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Commentary

Critical review of Clara cell protein: sound science?

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Introduction

For years, clinicians, toxicologists and epidemiologists have relied on respiratory symptoms and spirometry to assess the pulmonary toxicity of drugs, air pollutants and other chemicals. Clearly, these tests are not sensitive enough to detect early effects on the respiratory epithelium, which is the primary target of most lung toxicants. Lung function tests are also notoriously unreliable when applied on small children, the sick or the elderly. A more sensitive assessment of airway damage or inflammation can be achieved by measuring markers in the epithelial fluid collected by lung lavage but this implies an invasive technique unsuitable for most human studies.

Research in the field of biomarkers initiated in the early 1990s has opened new perspectives with the development of non-invasive approaches based on biomarkers in serum or exhaled breath. One approach, referred to as pneumoproteinaemia, relies on the measurement in serum of proteins specifically secreted by the respiratory epithelium (Hermans & Bernard 1998, 1999). A variety of clinical and experimental studies have shown that lung-specific proteins or pneumoproteins can serve as peripheral markers of lung epithelium integrity, reflecting the number of cells lining the airways or the permeability of the alveolar–capillary barrier. One of the most studied biomarkers based on this concept is the 16-kDa Clara cell specific protein (CC16, CC10 or CCSP). This protein indeed presents two features that make it particularly sensitive to airways injury. The first feature is that CC16 is produced by the Clara cell, a non-ciliated cell known for its high vulnerability to inhaled or systemic lung toxicants. The second feature is that CC16 is a short-lived plasma protein that responds very quickly to permeability changes in the bronchoalveolar capillary barrier. The interest of this biomarker might, however, go beyond the mere detection of lung epithelial damage. Several studies have indeed shown that low levels of this protein in serum are associated with poorer respiratory health in children (Bernard et al. 2007,

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Yang et al. 2007) or are predictive of unfavourable outcomes in critically ill patients (Nord et al. 2002, Mattsson et al. 2005, Lesur et al. 2006).

While interest in non-invasive lung markers such as serum CC16 is increasing, LaKind et al. (2007) have recently published in *Biomarkers* a review concluding that this test has a limited utility. The objective of this commentary is to try to understand how LaKind and colleagues have reached this conclusion, which contrasts with that drawn by most researchers having used this test.

The specificity of Clara cell protein

Biomarkers are molecular tools that can be used to improve the assessment of exposure or of early effects of toxic chemicals. As for any tool, one cannot make a valid judgment of the utility of a biomarker without having a clear view of the purpose for which it was developed. LaKind et al. (2007) are giving to serum CC16 meanings that vary through their review and that do not really correspond to the meaning given by other researchers. I think this is certainly the most confusing aspect of this review. For instance, in the abstract, LaKind and colleagues present serum CC16 first as a 'candidate marker of asthma' and then a few lines later as an exposure marker by concluding that 'the lack of specificity of serum CC16 limits its use as a biomarker of specific exposure'. Further confusing the situation, the authors state at the end of their paper that CC16 is 'a protein of limited utility as a single biomarker of effect'. As all this would not be sufficient, the review was published under the section of disease markers. Evaluating the utility of a marker without clearly defining its purpose is of course totally misleading. Biomarkers must indeed meet different criteria according to whether or not they are intended to evaluate exposure, response or a disease stage. To be useful, an exposure marker must be specific for a given exposure whereas an effect or response marker must be specific for a given tissue or cell target.

Researchers who developed the test of serum CC16 made it clear from the very beginning by presenting serum CC16 as a potential marker of lung epithelium damage or dysfunction caused by toxic chemicals (see the first review about CC16 published in *Biomarkers* in 1996 by Hermans & Bernard). They never claimed that this biomarker could be used for asthma diagnosis or for exposure assessment. Concluding, as LaKind and colleagues do, that serum CC16 is not specific for a given exposure is thus a self-evident statement that does need to be substantiated by a long review. Actually, the lack of specificity of CC16 as exposure marker is not a limitation but on the contrary an advantage that makes it all the more interesting. This means indeed that serum CC16 can be used to detect effects on the lung epithelium whatever the nature of the causal agent and probably also whatever the underlying mechanism of toxicity. This also means that this biomarker has the potential to detect damage to the epithelium caused by new chemicals as well as integrate damage caused by complex mixtures of air pollutants.

Confounding

Given the multitude of factors that can affect any biological system, confounding is unavoidable when using biomarkers. Gender, age, body mass index (BMI), exercise, diurnal variation and renal function are potential confounders of most biomarkers that of course were also considered by researchers who used serum CC16. The conclusion

of LaKind and colleagues that factors influencing serum CC16 have not been sufficiently investigated is not an exact account of the state of the art. For instance, when LaKind and colleagues state that only one study has examined the influence of gender, they overlook at least six other studies which found no difference related to this factor (Bernard et al. 1993, 1994a, 2003, 2006, Robin et al. 2002, Steiner et al. 2005). Time of sampling is another potential confounder that has also been highlighted by LaKind and colleagues but again by considering as discordant results that were obtained under different experimental conditions. In the study by Helleday et al. (2006), the first blood sample was taken at 07:00 while in the study by Bernard et al. (1997) it was taken at 10:00, which explains why the diurnal decrease of serum was statistically significant only in the former study, which covered a longer period of time. Likewise, LaKind and colleagues have presented the study Nomori et al. (1996), reporting an association between serum CC16 and lipids as inconsistent with the other studies. They knew, however, (footnote in Table 1) that the association between CC16 and lipids in serum published by Nomori et al. (1996) was an artefact due to the interference of lipids with their turbidimetric assay.

Because of its small size, CC16 is rapidly cleared from plasma by glomerular filtration. As a corollary, the concentration of CC16 in plasma or serum unavoidably increases as the glomerular filtration rate (GFR) decreases. Although this is indeed an important limitation of serum CC16, in practice, confounding by renal function is rarely a problem. Because of the hyperbolic nature of the relationship linking serum CC16 to the GFR, a reduction of the renal function by more than 40% is necessary for serum CC16 to rise significantly. Such a fall in GFR is unlikely to be observed in most toxicological or epidemiological studies on subjects exposed to air pollutants. If a study involved subjects with an impaired renal function, it suffices to divide the concentrations of CC16 in serum by that of creatinine or cystatin C to adjust for changes in the GFR.

Actually, if we compare serum CC16 to other markers, the situation is not as bad as depicted by LaKind and colleagues. Some biomarkers that are routinely in clinical practice are more sensitive to confounding factors than serum CC16. For instance, the exhaled NO test used to assess inflammation in asthma is influenced by many more variables than serum CC16 (gender, BMI, exercise, atopy, smoking, diurnal variation, for example).

The necessary reference to the exposure conditions

The basic principle underlying any toxicological evaluation, including that based on biomarkers, is that the response must be related to the dose. Dose–response relationships observed with most toxicants are usually characterized by a threshold below which there is no detectable response and above which the response increases proportionally to the dose. This is an aspect that has been neglected by LaKind and colleagues who classified as inconsistent those results that in reality were obtained under different exposure conditions. For instance, in the case of ozone, there is nothing inconsistent in the fact that serum CC16 increased significantly in exercising adults exposed to high concentrations of ozone (Broeckaert et al. 1999, 2000, Bergamaschi et al. 2001, Blomberg et al. 2003) but not in non-exercising children (Bernard et al. 2005) exposed to lower ozone concentrations (Lagerkvist et al. 2004). The review also overlooked the experimental studies that have carefully validated

serum CC16 as a marker of ozone-induced epithelial damage (Arsalane et al. 1999, Broeckaert et al. 2003). These studies carried out on different species and strains have demonstrated that ozone produces a dose-dependent intravascular leakage of lung CC16 that closely correlated with the leakage of plasma albumin into the lung. Similarly, results obtained during exercise were qualified as inconsistent while they corresponded to different levels of exercise. There is indeed no possible comparison between the level of exercise of children playing outdoors (Lagerkvist et al. 2004, Bernard et al. 2005) and of adults biking or swimming intensively at their maximal or submaximal capacity (Nanson et al. 2001, Carbonnelle et al. 2002). This lack of consideration for the exposure–response relationships is particular obvious when LaKind and colleagues state that the concentration of CC16 was not related to smoking habits. The dose-dependent decline of serum CC16 with the cumulative exposure to tobacco smoke (number of pack-years) is one of most consistent and strongest associations that was found with this marker (Bernard et al. 1992, 1994a, 1994b, Robin et al. 2002, Berthoin et al. 2002, Steiner et al. 2005, Mutti et al. 2006).

Latest developments

By definition, research is a never ending process. A fair evaluation of the potential of a new test cannot be done by focusing on the pioneering works and ignoring the latest developments. In the early 1990s, immunoassays for serum CC16 were based on standards that could not be accurately calibrated because of the lack of purified protein. Some assays such as the turbidimetric method of Nomori et al. (1996) were sensitive to interferences by lipids. All these difficulties, inevitable when developing a new protein marker, belong to the past, now that enzyme-linked immunosorbent assay methods and standards are commercially available. The main difficulty that persists with serum CC16 is the dual meaning of this biomarker which increases when the bronchoalveolar–capillary barrier is disrupted and decreases when Clara cells are damaged. LaKind and colleagues should have mentioned that researchers have solved this problem by adjusting the serum concentration of CC16 for that of surfactant-associated proteins such a SP-D. The CC16/SP-D ratio in serum appears now as one of the most specific and sensitive tests to detect lung epithelium damage caused by toxicants such tobacco smoke (Robin et al. 2002) or chlorination products (Bernard et al. 2007).

Conclusions

In their critical review of serum CC16, LaKind and colleagues conclude that this test has a limited utility because it is not specific for any exposure, disease or aetiology and that factors influencing serum levels of CC16 have not been sufficiently investigated. When making this conclusion, however, LaKind and colleagues failed to note the important differences between biomarkers of exposure and biomarkers of response, and among the latter between disease biomarkers and early-effect biomarkers. Clearly, serum CC16 was developed and validated as a biomarker of early effects on the lung epithelium, meaning that by definition the specificity of serum CC16 should be towards the lung epithelium (i.e. the target) and not towards a given agent, disease or aetiology. LaKind and colleagues have also frequently qualified as inconsistent results that actually were not really comparable because of differences in exposure conditions

or in the experimental protocol. When LaKind and colleagues claim that factors influencing serum CC16 have not been sufficiently investigated, they overlook several epidemiological or clinical studies that have carefully taken into consideration classical confounders such as age, gender or BMI. They also did not mention that confounding by renal function or lung epithelium permeability can be avoided or at least minimized by adjusting serum CC16 levels for other serum biomarkers such as cystatin C or creatinine for variations in renal function or surfactant-associated proteins for changes in lung epithelium permeability.

The ideal biomarker of course does not exist and this is particularly true with a peripheral lung biomarker such as serum CC16. However, in no way do the limitations of serum CC16, shared by many effect markers, justify the dark picture painted by LaKind and colleagues. The message conveyed by the review of LaKind and colleagues is that the serum CC16 test is insufficiently validated to be used for risk assessment and that consequently its application does not correspond to the 'sound science' that the chemical industry is frequently calling for. I am not sure, however, that this review is the best example of sound science one can give.

Toxicologists and epidemiologists, however, are committed to use the best available methods to detect early effects caused by chemicals, especially when assessing risks for particularly vulnerable populations. In the absence of any alternative method for detecting lung epithelium damage non-invasively, developing and applying sensitive peripheral lung biomarkers such as serum CC16 is currently one of best ways forward to protect public health from potentially harmful pollutants such as chlorine and volatile chlorination by-products.

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