

RESEARCH ARTICLE

Arginines Plasma Concentration and Oxidative Stress in Mild to Moderate COPD

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Abstract

Background

Elevated plasma concentrations of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) have been observed in respiratory conditions such as asthma and cystic fibrosis. Since oxidative stress has been shown to increase the activity of arginine methylating enzymes, hence increased ADMA synthesis, and to reduce ADMA degrading enzymes, hence increased ADMA concentrations, we assessed methylated arginines concentrations in chronic obstructive pulmonary disease (COPD), a disease characterized by increased oxidative stress.

Methods

Plasma arginine, ADMA and symmetric dimethylarginine (SDMA), oxidative stress markers (thiobarbituric acid reactive substances, TBARS, and plasma proteins SH, PSH) and antioxidants (taurine and paraoxonase 1, PON1, activity) were measured in 43 COPD patients with mild (n = 29) or moderate (n = 14) disease and 43 age- and sex-matched controls.

Results

TBARS significantly increased with COPD presence and severity (median 2.93 vs 3.18 vs 3.64 $\mu\text{mol/L}$, respectively in controls, mild and moderate group, $p < 0.0001$ by ANOVA) whereas PSH decreased (6.69 \pm 1.15 vs 6.04 \pm 0.85 vs 5.33 \pm 0.96 $\mu\text{mol/gr prot}$, $p < 0.0001$ by ANOVA). Increased ADMA/arginine ratio, primarily due to reduced arginine concentrations, was also observed with COPD presence and severity (median 0.0067 vs 0.0075 vs 0.0100, $p < 0.0001$ by ANOVA). In multiple logistic regression analysis, only TBARS (OR 0.44, 95% CI 0.25–0.77; $p = 0.0045$) and ADMA/Arginine ratio (OR 1.72, 95% CI 2.27–13.05; $p = 0.02$) were independently associated with COPD severity.

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Abbreviations: ADMA, asymmetric dimethylarginine; COPD, chronic obstructive pulmonary disease; NOS, nitric oxide synthase; OS, oxidative stress; PON1, paraoxonase 1, PSH, Proteins–SH; SDMA, symmetric dimethylarginine; TBARS, thiobarbituric acid reactive substances.

Conclusion

COPD presence and severity are associated with increased oxidative stress and alterations in arginine metabolism. The reduced arginine concentrations in COPD may offer a new target for therapeutic interventions increasing arginine availability.

Introduction

Chronic Obstructive Pulmonary Disease is a common respiratory condition characterized by progressive airflow limitation, persistent productive cough, mucous plugging and dyspnea [1–2]. Elevated concentrations of oxidative stress (OS) markers are commonly observed in this group [3–5]. The reduced ability of cellular antioxidant defenses to fully inactivate the reactive oxygen species (ROS) is a hallmark of OS. As a result, there is a functional impairment of several important biomolecules as lipids, proteins or nucleic acids, which can compromise cell health and viability. OS induces a variety of cellular responses through generation of secondary reactive species, leading to cell death by necrosis or apoptosis and, consequently, disease onset and progression. Moreover, activity of several enzymes can be influenced by redox regulation [6], including enzymes involved in the formation and degradation of asymmetric dimethylarginine (ADMA), such as protein arginine N-methyltransferases (PRMTs) and dimethylarginine dimethylaminohydrolase (DDAH) [7–8]. ADMA is an effective endogenous inhibitor of nitric oxide synthase (NOS). Its accumulation has been reported in renal failure [9–10], cardiovascular disease [11–12] and, only recently, lung disease [13–14]. ADMA synthesis is catalysed by PRMTs through the addition of one or two methyl groups to the terminal nitrogen atom of protein arginine. Human PRMTs are classified on the basis of their specific catalytic activities in type I and type II. In the first step, both enzymes catalyse monomethylarginine formation. During the second step type I enzymes produce ADMA, whereas type II enzymes lead to the formation of SDMA [15]. During proteolysis, ADMA and SDMA are released into the cytosol where free ADMA, but not free SDMA, is further degraded to citrulline and dimethylamine by DDAH15. Studies have shown that PRMT1 RNA or protein expression is increased, and DDAH activity is decreased, under OS stimuli [16–18]. While OS is well characterized, little information is available on methylated arginine concentrations in COPD. Available data principally focus on arginine and methylated arginines in sputum or exhaled breath condensate [19–22], while only one report describes plasma concentrations in COPD subjects [22]. Therefore, we tested the hypothesis that a) methylated arginines are associated with COPD presence and severity and b) such alterations are associated with OS markers (thiobarbituric acid reactive substances and Proteins–SH) and antioxidants (taurine and paraoxonase 1 activity).

Methods

Subjects

Forty-three consecutive COPD patients (29 mild and 14 moderate) without a previous diagnosis of COPD, were enrolled from the Respiratory Unit of the University of Sassari. Each patient underwent physical examination, chest radiographs, routine blood tests and respiratory function tests. The latter included forced expiratory volume in 1 sec (FEV1), forced vital capacity, and FEV₁/FVC ratio. A structured questionnaire was administered to obtain demographic and clinical information including age, sex, body mass index (BMI) and smoking status. No patient was treated with long-acting muscarinic antagonists, long or short acting β -agonists at the time

of the assessments. Moreover, no patient received inhaled corticosteroids within four weeks prior to the study. COPD patients with significant symptom deterioration within the last three months, indicative of disease exacerbation, were excluded.

COPD diagnosis and severity were assessed according to physical examination, spirometric results,

smoking history and respiratory symptoms based on the Global Initiative for Chronic Obstructive Lung Disease criteria [23]. In particular, classification of COPD severity was based on spirometric values reported in Table 1.

A group of 43 age and sex-matched healthy controls was also included in the study. Exclusion criteria included the presence of concomitant inflammatory disease such as autoimmune disorders and infections, liver, kidney, heart disease and cancer.

This study was approved by the Institutional Local Ethics Committee (Azienda Sanitaria Locale n°1 di Sassari (Italy) (prot. 2175/CE del 21/04/2015), and was in accordance with the principles of Declaration of Helsinki. All subjects provided written informed consent.

Biochemical analysis

Arginine, ADMA, SDMA and taurine were determined by capillary electrophoresis UV detection as previously described [24–25]. As inadequate precision of the assay used for the analysis of ADMA may increase the chance of statistical type 2 errors in clinical studies and may also lead to a severe underestimation of the strength of the association between ADMA and other biochemical or clinical variables [26], we used a capillary electrophoresis method that give inter-assay CV between 2 and 3% for Arginine, ADMA and SDMA measurement.

TBARS were determined according to the method described by Esterbauer and Cheeseman [27]. TBARS methodology measures MDA and other aldehydes produced by lipid peroxidation induced by hydroxyl free radicals. Plasma was mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid and heated at 95°C in a thermoblock heater for 25 min. TBARS were determined by measuring the absorbance at 535 nm. A calibration curve was obtained using standard MDA and each curve point was subjected to the same treatment as that of the samples. Paraoxonase activity was determined by measuring the increase in absorbance at 412 nm (formation of 4-nitrophenol) using paraoxon (O,O diethyl-O-p-nitrophenyl phosphate) as a substrate [28]. Enzyme activity was calculated by using the molar extinction coefficient of 17,100 M⁻¹cm⁻¹ and one unit (U) of paraoxonase activity was defined as 1 nmol of 4-nitrophenol formed per minute. Plasma PSH determination was performed by spectrophotometry with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as titrating agent by measuring the absorbance of conjugate at 405 nm [29]. Concentration in samples was determined from a GSH standard curve.

Statistical analysis

All results are expressed as mean values (mean ± SD) or median values (median and range). Variables distribution was assessed by the Kolmogorov-Smirnov test. Statistical differences

Table 1. Classification of COPD severity on the basis of spirometry values.

Severity of obstruction	Post bronchodilator FEV ₁ /FVC	FEV ₁ % PRED
MILD COPD	< 0.7	> 80%
MODERATE COPD	< 0.7	50–80%
SEVERE COPD	< 0.7	30–50%
VERY SEVERE	< 0.7	< 30%

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between groups were compared using unpaired Student’s t-test or Mann-Whitney rank sum test, as appropriate. Correlation analysis between variables was performed by Pearson’s correlation or Spearman’s correlation as appropriate. Multiple comparisons were performed by one-way ANOVA. Levene’s test for equality of error variances was employed, while student-Newman-Keuls test for all pairwise comparisons was used. Non-normally distributed variables were log10-transformed prior to being used with parametric tests. Normal distribution of the residuals was checked to assess the goodness of fit of the transformations.

Logistic regression analysis with COPD absence vs. presence as dependent variable was conducted to determine associations between variables potentially involved in disease development. A further logistic regression analysis with mild or moderate condition as dependent variable was conducted to determine associations between COPD severity and variables potentially involved in disease progression.

Statistical analyses were performed using MedCalc for Windows, version 15.4 64 bit (MedCalc Software, Ostend, Belgium) and SPSS for Windows, version 14.0 32 bit (IBM Corporation; Armonk, NY, USA).

Results

Table 2 describes the clinical and demographic characteristics of COPD patients and age- and sex-matched controls. FEV₁ decreased significantly from 2.75±0.59 L in controls to 2.24±0.56 L in mild and 1.56±0.32 L in moderate COPD patients (p<0.001), whereas FVC was 3.40±0.73 L, 3.18±0.77 L and 2.44±0.54 L, respectively (p<0.001). FEV₁/FVC ratio was 80.4±4.9% in controls, 70.2±3.12% in mild, and 64.8±7.9% in moderate COPD patients, respectively (p<0.001).

Table 2. Clinical, functional and biochemical parameters of healthy subjects and COPD patients.

Characteristics	Controls (n = 43)	Mild COPD (n = 29)	Moderate COPD (n = 14)	p value
Age (years)	73.4±6.9	75.4±4.8	73.4±7.7	NS
Sex (F/M)	9/34	7/22	2/12	NS
BMI (kg/m ²)	26.4±3.6	27.4±3.4	27.4±4.5	NS
Current smokers	3 (7%)	2 (6.9%)	1 (7.1%)	NS
Never smoked	14 (32.6%)	8 (27.6%)	2 (14.2%)	NS
Ex smokers	26 (60.4%)	19 (65.5%)	11 (78.6%)	NS
FEV ₁ (L)	2.75±0.59	2.24±0.56***	1.56 ± 0.32***°°°	<0.001
FVC (L)	3.40±0.73	3.18±0.77	2.44±0.54***°°	<0.001
FEV ₁ /FVC	80.8±4.9	70.2±3.1***	64.8±7.9***°°°	<0.001
TBARS (µmol/L)	2.93 (2.46–3.23)	3.18 (2.50–3.54)	3.64 (3.16–4.38)**°	0.003
PSH (µmol/ g prot)	6.69±1.15	6.04±0.85	5.33±0.96***°	<0.001
PON1(U/L)	253 (147–340)	230 (154–376)	211(157–284)	NS
Taurine (µmol/L)	55.8 (47.7–72.1)	59.3 (49.0–76.8)	57.6 (50.8–75.3)	NS
Arginine (µmol/L)	79.8 (68.3–90.4)	70.4 (60.3–78.2)*	53.4 (41.4–59.8)***°°	<0.001
ADMA (µmol/L)	0.488 (0.454–0.544)	0.505 (0.432–0.588)	0.513 (0.412–0.625)	NS
SDMA (µmol/L)	0.460 (0.395–0.590)	0.513 (0.429–0.594)	0.485 (0.456–0.577)	NS
ADMA/arginine	0.0067 (0.0056–0.0077)	0.0075 (0.0053–0.0098)	0.0100 (0.0079–0.0117)***°°°	<0.001
ADMA/SDMA	1.07 (0.80–1.28)	0.98 (0.81–1.31)	1.12 (0.86–1.25)	NS

*P<0.05

**p<0.01

***p<0.001 vs Controls;

°P<0.05

°°p<0.01

°°°p<0.001 vs mild COPD obtained by ANOVA (Student-Newman-Keuls test for all pairwise comparisons or Krustall-Wallis test as appropriate)

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Table 3. Linear regression analysis between FEV1 and some demographic and biochemical variables in controls and COPD patients.

	Controls (n = 43)		COPD (n = 43)	
	r or rho	p-value	r or rho	p-value
Age	-0.34	0.036	-0.31	0.043
Sex	-0.55	<0.0001	–	–
PSH	–	–	0.36	0.016
ADMA/Arginine	–	–	-0.43	<0.0001

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TBARS plasma concentrations increased significantly with COPD presence and severity ($p < 0.001$ by ANOVA). In particular we found a significant difference between controls and patients with moderate COPD (median 2.93 vs 3.64 $\mu\text{mol/L}$, $p < 0.01$) and between mild and moderate COPD patients (median 3.18 vs 3.64 $\mu\text{mol/L}$, $p < 0.05$). By contrast, a significant decrease in plasma PSH concentrations was observed with COPD presence and severity ($p < 0.001$). Multiple comparisons by ANOVA showed significant differences in PSH mean values between controls and moderate COPD ($6.69 \pm 1.15 \mu\text{mol/g prot}$ vs $5.33 \pm 0.96 \mu\text{mol/g prot}$, $p < 0.001$) and between mild and moderate COPD (6.04 ± 0.85 vs $5.33 \pm 0.96 \mu\text{mol/g prot}$, $p < 0.001$). ADMA and SDMA plasma concentrations were not significantly different between controls and COPD patients. By contrast, median arginine concentrations were progressively lower in controls (79.8 $\mu\text{mol/L}$), mild (70.4 $\mu\text{mol/L}$) and moderate COPD patients (53.4 $\mu\text{mol/L}$, $p < 0.001$). As consequence also ADMA/arginine showed significant differences according to COPD presence and severity ($p = 0.0001$). Multiple comparisons demonstrated a significant difference between controls and patients with moderate COPD (median 0.0067 vs 0.0100, $p < 0.001$) and between mild and moderate group (median 0.0075 vs 0.0100, $p < 0.05$).

As reported in Table 3, univariate analysis in COPD patients showed that FEV1 was correlated with age ($\rho = -0.31$, $p = 0.043$), PSH ($\rho = 0.36$, $p = 0.016$) and ADMA/arginine ratio ($\rho = -0.43$, $p = 0.0001$). In controls FEV1 was correlated only with age ($\rho = -0.34$; $p = 0.036$) and sex ($\rho = -0.55$, $p < 0.0001$). Table 4 report as, after adjusting for age, sex, BMI, smoking status, TBARS, PSH and ADMA/arginine ratio, sex ($\beta = -0.44$, $p = 0.007$), PSH ($\beta = 0.33$, $p = 0.047$), and ADMA/arginine ratio ($\beta = -0.45$, $p = 0.005$) were independently associated with FEV1 in COPD patients in regression analysis. In controls, only age ($\beta = -0.38$, $p = 0.009$) and sex ($\beta = -0.68$, $p < 0.0001$) were independently associated with FEV1.

When considering controls and COPD patients together, a negative relationship between PSH and ADMA/arginine ratio was also observed ($\rho = -0.23$, $p = 0.033$). In multiple logistic regression analysis of the total population (COPD and controls), after adjusting for age, sex, BMI, smoking status, ADMA/arginine ratio, TBARS, PSH, PON and taurine, only PSH (OR 0.44, 95% CI 0.25–0.77; $p = 0.004$) and ADMA/Arginine ratio (OR 172, 95% CI 2.27–13,055; $p = 0.02$) were independently associated with presence of COPD.

Table 4. Multiple regression analysis with FEV1 as dependent variable and age, sex, BMI, smoking status, TBARS, PSH and ADMA/arginine ratio as independent variables, in controls and COPD patients.

	Controls (n = 43)		COPD (n = 43)	
	β	p-value	β	p-value
Age	-0.38	0.009	–	–
Sex	-0.68	<0.0001	-0.44	0.007
PSH	–	–	0.33	0.047
ADMA/Arginine	–	–	-0.45	0.005

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Table 5. Logistic regression analysis (including TBARS, PSH and ADMA/arginine ratio) showing ORs for moderate disease.

Factor	Moderate disease		p-value
	OR	95%CI	
TBARS	481 x10 ¹²	26–9x10 ²⁷	0.030
PSH	0.0125	0.0003–0.4731	0.018
ADMA/arginine	49x10 ⁶	25–96x10 ¹²	0.016

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Data of multiple logistic regression analysis performed on 43 COPD patients according to disease severity (mild vs. moderate), after adjusting for age, sex, BMI, smoking status, ADMA/arginine ratio, TBARS, PSH, PON and taurine are reported in Table 5. Lower plasma PSH and higher TBARS and ADMA/arginine ratio were independently associated with disease severity.

Discussion

Chronic obstructive pulmonary disease is a major and increasing global health problem. According to the World Health Organization COPD will become the third leading cause of death and the fifth leading cause of disability in the world by 2020 [30]. Despite increasing awareness, the pathogenesis of COPD has received relatively little attention from clinicians, researchers, and the pharmaceutical industry [31]. This is likely due because COPD is viewed as self-inflicted (by smoking) and also because the underlying disease process is generally considered to be irreversible. Consequently, there is a fundamental lack of knowledge about the cellular, molecular, and genetic mechanisms of this pathology. COPD is associated with a chronic inflammatory response, predominantly in small airways and lung parenchyma, which is characterized by an increase of activated neutrophils and macrophages and increased numbers of inflammatory mediators in the airways [32–33]. It has been proposed that in COPD the increased oxidant burden may not be adequately counterbalanced by the lung antioxidant systems, resulting in OS. Increased OS may be induced both directly, as a result of smoking, or indirectly by the increased release of reactive oxygen species from airspace inflammatory cells stimulated by noxious particles and gases. Analysis of cell profile in alveoli and small airways of COPD patients shows, in fact, an increase in several inflammatory cell types, including macrophages, T lymphocytes, B lymphocytes, and neutrophils [34]. These cells, once activated, can generate anion superoxide (O₂⁻) probably through reduced nicotinamide adenine dinucleotide phosphate oxidase pathway. In addition, the impaired ventilation may result in a decreased hemoglobin oxygen saturation level (hypoxemia), resulting in local tissue hypoxia [35]. Experimental evidence suggests that hypoxaemia enhances OS in COPD [36] and that the source of ROS production in hypoxia is likely to be the mitochondria at the respiratory chain level [37]. In support of these observations we found increased concentrations of TBARS (+13.2%) and reduced concentrations of PSH (-13.3%) in COPD subjects vs. controls, indicating the presence of significant OS. PSH assay, in fact, provide a measure of total protein sulfhydryl groups in plasma. The most representative -SH group in plasma is that of human serum albumin, due to its high concentrations. The Cys³⁴ -SH group of HSA represents ~80% of all reduced thiols in human plasma. It is an important scavenger of reactive oxygen and nitrogen species in blood acting as an effective redox buffer in the vascular compartment [38]. When oxidative stress compromise this redox buffer system, ROS are free to attack lipids, thus yielding some products of lipid peroxidation as malondialdehyde, measurable by TBARS assay. Interestingly, simple linear regression suggests that in COPD patients, FEV₁ is associated with age, PSH and ADMA/Arginine ratio, while in controls only age and sex were related to FEV₁.

These data, further supported by multiple linear regression analysis after correction for other important variables, confirms that increased OS is strictly linked to deterioration in lung function. Moreover, similarly to our previous observations in the general older population [39], we found a significant negative association between ADMA/arginine ratio and FEV₁, suggesting a detrimental effect of arginine methylation on key lung functional parameters. This observation was further confirmed by multiple logistic regression analysis indicating that PSH and ADMA/arginine ratio were independently associated with COPD development. When analyzing the factors independently associated with COPD severity both OS markers, PSH and TBARS, and ADMA/arginine ratio showed significant associations. Arginine metabolism plays an important role in the maintenance of airways tone and function by production of nitric oxide via the NOS pathway [40]. Dysregulation of the competing enzymes has been shown to contribute to airway obstruction in asthma and in patients with cystic fibrosis [13–14,21]. Bode-Boger and coworkers recently proposed the calculation of the ADMA/arginine ratio as an index reflective of NOS imbalances activity caused by the accumulation of ADMA. As such, a “normal” ADMA/arginine ratio is in the range of 0.0044–0.0076 [40], consistent with our reported values in the control group, while COPD subjects had higher values in accordance with recent data reported by Aydin et al. [22]. In this last report, however, there is no information regarding the impact of disease severity on ADMA concentrations. We found that, when categorizing on the basis of disease progression, only moderate COPD patients had values above the normal range, whereas mild COPD patients had median values in the normal range, further supporting the hypothesis that ADMA and arginine could be involved primarily during disease worsening. Pending additional evidence from experimental and human studies, it is plausible to speculate that COPD disease exacerbation states are associated with further increases both in oxidative stress and in the ADMA/Arginine ratio. The mechanisms responsible for the reported imbalance between ADMA and arginine may be related to OS. The reduction in arginine concentrations observed in COPD patients is likely due to the well-known increase of arginase activity stimulated by OS [41]. Moreover, the increase of neutrophil numbers typical of COPD may contribute to arginine depletion since these cells constitutively express high levels of arginase I in azurophilic granules. These granules may be released in patients with COPD together with other constituents of the granules such as elastase [42]. It is also known that neutrophil numbers increase as COPD worsens [43]. This might further explain the further reduction of arginine concentrations observed in moderate vs. mild forms of disease.

Moreover, the activity of the enzymes involved in the formation and degradation of ADMA such as PRMTs and DDAH is regulated in a redox-sensitive fashion [16–18]. Studies in cultured endothelial cells have reported that the gene expression of PRMTs is increased by oxLDL in a concentration-dependent manner [7]. There is growing body of evidence that OS decreases the activity of the ADMA demethylating enzyme, DDAH [8]. The presence of a reactive cysteine residue (Cys²⁴⁹) in the active site of DDAH leads to diminished activity of the enzyme in presence of ROS. Thus, OS, through DDAH inhibition, PRMTs synthesis stimulation and arginase increase activity might be primarily responsible for an imbalance of ADMA/arginine ratio. This hypothesis is also supported by the significant negative correlation observed between ADMA/arginine ratio and PSH in the analyzed subjects. In our COPD cohort, ADMA/arginine ratio is altered mainly because of a reduction in arginine concentrations, even if a non significant increase in ADMA levels of about 3.5% has been found in all COPD patients with a rise of about 5.2% in moderate COPD. It will be interesting to evaluate if ADMA concentrations are further increased in patients with more severe symptoms (COPD stages 3 and 4). SDMA concentrations and ADMA/SDMA ratios were also similar in controls and COPD patients. Obtained ADMA/SDMA values were besides in accordance to that reported by Bulau et al [42].

Therefore, our data indicate that OS and ADMA/arginine ratios are related in COPD patients, confirming previous reports showing reduced PRMT RNA or protein expression, arginase and DDAH activity under OS stimuli [16–18, 43–44]. Both PRMT and DDAH are widely expressed in lung tissue. Recent evidence also suggest that methylarginine metabolism in the lung may significantly contribute to circulating ADMA and SDMA concentrations [42]. In particular, pulmonary DDAH-1 is actively involved in ADMA degradation while PRMT-1 pulmonary expression is related to increased protein arginine methylation of the lung proteome. Moreover, as previously discussed, neutrophils significantly contribute to arginine decrease in the lung through the release of arginase I. Although no specific assessment of the expression and activity of these enzymes in lung tissue was performed in our study, it is plausible that the structural and functional lung alterations in COPD may lead to changes in arginine metabolites plasma concentration.

In conclusion our data, while confirming the role of OS and imbalanced arginine concentrations in COPD patients, show for the first time that increased ADMA/arginine ratio is independently associated with OS and COPD severity. Further studies, with a larger number of subjects covering all stages of COPD disease, are required to fully characterize the impact of arginine and ADMA on disease worsening.

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Author Contributions

Conceived and designed the experiments: AZ AGF SS PP CC. Performed the experiments: AZ AGF SS ES EZ FB PP CC. Analyzed the data: AZ SS AAM. Contributed reagents/materials/analysis tools: AZ SS PP CC. Wrote the paper: AZ AGF SS AAM PP CC.

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