General Discussion
In this thesis we aimed to study the potential role of vitamin D in inflammation, pulmonary function and as a potential therapeutic agent in COPD. In this chapter we will summarize the main findings of the previous chapters and discuss the results, methodological issues, clinical implications and perspectives for future research.

**Summary of main findings**

**Relationship of serum 25(OH)D with inflammation and the role of adiposity**

In Chapter 2 we found that the relationship between different body fat deposits and 25(OH)D concentrations was different for men and women. In women, total body fat and visceral adipose tissue were inversely related to 25(OH)D concentrations. In men, visceral adipose tissue and hepatic fat were inversely related to 25(OH)D concentrations. In both men and women, visceral adipose tissue was most strongly associated with 25(OH)D concentrations. In Chapter 3 we found that serum 25(OH)D was negatively associated with markers related to a pro-inflammatory state (CRP and leptin) and positively associated with markers related to an anti-inflammatory state (adiponectin). This relationship was largely explained by adiposity measures. After adjustment for total body fat and waist circumference the associations of 25(OH)D concentrations with serum CRP and leptin disappeared, and the association with serum adiponectin attenuated.

**Relationship of serum 25(OH)D with pulmonary function**

In Chapter 4 we found an association of serum 25(OH)D concentrations with pulmonary function and airway inflammation in participants with a BMI ≥ 30 kg/m², but not in participants with a BMI < 30 kg/m². In participants with a BMI ≥ 30 kg/m², we observed that higher serum 25(OH)D concentrations were associated with a better pulmonary function and lower amount of airway inflammation. Serum 25(OH)D concentrations were not associated with the occurrence of common colds in the last month, irrespective of BMI. In Chapter 5, however, we found that serum 25(OH)D concentrations were associated with pulmonary function in men, but not in women. We did not find an effect of BMI in this study. We did perform a mediation analysis investigating the role of physical performance and inflammation. Physical performance score, hand grip strength, CRP and IL-6 concentrations did not mediate the relationship between 25(OH)D and pulmonary function. In addition, smoking was not an effect modifier in this relationship.
Relationship of serum 25(OH)D with quality of life and effects in COPD

In Chapter 6 we found that lower serum 25(OH)D concentrations were associated with lower scores on the physical component of the SF-12 and self-rated health. Physical performance, number of chronic diseases and depressive symptoms acted as mediators and largely explained the relationship between vitamin D and quality of life. In Chapter 7 we studied the effects of vitamin D supplementation in vitamin D deficient patients with COPD. We did not find an effect of vitamin D supplementation on respiratory muscle strength and physical performance. In addition, we did not find any effects on the secondary outcomes pulmonary function, hand grip strength, exacerbation rate and quality of life. This pilot trial did point out several issues we aimed to address in a new trial, in Chapter 8. In this chapter we described the design of our multicenter RCT on the effect of vitamin D supplementation in COPD-patients with vitamin D deficiency on exacerbation rate and both pulmonary and physical function. Finally, in Chapter 9 we presented an individual participants data meta-analysis, including our pilot trial. This study found that vitamin D supplementation did not affect overall exacerbation rate, but did reduce the number of exacerbations in participants with a baseline 25(OH)D concentration < 25 nmol/L.
Table 1. Summary of main findings

<table>
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<tr>
<th>Chapter</th>
<th>Study / Design</th>
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<th>Determinant / Intervention</th>
<th>Outcome</th>
<th>Effect modification / Mediation</th>
<th>Results</th>
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<td>NEO-study</td>
<td>6287 men and women aged 45-65 years</td>
<td>Serum 25(OH)D</td>
<td>Leptin, CRP and adiponectin concentrations</td>
<td>Effect modification: BMI, waist circumference, total body fat</td>
<td>Higher serum 25(OH)D associated with lower CRP and leptin, and higher adiponectin. Associations largely explained by adiposity measures.</td>
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<tr>
<td>4</td>
<td>NEO-study</td>
<td>6138 men and women aged 45-65 years</td>
<td>Serum 25(OH)D</td>
<td>FEV₁, FVC, FeNO, common colds</td>
<td>Effect modification: BMI</td>
<td>BMI ≥ 30: Higher serum 25(OH)D associated with a better pulmonary function and lower airway inflammation. BMI &lt; 30: No association. No association of serum 25(OH) with occurrence of common cold in the last month.</td>
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<td>5</td>
<td>LASA</td>
<td>542 men and women aged 55-65 years</td>
<td>Serum 25(OH)D</td>
<td>FEV₁, FVC</td>
<td>Effect modification: sex, smoking</td>
<td>☀: No association of serum 25(OH)D with pulmonary function ☢: Serum 25(OH)D positively associated with pulmonary function. ☢☀: No mediation by physical performance and inflammation</td>
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<td>6</td>
<td>LASA</td>
<td>1248 men and women aged 65 years and older</td>
<td>Serum 25(OH)D</td>
<td>SF-12 scores, self-rated health</td>
<td>Effect modification: physical performance score, grip strength, CRP and IL-6 concentrations</td>
<td>Lower serum 25(OH)D associated with lower scores on the physical component of the SF-12 and self-rated health. Physical performance, number of chronic diseases and depressive symptoms acted as mediators and largely explained the relationship between vitamin D and quality of life.</td>
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<tr>
<td>7</td>
<td>Pilot trial RCT</td>
<td>50 vitamin D-deficient COPD-patients</td>
<td>1200 IU vitamin D or placebo per day</td>
<td>Respiratory muscle strength and physical performance</td>
<td>-</td>
<td>No effect of vitamin D supplementation on respiratory muscle strength and physical performance</td>
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<td>8</td>
<td>PRECOVID Study protocol RCT</td>
<td>240 vitamin D-deficient COPD-patients</td>
<td>16.8000 IU vitamin D or placebo per week</td>
<td>Exacerbation rate</td>
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<td>-</td>
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<td></td>
<td>IPD-meta-analysis of RCTs</td>
<td>472 COPD-patients</td>
<td>100.000 IU monthly / 200.000 IU two-monthly / 1200 IU per day vs. placebo</td>
<td>Exacerbation rate</td>
<td>Effect modification: Baseline 25(OH)D, GOLD spirometric grade, use of inhaled corticosteroids, BMI, frequency of dosing, genotype</td>
<td>25(OH)D &lt; 25 nmol/L: Vitamin D supplementation led to a reduction in exacerbation rate 25(OH)D ≥ 25 nmol/L: No effect of vitamin D supplementation.</td>
</tr>
</tbody>
</table>

**Abbreviations:** NEO-study: Netherlands Epidemiology of Obesity study; LASA: Longitudinal Aging Study Amsterdam; RCT: randomized clinical trial; IPD: Individual Participant Data; IU: International Units; 25(OH)D: 25-hydroxyvitamin D; FEV1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; FeNO: Fractional Exhaled Nitric Oxide; BMI: Body Mass Index; GOLD: Global Initiative for Chronic Obstructive Lung Disease.
Discussion

In this thesis we aimed to study the potential role of vitamin D in inflammation, pulmonary function and as a potential therapeutic in COPD. To help understand potential underlying mechanisms we also studied several associated outcomes.

Relationship of serum 25(OH)D with inflammation and the role of adiposity

Higher vitamin D levels have been associated with both an anti-inflammatory profile and a lower prevalence of adiposity (1, 2). Adiposity and inflammation, on their turn, have also been associated with each other, with adiposity being related to a state of chronic low-grade inflammation. The interplay between these three factors is largely unknown. In the first part of this thesis we aimed to study these relationships.

Several hypotheses have been proposed for the inverse relationship between serum 25(OH)D concentrations and adiposity (3). As vitamin D is a fat-soluble molecule, it is sequestered in adipose tissue. One theory is that in people with obesity, the sequestration of vitamin D is increased due to a larger amount of adipose tissue. This leads to a decrease in the amount of circulating 25(OH)D concentrations. According to this theory, subcutaneous adipose tissue should be strongly related to serum 25(OH)D concentrations, as it forms the largest fat deposit in the body, representing 85% of total body fat. However, in our study, we did not find a relationship between serum 25(OH)D concentrations and subcutaneous adipose tissue. A second hypothesis is that the elimination of vitamin D in persons with obesity is increased due to obesity-associated inflammation. Obesity is associated with a state of chronic low-grade inflammation, which visceral adipose tissue attributes the most to (4). In contrast to subcutaneous adipose tissue, visceral adipose tissue secretes more pro-inflammatory and less anti-inflammatory adipokines. This hypothesis is in line with our finding that in linear regressions with standardized estimates, visceral adipose tissue was most strongly related to serum 25(OH)D concentrations. Therefore, we also studied the relationship of serum 25(OH)D concentrations with CRP and leptin, as pro-inflammatory markers and adiponectin as an anti-inflammatory marker. We found that, when adjusting for adiposity measures, the relationship with CRP and leptin largely disappeared, and the relationship with adiponectin largely attenuated. This finding suggests that adiposity is the common denominator of both lower 25(OH)D concentrations and a pro-inflammatory profile. An interesting finding was that, although the relationship of serum 25(OH)D with CRP and leptin largely disappeared, the relationship with adiponectin persisted. This finding could be coincidental or one of residual confounding. However, if a true finding, it suggests that the effects of vitamin D on the immune system might be explained by its anti-inflammatory effects, rather than an inhibition of the pro-inflammatory effects.
Relationship of serum 25(OH)D with pulmonary function

In the second part of this thesis, we studied the relationship between serum 25(OH)D concentrations and pulmonary function. Previous studies on the relationship between serum 25(OH)D and pulmonary function have shown inconsistent results. One study showed an association only in participants with obesity (5), while another study found sex differences (6). We therefore aimed to study whether BMI and sex are effect modifiers in this relationship. In the NEO-study, we found no associations of serum 25(OH)D concentrations with pulmonary function in participants with a BMI <30 kg/m². However, in participants with a BMI ≥ 30 kg/m², we observed that higher serum 25(OH)D concentrations were associated with a better pulmonary function. In the LASA-study, no effect modification of BMI was found. The differences in results between these studies in influence of BMI are difficult to explain. Both studies are population-based cohorts, representative of the general older population. Participants from both studies, however, were recruited in different ways. In the NEO-study participants were all recruited from one area in the West of the Netherlands, which is an urban region. In the LASA-study, participants were recruited in three different regions, including both urbanized and rural areas. Participants of the LASA-cohort were slightly older (mean(SD) age: 60 (3) years) than the NEO-cohort (56 (7) years). BMI values were comparable in the two cohorts. In the LASA-cohort mean (SD) BMI was 27.0 (4.4) and in the NEO-cohort the weighted mean was 26.3 (4.4) kg/m². Differences in BMI could therefore not explain effect modification of BMI. Serum 25(OH)D concentrations were also comparable in the two cohorts (LASA: 68.5 (22.1) nmol/L and NEO: 70.8 (24.9) nmol/L). Spirometry measures, however, were higher in the NEO-cohort compared to the LASA-cohort. Mean (SD) FEV₁ and FVC in the NEO-cohort were 107.8 (16.5) and 116.6 (17.3) % predicted, and in the LASA-cohort 98.0 (16.3) and 97.5 (13.4) % predicted, respectively. The reason and relevance of this difference is unclear.

Several theories could be suggested of how obesity might affect the relationship between serum 25(OH)D concentrations and pulmonary function (7). Obesity might affect the relationship due to its effects on systemic inflammation. As stated before, obesity is associated with a state of chronic low-grade inflammation, with higher levels of interleukin-6, tumor necrosis factor alpha, which might affect pulmonary function negatively (8, 9). Secondly, as stated in the first part of this thesis, lower levels of vitamin D are associated with higher levels of visceral adipose tissue. A higher amount of truncal fat can lead to a higher level of mechanical load and therefore reduce chest wall compliance and respiratory muscle strength (7). Another explanation might be that individuals with obesity were more vitamin D deficient. The relationship may only be present in people with lower levels of vitamin D, leading to an effect only in this group. A sensitivity analysis in a small subset of participants with vitamin D deficiency, however, did not show an association which renders this explanation unlikely.
In the LASA-study, we performed a mediation analysis to study the role of inflammation in the association between vitamin D and pulmonary function. An inverse association between systemic inflammatory markers and pulmonary function has been shown in several previous studies (10, 11). In addition, we also studied whether physical performance was a mediator as vitamin D supplementation has been proven to have beneficial effects on skeletal muscle function and physical function (12-14). Vitamin D might therefore affect pulmonary function through effects on muscle strength. In our study, however, we did not find a mediating effect of physical performance and inflammation markers. Because of the population-based nature of the study, physical performance scores were relatively good and concentrations of the inflammation markers were relatively low in our population. This might have affected our results, as a potential effect might only be seen in a more compromised population. The clinical relevance for the general population might thus be limited.

In the LASA-study we did find an inverse relationship between serum 25(OH)D concentrations and pulmonary function in men, but not in women. One previous study in LASA of a different cohort found a similar gender difference, but with peak expiratory flow rate and not FEV₁ and FVC as the outcome (6). The explanation for the gender difference remains unclear. No other studies have mentioned potential effect modification of sex. It is unclear whether previous studies did not find an effect or did not study this as a potential effect.

Relationship of serum 25(OH)D with quality of life

Vitamin D has been shown to be independently related to quality of life in persons with chronic diseases in previous studies (15). In our study, we found that lower serum concentrations 25(OH)D were associated with lower scores on the physical component of the SF-12 and self-rated health in the general population. Interestingly, we observed that this relationship was only present until levels of serum 25(OH)D around 60 nmol/L. This suggests that this relationship is only applicable to individuals with a vitamin D deficiency. The finding that vitamin D status was related to the physical component of the SF-12, and not the mental component, points towards a potential relationship due to effects on physical function. Indeed, we found that physical performance and number of chronic diseases acted as mediators and largely explained the relationship between vitamin D and quality of life.

Effects of vitamin D supplementation in patients with COPD

The large amount of evidence pointing towards a beneficial role of vitamin D in inflammation and pulmonary function, have led us to study the effects of vitamin D supplementation in patients with COPD. In our pilot trial we only included patients with a vitamin D deficiency, because a previous trial showed effects only in participants with
low levels of vitamin D. In our trial we did not find an effect of vitamin D supplementation on physical performance score or respiratory muscle strength. In addition, we did not find an effect on any of the secondary outcomes including pulmonary function, number of exacerbations and quality of life. This finding is in line with two other trials that also did not find an effect of vitamin D supplementation on physical function in patients with COPD (16, 17). Compared to other studies, participants in our pilot trial received a relatively low dose of 1200 IU per day. Furthermore, participants had relatively lower disease activity, as we also included participants not under treatment in a hospital. Finally, our follow-up duration was 6 months, which might have been too short to find an effect of vitamin D supplementation. The results of this pilot trial were later included in an individual participants data meta-analysis, assessing the effects of vitamin D supplementation on exacerbation rate. In this study, no overall effect of vitamin D supplementation was found. However, in prespecified subgroup analyses in patients with serum 25(OH)D levels < 25 nmol/L, a reduction in exacerbation rate was found. The results of this study suggest that vitamin D supplementation is only beneficial in participants with a severe vitamin D deficiency. This study also showed that vitamin D supplementation is safe, as no differences were found in adverse event rates.

In the PRECOVID-trial we addressed several of the issues found in the pilot trial. To select a population with more disease activity, we only included participants with an exacerbation in the previous year and who are under treatment by a pulmonologist. In addition, we increased the follow-up duration to one year. Finally, the supplementation dose was raised to 16,800 IU per week. This trial is momentarily still ongoing and the results are expected in 2020 (18).

Methodological issues

Strengths
In this thesis we used data from two large population-based cohorts. The cohorts were well phenotyped, which allowed us to correct for several relevant confounders and test potential effect modification and mediation. This helped us to better define the studied the relationships. The population-based nature of the studies increased the generalizability of our findings.

Limitations
In the NEO-study three different methods were used to measure serum 25-hydroxyvitamin D during the study period. This could have led to variations in the measurements. To minimize these possible variations, we calibrated our serum 25(OH)D measurements to the golden
standard LC-MS/MS. Because of the population-based nature of the studies, 70-80 % of the participants were vitamin D sufficient (serum 25(OH)D > nmol/L). Previous studies have shown that potential effects of vitamin D are larger in individuals with vitamin D deficiency. In addition, the outcomes used, including inflammation markers, pulmonary function and physical performance, were also in the normal range, and had a small variability. This might have affected our results, as this makes it more difficult to find an association. Another limitation of our studies is that the study populations primarily consisted of participants of white European origin. Previous studies have shown ethnic differences in vitamin D metabolism which might have affected our results and the generalisability of the studies.

Observational vs. interventional studies

In this thesis we studied the relationship between serum 25(OH)D concentrations and a variety of health outcomes in several cross-sectional studies. An important question is whether vitamin D is a cause or consequence of these outcomes. According to the Bradford Hill criteria (19), temporality is an important criterion to establish causality. As the exposure has to precede the effect, causality cannot be established in a cross-sectional design. In medicine, RCTs are currently seen as the golden standard to establish evidence for a causal effect. However, RCTs are not always feasible due to their expensive and time-consuming nature. Observational studies can then be used to establish enough evidence to justify the design of RCTs. Furthermore, RCTs do have some limitations. To achieve enough power to establish an effect, large sample sizes are often needed. Due to the strict in- and exclusion criteria that are often applied in RCTs, generalizability is decreased. Results could be further biased by low response rates with selection of healthier people responding to invitations. Finally, there are ethical and practical constrictions to the type of exposure that can be studied in an RCT, such as the effect of smoking and adiposity. Studying rare and long-term effects could be hard in an RCT as this warrants very large sample sizes and long follow-up duration. These are, however, factors that can be addressed in observational studies by designing large, well-phenotyped cohort studies. In a population-based cohort, several research topics can be addressed and findings can be directly generalized to the general population. By well phenotyping the population, confounders can be identified and adjusted for in statistical analyses. In addition, mediation analyses can help understand potential underlying mechanisms. In this way, observational studies can play an important role in scientific discovery.
Clinical implications

In this thesis we found that serum 25(OH)D concentrations are associated with several health-related outcomes. As stated before, because of the observational nature of the cohort studies, no inferences can be made about causality. The question whether vitamin D plays a causal role in these health outcomes, and therefore whether supplementation is beneficial, cannot be answered. However, taking this limitation into account, a few conclusions can be drawn from the studies. We found that visceral adiposity is most strongly related to serum 25(OH)D concentrations. This suggests that individuals with a larger amount of visceral adipose tissue are at a higher risk of lower vitamin D concentrations. As vitamin D deficiency is highly prevalent in individuals with obesity, specific attention to persons with a large amount of visceral adiposity should be given in the identification of vitamin D deficiency. In addition, we found that in participants with obesity, lower 25(OH)D concentrations were associated with worse pulmonary function and airway inflammation. This suggests that individuals with obesity and lower 25(OH)D concentrations are at higher risk for having worse pulmonary function and higher levels of airway inflammation. The differences found in this study, however, were very small and this makes it difficult to assess the clinical relevance of the findings. Finally, when assessing the effect of vitamin D supplementation in vitamin D-deficient patients with COPD, we did not find an effect on physical performance or respiratory muscle strength. The IPD meta-analysis, however, showed an effect of vitamin D supplementation on exacerbation rate in patients with serum 25(OH)D concentrations <25 nmol/L. These results are promising, and suggest that vitamin D supplementation is beneficial in COPD patients with a vitamin D deficiency. In the ongoing PRECOVID-trial, the effects of vitamin D supplementation on exacerbation rate are further studied. If indeed effective, vitamin D supplementation might be an easy, safe and low-cost therapy in the prevention of COPD exacerbations.

Implications for future research

This thesis identified two large research themes that should be addressed in future research. The first theme includes the role of adiposity. We found that visceral adipose tissue was most strongly associated with serum 25(OH)D concentrations compared to the other fat depots. While this finding suggests that individuals with a larger amount of visceral adipose tissue are at a higher risk of lower vitamin D concentrations, future studies should assess the clinical relevance of this finding. Do persons with a larger amount of visceral adipose tissue score less in vitamin D-related outcomes such as bone health, compared to individuals with a different fat distribution? Also, as vitamin D is potentially differently metabolized,
the question arises whether supplementation regimens should be adjusted for persons with obesity. As stated before, vitamin D might me be more sequestered in persons with obesity, and is potentially cleared sooner due to inflammation. Dosing regimens based on the general population may therefore not apply to persons with obesity. Furthermore, in studies assessing the clinical outcomes of vitamin D supplementation, specific attention should be given to the effects in persons with obesity. In this thesis, for example, we found that adiposity plays an important role in the associations found between serum 25(OH)D concentrations and pulmonary function. In future studies on the effect of vitamin D supplementation on pulmonary function, it should therefore be studied whether these effects are the same in persons with obesity compared to persons without obesity. Besides the clinical relevance, the underlying mechanism for the relationship found between serum 25(OH)D and visceral adiposity is unclear. In vitro studies are needed to study potential differences in vitamin D metabolism and storage in the different fat deposits.

The second theme concerns the causality of the relationships studied. In this thesis, several observational studies have been performed, which calls for the need of RCTs to prove causality. To improve the validity of these trials, however, several factors should be taken into account in the design of future trials. Several studies have shown a beneficial effect of vitamin D supplementation to be only present in participants with a vitamin D deficiency (20). Therefore, future clinical studies should include sufficient vitamin D deficient participants as a potential therapeutic effect is expected to be largest in this group. Second, several studies have shown that large-interval bolus dosing of vitamin D is less efficient in increasing serum 25(OH)D levels compared to a more continuous weekly or daily dosing (21). These effects are probably caused by more fluctuating vitamin D levels due to a short half-time. In addition, several studies have shown an increased risk of falls in high intermittent dosing (22, 23). Finally, follow-up duration should be long enough to find outcomes that need long lead-times to become manifest, such as the development of obesity and pulmonary diseases.

Some of these issues could be addressed in Mendelian randomisation studies. In Mendelian randomisation studies genetic variation is used as a proxy for the variable of interest. Because alleles are randomly allocated at conception, no effect of confounding or reverse causation can take place. Different single nucleotide polymorphisms (SNP’s) have been identified that are related to vitamin D metabolism (24). These known SNP’s are found to explain 7,5% of heritability of serum 25(OH)D concentrations. Twin studies, however, have found heritability estimates of 40-80 %, leaving a large amount yet to be explained (25-27). As these SNP’s are involved in different aspects of vitamin D metabolism (VDR, hydroxylation and inactivation) it could be hypothesized that these SNP’s might be independently associated with different health outcomes and used to study a causative role of vitamin D.
Conclusions

This thesis showed a complex role of vitamin D in relationship to inflammation, pulmonary function and COPD. In the first part we found that lower vitamin D concentrations are mostly related to the amount of visceral adipose tissue. We also found that adiposity largely explains the relationship between vitamin D concentrations and inflammation markers. These findings suggest an important role of adipose tissue in vitamin D metabolism and warrant future studies specifically in persons in obesity and in vitro studies of vitamin D metabolism in adipose tissue. In the relationship with pulmonary function we found conflicting results. In the NEO-study we found that lower vitamin D concentrations were related to lower pulmonary function and airway inflammation in persons with obesity, but not in persons without obesity. In the LASA study, we did not find effect modification of BMI, but did find effect modification by sex. The reason for this underlying difference could not be fully explained. The results of our observational studies affirmed the need for further research, which led to the set-up of an RCT. In our pilot trial, no effect of vitamin D supplementation was found on physical function, or respiratory muscle strength. The results of this study, however, were included in a meta-analysis, which showed a beneficial effect of vitamin D supplementation on exacerbation rate. These findings are promising results, which will be further studied in the still ongoing PRECOVID-trial.
References


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