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Article *in* Journal of clinical pathology · July 2013

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The impact of chronic untreated hyperglycaemia on the long-term stability of paraoxonase 1 (PON1) and antioxidant status in human sera

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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jclinpath-2013-201646).

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Received 27 March 2013
Revised 29 May 2013
Accepted 11 July 2013
Published Online First 31 July 2013

To cite: Macharia M, Kengne AP, Blackhurst DM, *et al*. *J Clin Pathol* 2014;**67**:55–59.

ABSTRACT

Aims Paraoxonase 1 (PON1) is increasingly measured on samples that have been stored for extended durations. The impact of storage and baseline conditions on the stability of the enzyme is however not well documented. We investigated the influence of hyperglycaemia on the stability of PON1 activity and antioxidant status in human sera stored for 12 months.

Methods Blood was collected from 60 individuals aged 35–80 years with chronic hyperglycaemia (HbA1c≥6.5%) or normoglycaemia (HbA1c<6.5%) in Cape Town. At baseline and after 12 months at −80°C, levels of PON1 activity (paraoxoase and arylesterase), antioxidant activity (ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC)) and lipid peroxidation (malondialdehyde and oxidised low density lipoprotein (ox-LDL)) were measured and compared.

Results In normoglycaemic samples, 12-month storage led to minor alterations of <10% for the six target variables. In hyperglycaemic samples, alterations ranged from 13% for AREase activity to about 23% for ox-LDLs indicating a twofold to fourfold difference between the two groups in the variables assessed. Changes in levels of FRAP, TEAC and ox-LDL were both statistically and clinically significant. Furthermore, there was evidence of significant statistical interaction by baseline glycaemic status on the alteration of FRAP, TEAC, thiobarbituric acid reactive substances and ox-LDL, but not for PON1 activity.

Conclusions The results indicate that baseline glycaemic status may contribute to a decline in the stability of antioxidant activity and extent of lipid peroxidation but not PON activity.

INTRODUCTION

Biorepositories have become an integral part of many clinical and epidemiological studies. Stored samples are very precious when fiscal or logistical reasons necessitate delayed or batch analyses. They provide an effective and economical avenue to increase the range of measured analytes as the study progresses or as new information and risk factors emerge. Ideally, the analyte of interest should either be completely stable during the storage period or the rate and extent of deterioration should be known. This often is not the case as the samples may be used to measure components for which the long-term stability during storage has not been well established such as paraoxonase 1 (PON1).

PON1 is a calcium-dependent esterase that is widely distributed in various tissues including liver, kidney, intestine and serum where it is associated with high density lipoprotein (HDL).[1] Although PON1 can protect against the toxicity of some organophosphates, its precise physiological role is yet to be firmly established. Increasing evidence from in vitro and animal models suggest that PON1 is an antioxidant enzyme that protects HDL and low density lipoprotein (LDL) against oxidative damage.[2–4] Since the oxidation of LDL is a key process in the pathophysiology of atherosclerosis and cardiovascular disease, it is not surprising that the enzyme has attracted immense scientific interest in the last two decades. In addition to cardiovascular health, PON1 has been widely investigated in many other areas including, but not limited to, drug metabolism,[5] nephrology, neurobiology, cancer biology, autoimmunity and ageing (reviewed in [6]).

Measurement of PON1 status is a component of an increasing number of studies, many of which may involve samples that have been stored for months or years even though only a handful of studies have evaluated the impact of storage on PON1 status.[7–10] Lower storage temperatures sustain the enzyme activity which nonetheless declines with extended storage. For example, AREase and POase activities were 17.1% and 37.6% lower respectively in the batch of samples stored for 7 years compared with those stored for 2 years.[8] There are no data regarding the stability of the antioxidant PON1 in relation to the samples' baseline antioxidant status and pro-oxidant states such as hyperglycaemia in which the functions of HDL and PON1 are impaired.[11 12]

In this study, we investigated the impact of long-term sample storage at −80°C on the stability of PON1 activity and markers of oxidative status in samples from individuals with hyperglycaemia and normorglycaemia.

MATERIALS AND METHODS

Study setting and population

The study setting has been described previously.[13 14] Study participants (n=60) were members of a cohort study conducted in a mixed ancestry urban community from Cape Town, South Africa. The study was approved by the research ethics committees of Stellenbosch University (Reference Number: N10/04/118) and the Cape Peninsula University of Technology (CPUT/HW-REC 2010/H017). The study was conducted according to the Code of Ethics of the World Medical Association

(Declaration of Helsinki) and all participants provided a written informed consent prior to the commencement of the study.

Blood collection and processing

Venous blood was collected after overnight fasting. Within 2 h of bleeding, serum and plasma were obtained by low speed centrifugation and aliquoted. One aliquot for each sample was left at 4°C for baseline analysis within 48 h while the rest were stored at −80°C. With the exception of screen-detected diabetes, all participants were non-pregnant and free of any known infections, malignant disease, dementia or addiction to alcohol.

Routine determinations on a Cobas 6000 immunometric analyser (Roche Diagnostics) were used to measure levels of plasma glucose, glycated haemoglobin (HbA1c), total cholesterol, HDL cholesterol, triglycerides and γ-glutamyl transferase (GGT), alanine aminotransferase, uric acid and lipid profile. LDL cholesterol was calculated using the formula of Friedewald and coworkers.[15] The HbA1c method used is National Glycohaemoglobin Standardization Program certified according to Roche Diagnostics. Chronic untreated hyperglycaemia was defined by HbA1c levels≥6.5%. Analyses at baseline and after 12 months in storage were done for total antioxidant capacity, PON activity and lipid peroxidation (LPO).

Total antioxidant capacity

The total antioxidant capacity in plasma samples was assessed using the ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays. FRAP was done according to the method of Benzie and Strain.[16] Briefly, plasma samples were mixed with FRAP reagent, incubated for 30 min at 37°C, and the absorbance at 593 nm recorded using a spectrophotometer (Spectramax plus384 Molecular devices, USA). The TEAC assay was according to Re *et al*[17] and is based on monitoring (at 734 nm) the discolouration of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS) cation formed by reacting ABTS and potassium persulfate. We used distilled water instead of PBS to dilute the $ABTS^{+}$ radical solution.

PONnase activity

Paraoxonase (PONase) and arylesterase (AREase) activities were measured using paraoxon and phenylacetate (Sigma Aldrich, South Africa) as substrates, respectively. PONase activity was measured using the method of Richter and Furlong[18] from the initial velocity of p-nitrophenol production at 37°C, and the increased absorbance at 405 nm was monitored using a spectrophotometer (Spectramax plus384, Molecular devices, USA). Each serum sample was incubated with 5-mM eserine (Sigma Aldrich) for 15 min at room temperature to inhibit serum cholinesterase activity which is markedly elevated in diabetes and would otherwise interfere with the determination of PONase activity in serum from diabetic individuals. PON1 activity of 1 U/L was defined as 1 μmol of p-nitrophenol hydrolysed per minute. The method of Browne *et al*[19] was modified to measure AREase activity. The working reagent consisted of 20 mmol/L Tris-HCl, 4 mmol/L phenyl acetate, pH 8.0, with 1.0 mmol/L $CaCl_2$ (Sigma Aldrich). The reaction was initiated by adding 5 μL of 40-fold tris-diluted samples to 345 μL of the working reagent at 25°C. The change in absorbance at 270 nm was recorded for 60 min after a 20 s lag time on a Spectramax plus384 spectrophotometer. The activity, expressed as kU/L, was based on the molar absorptivity (1310) of phenol at 270 nm. In both assays, the rates used to generate the data points were derived from the linear portions of the rate versus time plots.

Lipid peroxidation

Malondialdehyde (MDA) and oxidised LDL (ox-LDL) were used as markers of LPO. The method of Jentzsch *et al*[20] was used to estimate the thiobarbituric acid reactive substances (TBARS) which reflect the production of MDA. Plasma ox-LDLs were determined using an ELISA procedure (Cellbiolabs, San Diego, California, USA). For FRAP, TEAC and ox-LDL assays, the standards to generate the standard curves were done in triplicate. Reproducibility was checked by additionally running selected standards in triplicate at two different points on each plate. For the other three assays which make use of extinction coefficients, a random sample was done in duplicate at two different points on each plate.

Statistical analysis

Data were analysed using the R statistical software V.2.13.0 (13-04-2011) (The R Foundation for Statistical Computing, Vienna, Austria). Variables are summarised as mean and SD, median and 25th–75th percentiles, and count and percentages. The Shapiro–Wilk W test was employed to determine whether the analytes of interest were normally distributed based on probability thresholds of p≥0.1. Student t test, Mann–Whitney U test and χ^2 test were used to compare baseline characteristics across glycaemic subgroups at baseline. Measurements at baseline served as the reference for all comparisons between baseline and 12-month measurements. The continuous association between baseline and 12-month measurements was assessed using Spearman's correlation test. Systematic bias in the two measurements (baseline and 12 months later) at any given level of the target variable was then examined using Bland and Altman plots,[21] implemented with the use of *ResearchMethods* package of R. Paired-sample t test and Wilcoxon test were used to compare differences in means of baseline and 12-month measurements overall and within glycaemic subgroups. Then, the general linear models for repeated measurements were used to investigate the effects of baseline glycaemic status on the changes in levels of target variables between measurements at baseline and 12 months later.

Clinical significance

To assess the clinical importance of statistically significant differences between baseline and 12-month measurements, we used the total allowable error (TEa). For PONase and ox-LDL, the specifications of TEa were based on within- and between-subject variation.[22] For other analytes, we used the significant change limit (SCL)[23] which was calculated as SCL=reference value ±2.8*USD, where USD is the usual SD. The USD in the current study was obtained by averaging the quality control data over the period of analysis. The mean of the 12 months was then compared with the reference (baseline measurement) and should be within clinical range of reference mean±TEa. The percentage difference was calculated as 100×((12-month −baseline measurements)/baseline measurement).

RESULTS

Participants' profile and baseline measures

The samples used were from 60 subjects who were grouped as having either hyperglycaemia (HbA1c≥6.5%) or normoglycaemia (HbA1c<6.5%). The baseline characteristics of the two groups were comparable in term of age and gender distribution, blood pressure and heart rate, total cholesterol and LDL cholesterol, C reactive protein, GGT, creatine and estimated glomerular filtration rate (table 1). As expected, baseline levels of

Table 1 Participants' profile and baseline measures

Variables	Overall	HbA1c<6.5%	HbA1c≥6.5%	p Value
N	60	30	30	
Age (years)	60.6 (13.3)	60.1 (13.5)	61.2 (13.5)	0.75
Female, n (%)	43 (72)	21 (70)	22 (73)	0.77
Body mass index (kg/m^2)	32.8 (9.8)	30.0 (8.1)	35.6 (10.7)	0.03
Waist circumference (cm)	96 (14)	90 (14)	101 (12)	0.001
Waist to hip ratio	0.90 (0.07)	0.86 (0.05)	0.93 (0.08)	0.0003
Systolic blood pressure (mm Hg)	148 (29)	146 (28)	151 (31)	0.54
Diastolic blood pressure (mm Hg)	83 (13)	82 (12)	84 (14)	0.51
Pulse rate (beat/min)	72 (12)	71 (12)	73 (11)	0.62
Fasting plasma glucose (mmol/L)	8.4 (4.2)	5.0 (0.6)	11.8 (4.1)	<0.0001
HbA1c (%)	7.3 (2.2)	5.7 (0.3)	8.9 (2.1)	<0.0001
Fasting insulin (μU/mL)	14.1 (6.1–22.0)	8.2 (4.0–15.3)	18.3 (12.4–26.5)	0.001
Glucose to insulin ratio	0.65 (0.33–0.99)	0.67 (0.32–1.16)	0.65 (0.35–0.86)	0.97
Creatine (μmol/L)	80.1 (28.8)	80.2 (30.2)	77.0 (27.56)	0.41
eGFR (mL/min)	82.3 (29.1)	77.8 (23)	86.8 (34)	0.24
CRP (mg/L)	6.0 (2.0–11.0)	6.1 (1.9–13.4)	6.0 (2.5–10.4)	0.74
GGT (IU/L)	31.0 (24.0–44.5)	31.0 (23.2–43.2)	31.5 (25.2–53.2)	0.63
Total cholesterol (mmol/L)	5.4 (1.2)	5.2 (1.0)	5.5 (1.3)	0.43
HDL cholesterol (mmol/L)	1.4 (0.5)	1.6 (0.5)	1.3 (0.3)	0.007
LDL cholesterol (mmol/L)	3.2 (1.0)	3.1 (0.8)	3.4 (1.2)	0.35
Total to HDL cholesterol ratio	4.0 (1.2)	3.5 (0.9)	4.5 (1.3)	0.0006
Triglycerides (mmol/L)	1.5 (0.6)	1.1 (0.4)	1.8 (0.6)	<0.0001

CRP, C reactive protein; eGFR, estimated glomerular filtration rate; GGT, γ-glutamyl transferase; HDL, high density lipoprotein; LDL, low density lipoprotein.

HbA1c and fasting glucose were significantly higher in the hyperglycaemic group (p≤0.001). Furthermore, markers of adiposity, fasting insulin, total to HDL cholesterol ratio and triglyceride levels were higher in the hyperglycaemic group (all p≤0.03), while HDL cholesterol was significantly lower (p=0.007, table 1). The pattern was similar when comparisons were all based on non-parametric tests.

Description of targeted analytes

The median and mean values (and SD) for the baseline and 12-month measurements are summarised in online supplementary table S1. All variables were normally distributed, except for ox-LDL and AREase (baseline and 12 months) and FRAP (baseline). With the exception of TEAC and ox-LDL, values for the other four target variables differed significantly between the two groups at both time points. PON1 activities were higher while FRAP and TBARs were lower. Ox-LDL only differed significantly between the groups during the second measurement while TEAC did not differ at either time point.

Effect on stability

After 12 months storage at −80°C, all measures of PON activity and antioxidant status in the normoglycaemia group showed minimal changes below 10%. PONase and antioxidant activities decreased by between 2.5% and 9.0%, while the two markers of oxidative stress increased by 5.3% and 7.4% (see online supplementary table S1). A similar trend was observed in the hyperglycaemic group but the magnitude of alteration from baseline levels was twofold to fourfold higher compared with the normoglycaemic group (table 2). For instance, PONase and AREase activities dropped by 5.9% and 13.1% while ox-LDL and TBARS increased by 22.7% and 20.9%, respectively. In both groups, measures of PON activity and antioxidant status over the two time points were significantly and positively correlated (r=0.74–0.99, all p<0.0001) and also in good agreement as reflected on the Bland and Altman plots (see online supplementary figure S1). There was evidence of significant interaction by baseline glycaemic status on the deterioration of analytes levels in storage for FRAP (p=0.001), TEAC (p=0.001), ox-LDL (p<0.001), TBARS (p<0.001), but not for PONase (p=0.21) and AREase (p=0.31).

Clinical significance

The clinical relevance of statistically significant differences between baseline and 12-month measurements was evaluated using TEa (PONase and ox-LDL) and SCL (all six measures) approaches. Based on TEa method, the significant differences between baseline and 12 months for PONase and ox-LDL measures were clinically trivial as were TBARS, PONase and AREase on the SCL approach (table 2). However, differences between the two time points for FRAP, TEAC and ox-LDL were both statistically and clinically significant based on SCL, regardless of the baseline status for hyperglycaemia (table 2).

DISCUSSION

The results from this study point to the possibility that hyperglycaemia at baseline may lead to decreased PON1 and total antioxidant activities as well as an enhanced rate of LPO after storage −80°C for 12 months. In samples from participants with normoglycaemia, storage of up to 12 months at −80°C led to only minor alterations of less than 10% for PON1 activity and markers of oxidative status but in samples from participants with hyperglycaemia the magnitude of alteration was twofold to fourfold higher. However, the effect of hyperglycaemia was statistically and clinically significant only for FRAP, TEAC and ox-LDL but not PONase, AREase and TBARS. Furthermore, we found evidence of significant statistical interaction by baseline glycaemic status on the alteration of FRAP, TEAC, TBARS and ox-LDL upon storage.

Table 2 Clinical significance of difference in analytes based on SCL

Analyte	Subgroup	Measurement	n	Mean	SD	% Difference	Statistical significance	USD	2.8*USD	SCL Min	SCL Max	Clinical significance
PONase	Overall	Baseline	60	173.7	40.2	0	<0.0001	19.9	55.8	117.9	229.6	NS
		12 months	60	166.6	41.0	−4.1						
	Normoglycaemia	Baseline	30	191.3	40.4	0	0.04	19.9	55.8	135.5	247.2	NS
		12 months	30	186.3	42.0	−2.6						NS
	Hyperglycaemia	Baseline	30	156.1	29.6	0	0.0006	19.9	55.8	100.3	212	NS
		12 months	30	146.9	31.3	−5.9						NS
AREase	Overall	Baseline	60	68.5	26.6	0	<0.0001	18.7	52.4	16.1	120.9	NS
		12 months	60	62.3	24.8	−9.1						NS
	Normoglycaemia	Baseline	30	80.5	19.1	0	0.0004	18.7	52.4	28.1	132.9	NS
		12 months	30	75.4	18.3	−6.3						NS
	Hyperglycaemia	Baseline	30	56.6	27.9	0	0.0003	18.7	52.4	4.2	109	NS
		12 months	30	49.2	23.8	−13						
FRAP	Overall	Baseline	60	892.5	220.8	0	<0.0001	4.2	11.7	880.9	904.2	Clinically significant
		12 months	60	780.4	185.4	−12.6						
	Normoglycaemia	Baseline	30	789.3	166.2	0	0.0003	4.2	11.7	777.6	801	Clinically significant
		12 months	30	723.8	177.6	−8.3						
	Hyperglycaemia	Baseline	30	837.1	178.3	0	<0.0001	4.2	11.7	825.4	848.7	Clinically significant
		12 months	30	995.8	222.5	19						
TEAC	Overall	Baseline	60	1427.7	391.7	0	<0.0001	6.7	18.8	1408.9	1446.5	Clinically significant
		12 months	60	1197.8	323.9	−16.1						
	Normoglycaemia	Baseline	30	1353.6	359.3	0	0.002	6.7	18.8	1334.8	1372.4	Clinically significant
		12 months	30	1223.7	305.0	−9.6						
	Hyperglycaemia	Baseline	30	1501.7	414.4	0	<0.0001	6.7	18.8	1483	1520.5	Clinically significant
		12 months	30	1171.9	345.0	−22						
Ox-LDL	Overall	Baseline	60	3933.9	1852.5	0	<0.0001	13.9	39.0	3894.9	3972.9	Clinically significant
		12 months	60	4505.9	2105.1	14.5						
	Normoglycaemia	Baseline	30	3545.3	1895.2	0	0.0008	13.9	39.0	3506.3	3584.3	Clinically significant
		12 months	30	3709.1	1940.5	4.6						
	Hyperglycaemia	Baseline	30	4322.4	1754.4	0	<0.0001	13.9	39.0	4283.4	4361.4	Clinically significant
		12 months	30	5302.7	1984.0	22.7						
TBARS	Overall	Baseline	60	2361.9	1213.4	0	<0.0001	490.2	1372.5	989.4	3734.5	Not significant
		12 months	60	2708.4	1465.3	14.7						
	Normoglycaemia	Baseline	30	1806.3	648.9	0	<0.0001	490.2	1372.5	433.8	3178.8	Not significant
		12 months	30	1901.5	665.6	5.3						
	Hyperglycaemia	Baseline	30	2917.6	1391.4	0	<0.0001	490.2	1372.5	1545	4290.1	Not significant
		12 months	30	3515.3	1605.7	20.5						

AREase arylesterase, kU/L; FRAP, ferric reducing ability of plasma, μM; Ox-LDL, oxidised low density lipoprotein, ng/mL; PONase, paraoxonase, U/L; SCL, significant change limit; TBARS, thiobarbituric acid reactive substances, nM; TEAC, Trolox equivalent antioxidant capacity, μM TE/L; USD, usual SD.

Though previous studies[7–10] reported minimal changes in PON1 activity in samples stored at 80°C for up to years, this is the first report that has investigated the stability of PON1 activity and/or antioxidant status in relation to glycaemic status. Similar to our findings, Huen *et al*[8] reported minimal changes in PON1 activity in samples stored at 80°C for <2 years, but a considerable decrease after 7 years of sample storage. We are therefore inclined to speculate that the effect of hyperglycaemia would translate into differential clinical significance upon extended sample storage. The extensive glycoxidation that occurs in hyperglycaemia can lead to substantial decrease in the activity of both purified and HDL bound PON1 due to modifications of PON-phospholipids or PON-apoprotein interactions at the lipoprotein surface or due to inactivation by reactive oxygen species (ROS).[11]

A less common finding in our study was the elevated measures of total antioxidant status in hyperglycaemic individuals despite increased oxidative stress. In health, the production of ROS is balanced by the collective activities of antioxidants in plasma. There is evidence that persistent hyperglycaemia may disrupt this network of antioxidants by promoting ROS production via various mechanisms.[24 25] Excessive ROS can then lead to enhanced oxidation of biomolecules, inactivation of antioxidant enzymes and subsequent depletion of antioxidant

reserves.[25] However, a recent study in a European population also reported elevated total antioxidant status (ABTS-TEAC) and increased LPO (TBARS) in patients with uncomplicated diabetes.[26] We suspect that this maybe a reflection of a transient compensatory mechanism by the body in an attempt to counter the overwhelming ROS burden.

Our study has some limitations to be accounted for while interpreting the findings. We included only participants free of any known pathologies other than hyperglycaemia. Our results may therefore not necessarily be extrapolated to samples from individuals with other conditions. We also did not measure the 12-month levels of HDL though it has an important relationship with PON1.[1] Furthermore, the storage duration may not have been long enough to demonstrate the full spectrum of effects. Last, the method used to assess clinical significance in half of the assays was based on parameters derived from our sample which may affect estimation of the significance and thus have implications on reproducibility in other settings. Nonetheless, our study has several strengths including a good matching of the participants in terms of gender and age. Since PON1 is purported to function as an antioxidant, another key strength distinguishing this study from previous ones is the evaluation of its stability in the context of antioxidant status and a pro-oxidant state (hyperglycaemia). Unlike previous studies, we used extensive analytic methods to assess systematic bias in the two time-point measurements and the clinical relevance of the observed differences. In addition, we made use of two methods for each of the three aspects evaluated, that is, PON1, antioxidant activity and LPO. Besides allowing us to demonstrate the consistency of our findings, this approach reinforces antioxidant status results since the measured total antioxidant capacity of biological samples is known to be method-specific.[27] Furthermore, the non-specific nature of the MDA-TBARS method requires corroboration.

In summary, our results indicate that for samples stored at −80°C for 12 months, baseline glycaemic status may contribute to a decline in the stability of antioxidant activity and extent of LPO from a statistical standpoint. However, extended storage is needed to demonstrate differential effect, if any, on the clinical significance of the statistical differences.

Take home messages

- Sample storage of up to 12 months at −80°C altered PON1 activity and markers of oxidative status by less than 10% in samples from participants with normoglycaemia at baseline, but in samples from participants with hyperglycaemia the magnitude of alteration was twofold to fourfold higher.
- Presence of hyperglycaemic at baseline group but the magnitude of alteration from baseline levels was twofold to fourfold higher compared with the normoglycaemic group.

Acknowledgements We thank the Bellville South community for participating in the study.

Contributors MM: conception and design, acquisition and interpretation of data, preparation of the first draft and approval of final draft. APK: analysis and interpretation of data, revision for important intellectual content and approval of final draft. DMB: acquisition of data, revision for important intellectual content and approval of final draft. RTE: conception and design, revision for important intellectual content and approval of final draft. TEM: conception and design, interpretation of data, revision for important intellectual content and approval of final draft.

Funding Cape Peninsula University of Technology and South African Medical Research Council.

Competing interests None.

Ethics approval Stellenbosch University (Reference Number: N10/04/118) and the Cape Peninsula University of Technology (CPUT/HW-REC 2010/H017).

Provenance and peer review Not commissioned; externally peer reviewed.

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J Clin Pathol 2014 67: 55-59 originally published online July 31, 2013
doi: 10.1136/jclinpath-2013-201646

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