Within the tube type, there were no significant differences in hs-CRP concentration in samples taken into plain tubes and EDTA tubes over the 24-h period. In gel tubes, however, there were significant differences over time (*ANOVA* $P=0.03$), with hs-CRP concentration significantly lower ($P<0.05$) in the samples separated at 8 and 24 h, compared with that in the basal sample separated at 0.3 h. In gel tubes, hs-CRP concentration decreased by 9.7% over 24 h.

These results demonstrate a significant decline in hs-CRP concentration over time, when blood is collected into a gel tube, and support previous studies reporting the effect of gel tubes on other analytes such as therapeutic drugs[9] and intact parathyroid hormone.[10]

Several studies have proposed that hs-CRP be used as a prognostic indicator in acute coronary syndromes[1-3] or a predictor of future coronary events.[4,5] However, the significant decline in hs-CRP in gel collection tubes observed over 24 h in this study could lead to the misclassification of patients in samples left unseparated for eight or more hours. This may be particularly important for samples collected in the community for evaluation of CAD risk.

In conclusion, results from this study suggest that caution should be exercised in interpreting hs-CRP results when the sample is collected into a gel tube, as we report a significant decline in hs-CRP concentration over time. □

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Enzyme-linked immunosorbent assay for β_2-glycoprotein I quantitation: the importance of variability in the plastic support

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β_2-glycoprotein I (β2GPI) is a poorly understood plasma protein that is thought to be an important autoantigen in the antiphospholipid syndrome. In addition, β2GPI may be a key player in the phospholipid-dependent coagulation pathway, and in the clearance of liposomes, phosphatidylserine-expressing cells and foreign particles.[1-9] It shows a high propensity to bind negatively charged surfaces, including phospholipids and irradiated plastic plates.[10] Binding to phospholipids is accompanied *in vitro* by the inhibition of intrinsic coagulation pathway activation,[4] ADP-induced platelet aggregation,[5] prothrombinase activity of activated platelets,[6] as well as the anticoagulant activity of activated protein C.[11]

As relatively little is known about the pathophysiological role of β2GPI, a reliable assay to determine plasma levels would further our understanding of its functions in health and disease. However, non-specific binding of β2GPI to plastic surfaces could significantly decrease the accuracy and reliability of an enzyme-linked immunosorbent assay (ELISA) designed to measure it.

In order to study the effect of non-specific binding of β2GPI to plastic and to determine a normal range for β2GPI concentration in sera from female and male subjects, this study establishes a capture ELISA based on the protocol designed by McNally *et al*.[12] Nunc A/S (Kamstrup, Roskilde, Denmark) provides two commonly used sets of γ-irradiated plates; one certified for consistency in adsorption of protein, the other uncertified. Here, we test six batches of γ-irradiated Nunc Maxisorp 96-well flat-bottomed polystyrene ELISA plates for reproducibility; one of which was certified by the manufacturer for homogeneity in adsorption of IgG.

Briefly, the ELISA plates were incubated overnight at 4°C with 100 μL mouse monoclonal anti-human β2GPI (Chemicon International Inc., Temecula, CA, USA) at a

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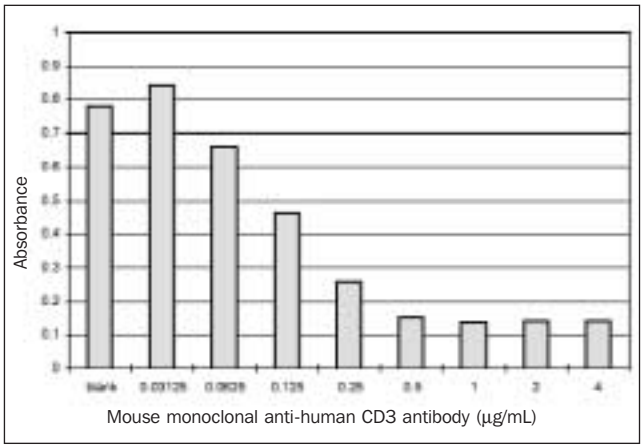

Fig. 1. *A* values of a normal serum on a certified Nunc Maxisorp ELISA plate coated with increasing concentrations of monoclonal anti-CD3 antibody.

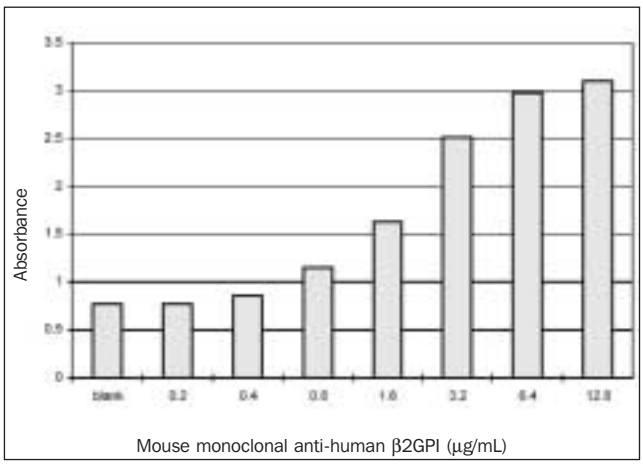

Fig. 2. *A* values of a normal serum on a certified Nunc Maxisorp ELISA plate coated with increasing concentrations of mouse monoclonal anti-β2GPI antibody.

concentration of 3.2 µg/mL in coating buffer (15.0 mmol/L Na_2CO_3, 34.9 mmol/L $NaHCO_3$ [pH 9.6]). The wells were washed (x4) with phosphate-buffered saline with Tween (PBST; 136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4 [pH 7.2], 0.05% v/v Tween 20).

A pooled normal serum sample calibrated against purified β2GPI (Crystal Chem Inc., Chicago, USA) was used as a standard. 100 µL diluted standards (from 1 in 320 to 1 in 40,960 in PBST) and 100 µL of test samples (at 1 in 3000 in PBST) were added in duplicate. Two wells with PBST alone were used as blanks. The plates were incubated at 37°C for 1 h. Following an additional wash with PBST, 100 µL horseradish peroxidase-conjugated rabbit polyclonal anti-human β2GPI (Dako A/S, Glostrup, Denmark), diluted at 1 in 1000 in PBST, was then added to each well and the plates were again incubated at 37°C for 1 h.

Following a wash with PBST, colour was developed with 100 µL orthophenylenediamine dihydrochloride with H_2O_2. After 5 min at room temperature, 100 µL 2.5 mol/L H_2SO_4 was added to stop colour development, and absorbance (*A*) was measured at 492 nm on a Titertek Multiskan Plus II plate reader. *A* values were processed through Multiscan Advanced software. β2GPI concentrations in control and test samples were calculated from the standard curve and expressed in µg/mL.

Several experiments were performed following procedures slightly modified from the protocol described above. The modifications included varying the coating antibody concentration and its antigenic specificity, or adding a blocking step.

Using certified and uncertified plates, wells that were not coated with capture antibody gave significant *A* values (up to 0.8 *A* unit). In addition, a striking variability in the results obtained with these uncoated wells was found between uncertified plates. Although the plates were supplied by the same manufacturer they were not certified for binding capacity and it was assumed that the plates probably displayed different charge densities following doses of radiation that differed from one batch to another. Owing to this non-specific binding of β2GPI to the plastic, the β2GPI assay required either a blocking agent or use of a surface-saturating concentration for the coating antibody to suppress non-specific binding.

Most blocking agents did not sufficiently or consistently suppress the non-specific binding of β2GPI – as was the case with bovine (DiaMed) and chicken (Sigma) albumin, gelatin (Merck and Sigma), glycine (Sigma) and agar (Merck and Sigma). In addition, some of the blocking agents – such as casein (Sigma) and carrageen (Sigma) – completely inhibited specific recognition of β2GPI by antibody (results not shown).

As attempts to block non-specific binding were unsuccessful, the study then focused on saturating a certified plate surface with the primary coating antibody. An irrelevant mouse anti-human CD3 monoclonal antibody (Becton Dickinson, San Jose, CA, USA) was used to determine the optimum concentration of monoclonal antibody required to suppress non-specific β2GPI adsorption. Increasing concentrations of mouse anti-human CD3 antibody were incubated in place of the mouse anti-human β2GPI antibody, keeping the remaining steps of the β2GPI ELISA protocol the same.

As can be seen in Figure 1, concentrations of anti-CD3 antibody >0.5 µg/mL significantly suppressed non-specific binding of β2GPI to the plates. Identical results were found with other mouse monoclonal antibodies (anti-human CD4, CD14, CD19 or HLA-DP; Becton Dickinson, San Jose, CA, USA [results not shown]). Therefore, it was concluded that when ELISA wells were coated with monoclonal antibody >0.5 µg/mL, there was negligible non-specific adsorption of β2GPI to the plastic surface.

Subsequently, capture anti-β2GPI antibody was titrated on certified plates to determine the optimum concentration for β2GPI detection. As can be seen in Figure 2, mouse monoclonal anti-β2GPI at concentrations ≥ 3.2 µg/mL allowed optimal detection of β2GPI. At concentrations >3.2 µg/mL, the *A* signal continued to rise, but not as substantially (suggesting that 3.2 µg/mL was close to the saturation concentration). Therefore, the coating antibody concentration was fixed at 3.2 µg/mL, which is in line with the theoretical calculation of 3.76 µg/mL for the saturating concentration of Nunc Maxisorp ELISA plates by IgG formulated by the manufacturer.[13]

Optimal dilution of the peroxidase-conjugated secondary antibody was determined by testing various dilutions, while keeping all other assay steps unchanged. A dilution of 1 in

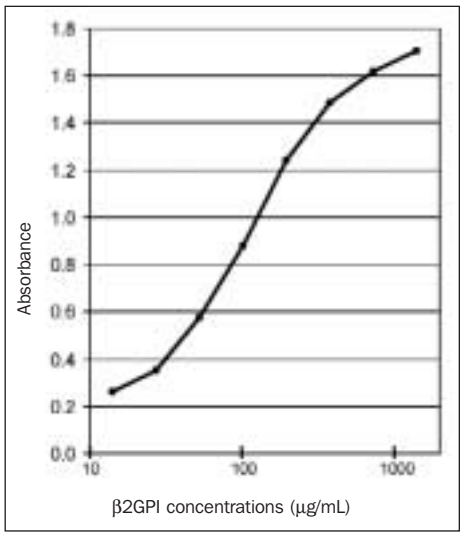

Fig. 3. Typical standard curve obtained with the pooled serum on a Nunc Maxisorp ELISA plate coated with 3.2 μg/mL mouse monoclonal anti-β2GPI antibody.

Table 1. Intra-assay average CV and inter-assay CV of samples A and B on six different Nunc Maxisorp ELISA plate batches coated with 3.2 μg/mL mouse monoclonal anti-β2GPI antibody. Batches 1 to 5 are non-certified and batch 6 is certified by the manufacturer for homogeneity in adsorption of IgG

	Plate batch					
	1	2	3	4	5	6
Intra-assay variation						
Sample A	8.3%	9.4%	8.8%	5.5%	23.4%	4.7%
Sample B	7.9%	5.7%	7.0%	6.2%	8.8%	3.4%
Inter-assay variation						
Sample A	14.9%	17.9%	15.1%	14.5%	36.1%	14.6%
Sample B	8.0%	6.6%	7.9%	9.8%	12.2%	13.9%

1000 was selected because it allowed a normal serum to achieve an *A* of approximately 0.8–1.0. As can be seen in Figure 3, a typical standard curve obtained from the pooled serum has a sigmoidal shape. β2GPI concentrations in the test samples were estimated against this standard curve provided that the A fell in the linear segment.

Two control serum samples (A and B) were tested on 45 and 55 occasions on separate plates from the same batch of certified Nunc Maxisorp ELISA plates. Mean β2GPI levels (±SD) were estimated at 244.2 (± 35.7) and 147.7 (± 20.6) μg/mL, respectively. Intra-assay coefficients of variation (CV) were 4.7% and 3.4%, respectively, and inter-assay CV 14.6% and 13.9%, respectively (Table 1, column 6).

In order to assess assay variability further between batches of non-certified Nunc Maxisorp ELISA plates, samples A and B were tested eight times, respectively, using five plates from each batch. Intra-assay CV was calculated for each plate and inter-assay CV was calculated for each plate batch (Table 1, columns 1 to 5). As can be seen in Table 1, batch 5 gave unacceptable reproducibility in measurements for β2GPI level.

When using an ELISA it is important to check for intra-batch variability in the plate support. Alternatively, the use of batches of plates certified by the manufacturer for consistency in adsorption should overcome this problem. This report is a timely reminder for those having problems with reproducibility with an ELISA to check that the plates being used are certified by the manufacturer for consistency in adsorption of proteins.

Having satisfactorily standardised the β2GPI ELISA and overcome reproducibility problems, we tested sera collected from 269 healthy volunteers (either laboratory and hospital employees or students: 202 females and 67 males, overall mean age ±SD: 30.7 ± 12.3 years). β2GPI concentration in this normal population ranged from 20.8–296.8 μg/mL, and there was no significant difference between β2GPI levels in female and male subjects (mean ± SD: 174.4 ± 45.3 and 180.9 ± 46.9 μg/mL, respectively; Mann-Whitney *P* value = 0.4280).

There is inconsistency in the literature regarding levels of β2GPI between female and male subjects,[12,14] which may reflect the difficulties that we encountered in the reliability of the ELISA. However, having successfully overcome these problems, this study shows that although males have slightly higher levels of β2GPI than females, the difference is not statistically significant. The relative levels of β2GPI in females and males is of interest as β2GPI is thought to be an inhibitor of the coagulation pathway, and females have a higher incidence of thrombotic episodes, both during pregnancy and while receiving oestrogen therapy.[15]

In summary, the non-specific binding properties of β2GPI to the plastic support can have adverse effects on immunoassay characteristics. The use of a saturating concentration for the capture antibody can prevent such non-specific binding. Extra care is required, however, when selecting a solid phase for the capture antibody immobilisation, as a great deal of variation in test results exists among the types of plates from different manufacturers, and even among different batches of specific types of plates. □

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TypA is a virulence regulator and is present in many pathogenic bacteria

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Tyrosine phosphorylation is rare in prokaryotes and, despite its pivotal function in the control of cell division and differentiation in eukaryotes, its biological role in bacteria is poorly understood. In bacteria it has been shown to play a minor role in protein phosphorylation, which suggests that it may have a more significant function elsewhere.[1-3] TypA has gained in interest recently due to the fact that it is tyrosine phosphorylated in certain *Escherichia coli*. Early work in an *E. coli* K-12 *typA* mutant showed that protein expression was altered, thereby suggesting a regulatory role for TypA.[4,5]

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Table 1. Sequence similarity between 39 of the 59 bacterial species known to contain TypA homologues

Species	*E. coli* identity (%)
Escherichia coli K-12 MG1655	100
Escherichia coli Enteropathogenic 0127:H7 strain E2348-69	100
Salmonella typhi	96.8
Salmonella typhimurium	95.9
Salmonella paratyphi	95.6
Escherichia coli 0157	94
Yersinia pestis	89.4
Pasteurella multocida PM70	84.1
Haemophilus influenzae Rd	82.1
Vibrio cholerae	77
Shewanella putrefaciens	76.9
Vibrio parahaemolytica	76.2
Pseudomonas aeruginosa	73.5
Neisseria gonorrhoeae	64
Stenotrophomonas maltophila	64
Neisseria meningitidis MC58	63.9
Bordetella pertussis	63.2
Acidothiobacillus ferrooxidans	62.3
Clostridium acetobutylicum	56.5
Streptococcus pneumoniae	56.3
Streptococcus pyogenes	55.9
Deinococcus radiodurans	55.5
Bacillus subtilis	54.9
Streptococcus mutans	54.9
Campylobacter jejuni NCTC 11168	54.7
Helicobacter pylori J99	54.2
Helicobacter pylori 26695	54.2
Chlorobium tepidum	53.8
Synechocystis PCC6803	53.3
Rickettsia prowazekii	53.2
Clostridium difficile	51.9
Streptococcus agalactiae	51
Porphyromonas gingivalis W83	50.3
Mycobacterium avium	49.1
Mycobacterium bovis	48.7
Mycobacterium tuberculosis H37 Rv	48.7
Mycobacterium tuberculosis CSU#93	48.7
Corynebacterium diphtheriae	48.5
Mycobacterium leprae	47.9

Interestingly, the expression of the global regulator H-NS is increased, as is the expression of the carbon starvation protein CspA and the universal stress protein UspA.[6] H-NS is an important global regulator and thus also plays a role in virulence.[7] CspA is important in responding to environmental stimuli,[8] while UspA is important in responding to various stress stimuli, including antibiotics.[9,10]

Further work in the enteropathogenic *E. coli* (EPEC) derivative MAR001 shows that *typA* has homology with members of the GTPase superfamily such as elongation