean age (years)	65	65.4
History and risk factors		
Previous CABG	5 (12.5%)	6 (15%)
Previous PTCA	10 (25%)	10 (25%)
Diabetes	8 (20%)	9 (22.5%)
Hypertension	31 (77.5%)	26 (65%)
Current smoker	6 (15%)	8 (20%)
Former smoker	12 (30%)	8 (20%)
Never smoker	22 (55%)	24 (60%)
Angiographic analysis		
Extension/severity score	6.1%	6.3%
One vessel disease	14 (35%)	18 (45%)
Two vessel disease	17 (42.5%)	13 (32.5%)
Three vessel disease	8 (20%)	9 (22.5%)
Left main disease	1 (2.5%)	0
Biochemistry (mean concentrations)		
Cholesterol (mmol/l)	5.29	5.43
LDL cholesterol (mmol/l)	3.22	3.35
HDL cholesterol (mmol/l)	1.23	1.12
Triglycerides (mmol/l)	1.77	1.91
Creatinine ( $\mu$ mol/l)	97.2	97.2
Left ventricular systolic function		
Normal	29 (72.5%)	31 (75.5%)
Mild impairment	8 (20.3%)	3 (7.5%)
Moderate impairment	3 (7.5%)	6 (15%)
Severe impairment	0	0

CABG; coronary artery bypass grafting; HDL, high density lipoprotein; LDL, low density lipoprotein; PTCA, percutaneous transluminal coronary angioplasty.

assessed according to vessel score, stenosis score, and extension score.<sup>13</sup>

### Laboratory analysis

Serum samples were obtained after clinical assessment grading the patient affected with stable or unstable angina and before cardiac catheterisation. Serum was then aliquoted and kept frozen. Circulating sCD95 and sCD95L were measured by a commercial enzyme linked immunosorbent assay (ELISA) with polyclonal antibodies against human CD95 (B&D Pharmingen, Franklin Lakes, New Jersey, USA) and CD95L (MBL Co Ltd, Nagoya, Japan). The concentration of TNF $\alpha$  and sTNFR1 (p55; CD120a) was measured by ELISA with polyclonal antibodies against human TNF receptor type 1 (TNFR1) and TNF $\alpha$  (R&D Systems Inc, McKinley, Minnesota, USA). ICE was measured in the serum by ELISA with monoclonal antibody to ICE 1 antibody (Bender MedSystems, Vienna, Austria). The sensitivity of the ELISA was 20 pg/ml for sCD95 and sCD95L, 3 pg/ml for sTNFR1, 80 pg/ml for TNF $\alpha$ , and 5 pg/ml for human ICE. The analytical unit of the biochemistry department following standard methods carried out all other biochemistry measurements.

**Table 2** Soluble death inducing receptors (sDIR) in patients with stable and unstable angina pectoris

sDIR and cytokines	Stable angina (n = 40)	Unstable angina (n = 40)	p Value
sCD95L (pg/m)	78.29 (1.2)	62.34 (10.3)	0.26
ICE/caspase 1 (pg/m)	46.94 (6.2)	78.21 (13.1)	0.023
sTNFR1 (ng/m)	1.72 (0.2)	1.42 (0.3)	0.39
TNF- $\alpha$ (pg/m)	15.55 (6.4)	17.43 (5.4)	0.83

Data are mean (SEM).

sCD95L, soluble CD95 ligand; ICE, interleukin 1 $\beta$  converting enzyme; TNF, tumour necrosis factor; sTNFR1, soluble TNF receptor type 1.

### Statistical analysis

Results are presented as mean (SEM) for continuous, normally distributed variables and as percentages for categorical data. Continuous variables were analysed by two tailed *t* test for unpaired observations. A probability value of *p* < 0.05 was considered significant. The statistical software package SPSS (SPSS Inc, Chicago, Illinois, USA) was used for all calculations.

## RESULTS

### Demographic and clinical features

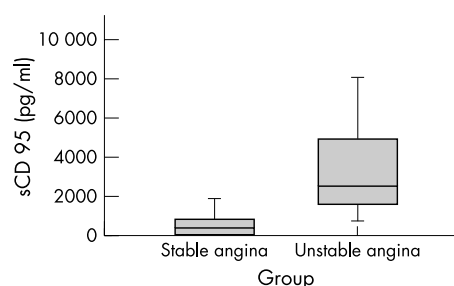
Table 1 presents baseline characteristics of patients with stable and unstable angina. In both groups, a similar proportion of patients had a history of myocardial infarction or previous coronary interventions (coronary artery bypass graft or percutaneous transluminal coronary angioplasty). Established risk factors for coronary artery disease were similar in the two groups. Total cholesterol concentrations did not differ significantly between the groups. Angiographic findings were similar in both groups. The chronic stable angina group had slightly higher extension scores but this did not reach significance.

### Soluble death inducing receptors and ICE concentrations

The results listed in the table 2 show a significant increase in the concentration of ICE in serum from patients with unstable angina (*p* < 0.023). The concentrations of sCD95L, TNF $\alpha$ , and sTNFR1 were not significantly different (table 2). Interestingly, as shown in fig 1, the concentration of sCD95 was greatly increased in the unstable angina group compared with the stable group (mean (SEM) 3235.78 (380.2) and 668.23 (143.1) pg/ml, respectively; *p* < 0.001).

## DISCUSSION

Our study showed for the first time that serum sCD95 and ICE concentrations are significantly higher in patients with unstable angina than in patients with stable angina. This



**Figure 1** Concentration of soluble CD95 (sCD95) in the serum of patients with stable and unstable angina. Box plot shows the median, quartiles, and extreme concentrations of sCD95. Differences were significant (*p* < 0.001).

suggests that increased apoptosis specific markers are associated with clinical instability. We have also found that the serum concentrations of TNF $\alpha$ , sTNFR1, and sCD95L were not significantly different in the study cohorts. These results indicate that patients with unstable angina may be in a paradoxical state of increased apoptotic turnover, as indicated by increased secretion of sCD95 and ICE/caspase 1.

Apoptosis is a highly organised, controlled, and energy dependent mechanism whereby a cell commits suicide. Distinct morphological features such as cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and formation of apoptotic bodies are characteristic of this cell death mechanism. A family of cysteine proteases, termed caspase, the mammalian homologue to the *Caenorhabditis elegans* cell death gene CED3,<sup>14</sup> play a pivotal part in apoptosis, particularly caspase 8, which initiate the caspase activation cascade through caspase 3 and caspase 9.<sup>7-15</sup> Caspases are present in the cell in an inactive form and are activated by autoproteolytic processing, oligomerisation, and heterocleavage by other caspases or granzyme B. Activation of caspase requires transduction of a signal from cell membrane receptors, such as CD95 and TNFR1. Binding of ligand to death inducing receptors leads to trimerisation and cross linking of the aggregated cytoplasmic domain of the receptor complex and recruitment of the so called Fas associated death domain (FADD; MORT1), TNFR1 associated death domain (TRADD), FADD-like ICE1 (FLICE1; MACH1; caspase 8), or FLICE2/caspase 10.<sup>7-8</sup>

The role of ICE in apoptosis and its potential to interfere in the activity of atherosclerotic lesions and plaque stability is not well understood. It has been shown that ICE cleaves the 31 kDa interleukin 1 $\beta$  precursor protein at Asp116-Ala117, whereby it creates the mature biologically active interleukin 1 $\beta$ , a key mediator of inflammation.<sup>16</sup> Interestingly, ICE can also cleave an inactive precursor of interferon  $\gamma$  inducing factor or interleukin 18<sup>17</sup>; thus, ICE regulates production of interferon  $\gamma$  and subsequently T cell activation. Although vascular smooth muscle cells express ICE, an ICE inhibitor suppresses the release of mature interleukin 1 $\beta$ .<sup>18</sup> This may be a novel regulatory mechanism in vascular biology and pathology.

The cell death machinery is tightly controlled by Bcl-2, a member of a large gene family encoding proteins that can either promote (for example, Bax, Bak, Bid, and Bad) or inhibit (for example, Bcl-2 and Bcl-H<sub>L</sub>) apoptosis.<sup>19</sup> The nuclear factor  $\kappa$ B pathway is a further key component of the cellular response to a variety of stimuli. The multiplicity of mechanisms whereby the transcription factor nuclear factor  $\kappa$ B serves the pro-apoptotic or anti-apoptotic function is complex and may be determined by the nature of the death stimulus rather than by the tissue origin.<sup>20</sup> Other important regulators of apoptosis act at the level of caspase. Such proteins include cellular FADD-like inhibitory protein and the inhibitor of the apoptosis family, which counteracts the effect of the caspase inhibitors themselves.<sup>21</sup> The roles of sCD95 and sCD95L as pro-apoptotic and anti-apoptotic molecules are not clearly defined. The CD95 antigen is expressed on T and B cells, granulocytes, monocytes, and natural killer cells, particularly on activated cells. CD95 is cleaved by proteolytic enzymes produced by inflammatory cells and sCD95 is generated by alternative mRNA splicing. It has been previously shown that supernatants containing sCD95 can inhibit anti-CD95 antibody induced apoptosis, hence indicating that sCD95 may block signal transduction and apoptosis.<sup>22</sup> Overexpression of sCD95 protects the integrity of the vessel wall from immune injury and attenuates transplant arteriosclerosis.<sup>23</sup> Furthermore, an increased concentration of sCD95 has been regarded as a predictor of active atherosclerosis in patients with end stage

renal disease.<sup>24</sup> CD95L is expressed on activated T cells, natural killer cells, and vascular endothelial cells.<sup>25</sup> CD95L is cleaved from the cell membrane by a metalloproteinase-like enzyme. sCD95L may be detected in the serum<sup>26</sup> and considered to be a marker of atherosclerosis.<sup>27</sup>

The coexistence of increased apoptotic turnover and secretion of the apoptosis prohibiting sCD95 molecule may be a desperate attempt by the immune system in unstable angina to overcome lifelong and constant triggering of autoreactive T cells by way of the T cell receptor. We are tempted to believe that sCD95, sCD95L, sTNFR1, and ICE are serological markers that may help to discriminate between stable and unstable angina. We conclude that our results showing increased concentrations of sCD95 and ICE in unstable angina may merely reflect inflammatory cell activation and the role in relation to apoptosis specific immune activation remains to be determined. However, to elucidate the role of these molecules in the pathogenesis, disease progression and destabilisation, diagnosis, and prognosis of acute coronary syndromes further clinical studies as well as new experimental designs are needed.

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## IMAGES IN CARDIOLOGY

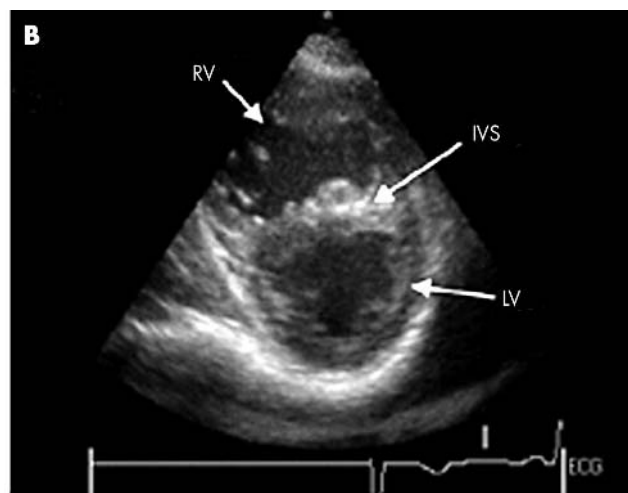
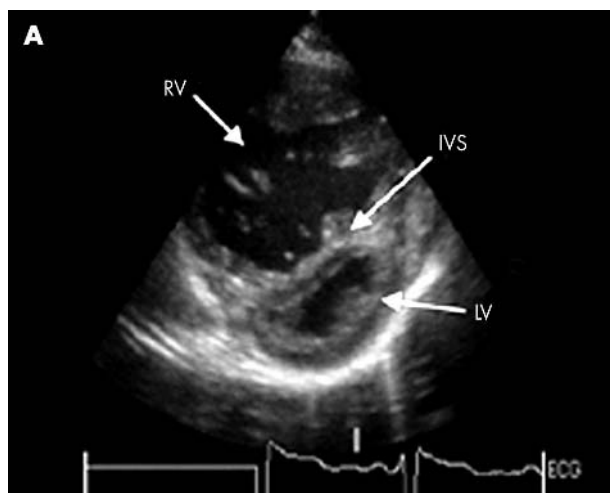
### New combined treatments avoided transplantation in a child with severe pulmonary hypertension

**T**herapeutic options for the management of pulmonary hypertension have been limited for many years. However, recent progresses in the understanding of its pathology have led to the introduction of new treatments that have changed its prognosis, such as the continuous infusion of prostacyclin. Several other treatments (oral, inhaled, or subcutaneous prostacyclin, phosphodiesterase inhibitors, and endothelin receptor blockers) have shown beneficial effects in recent trials. An 8 year old boy with severe primary pulmonary hypertension showed a dramatic improvement following combined treatment with inhaled iloprost (a prostacyclin analogue) and oral bosentan (a dual endothelin receptor blocker). In the six minute walk test he increased the distance from 295 m before bosentan to 400 m after three months of combined treatment. Dramatic changes were observed at echocardiography. Echocardiographic short axis views were taken at the same time in the cardiac cycle before combined treatment (panel A) and three months after combined treatment of iloprost and bosentan (panel B). There was a significant reduction in right ventricular (RV)

size and changes in interventricular septal curvature (IVS), which went from flat before treatment (panel A) to normal left to right on combined treatment (panel B). Echocardiography showed a decrease in right ventricular surface, from 39 cm<sup>2</sup> to 34 cm<sup>2</sup>. The left ventricle (LV) is squeezed by the dilated right ventricle before (panel A) and is almost normal after three months (panel B). This allowed the lung transplantation to be postponed and improved the patient's quality of life.

This case shows that combined therapies may offer further improvement in the therapeutic options for the treatment of pulmonary hypertension. Appropriate clinical trials should be performed to confirm this beneficial result.

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## New combined treatments avoided transplantation in a child with severe pulmonary hypertension

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