Correspondence

Modification of simple and specific test for measuring lipid peroxides in plasma

In our paper, we used and recommended an idiosometric (spectrophotometric) test to measure lipid peroxides in human plasma samples. The use of a commercially available colour reagent (CHOD-iodide; Merck, Germany) made the technique very simple and easy to use.

Not long after the paper had been published, we and others had difficulties performing the test as it was described. Basically, the saline treated (control) sample resulted in higher absorbance than the colour reagent (potted test) sample. This was due to some as yet unidentified changes in the CHOD-iodide reagent. The colour reagent presently available somehow quenches the original absorbance of plasma due to yellowish carotenoids. All our efforts to identify and eliminate this interference or change the protocol while measuring the formed I, at 360 nm have failed.

We therefore adopted a technique, which measures iodine in the presence of starch as a blue colour iodine-starch complex at 560 nm, where the natural yellowish colour of plasma does not interfere with the measurement. This modification has been in use for six months with different batches of CHOD-iodide and produced reliable estimate of the overall oxidative capacity of plasma.

Reagents:

CHOD-iodide (Merck, Germany).
Butylated hydroxy toluene (BHT): 0.572 mg/ml in ethanol.
Starch.

Sample solubilized from Sigma, No. S-2630: A 1% solution is made fresh in 6.7 mM sodium chloride (39.2 mg/100 ml) by immersing into boiling water for a few minutes. After centrifugation (5 min) at 12 000 rpm (Eppendorf centrifuge), the clear supernatant fluid is used the same day.

Procedure: Blood is taken into EDTA (1.5 mg/ml), centrifuged, and the plasma samples kept at 4°C for no longer than two days. In a solid phase, place 200 ml plasma, 1.0 ml colour reagent (CHOD), 10.0 ml BHT and 200 ml starch solution. Mix and transfer into a disposable cuvette and read the absorbance immediately at 560 nm against distilled water. Place the cuvette immediately into dark and then read the absorbance again at 60 minutes. The difference in the absorbance represents the peroxide content of plasma.

Direct calculation by using molar absorptivity is difficult due to the variation of the absorption with the source of starch. However, for one batch, the system can be calibrated by hydrogen peroxide, according to the stoichiometry of the reaction:

\[(1) \quad \text{H}_2\text{O}_2 + 2\text{I}^- + 2\text{H}^+ \rightarrow \text{I}_2 + \text{H}_2\text{O} \]

Absorption changes are linear between the amounts of peroxide added (0.2-1 nmol) and the I₂ produced.

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Parathyroid hormone related protein (PTHrP) in hypercalcaemia of lymphoproliferative disease

Bolo-Deoku et al have shown that PTHrP was not implicated in a case of hypercalcaemia in Hodgkin’s disease, thus expanding the very limited literature on PTHrP in lymphoproliferative disorders. We report a case of hypercalcaemia in chronic lymphocytic leukaemia (CLL) where the same conclusion was reached.

Eight years after presentation, a patient with stage IV C B-CLL developed acute low back pain due to vertebral body collapse. Generalised loss of bone density was apparent radiologically, but no discreet osteolytic lesions were seen. Corrected serum calcium concentration was 2.94 mmol/l (range 2.26-2.60), phosphate activity 1.31 mmol/l (range 0.8-1.4), alkaline phosphatase 84 IU/l (range 30-115), and renal function was normal. Vigorous intravenous hydration and intensified treatment with corticosteroids failed to control the hypercalcaemia and a peak corrected serum calcium of 3.29 mmol/l was reached before effective treatment with pamidronate sodium was started. Prolymphocytoid transformation and myeloma were excluded and there was no evidence of coincidental malignancy. Serum 25-hydroxycholecalciferol was 11.8 ng/ml (range 8-50), PTH 1.7 pg/ml (range 0.9-5.4), and PTH-related protein (PTHrP) was less than 0.23 pmol/l (normal).

While lymphotoxin (tumour necrosis factor β), interferons 1 and 6, and prosta-
glandins have a well defined role in the bone disease of myelomatosis, the mechanisms underlying dysfunctional osteoclastic activity in other lymphoproliferative disorders are unknown. Although larger studies must be performed, it seems unlikely that PTHrP has an important role.

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3 Rossi JF, Chapeau R, Zech G, et al. Micro-
oclasts resorption as a characteristic feature of B-cell malignancies other than multiple myeloma. Br J Haematol 1990; 76:469-75.

Value of PCNA and Ki67 staining in breast cancer

The failure of PCNA to reflect proliferative activity, as measured by Ki67 or mitotic counting in breast tumours, documented by Leonardi and colleagues, is disappointing. We have found a weak positive correlation between PCNA staining and Ki67 staining in both benign and malignant breast tumours (personal observation).

To some degree such observations may be related to the techniques used. Our practice with both Ki67 and PCNA is to count areas of highest antigen expression after scanning the slide at low magnification. This gives a measure of the maximum proliferative activity in any given tumour and obviates the need to decide how many areas of low to medium enrichment concentration to include (not to mention the intermediate areas).

Using this method, we found no correlation between PCNA and mitotic count in cycle only. PCNA must therefore persist in non-cycling cells but in a variable manner, losing its association with mitotic activity and making its correlation with Ki67 expression tenous.

This suggests that although PCNA is to some extent comparable with Ki67 it is influenced by factors as yet undefined to such an extent that it loses its usefulness in quantifying proliferative activity.

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1 Leonardi E, Girlando S, Serio G, et al. PCNA and Ki67 expression in breast carcinoma; Correlations with clinical and biological as-
4 Barrett S, Anderson TJ. Determination of prolifer-

Dr Barbarecchi comments:

Dr Sullivan’s letter, which suggests a weak correlation between PCNA and Ki67 immunostaining in benign and malignant breast tumours, agrees with our paper where we concluded that in breast cancer PCNA immunostaining is not a substitute for Ki67
labelling. PCNA immunohistochemical expression (evaluated with the PC10 mono-
clonal antibody) seems to be related to cellular proliferation in many normal tissues and to the AgNOR silver staining in some neoplasms, such as gastro-
intestinal lymphomas, central nervous system tumours, lung neuroendocrine neoplasms, and prostatic carcinomas. However, in other tumours, like breast and gas-
trointestinal carcinomas, PCNA (PC10) expression seems aberrant and not strictly related to proliferative activity.1,3,8

Various factors unrelated to cell proliferation may influence the immunohistochemical expression of PCNA, including de
-transcriptional regulation and (deregulation) of the PCNA gene,9 long half-life of the PCNA protein,10 involvement of PCNA pro-	ein in DNA repair synthesis,11 and tissue and section processing—type and strength of the fixatives, fixation time, section staining, immunohistochemical techni-
ques.12,13

Further problems in PCNA immunohisto-
chemical staining, as in other kinetic quan-
titative immunohistochemical studies, concern evaluation and scoring methods.14,15 Should we use quantitative or semiquantita-
tive surrogates or should the counts be counted? Which tumour areas should be evaluated (the most positive or random selec-
ted areas)? Which immunoreactive cells should be evaluated (all positive cells or only the most intensely stained)?

Particular attention should be also drawn to the kind of antibody used to localise PCNA. Different staining patterns may be seen with different antibodies, and this may add to confusing and confusing results.

In our opinion PCNA immunostaining should be evaluated with great caution and in some fields even with scepticism. More work is needed to assess the extent and range of PCNA staining in different tissues and lesions (neoplastic and non-neoplastic). PCNA counts should be evaluated concurrently with the different anti-PCNA available antibodies and the results should be compared with the "proliferation index" and especially with clinical data. The possi-
bility that PCNA immunostaining may have diagnostic or prognostic value is intriguing and carefully performed clinicopathological studies are needed to assess this possibility further. This will be the only way to know if we are faced with an interesting but clinically worthless tool or with an important test to be added to the routine evaluation of neo-
plasms.

1 Leonardo E, Girlando S, Mauri FA, Dalla Palma P, Barbaresci M, PCNA and Ki67 expres-
3 Woods AL, Hall PA, Shepherd NA, et al. The assessment of proliferating cell nuclear anti-

AgNOR quantification in tumour pathology: What is actually evaluated?

The interest of pathologists in interphase silver stained nucleolar organiser regions (AgNORs) has grown significantly.2 If it was shown that malignant cells frequently have higher AgNOR numbers compared with corre-
sponding benign or normal cells. Moreover, interphase AgNOR counts are closely related to cell proliferative activity, suggesting that this parameter might also have prog-
nostic importance.

Nucleolar organiser regions (NORs) are chromosome transcriptional control areas or ribo-
somal genes. NORs are associated with a group of argyrophilic proteins, and can be visualised by silver staining in routinely pro-
cessed cytological and histological samples. At light microscopy, AgNORs appear as well defined black dots, which in interphase cells are exclusively distributed throughout the lighter stained nuclei. Each black dot corres-
ponds, at the ultrastructural level, to a fibrillar centre with the surrounding dense fibrillar component. The number of AgNORs in quiescent cells is generally low (most lymphocytes or stromal cells have only one), while in proliferating cells, such as cancer cells, a high AgNOR number is present.

Over the past six years the silver staining technique has become widespread among pathologists, but the lack of a standardised staining protocol has led to misinterpretation of structures by different authors.3,4 Looking in fact at the micrographs repor-
ted—for example, by Giri et al (breast carci-
noma)5 Offner et al, (colonic carcinoma)6 Cheville et al, (renal cell carcinoma)7 and Kaneko et al (lung carcinoma)8—it is evident that not just the AgNORs, but the whole nuclei have been stained by silver and counted as AgNORs.

The selective visualisation of AgNORs is subject, apart from the fixative used, to the temperature and temporal length of the staining reaction. These two variables are inversely related to each other: the higher the temperature, the shorter the time required for AgNOR silver staining. The staining reaction is prolonged beyond the time for selective visualisation of NORs, all the other nuclear structures are progressively stained, until the whole nucleus appears homogeneously stained.6 Therefore evident that different nuclear struc-
tures have been stained and counted in various laboratories, and this has caused disagreement about AgNOR numbers report-
ed in individual studies on the same neo-
plastic lesions.

In a recent investigation it was shown that the total interphase AgNOR area was closely related to the whole nucleolar area stained by silver when staining was prolonged beyond the optimal time for selective interphase NOR visualisation.9

To obtain comparable data between differ-
ent laboratories the whole nucleolar ought to be silver stained and the area occupied by the silver stained nucleoli per cell measured using image analysis instead of AgNOR counting. Because AgNOR area and nucleo-
lar area have different values,1,4 it is evident that the morphometric analysis of silver stained nucleoli will certainly have the same clinical and biological relevance demonstrated for interphase AgNORs.

Method for grading breast cancer

Parham and colleagues have proposed a new and "simplified" method for grading breast cancer.6 They claim that it is superior to the method of Bloom and Richardson,7 which they rightly criticise for its lack of precision. We agree entirely with this criticism, but are rather surprised that they do not refer to our recent publication in which, for precisely this reason, we have devised modifications which provide objective criteria for the evaluation of the three morphological components of his-
tron grade.8 We have shown in a study of over 1500 patients that histological grade, using this method, provides powerful prog-
nostic information, and in combination with tumour size and lymph node status (the Nottingham Prognostic Index which can be used by clinicians to stratify patients for

6 Derenzini M, Farabegoli F, Trerè D. Relationship between interphase AgNOR distribution and nucleolar size in cancer cells. Histochem J (in press).
Value of PCNA and Ki67 staining in breast cancer.

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