MUT/2016/01

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

Assays used to evaluate germ cell DNA integrity in human fertility investigations

Members considered a paper on germ cell mutagenesis and aging, and recent developments in germ cell mutagenicity testing at the meeting in October 2015.

The suggestion that air pollution should be classified as a human germ cell mutagen was considered. It is noted that the studies used to underpin this suggestion use DNA integrity assays developed to assist in fertility assessments in assisted reproductive technologies (ART) as a marker of sperm DNA damage. Currently it is not clear what value these assays have in investigating potential germ cell mutagenesis in humans. Therefore an overview of the two methods commonly used, the sperm chromatin structure assay (SCSA) and the TUNEL assay, has been prepared with view to establishing their use in human bio-monitoring studies or for evaluating human germ cell mutagenesis.

Questions are provided at the end of the paper for Members consideration.

Secretariat/PHE Toxicology Unit

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ANNEX 2:

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Assays used to evaluate germ cell DNA integrity in human fertility investigations

Introduction:

1) There is increasing focus on identifying whether chemicals are potential germ cell mutagens with a view to ensuring protection of human health. Whilst the assumption that 'somatic cell mutagen = germ cell mutagen' is currently made when assessing genotoxicity test data for decision making and classification purposes, how that translates to actual human health effects is not well defined. There is also concern that not all germ cell mutagens will be positive in assays for somatic cell mutagens.

2) The Committee considered germ cell mutagenesis, in the context of increasing mutation frequency in aging males, in October 2015. Brief abstracts of some studies investigating the impact of air pollution were presented as an example of an environmental exposure which could potentially give rise to human germ cell mutagenesis (Annex 1). In a paper 'Declaring the existence of human germ-cell mutagens', DeMarini (2012) suggested that there is a need to investigate and advise on the existence and implications of human germ cell mutagens, in a manner similar to that undertaken by IARC for human carcinogens. In such a scheme, it is proposed that ionizing radiation, cancer chemotherapeutic agents, smoking and air pollution should be considered as Group 1 germ cell mutagens.

3) Methods for investigating germ cell mutagenesis were reviewed by COM in November 2013 (MUT/2013/05) and most of these are also described and evaluated in detail in a report from the IWGT work shop on germ cell assays (Yauk et al 2015 – Annex 2). It is noteworthy that the majority of these are established methods in animal models (eg transgenic rodent germ cell mutation assay, sperm comet assay, sperm FISH assay). A few studies using methods adapted for use with human sperm were included in the COM review (eg human comet assay, human sperm FISH assay). However the work quoted by DeMarini (2012) to substantiate the suggestion that air pollution is a human germ cell mutagen (Rubes et al 2005; Somers 2011 -Annex 2) utilised routine semen characteristics/parameters (WHO parameters: semen volume, sperm concentration, sperm morphology, sperm motility) and an assay of sperm DNA integrity, not evaluated by COM, as evidence of male germ cell DNA damage. These assays are most often used to investigate human fertility.

4) A substantial amount of information on germ cell DNA damage in humans has been obtained from studies investigating infertility or those aiming to improve the outcome of assisted reproduction technologies (ART). The principle assays used are the sperm chromatin structure assay (SCSA) and the TUNEL (terminal deoxynucleotidyl transferase mediated d-UTP nick end labelling) assay. Yauk et al (2015) described these assays briefly and noted that, although they generally correlate well with one another, they measure different aspects of DNA integrity and therefore will differ in sensitivity. No detailed description or analysis of the development and validation of these assays were provided in this IWGT report.

5) To explore further DeMarini's suggestion that certain agents such as air pollution should be classified as Group 1 human germ cell mutagens, it seemed pertinent to first evaluate the techniques used in assessing germ cell DNA damage in the clinic with a view to establishing their value as biomarkers of germ cell genotoxicity. This will facilitate interpretation of the assays' results if they are used in environmental or occupational exposure studies. The literature on DNA damage in sperm and fertility is vast and the included studies are representative, not exhaustive.

6) Also of value is deliberation of the following:

- What information is needed to conclusively determine whether a chemical is a human germ cell mutagen?
- If cancer is the ultimate consequence of DNA damage in somatic cells, what is the equivalent consequence of DNA damage in germ cells?

Methodologies for examining the integrity of germ cell DNA in humans

7) Older tests for germ cell effects, such as the dominant lethal assay in rodents, were based on a chemical impacting on the ability of an embryo to develop (pre-implantation loss) or on fertility outcomes, thus indicating the association between germ cell mutagenesis and fertility. Accordingly, the widespread use of ART has given rise to extensive investigations of the biological and clinical significance of DNA damage in male germ cells, and its close link to human fertility, including poor fertilization, pre-implantation loss, and miscarriage (Aitken and Iullius 2007; Aitken et al 2008 = Annex 2). These reviews focus on the importance of oxidative stress as a causal mechanism in inducing sperm DNA damage and a decrease in male reproductive health. Consideration is not given to other possible mechanisms which may lead to DNA damage in sperm. The link between paternal age and disorders attributed to mutations in sperm (achondroplasia and Apert syndrome) and the weak association with other effects such as congenital abnormalities, or Down syndrome, have been discussed. The authors suggest that DNA damage in the male germ line should be regarded as a risk factor for poor reproductive outcomes in much the same way that moderate smoking is a risk factor in developing lung cancer (they claim that the relative risk of spontaneous abortion because of DNA damage in sperm of men older than 40 is of a similar magnitude of a male developing lung cancer if he smokes 20 cigarettes a day (Kleinhaus et al 2006)).

8) Normal WHO sperm parameters are poor predictors of ART outcome and thus the development of a number of other tests using DNA damage parameters offered a means to more accurately predict the consequences for fertility outcome (Zini et al 2014).

Sperm Chromatin Structure Assay (SCSA®)

9) One of the most widespread effects used to assess fertility outcome in ART is sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA®) (Evenson 2006 – Annex 2) and there are also some publications on its

use in examining environmental causes of infertility (Eveson and Wixon 2005; Somers 2011). The methodology was originally developed to evaluate sperm DNA integrity as a potential indicator of fertility. The original assay assessed a number of parameters derived following flow cytometry such as nuclear shape, susceptibility to DNA denaturation when heated and chromatin condensation, and these were compared to known effects on fertility in mice, bulls and humans with the aim of selecting the parameters which best predicted fertility (Eveson 1980). The assay measures abnormal chromatin structure and involves staining with acridine orange which intercalates in double stranded DNA and stacks in single stranded DNA which causes differential shifts in fluorescence in a flow cytometer. Differences in ratios of red and green within the acceptable cell population represents susceptibility to DNA denaturation (Evenson et al 1999). The red population are considered to be those with denatured DNA and provide the DNA fragmentation index (%DFI) whilst the green coloured are high DNA stainable (HDS) sperm (Evenson 2006; Bungum et al 2011).

10) There are a large number of studies that have evaluated DNA fragmentation and pregnancy outcome in the context of normal fertility, or use of the ART techniques IVF (*in vitro* fertilisation), IUI (intrauterine insemination) and ICSI (intracytoplasmic sperm injection). These studies have been evaluated with a view to determining the relationship between results of the assay and human fertility.

11) In an initial study, the male partners from 73 couples achieving pregnancy within 3 months were used as 'a standard'; their DNA integrity was considered consistent with high fertility. This was significantly different from those who did not conceive until months 4-12 (40 couples) or those not achieving pregnancy (31 couples) (Evenson et al 1999). From these initial studies cut-off values of<15%, 15-30 and >30%, emerged as representing excellent, good and poor sperm DNA integrity respectively (formerly claimed to represent infertile, subfertile and fertile sperm). However it is noted that low values are not directly predictive of fertility as there are many other sperm parameters that impact, including sperm morphology and sperm motility.

12) Bungum et al (2004) examined whether SCSA parameters could be used to predict ART outcome and whether early pregnancy loss was associated with the use of sperm samples with high DFI. It was concluded that thresholds could be applied in a clinic setting, and identified an infertility DFI of 27-30% - couples in whom intrauterine insemination (IUI) was used, were 8.7 times more likely to conceive if the DFI was <27%.

13) However results from the use of SCSA in predicting success in ART are mixed. Evenson et al (2006) performed a meta-analysis of studies using SCSA in IUI, IVF and ICSI. All studies had cited the male as the cause of the possible infertility. The average OR for couples with DFI <30% to achieve pregnancy was 7.3 (10 studies - 4 studies had OR 6.5-10; 6 studies OR 0.5-2) by IUI, but only approx 2-fold more likely to become pregnant by IVF. It was concluded that the results of SCSA in different assisted reproduction procedures should be assessed separately. This analysis concluded that whilst a DFI >30% does not preclude a normal pregnancy, the likelihood of a successful pregnancy was statistically significantly reduced with spermatozoa with DFI >30% and HDS >15%. (If DFI >30%, it was

recommended that IUI should not be considered and the couple should move to IVF or ICSI procedures).

14) Collins et al (2008) examined 22 studies in which SCSA DNA integrity measurements were used as a predictive measure for pregnancy outcome in IVF. It was concluded that the small but statistically significant association between impaired sperm DNA integrity and an unsuccessful pregnancy in IVF and ICSI cycles is not strong enough to justify the routine clinical use of these tests in infertility evaluation of men. In investigations of 108 couples experiencing recurrent miscarriages, 30% of men had high DFI (>15%). The group with DFI >30% comprised 7 subjects out of 108 (perhaps those with >30% are infertile rather than spontaneous miscarriage?) (Leach et al 2015).

15) Lin et al (2008) reviewed pregnancy outcomes of 137 couples undergoing IVF and 86 undergoing ICSI in which SCSA was used to examine sperm viability. There were no significant differences in IVF and ICSI fertilization rate, good embryo rate, and pregnancy rate (PR) between high, moderate, and low DFI or HDS groups. Men with HDS >15% had significantly higher IVF abortion rates. There was a statistically insignificant trend toward an increased abortion rate in the high DFI (>27%) group. The DFI correlated negatively with sperm motility, and HDS correlated negatively with sperm morphology and concentration.

16) It is suggested that up to 40% of unexplained infertility cases are related to high DFI (>30%) (Bungum et al 2011 –Annex 2). These authors also indicated that the association between the DFI and WHO semen parameters is only weak to moderate. It is estimated that 25-40% of infertile men have normal WHO parameters but generate a DFI of 20-30% in the SCSA assay. It was suggested that the presence of damaged sperm has a significant impact on fertilization rate and embryo cleavage/development in IVF and ICSI procedures.

17) However, a previous study of 998 couples (387 IUI; 388 IVF; 233 ICSI) showed no relationship between sperm DNA fragmentation and implantation rate or early pregnancy loss (Bungum et al 2007).

18) A recent meta-analysis of 27 studies which had evaluated three groups of DFI cut-off levels (>27%, 15-27% and <15%) failed to establish the SCSA assay as a positive indicator of predicting ART outcome following IVF or ICSI (Zhang et al 2015).

19) The use of SCSA in ART appear to assign a cut-off point of >30% DFI to designate infertility. However, on the whole, studies do not consistently show a clear association between fertility and DNA fragmentation.

The TUNEL assay

20) The TUNEL assay is based on the labelling and detection of double and single strand breaks in DNA, using terminal deoxynucleotidyl transferase (TdT). Damage is quantified using fluorescence detection by microscopy or flow cytometry. The assay has been widely utilised to detect apoptosis but has also been developed

for clinical investigations to examine sperm DNA damage with respect to infertility. During apoptotic cleavage, TdT activity principally detects double DNA strand breaks. This is considered to be a specific marker of apoptosis in somatic cells (Negoeso et al 1998). However, this has not been confirmed in germ cells, in which apoptosis is only one of the possible mechanisms causing sperm DNA fragmentation (Muratori et al 2010). Hence, the TUNEL assay may have application in detecting genotoxic damage in sperm cells.

21) An initial evaluation in sperm cells identified an increased DNA sensitivity to denaturation which correlated with extensive DNA strand breakage (Gorczya et al 1993). However, doubt about the usefulness of the assay was raised because the method may be specific to phosphodiester strand breaks in somatic cells which are not demonstrated in germ cells (Lewis et al 2013). Furthermore, several investigators claim the TUNEL assay to be insensitive based on the fact that the terminal transferase cannot access the DNA in sperm in the way it would in a somatic cell due to the nature of the highly compacted structure of chromatin in such cells (Mitchell et al 2011; Lewis et al 2008). A published modification to the TUNEL assay involves incubation with dithiothreitol (DTT) prior to fixation. This step was demonstrated to increase the detection of DNA damage in animal and human sperm (Mitchell et al, 2008).

22) Sharma et al (2010) aimed to standardize the TUNEL assay in sperm, examining samples across and within laboratories to establish inter- and intra-laboratory variability. Both of these were determined to be low (<10%). From these data, reference ranges were determined for DNA damage across 25 healthy donors and 194 infertile men. A cut off of 19.5% was established as a value which acknowledged sperm DNA damage as a factor contributing to infertility for fertility screenings. A standardised protocol has been published (Sharma et al 2013).

23) Aitken et al (2010) evaluated the flow cytometric TUNEL assay in human sperm and observed a correlation between 8-hydroxy-2'-deoxyguanosine formation and DNA fragmentation. The authors suggest that DNA damage in sperm is largely associated with oxidative stress.

24) In a relatively small study, 100 infertile men undergoing ICSI and 61 fertile men were tested for sperm parameters, and evaluated using chromatin assays (not SCSA) and TUNEL. Despite there being significant differences between the results from sperm from infertile and fertile men in the chromatin and TUNEL assays, neither assay distinguished the sperm samples which had eventual positive ICSI outcomes (Garolla et al 2015).

25) In a critical examination of aspects of sperm DNA fragmentation by TUNEL, it was noted that the flow cytometric and fluorescence detection methods yielded different results. Whilst it was considered that TUNEL is a precise technique, it was recommended that a standardized procedure was implemented (Muratori et al 2010) .

26) In a group of 433 infertile men a correlation between bulky DNA adducts, WHO semen parameters and DNA fragmentation as detected by the TUNEL assay was demonstrated (Li et al 2013).

27) There are several reports comparing SCSA and TUNEL assays or providing meta-analysis of TUNEL, SCD and Comet assays. Chohan et al (2006) compared the results of 4 sperm chromatin assays from 60 infertile men and 7 fertile donors including SCSA and TUNEL and demonstrated similar levels of DNA fragmentation (SCSA 11.8± 1.4 and 22.0± 1.6 : TUNEL 11.1 ± 0.9 and 19.5 ±1.3 for fertile and infertile sperm respectively).

28) A meta-analysis was performed of spontaneous pregnancy loss after IVF or ICSI and the influence of sperm DNA damage as measured by TUNEL or SCSA (Zini et al 2008). In total, these studies looked at 1549 cycles of treatment, 640 pregnancies and 122 pregnancy losses. With TUNEL (five estimates) the OR for an association between pregnancy loss and DNA damage (>30%) was 7.04 (95% CI 2.81, 17.67; P < 0.001), whereas using SCSA (six estimates) the OR was only 1.77 (95% CI, 1.01, 3.13; P < 0.05). The meta-regression analysis revealed that the difference in OR estimates between the two assays was stastically significant (P = 0.012).The combined OR for the two assays was 2.48 (95% CI 1.52, 4.04; P < 0.0001).

29) Ribas-Maynou (2013) undertook a comprehensive review of the different methodologies available to assess sperm DNA integrity. They report statistical differences between infertile and fertile patients in SCSA and TUNEL and SCD assays with high correlations between all assays (p<0.001). The strongest correlation is between SCSA and TUNEL and it is commented that weaker correlations were reported for the alkaline comet assay and it was claimed that the neutral comet assay did not differentiate between fertile and non-fertile subjects. The TUNEL assay threshold for infertility was fragmentation value of 20.05%.

27) Palermo et al (2014- Annex 2) reviewed the DNA fragmentation assays in the context of ART and made the following observations: Men with high DFI (>30%) had significantly lower sperm concentrations than controls and an increase in DFI correlated with increasing age. However fertilization, embryonic cleavage and implantation rates did not differ between groups with low (<30%) and high (>30%) DFI.

28) In a recent study, Stahl et al (2015) evaluated sperm from 212 men attending for fertility assessment and compared SCSA and TUNEL assays with normal sperm parameters (semen volume, sperm concentration, %motility and morphology). Only a moderate correlation between SCSA DFI and the % TUNEL-positive sperm was found (r =0.314, P<.00001), and it is noted that this is a weaker correlation than previously reported. The discordance rate between SCSA DFI and the % of TUNEL-positive sperm in classifying patients as normal by one assay or abnormal by the other was 86 of 212 (40.6%). Correlations between WHO parameters and the TUNEL assays were reported as being weak or negligible whilst the correlation with SCSA was high. The authors note that the end points for SCSA and TUNEL are different and suggest that the assays should not be used interchangeably.

Discussion:

29) This paper provides an overview of the development and use of two DNA fragmentation assays, SCSA and TUNEL assay, and their use in ART. Both fragmentation assays gave variable results although the SCSA appears to perform more consistently. Opinions of their usefulness in ART are mixed.

30) Whilst both claim to measure DNA fragmentation, the endpoints are significantly different from one another and this could be of importance when evaluating the usefulness of the assay. It has been suggested that the SCSA assay measures DNA fragmentation arising from abasic sites created by OGG1, which will destabilize the DNA making it more vulnerable to acid hydrolysis and expression of single-stranded breaks. TUNEL measures DNA strand breaks which may arise from either free radical attack (oxidative damge) or enzymatic cleavage during apoptosis. It is likely that the TUNEL assay reflects oxidative damage to DNA in sperm. It is feasible that sperm can be SCSA-positive but TUNEL-negative.

31) The assays are discussed principally in the context of the oxidative stress hypothesis. There are no reports examining other mechanisms which could cause sperm DNA fragmentation. Therefore it is not clear whether these assays could be used as biomarkers of genotoxicity cause by environmental or lifestyle factors. There is evidence that DNA fragmentation in sperm can be factor in reduced fertility, but it is likely that other factors are involved.

Questions to Members

- What types of DNA damage do the SCSA and TUNEL assays measure?
- Could these assays be used to reliably detect DNA damage caused by genotoxic chemicals?
- Do studies using these assays corroborate a link between DNA damage in sperm and fertility?
- Can Members envisage these assays being useful in studies looking at the impact of environmental exposures on male germ cells in humans?
- Are there any modifications or validations that would increase their usefulness?
- Do Members wish to undertake a detailed evaluation of an environmental exposure as discussed by DeMarini (2013) (smoking, air pollution, chemotherapy)

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ANNEX 1

Studies on air pollution:

Studies in humans evaluating DNA damage parameters

Sram et al (1999) provides an overview of the programme initiated to investigate the impact of air pollution and lifestyle on a wide variety of health parameters on people living in Teplice Czech Republic. This area has high pollution levels as a result of intensive mining and brown coal combustion in the area compared to low pollution areas in California. Questionnaires were completed and biomarkers and air pollution levels were monitored (SO_2, PM_{10}, PAH's). Low birth weight and intrauterine growth retardation were observed. – Sperm parameters most notably affected (compared to the control group) were sperm morphology and X, x-y and 8 sperm disomy.

A study from the Teplice programme of work analysed donated sperm from 154 eighteen-year old men from Teplice and 118 from a rural area (Prachatice) as controls (Selevan et al 2000). Routine semen analysis, included sperm motion analysis, semen volume, motility and motion and also incorporated the SCSA. Air pollution levels were analysed for the two locations (PM_{10}, PM-TSP – total suspended particulates, SO_2, NO_x CO) at different times of years. Sperm parameter analyses were made accounting for high and low pollution seasons (low, medium high definitions unclear in paper). Significant changes in some sperm parameters (decreased motility, decreased normal morphology) and the following values for SCSA (COMPα) were reported.

Area	n	DFI% Mean ± SD	Range
Prachatice	118	19.8 ± 12.1	2.7-57.6
4.5 Teplice	154	20.5 ±15.4	2.0-81.0
Pollution Low	158	19.2 ±12.2	2.7-67.1
Medium	61	16.2 ±9.3	2.0-45.6
High	47	28.8 ±20.4	2.9-81.0

High vs Low values were statistically significant when adjusted in regression analyses but there were no differences between Teplice and Prachatice. The authors suggest further consideration of the impact of air pollution and/smoking on genetic integrity of sperm.

Sperm from 212 men from infertility clinics in Poland was analysed for aneuploidy using FISH procedures specific for X,Y,18, (centromic probes) and 13, 21 (locus specific probes) (Juerwicz et al 2015). Exposure to pollutants, $PM_{2.5}$, PM_{10} and SO_2, CO and NO_x was measured. The following positive associations were noted - $PM_{2.5}$ with disomy Y, sex chromosome disomy and disomy 21; and PM_{10} with disomy 21.

A study investigating the impact of traffic pollution on sperm chromatin and DNA integrity of traffic tollgate workers (36) and controls (32) in Italy (Calogero et al 2011)

measured some specific pollutants (Pb, NOx and SO_2). Measurements included hormones, sperm analyses including sperm chromatin integrity (PI flow cytometry) and DNA fragmentation (TUNEL assay). The % of spermatozoa with damaged chromatin in controls was statistically significant different compared to the tollgate workers (11.3 ± 1.2 compared to 18.3 ±1.4; p<00.1) and sperm with fragmented DNA (4.5 ± 0.4 compared to 9.3 ±0.9; p<0.001). There are no apparent attempts to correlate the results with pollution levels

The impact of different genetic polymorphisms on the susceptibility of sperm to air-pollution induced DNA damage was investigated in 47 urban policeman in the Czech Republic (Rubes et al 2010). Polymorphisms in metabolic genes (CYP1A1, EPHX1, GSTM1, GSTP1, GSTT1), folic acid metabolism genes (MTR, MTHFR) and DNA repair genes (XRCC1, XPD6, XPD23, hOGG1) were evaluated. Markers of sperm damage were assessed using SCSA and samples were assigned a DNA fragmentation index (DFI) - detectable, moderate or high (cut off points not described) . Air pollutants were measured (SO_2, NOx, CO, $PM_{2.5}$,O_3) and benzo(a)pyrene (B(a)P), polyaromatic hydrocarbons (cPAH) and benzene levels were determined in samples collected via personal monitors at two different times of year. Significantly higher levels of all pollutants were apparent during February compared to May but no differences in semen quality parameters were noted between seasons. However, higher DFI's were observed in the period of greater pollution, and were in particular associated with B(a)P and benzene levels. Associations between degree of DFI and genetic polymorphisms were also reported – of note were those with CYP1A1Mspl, XPD6 and XPD23 status and cPAHs.

Semen samples from a study population of 327 men attending an infertility clinic in Poland were analysed for normal semen quality parameters (Radwan et al 2015). SCSA was used to evaluate DNA fragmentation (%DFI) and high DNA stainability (HDS% - considered to be immature sperm with incomplete chromatin condensation). Air pollution levels of PM_{10}, $PM_{2.5}$, SO_2, NO_x, CO, were measured and associations between air pollution and semen quality were measured following adjustments for age, smoking, past diseases, sexual abstinence period and season . Exposure to all air pollutants increased abnormal sperm, morphology and there was a positive association of HDS% with PM_{10} and $PM_{2.5}$ but a similar correlation was not observed with DFI%. *NB: This study showed that sexual abstinence increased the % of sperm with DFI – is this a factor that needs to be taken into account? Chromatin mean and ranges for DFI% and HDS appear to be highly variable*

Chromatin	*Mean ±SD*	*Median*	*Range*	*N*
DFI%	*16.2 ± 11.0*	*13.3*	*2.7 -71.2*	*285*
HDS%	*8.5 ± 4.3*	*8.0*	*0.7 – 30.6*	*285*

Ji et al (2013) investigated the relationship of PAH DNA adducts and sperm DNA fragmentation in 443 infertile men from a Han Chinese population in the context of examining DNA repair gene polymorphisms. Those exposed to PAH's occupationally were excluded; there were 42.5% never smokers, 49.4% smokers and 8.1% ex-smokers. PAH adducts were determined using immunoflourescence staining and DNA fragmentation by TUNEL assay. Mean DFI% was 18.7 ± 11.5 with

21.9% of subjects >30% TUNEL. Adduct distribution was considered in relation to semen quality parameters, semen motion parameters and DFI. For each equal tertile, adjusted for confounders, several sperm quality parameters and PAH-adducts were associated with an increasing trend in DFI (P for trend <0.001). The authors do not examine the associated changes with smoking status

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