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SHORT COMMUNICATION

Serum Clara cell protein (CC16) in healthy young smokers

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The CC16 microprotein is the main secretory product of Clara cells, which are epithelial cells lining lung airways. In crossing through the bronchoalveolar/blood barrier, CC16 diffuses passively into plasma. Serum CC16 (sCC16) has recently been proposed as a biomarker for detecting Clara cell impairments. The aim of this study was to assess if sCC16 concentrations are reduced in a group of healthy young smokers. A group of 118 healthy young males volunteered to take part in the study. Each subject answered a questionnaire, and provided blood and urine samples. Serum CC16, urinary cotinine and creatinine were measured. Median serum CC16 concentrations were lower in smokers than in non-smokers (11.3 μg l^{-1} vs 14.6 μg l^{-1}; $p = 0.005$; $N = 89$ and 29, respectively) but did not correlate with either the daily or the life-time cigarette consumption, or with urinary cotinine concentrations. sCC16 did not correlate with age or body mass index in the whole study population or in the groups of smokers and non-smokers. These results suggest the reduction in sCC16 concentrations in a group of healthy young smokers may be an early effect of cigarette smoking.

Keywords: Clara cell protein (CC16-CC10-CCSP), lung biomarker, smoking.

Introduction

The CC16 microprotein is the main secretory product of human Clara cells, which are non-ciliated epithelial cells lining lung airways (Singh and Katyal 1984, Singh *et al.* 1985). The acronym CC16 is related to the molecular mass of the protein which, as determined by electrospray/mass spectrometry, is 15840 Da (Bernard *et al.* 1993). CC16 has also been designated by other terms, such as CC10 (on the basis of the less accurate molecular mass determination by SDS–PAGE) or the Clara cell secretory protein. CC16 is identical to human protein 1, a microprotein detected in urine from patients with tubular proteinuria (Bernard *et al.* 1992a, Itoh *et al.* 1993).

CC16 is secreted from Clara cells into the airways. In bronchoalveolar lavage fluids from healthy subjects, the CC16 concentration is on average 2 % of the total

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protein concentration. In crossing through the bronchoalveolar/blood barrier, CC16 diffuses passively into plasma, where concentrations are about 50 times lower than in bronchoalveolar lavage fluid (Bernard *et al.* 1992b). CC16 is then rapidly cleared from plasma by glomerular filtration, before being reabsorbed and catabolized into the renal tubules. Thus, several factors such as Clara cell number and functioning, the integrity of the bronchoalveolar/blood barrier and the glomerular filtration rate may cause serum CC16 (sCC16) concentrations to vary (Bernard *et al.* 1989, Hermans and Bernard 1996).

Serum CC16 may be useful for detecting impairments in Clara cells (Hermans and Bernard 1996, 1998, 1999), as few and/or poorly functioning Clara cells may reduce CC16 synthesis and secretion in the airways, thus lowering sCC16 concentrations (Hermans and Bernard 1996).

Histological and immunohistochemical studies have shown the number of Clara cells in lung epithelium is markedly reduced in smokers, and consequently CC16 synthesis is decreased (Lumsden *et al.* 1984, Shijubo *et al.* 1997). Furthermore, sCC16 concentrations are reported to be low in middle-aged smokers (Bernard *et al.* 1994, Shijubo *et al.* 1997, Hermans *et al.* 1998).

As little information is available on younger subjects with a low lifetime consumption of cigarettes, this study determined sCC16 concentrations in healthy young male smokers.

Materials and methods

A group of 119 young men doing their National Service in a small country town in Umbria, central Italy, volunteered to take part in the study. The town was free of sources of environmental or industrial pollution. At the National Service medical examination less than 12 months previously all men had undergone a clinical examination, routine blood and urine tests, and spirometry. All results were normal.

Each soldier answered a questionnaire on personal information, smoking habit, and civilian and military occupations. Height and weight were measured to calculate body mass index (weight/height^2 expressed as kg m^{-2}).

Each volunteer provided a blood sample for the determinations of sCC16 concentrations and a urine sample for cotinine and creatinine levels.

Study population

None of the subjects had been affected by respiratory infections in the month before enrolment in the study. No one had bronchial asthma or chronic pulmonary diseases.

Ninety of the 119 soldiers declared they were smokers. Data from one smoker were omitted from analysis because of blood sample haemolysis. All figures refer to 89 smokers out a population of 118. The median number of cigarettes daily was 20 (range 2–50) and the median of pack-years [(number of cigarettes daily/20) × years smoking] was 3.8 (0.1–12.4). The mean age of the 89 smokers was 21.1 (SD: 2.2; range 18.6–27.4) years and of the 39 non-smokers 23.4 (3.2; 8.9–29.0) years.

Mean body mass index in smokers was 24.7 (3.5; 18.7–34.3) kg m^{-2} and in non-smokers 24.5 (2.9; 19.4–34.8) kg m^{-2}.

Median urinary cotinine concentration was 572.5 (range 9.8–2350.0) ng mg^{-1} creatinine in smokers and 18.1 (8.9–124.0) ng mg^{-1} creatinine in non-smokers.

Analytical methods

All samples were collected in the afternoon (2–4 p.m.) and frozen at –80°C except for the aliquot for urinary creatinine, which was stored at 4 °C and analysed the following morning.

Serum and urine samples were shipped to the Unité de Toxicologie Industrielle, Université Catholique de Louvain, Brussels, in dry ice for sCC16 and cotinine determination, as described in detail elsewhere (Haufroid and Lison 1998, Hermans *et al.* 1998).

Briefly, sCC16 was measured by an immunoassay based upon agglutination of latex particles coated with polyclonal anti-CC16 antibodies and quantification of residual unagglutinated particles with a Technicon Autocounter. Serum samples were decomplemented and delipidated by heating and by adding polyethylene glycol and trichloroacetic acid before analysis, because chylomicrons may be

counted as latex particles in the assay (Bernard and Lauwerys 1983, Hermans *et al*. 1998). Cotinine was measured by capillary column gas–liquid chromatography coupled with nitrogen-phosphorous detector after extracting alkalinized urine in chloroform (Feyerabend and Russell 1990).

For the statistical analysis, the value of 25 ng ml^{-1} was attributed to urinary cotinine concentrations below the detection limit (50 ng ml^{-1}). Cotinine concentrations were then corrected for creatinine content.

Statistical analysis

We used non-parametric methods as most variables were not normally distributed. The Mann–Whitney *U* test was used for comparisons between two groups and the Spearman correlation coefficient to correlate sCC16 with other variables. SPSS software was used for the statistical analysis.

Results

Serum CC16 concentrations were significantly lower in smokers than in non-smokers, median values being 11.3 (range 2.3–32.9) μg l^{-1} and 14.6 (7.5–31) μg l^{-1}, respectively ($p = 0.005$).

Median sCC16 values were 12.2 μg l^{-1} in moderate smokers (i.e. subjects smoking 1–19 cigarettes day^{-1}) and 10.1 μg l^{-1} in heavy smokers (i.e. subjects smoking 20 or more cigarettes day^{-1}) (figure 1). In subjects who had smoked less than 3.8 pack-years, median sCC16 levels were 11.4 μg l^{-1} which fell to 11.0 μg l^{-1} when pack-years were more than 3.8 (p = n.s.)

In smokers, sCC16 concentrations did not correlate with either the daily or life-time cigarette consumption ($r_s = -0.04$, $p = 0{,}70$; and $r_s = 0.06$, $p = 0.60$, respectively).

When urinary cotinine was assumed as an estimate of current smoking, sCC16 did not correlate with urinary cotinine concentrations either in the total population

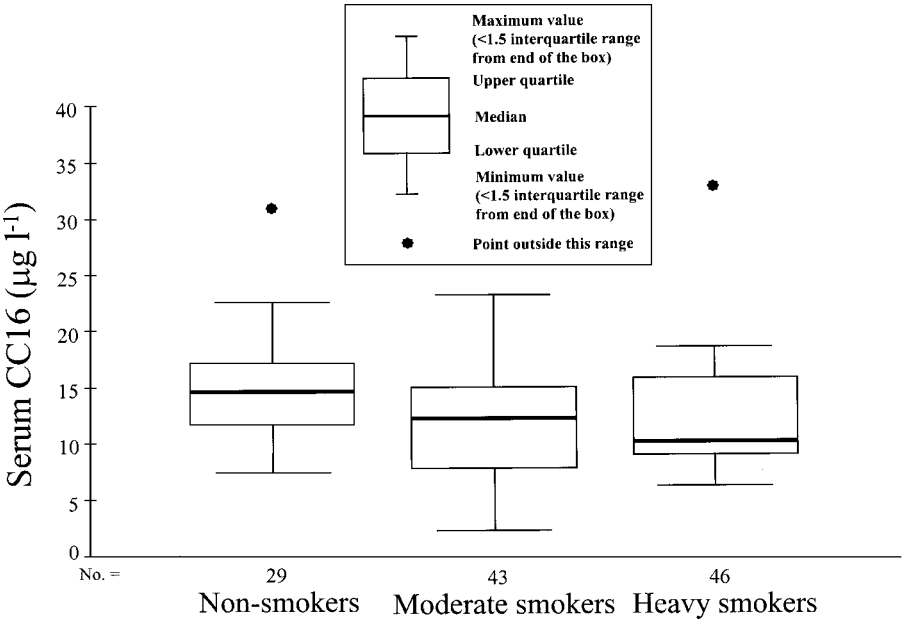

Figure 1. Box and whisker plot of serum CC16 concentrations in male non-smokers, moderate smokers (1–19 cigarettes daily) and heavy smokers (20 or more cigarettes daily) all under 29 years of age who were not affected by any disease *(*moderate smokers *vs* heavy smokers: $p = 0.65$*)*. The number of subjects is shown on the *x*-axis.

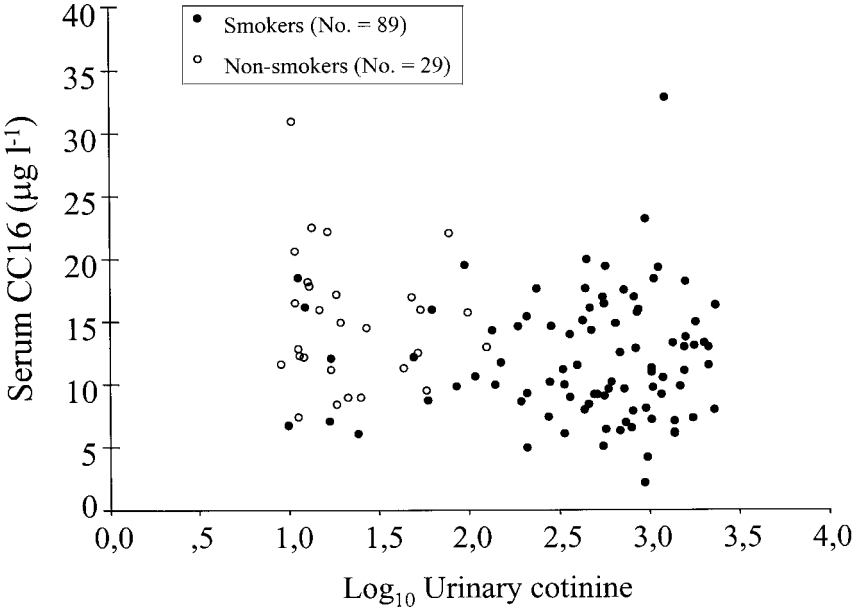

Figure 2. Scatterplot of urinary cotinine and serum CC16 concentrations in healthy male smokers and non-smokers under 29 years of age. No correlation emerged in the whole population (r_s=–0.16; P = 0.08) or in the sub-group of smokers (r_s = 0.02; P = 0.84).

or in the group of smokers (r_s = –0.16, p = 0.08; and, r_s = 0.02, p = 0.84, respectively) (figure 2).

Serum CC16 concentrations did not correlate with age or body mass index in the whole study population or in the groups of smokers and non-smokers.

Discussion

We found significantly reduced sCC16 concentrations in these healthy young male smokers who were not occupationally or environmentally exposed to lung toxicants and who were not affected by lung and kidney diseases which may modify sCC16 concentrations (Hermans and Bernard 1996, Bernard and Hermans 1997, Bernard *et al.* 1998). We believe smoking habit misclassification bias was avoided in our study, as we used both self-reported cigarette consumption and a well-validated biomarker, i.e. urinary cotinine, to assess the extent of the smoking habit. Even though self-reports of smoking are considered reasonably accurate, measurements of urinary cotinine may detect smoking in subjects who deny it (Patrick *et al.* 1994, Haufroid and Lison 1998). In our subjects who denied smoking, concentrations of urinary cotinine did not exceed 150 ng mg^{-1} creatinine, the proposed cut-off between active smokers and non-smokers who are exposed to environmental tobacco smoke (Riboli *et al.* 1995).

Our finding of reduced sCC16 concentrations in smokers diverges from Nomori *et al.* (1996), who reported slightly increased sCC16 concentrations in 35 smokers compared with 61 non-smokers. We suspect the discrepancy is due to serum lipid interference in the sCC16 determination when the sCC16 analysis is performed by nephelometric latex immunoassay, as in the study by Nomori *et al.*

(Hermans *et al.* 1998). Concurring with us, sCC16 levels were significantly reduced in other studies on different groups of smokers with a markedly higher life-time cigarette consumption than our volunteers (Bernard *et al.* 1994, Shijubo *et al.* 1997, Hermans *et al.* 1998). In fact, the geometric mean was 17.9 (range: 0.8–126) pack-years in a group of male Belgian smokers (Bernard *et al.* 1994), 13.7 (2–42) pack-years in a group of Belgian blood-donors (Hermans *et al.* 1998), and the arithmetic mean 28.4 (SD: 16.7) pack-years in a group of Japanese smokers (Shijubo *et al.* 1997), as compared with a median life-time cigarette consumption of only 3.8 (range: 0.1–12.4) pack-years in our group. Thus, our results suggest the drop in sCC16 is an early effect of smoking. This hypothesis is supported by the fact that, in our study, the median sCC16 concentration was only about 24 % lower in young smokers than in non-smokers, whereas average values were about 30–35 % lower in groups of older smokers with higher cumulative cigarette consumption than in age-matched non-smoking referents (Bernard *et al.* 1994, Shijubo *et al.* 1997, Hermans *et al.* 1998).

In smokers, we did not find a dose–response relationship between sCC16 concentrations and current or cumulative degree of smoking. Our findings do not concur with those of Bernard *et al.*, showing a negative association between sCC16 concentrations (age-adjusted values) and several indices of cigarette consumption, in particular life-time cigarette smoking (Bernard *et al.* 1994). Lifetime-cigarette consumption was on average four-fold, and up to ten-fold, higher in the Belgian male population than in ours. So we may presume dose-related toxic effects are detectable only when cumulative consumption is high.

Reduced sCC16 concentrations in smokers appear to be related to decreased CC16 production in the lung. In fact, a recent study showed that the proportion of CC16-positive cells in lung biopsy specimens was lower in healthy smokers (i.e. without ventilatory lung impairment) compared with non-smokers, and was associated with decreased CC16 levels in bronchoalveolar lavage fluid and serum (Shijubo *et al.* 1997).

Individual factors which modulate Clara cell sensibility to tobacco smoke remain unknown. Many potential lung toxicants have been detected in tobacco smoke, and several need to be activated by cytochrome P_{450}-dependent enzymes before exerting their effects (Stripp *et al.* 1995, Van Winkle *et al.* 1995). With their cytochrome P_{450} activity, Clara cells may activate these pneumotoxicants (Boyd 1977, Massaro *et al.* 1994, Plopper *et al.* 1997), and inter-individual variations in Clara cells cytochrome P_{450} activity might be related to inter-individual Clara cell sensibility to tobacco smoke.

The roles of Clara cells and CC16 in physiological and pathological lung processes, and the health consequences of their reduction are still unclear. There is evidence to suggest Clara cells play a role in repairing damaged bronchial epithelium (Singh and Katyal 1997). Furthermore CC16 appears to modulate inflammatory processes in the lung by feeding back with β-interferon (Miele *et al.* 1987, Dierynck *et al.* 1995, Magdaleno *et al.* 1997, Yao *et al.* 1998a, b). Low sCC16 levels are indicative of fewer Clara cells and reduced CC16 pulmonary production, and subjects with them might well be at higher risk of epithelial damage and inflammatory processes in the lung.

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References

BERNARD, A. and HERMANS, C. 1997, Biomonitoring of early effects on the kidney or the lung. *Science of the Total Environment*, **199**, 205–211.

BERNARD, A., ROELS, H., LAUWERYS, R., WITTERS, R., GIELENS, C., SOUMILLION, A., VAN DAMME, J. and DE LEY, M. 1992a, Protein 1 is a secretory protein of the respiratory and urogenital tracts identical to the Clara cell protein. *Clinical Chemistry*, **38**, 434–435.

BERNARD, A., MARCHANDISE, F. X., DEPELCHIN, S., LAUWERYS, R. and SIBILLE, Y. 1992b, Clara cell protein in serum and bronchoalveolar lavage. *European Respiratory Journal*, **5**, 1231–1238.

BERNARD, A., DUMONT, X., ROELS, H., LAUWERYS, R., DIERYNCK, I., DE LEY, M., STROOBANT, V. and DE HOFFMANN, E. 1993, The molecular mass and concentrations of protein 1 or Clara cell protein in biological fluids: a reappraisal [letter]. *Clinica Chimica Acta*, **223**, 189–191.

BERNARD, A., BROECKAERT, F., HERMANS, C. and KNOOPS, B. 1998, The Clara cell protein, CC16: a biomarker of pulmonary toxicity. In *Biomarkers. Medical and Workplace Applications,* M.L. Mendelsohn, L.C. Mohr and J.P. Peeters, eds (Washington, DC: Joseph Henry Press), pp. 273–283.

BERNARD, A. M. and LAUWERYS, R. R. 1983, Continuous-flow system for automation of latex immunoassay by particle counting. *Clinical Chemistry*, **29**, 1007–1011.

BERNARD, A. M., LAUWERYS, R. R., NOEL, A., VANDELEENE, B. and LAMBERT, A. 1989, Urine protein 1: a sex-dependent marker of tubular or glomerular dysfunction. *Clinical Chemistry*, **35**, 2141–2142.

BERNARD, A. M., ROELS, H. A., BUCHET, J. P. and LAUWERYS, R. R. 1994, Serum Clara cell protein: an indicator of bronchial cell dysfunction caused by tobacco smoking. *Environmental Research*, **66**, 96–104.

BOYD, M. R. 1977, Evidence for the Clara cell as a site of cytochrome P450-dependent mixed-function oxidase activity in lung. *Nature*, **269**, 713–715.

DIERYNCK, I., BERNARD, A., ROELS, H. and DE LEY, M. 1995, Potent inhibition of both human interferon-gamma production and biologic activity by the Clara cell protein CC16. *American Journal of Respiratory Cell and Molecular Biology*, **12**, 205–210.

FEYARABEND, C. and RUSSELL, M. A. H. 1990, A rapid gas–liquid chromatographic method for the determination of cotinine and nicotine in biological fluids. *Journal of Pharmacy and Pharmacology*, **42**, 450–452.

HAUFROID, V. and LISON, D. 1998, Urinary cotinine as a tobacco-smoke exposure index: a minireview. *International Archives of Occupational and Environmental Health*, **71**, 162–168.

HERMANS, C. and BERNARD, A. 1996, The Clara cell protein (CC16): characteristics and potential appications as biomarker of lung toxicity. *Biomarkers*, **1**, 3–8.

HERMANS, C. and BERNARD, A. 1998, Pneumoproteinaemia: a new perpsective in the assessment of lung disorders. *European Respiratory Journal*, **11**, 801–803.

HERMANS, C. and BERNARD, A. 1999, Lung epithelium-specific proteins. Characteristics and potential applications as markers. State-of-the-Art. *American Journal of Respiratory and Critical Care Medicine*, **159**, 646–678.

HERMANS, C., OSMAN ALY, NYBERG, B. I., PETERSON, C. and BERNARD, A. 1998, Determinants of Clara cell protein (CC16) concentration in serum: a reassessment with two different immunoassays. *Clinica Chimica Acta*, **272**, 101–110.

ITOH, Y., ISHII, S., OKUTANI, R., ASANO, Y. and KAWAI, T. 1993, Protein 1: its purification and application in clinical medicine. *Journal of Clinical Laboratory Analysis*, **7**, 394–400.

LUMSDEN, A. B., MCLEAN, A. and LAMB, D. 1984, Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax*, **39**, 844–849.

MAGDALENO, S. M., WANG, G., JACKSON, K. J., RAY, M. K., WELTY, S., COSTA, R. H. and DEMAYO, F. J. 1997, Interferon-gamma regulation of Clara cell gene expression: *in vivo* and *in vitro*. *American Journal of Physiology*, **272**, L1142–L1151.

MASSARO, G. D., SINGH, G., MASON, R., PLOPPER, C. G., MALKINSON, A. M. and GAIL, D. B. 1994, Biology of the Clara cell. *American Journal of Physiology*, **266**, L101–L106.

MIELE, L., CORDELLA MIELE, E. and MUKHERJEE, A. B. 1987, Uteroglobin: structure, molecular biology, and new perspectives on its function as a phospholipase A2 inhibitor. *Endocrine Reviews*, **8**, 474–490.

NOMORI, H., HORIO, H., TAKAGI, M., KOBAYASHI, R. and HIRABAYASHI, Y. 1996, Clara cell protein correlation with hyperlipidemia [see comments]. *Chest*, **110**, 680–684.

PATRICK, D. L., CHEADLE, A., THOMPSON, D. C., DIEHR, P., KOEPSELL, T. and KINNE, S. 1994, The validity of self-reported smoking: a review and meta-analysis. *American Journal of Public Health*, **84**, 1086–1093.

PLOPPER, C. G., HYDE, D. M. and BUCKPITT, A. R. 1997, Clara cells. In *The Lung Scientific Foundation,* R. G. Crystal, J. B. West, E. R. Weibel and P. J. Barnes, eds (New-York: Lippincott-Raven), pp. 517–533.

RIBOLI, E., HALEY, N.J., DE WAARD, F. and SARACCI, R. 1995, Validity of urinary biomarkers of exposure to tobacco smoke following prolonged storage. *International Journal of Epidemiology*, **24**, 354–358.

SHIJUBO, N., ITOH, Y., YAMAGUCHI, T., SHIBUYA, Y., MORITA, Y., HIRASAWA, M., OKUTANI, R., KAWAI, T. and ABE, S. 1997, Serum and BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers. *European Respiratory Journal*, **10**, 1108–1114.

SINGH, G. and KATYAL, S. L. 1984, An immunologic study of the secretory products of rat Clara cells. *Journal of Histochemistry and Cytochemistry*, **32**, 49–54.

SINGH, G. and KATYAL, S. L. 1997, Clara cells and Clara cell 10 kD protein (CC10). *American Journal of Respiratory Cell and Molecular Biology*, **17**, 141–143.

SINGH, G., KATYAL, S. L., WARD, J. M., GOTTRON, S. A., WONG CHONG, M. L. and RILEY, E. J. 1985, Secretory proteins of the lung in rodents: immunocytochemistry. *Journal of Histochemistry and Cytochemistry*, **33**, 564–568.

STRIPP, B. R., MAXSON, K., MERA, R. and SINGH, G. 1995, Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. *American Journal of Physiology*, **269**, L791–L799.

VAN WINKLE, L. S., BUCKPITT, A. R., NISHIO, S. J., ISAAC, J. M. and PLOPPER, C. G. 1995, Cellular response in naphthalene-induced Clara cell injury and bronchiolar epithelial repair in mice. *American Journal of Physiology*, **269**, L800–L818.

YAO, X. L., IKEZONO, T., COWAN, M., LOGUN, C., ANGUS, C. W. and SHELHAMER, J. H. 1998a, Interferon-gamma stimulates human Clara cell secretory protein production by human airway epithelial cells. *American Journal of Physiology,* **274**, L864–L869.

YAO, X. L., LEVINE, S. J., COWAN, M. J., LOGUN, C. and SHELHAMER, J. H. 1998b, Tumor necrosis factor-alpha stimulates human Clara cell secretory protein production by human airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology*, **19**, 629–635.