Summary and General Discussion
SUMMARY

The scope of this thesis was on recognizing the ‘susceptible smoker’. Smoking is the main cause of COPD, a disease which is characterized by chronic airflow limitation which is generally progressive and associated with enhanced inflammatory responses in the airways and lungs. COPD is a leading cause of morbidity and mortality worldwide, with increasing rates over time. Importantly, not all individuals with the same smoking history will develop COPD, i.e. only 15-20% of all smokers. It is still unclear why COPD is manifested only in this small proportion of smokers. Therefore we aimed to investigate several biomarkers which might help to identify the susceptible smoker, and to provide more insight in the origins and pathogenesis of COPD. To this aim, an acute smoking study was performed in old and young individuals susceptible and non-susceptible to COPD. Furthermore, advanced glycation end products (AGEs) and their receptor (RAGE) were investigated in COPD patients and healthy smoking and never-smoking controls. Formation of AGEs is accelerated in conditions of inflammation and oxidative stress, and accumulated deposition of AGEs in tissue may have harmful effects. Finally, corticosteroid (in)sensitivity was investigated in the skin as a potential characteristic of COPD. Additionally, the role of smoking on the anti-inflammatory responses to ICS-treatment was investigated in smokers and ex-smokers with COPD.

Study design

Chapter 2 describes the outlines of the cross-sectional study that was performed at the University Medical Centers Groningen and Utrecht. The overall aim of this clinical study was to investigate the underlying local and systemic inflammation that is present in different clinical COPD phenotypes, and the acute effects of cigarette smoke exposure in individuals who are susceptible and non-susceptible for the development of COPD. To this aim, COPD patients GOLD stage I-IV and healthy controls (both groups aged >40 years) were recruited, being susceptible and non-susceptible (i.e. they smoked but did not develop COPD) to develop COPD respectively. In addition, young healthy subjects, susceptible and non-susceptible to develop COPD (both groups aged <40 years) were recruited. In these young individuals susceptibility was based on their family history, i.e. children in families where many family members smoked and either developed COPD (hence susceptible offspring) or not developed COPD (non-susceptible). All subjects were characterized in terms of clinical, physiological, immunological and radiographical measurements. In addition, young and old susceptible and non-susceptible groups performed the acute smoking procedure, which is extensively described in Chapter 3.

Acute smoking model in young and old susceptible or non-susceptible individuals

In Chapter 3 we investigated the effects of acute smoking in young and old individuals being susceptible or non-susceptible to the development of COPD. After smoking of 3 cigarettes in one hour inflammatory responses in peripheral blood (after 3 hours) and bronchial biopsies (after 24 hours) were analyzed within and between susceptible and non-susceptible groups. This study showed that activation markers A17 and A27 on circulating neutrophils in young susceptible individuals were significantly more increased with smoking than in non-susceptible
subjects. No effects of acute smoking were present in mediators in blood or inflammatory cell counts in bronchial biopsies. In the old group, the effects of acute smoking were comparable between healthy controls and COPD patients. In conclusion, we showed for the first time that susceptibility to COPD at young age associates with an increased innate immune response to a disease-specific challenge. This suggests that systemic inflammation may play a role in the early induction phase of COPD.

**Advanced glycation endproducts (AGEs) and their receptor**

In Chapter 4, we questioned whether AGEs accumulation in the skin is associated with COPD, measured by skin autofluorescence (SAF). A total of 202 mild-to-very severe COPD patients, 83 smoking and never-smoking healthy controls (aged >40 years), and 110 younger smoking and never-smoking controls (aged <40 years) were included. We demonstrated that SAF values were significantly elevated in COPD patients compared with old and young healthy controls. Interestingly, SAF did not significantly differ between the four disease severity stages in COPD patients. Lower lung function values (FEV$_1$/FVC, MEF$_{50}$/FVC, RV/TLC) and a higher number of packyears were associated with higher SAF values in the total population. Since SAF was higher in COPD patients than in healthy controls, but was not associated with disease severity within the COPD group, we concluded that AGEs may play a role in the induction phase of COPD in susceptible smokers.

We further investigated the accumulation of AGEs in other body compartments in Chapter 5. In this study we examined the levels of AGEs (CEL, CML, and pentosidine) in plasma, induced sputum, bronchial biopsies and the skin. In addition, we studied the expression of the receptor for AGEs (RAGE) in these compartments. We included 97 COPD patients (GOLD I-IV), 83 smoking and never-smoking controls (>40 years), and 108 younger smoking and never-smoking controls (<40 years). In this population we confirmed that SAF was increased in COPD patients compared with healthy controls. Furthermore, sRAGE levels in plasma were significantly decreased in COPD patients, and this decrease was significantly associated with an increase in SAF. No further differences of AGEs and RAGE levels were detected in bronchial biopsies, sputum supernatant and plasma levels. We conclude that AGEs accumulation is not necessarily associated between different body tissues in COPD. This might be due to differences in tissue turnover rates of AGEs. Lower sRAGE levels in plasma in COPD might indicate an impaired protective mechanism, reducing the clearance ability of circulating AGEs.

**Corticosteroid (in)sensitivity**

In Chapter 6 we assessed whether corticosteroid sensitivity was lower in COPD patients than in smoking and never-smoking healthy controls, by performing the corticosteroid skin blanching test. We included 89 COPD patients (GOLD I-IV), 28 never-smokers and 56 smokers. We demonstrated that GOLD III and IV COPD patients showed lower skin blanching responses than healthy never-smokers and smokers, as well as GOLD I and GOLD II patients despite the fact that they had comparable age. Additionally we showed that lower FEV$_1$, values and higher RV/TLC ratios were significantly associated with lower skin blanching responses. These findings
suggest that the reduced skin blanching response fits with a subgroup of COPD patients that has an early-onset COPD phenotype.

In Chapter 7 we questioned whether anti-inflammatory effects of ICS are different in 41 persistent smokers and 31 ex-smokers in a post-hoc analysis of the GLUCOLD study. We investigated if there were differences in treatment effects compared with placebo, by analyzing changes in lung function, hyperresponsiveness, and inflammatory cell counts in sputum and bronchial biopsies during short-term (0-6 months) and long-term (6-30 months) treatment with ICS. We showed that mast cells were reduced by short-term and long-term ICS treatment in both smokers and ex-smokers. In addition, sputum neutrophils and lymphocytes, and bronchial CD8⁺ cells were reduced after long-term treatment in ex-smokers only. The clinical implication of these findings is that, even in the presence of smoking, long-term ICS treatment may lead to anti-inflammatory effects in the lung.
GENERAL DISCUSSION

We investigated different biomarkers to identify susceptible smokers in an early phase of COPD and to gain more insight in the underlying mechanisms driving this development towards COPD. Familial presence of COPD is an important risk factor of COPD susceptibility, but more discriminative biomarkers are needed to recognize individuals susceptible to develop COPD later in life, and to better understand the pathogenesis of the disease. In this section we reflect our findings presented in this thesis and discuss their implications, limitations and future perspectives.

Biomarkers of COPD susceptibility in young individuals

Below we will discuss the potential biomarkers that were investigated in this thesis, in relation to their ability to be a ‘diagnostic’ parameter of COPD susceptibility at young age.

Inflammatory biomarkers with acute smoking

An appropriate method to differentiate susceptible and non-susceptible individuals might be to investigate their initial response to cigarette smoking, assuming that there is a differential response. The number of acute smoking studies that has been performed in humans is limited, although the acute smoke model has been postulated as a useful method to better understand the inflammatory and oxidative stress responses to smoke (1). In Chapter 3 we investigated responses of local and systemic inflammatory biomarkers to this disease-specific challenge in susceptible and non-susceptible subjects. We showed that young susceptible individuals could be recognized by a higher activation state of circulating neutrophils as a result from smoking. The expression of the markers A17 and A27, which both specifically recognize the active form of FCRγII (2,3), was more upregulated in young susceptible individuals when compared with young non-susceptible subjects. The associations with COPD susceptibility were present after adjustment for age, smoking status and expression of the markers at baseline. This suggests they might be potential biomarkers of COPD susceptibility. On the other hand, the changes were subtle and not uniform, which poses questions about the sensitivity and specificity of such a potential biomarker. Since this biomarker can only be recognized as a response to an activating stimulus like smoking, and smoking of 3 cigarettes within one hour is not very attractive in the frame of a diagnostic test, a more easy diagnostic test is needed to recognize susceptible individuals.

One way to develop such a test is by isolating neutrophils from individuals, treating these cells with cigarette smoke extract or an LPS challenge in vitro, and measure their change in activation status. In this way, some in vitro studies have shown that circulating neutrophils of smokers are pre-activated compared with never-smokers, as they have a higher capacity to migrate towards chemotactic stimuli or are more responsive to activating agents (4,5). Perhaps it is possible to develop a reliable in vitro test that discriminates between high and low responders on in vitro challenge of leucocytes with cigarette smoke or a surrogate of cigarette smoke. Another approach might be to challenge participants with a surrogate of cigarette smoke in vivo, for example challenging participants with lipopolysaccharide (LPS) (6,7).
However, this is also an invasive procedure with potential side effects. The next step would then be to validate this in the perspective of COPD development, which is not very attractive in the light of a disease with a 30-year latency time. We conclude that a reliable biomarker test on susceptibility to develop COPD is still far away.

We did not find differential responses with the other neutrophils markers between groups, but significant ‘within’ group differences were observed for several markers. For example, the adhesion molecule ICAM-1 significantly decreased in susceptible subjects, while no significant effects was present in the non-susceptible group. These markers need further investigation, since differential effects may be detected in a larger population or a population with objectively defined COPD as a marker of familial susceptibility.

**AGE-reader**

In Chapter 4 we have demonstrated that AGEs accumulation in the skin, as measured by SAF, is elevated in COPD patients. A logical explanation is that the ongoing inflammation and oxidative stress that is present in COPD has contributed to this elevated AGEs levels over time. Interestingly, SAF values were comparable between the different disease severity stages who had the same age. This suggests that AGEs accumulation is not associated with disease progression, but might take place during the developmental phase of COPD. We therefore investigated SAF in our young population containing susceptible and non-susceptible individuals, as presented in the Addendum of Chapter 4. We could not demonstrate that SAF values were higher in susceptible than non-susceptible subjects, also not after adjustment for gender, age and smoking. An explanation for this negative finding might be that accumulation of AGEs takes some years, in other words, maybe our young subjects were too young to show differences between the two groups. A comparison between susceptible and non-susceptible individuals aged 35-45 years was also negative (data not shown); however the two groups had a small sample size. Although a larger study might be able to detect differences in SAF in such individuals, we believe the overlap between the two groups is too large to become a discriminative tool at an individual level. On the other hand, we cannot exclude that SAF in combination with determination of AGE-receptor expression or with acute rises of AGEs in plasma after smoking would be worthwhile. At the moment we have to conclude that SAF is not suitable as a biomarker of COPD susceptibility, and potentially suitable as a biomarker of COPD.

**Corticosteroid (in)sensitivity**

The corticosteroid skin blanching test is an easy non-invasive test to assess responses to corticosteroids, and might reflect corticosteroid sensitivity in the airways. Interestingly, we observed that the young susceptible group had a lower corticosteroid response to topically applied budesonide in the corticosteroid skin blanching test than non-susceptible individuals, as presented in the Addendum of Chapter 6. This finding suggests that reduced corticosteroid sensitivity might be a characteristic, particularly present in subjects with established COPD or with susceptibility of COPD. However, susceptibility remained no predictor of skin blanching response after adjustment for age, packyears, gender and corticosteroid use. Also here we
have to realize that there is a large variability in the skin blanching outcomes, which makes it hard to compare between individuals. Moreover, the read-out is subjective. In the past, several more objective techniques like Doppler velocimetry and reflectance spectrophotometry were compared with this visual scoring system, but visual assessment of the skin blanching response appeared to be the most sensitive tool (8). Although, determination of individual steroid responsiveness might be improved in the future, it is difficult to imagine that a corticosteroid test would be discriminative to identify subjects with such a multifactorial and heterogeneous disease like COPD.

Mechanisms underlying COPD susceptibility

Inflammatory responses to acute smoking
How can we explain increased activity of the innate immune response as a result from smoking in young susceptible individuals and in which way could this response contribute to development of COPD at higher age? We speculated that neutrophils of susceptible individuals are more easily to prime, and therefore more sensitivity for inflammatory triggers. The activation of neutrophils is a multi-step process, which generally starts with priming (pre-activation) caused by chemotaxins or cytokines, leading to upregulation of integrins and adhesion molecules like CD11b or ICAM-1 (9,10). Acute smoking might have triggered local inflammation, thereby increasing the expression of activation molecules on neutrophils. Our data indicate that circulating neutrophils in susceptible individuals are more sensitive to this response, resulting in a faster influx of neutrophils to the site of inflammation. The trend we showed in decreased expression of the adhesion molecule ICAM-1 supports this idea, since neutrophils with increased adhesion molecules might have left the circulation by migration into the lung tissue. This may lead to a more intense inflammatory response in the airways of susceptible individuals, which may contribute to lung tissue damage at higher age. It is contradictory that we were not able to detect a higher release of mediators in peripheral blood, which was measured at the same time point. Additionally, no increased influx of neutrophils was found in bronchial biopsies, which can be explained e.g. by the fact that biopsies were taken 24 hours after smoking. In general, the inflammation in COPD is predominantly characterized by a neutrophilic inflammation type, which might further strengthen our hypothesis.

Role of AGEs
We concluded before that AGEs might be involved in the induction phase of COPD as AGE accumulation is elevated in the skin of COPD patients, but did not associate with COPD severity. We hypothesize that AGEs in non-smokers are slowly formed with aging, and that accumulation of AGEs in smokers is slightly increased due to smoking as an additional source of AGEs formation (11,12). Probably, in ‘susceptible’ smokers AGEs formation and accumulation is increased during COPD development, as a result of chronically increased oxidative stress due to smoking (exogenous factor) and systemic inflammation (endogenous factor). A contributing factor might be a reduction in levels of the soluble form of the receptor for AGEs (sRAGE), which is lower in COPD patients as we show in Chapter 5 and as presented by previous research (13-16). sRAGE can act as a decoy receptor, by scavenging AGEs from the circulation.
In this way, sRAGE facilitates clearance of AGEs and prevents AGEs to bind cellular bound RAGE which otherwise would activate pro-inflammatory intracellular pathways. This protective mechanism probably is impaired in COPD patients and might be genetically determined, as polymorphisms in the advanced glycosylation end product-specific receptor (AGER) gene are associated with lower lung function (FEV₁ and FEV₁/FVC) (17,18). These polymorphisms can affect the proportion of sRAGE expression and thereby influence their protective function.

Role of corticosteroid (in)sensitivity
While it might be hard to recognize susceptible individuals using the skin blanching test, this test can give more insight in the potential role of corticosteroid sensitivity in the pathogenesis of COPD. It is generally accepted that the majority of COPD patients are poor-responders to corticosteroid treatment, as inhaled corticosteroids have generally no or little effects on disease progression or mortality. It is unclear if this reduced corticosteroid sensitivity it is a general characteristic of the disease or if it contributes to disease development.

In Chapter 6 we showed that patients with a more severe COPD (GOLD stage III and IV) have lower corticosteroid responses compared with healthy controls, while milder disease stages (GOLD stage I and II) showed comparable levels of skin blanching. Because all patients were age-matched and smoked the same high number of packyears, it suggests that the skin blanching response identifies a subgroup or subphenotype of COPD patients with a reduced corticosteroid sensitivity. In Chapter 7 we investigated the effect of smoking on anti-inflammatory effects of inhaled corticosteroid treatment (ICS) in the GLUCOLD cohort, as there are indications that smoking may induce resistance to the anti-inflammatory effects of corticosteroids (19-21). Here we showed that some anti-inflammatory effects were present in the airways after short-term (0-6 months) and long-term treatment (6-30 months) with inhaled corticosteroid (ICS) treatment, in both persistent smokers and ex-smokers of the GLUCOLD cohort. So apparently, even in the presence of smoking ICS treatment may lead to some anti-inflammatory effects in the lung. From both studies we have learned that reduced corticosteroid responsiveness is not a general characteristic of the disease, but is variable between patients and might be associated with different COPD phenotypes.

We hypothesize that this variability in corticosteroid responses might have its origins earlier in life. If the corticosteroid response in susceptible individuals at young age is already reduced, as suggested earlier in this section, one can imagine this may have large consequences at higher age after many years of smoking. One mechanism might be via reduced glucocorticosteroid receptor function (GR). The skin blanching has been shown to be regulated by vascular smooth muscle GRs (22-24). SNPs in the GR gene affect receptor structure and have been shown to be associated with a lower skin blanching response (24,25). Since endogenous corticosteroids are of importance during lung development in fetal growth (26), even small changes in the GR structure might affect lung growth. And lower lung function values at birth or in early childhood have been shown to increase the risk of COPD development (27). Another consequence of lower steroid responsiveness might be that endogenous anti-inflammatory pathways are suppressed, leading to increased local inflammation resulting in tissue damage, particularly after many years of chronic smoking.
Other mechanisms underlying COPD susceptibility

In this thesis, our main focus was on differential mechanisms that are present in susceptible individuals which may contribute to COPD development. This focus on disease processes is in line with the normal strategy to investigate diseases in general. Another strategy might be to focus on the disease-protective processes, in other words to pose the question why subjects stay healthy. Regarding COPD it might be worthwhile to extend our work by a more critical view on the non-susceptible individuals, and how they are protected against the damaging effects of cigarette smoke. For example, it may be that non-susceptible smokers have less depletion of the anti-oxidant capacity as a response to smoking is present in the airways, because of a different oxidant/anti-oxidant balance. This might be an important focus in future studies.

Future studies

Acute smoking study design

In the acute smoking study we observed that, besides neutrophil activation markers, the majority of the measured inflammatory markers were not affected by acute smoking at all. This lack of response can be explained by several causes.

First of all, the number of cigarettes smoked may have been insufficient. Previous acute smoking studies in human used very diverse study designs regarding smoke exposure (1), varying from 1-2 cigarette in 10 minutes, to 4 cigarettes in 1 hour, to 24 in 8 hours, or even 24 cigarettes in 8 hours (28-31). Smoking 3 cigarettes within one hour was the lowest dose that had an a priori good chance to elicit an inflammatory response in bronchial biopsies without inducing cigarette smoke addiction. However, not one inflammatory marker in our bronchial biopsies responded, perhaps because we projected the cigarette dose from animal studies. Indeed future studies might increase the number of cigarettes and number of days of smoking, but this seems not very ethical.

We asked participants to refrain from smoking during two days prior to the acute smoking procedure. This might be a relatively short wash-out period, since the variation in smoking behavior between participating subjects was high. In future studies this period might be extended to for example ten days. On the other hand, this might be difficult to verify. In our study we used exhaled CO measurements to control if subjects adequately refrained from smoking, however this method can only detect smoking during the last 6 hours. Another method is measuring urinary cotinine levels, which gives information of the last 10 days. This might be more precise, but is time consuming.

As the number of human studies is small and diverse in their design, it was hard to decide at what time point after smoking samples should be obtained. This was particularly true for bronchial biopsies, because no previous studies investigated this tissue in the context of acute smoking effects. Cell influx in the lungs occurs very quickly after smoking in BALF. Therefore 24 hours may have been too long after smoking. In addition, there was a minimum of 6 weeks between the bronchoscopy after smoking and the bronchoscopy at baseline. We chose for this time span for comfort and safety reasons of the patient. However, this is a relatively long period of time and immunological events like airway infections may have occurred, in this way
affecting our results. Future studies should consider to take biopsies somewhat earlier after the acute smoking event and to shorten the time period between the two bronchoscopies, for example doing a bronchoscopy 6 hours after smoking with a minimum interval of two weeks between the two bronchoscopies.

Furthermore, we can question if we used the reliable tools to investigate acute smoke responses. From our study, assuming that we chose the right time points, it turned out that bronchial biopsies are not very sensitive and specific to investigate the acute inflammatory response to cigarette smoking. It could be that these responses mainly take place in the more peripheral airways. Taking transbronchial biopsies might be an alternative, however quantification of inflammatory responses in this material is very complicated and inaccurate, leading to the need of high sample sizes, and many more people at risk for this invasive procedure.

To end, we selected our young susceptible individuals based on prevalence of COPD in their families, as we know that COPD has a genetic component and family studies have shown that the combination of smoking and familial clustering of COPD associates with the risk to develop COPD (32-35). We gained this information based on an extensive questionnaire. We did not invite their family members to perform flow volume measurements to confirm the prevalence of COPD. Maybe even more importantly, as COPD still is an underdiagnosed disease, we did not confirm that smoking family members did not develop COPD. This may have affected the correct categorization of susceptible and non-susceptible family members and thus the correct categorization of subjects participating in this study. Of course, performing flow volume measurements in all family members is time consuming and logistically a challenge. Another way to improve selection of the young susceptible group is to define more strict inclusion criteria and to select ‘very’ susceptible subjects, e.g. by selecting children of very severe early-onset COPD patients. One disadvantage of this approach is that the study population might not represent “normal” COPD but a special subphenotype of COPD, e.g. early onset only.

**Follow-up**

One limiting factor in studying the ‘susceptible’ smoker is that COPD is manifested relatively late in life, around the age of 50-60 years. Ideally, young susceptible individuals should be followed-up in longitudinal studies to see if COPD is actually developed at higher age. It would be interesting to link responses to cigarette smoke in early life with clinical and pathological manifestations in later life. Ideally, this should be combined with genetic profiling and gene expression in different tissues. In this way, future research may lead to hopefully better understanding the mechanisms underlying smoking-induced COPD, leading to an earlier diagnosis of COPD, and to new treatment targets of this disabling disease.
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