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General discussion and future perspectives
SUMMARY OF THESIS

The ability to predict the rate of disease progression in patients with autosomal dominant polycystic kidney disease (ADPKD) is important, as it would help patients and caregivers alike in treatment related decisions. Patients with a higher rate of disease progression will probably benefit the most from therapy, since in these patients the benefit to risk ratio of treatment is expected to be better, especially when treatment is started early. It is difficult to predict the rate of disease progression in patients with ADPKD, as the disease course shows large interindividual variability. Especially early in the disease course it is difficult to predict prognosis, as kidney function remains relatively stable in the near-normal range for prolonged periods of time. Currently used predictors of prognosis, as total kidney volume (TKV) and \( PKD \) mutation, have limited sensitivity and specificity. Furthermore, their assessments are expensive and laborious and therefore often not available in routine clinical care. The general aim of this thesis was to study if current risk markers that predict the rate of disease progression in ADPKD could be improved, and to search for new markers that may predict the rate of disease progression beyond currently used risk markers.

Volumetry by manual tracing on MR images is the gold standard method to assess TKV in patients with ADPKD. This is a laborious, time consuming assessment and therefore not feasible in routine clinical care. In chapter 2 we investigated in patients with ADPKD, using MR images obtained during a clinical trial, if TKV (\( n=220 \)) and growth in TKV (\( n=48 \)) could be assessed by easier to use estimation methods instead. We found that the midslice and ellipsoid methods had comparable performances in terms of bias, precision and accuracy to that of the gold standard manual tracing method to assess TKV and growth in TKV. As the ellipsoid method could be performed in less time without the need for special software, this estimation method may be preferred over the midslice method for clinical care.

Besides the methodology, the quality of the MR images is important for accurate measurement of TKV. Historically, gadolinium enhanced T1 weighted images were the preferred sequence for the measurement of TKV. The use of gadolinium is nowadays discouraged because of potential side effects. When not using gadolinium contrast, T2 weighted images might be preferred over T1 weighted images for the measurement of the TKV, because this technique shows high kidney tissue-contrast and hyperintense renal cysts, that may help to better delineate the kidney boundaries.
against background tissue. In the past T2 weighted imaging required longer scanning time and multiple breath-hold scanning, and was more prone to misregistration, motion artifacts and heterogeneous tissue signal intensities leading to high variation in scanning quality. For that reason non-gadolinium enhanced T1-MR imaging has become the preferred method to assess TKV. However, the single-shot T2 weighted techniques have evolved over the last decades, making T2 weighted imaging potentially preferable over T1 weighted imaging for TKV measurement. In chapter 3 we compared the performance of T2 weighted images with T1 weighted images for TKV measurement with the gold standard manual tracing method in 40 ADPKD patients. We found that TKV and growth in TKV could be assessed as reliable and reproducible when using T2 weighted images. T2 weighted images can therefore be used for TKV measurement in patients with ADPKD. These images had minor advantages over T1 weighted images as there was a slightly lower intra- and interreader variability in TKV when using T2 weighted images and T2 weighted images were more often of sufficient quality for volume measurement.

Since ADPKD is a tubular disease with an inflammatory component, measurement of urinary tubular damage and inflammation markers was of interest as possible new risk markers to predict the rate disease progression, especially because these markers are relatively inexpensive and easy to measure. In chapter 4 we investigated, in a longitudinal study of 104 patients with ADPKD, who were 40 ± 11 years of age and had a baseline eGFR of 77 ± 30 ml/min/1.73m², whether 24 hour urinary excretion of certain markers was associated with ADPKD disease progression during a follow-up of 3.82 ± 1.23 years. We found that especially urinary β2MG and MCP-1 excretion were associated with kidney function decline independent of baseline eGFR, TKV and PKD mutation. Importantly, these markers were also associated with kidney function decline when only patients were taken into account with a relatively preserved kidney function. We furthermore demonstrated that these urinary biomarkers can even be of value to predict kidney function decline beyond TKV and PKD mutation. Therefore urinary tubular damage and inflammation markers are promising to serve as a clinical tool to identify patients with rapidly progressive disease for treatment selection. To corroborate these results, we investigated in chapter 5, in an independent cohort, whether urinary markers could be used to select ADPKD patients with rapidly progressive disease. This cohort consisted of 152 patients, with an average age of 49 ± 7 years and an eGFR of 51 ± 11 ml/min/1.73m², participating in a study investigating the therapeutic efficacy of lanreotide in ADPKD. We found that urinary excretion of in
particular β2MG and MCP-1 predicted rapidly progressive disease (annual change in eGFR ≤-3.5 ml/min/1.73m², during a follow-up 2.43 ± 0.41 years) independent of TKV and PKD mutation. The predictive value of a urinary biomarker score (based on tertiles of β2MG and MCP-1 excretion) was higher compared to a score using a single TKV in conjunction with age (Mayo htTKV classification) and equal to a score based on sex, the occurrence of hypertension and urological events before the age of 35 and PKD mutation (PROPKD score). Easy and inexpensive to measure urinary β2MG and MCP-1 excretion therefore hold promise to help identify patients with rapidly progressive disease instead of more expensive and laborious risk markers. Remarkably, urinary biomarker excretion was in neither of these studies associated with kidney growth. This could indicate that kidney growth may represent another pathophysiological phenomenon than kidney function decline in terms of urinary biomarkers.

It is assumed that renal function stays stable in the early stages of ADPKD while cysts are progressively formed and nephrons are lost. GFR indexed for age is therefore not a reliable marker of disease severity and future disease progression in early stage ADPKD. It is hypothesized that in this stage kidney function remains stable because of compensatory hyperfiltration of remnant nephrons. The extent to which a patient is hyperfiltrating may therefore be the earliest marker of disease severity and predictor of future disease progression. Glomerular hyperfiltration cannot be directly measured in humans. Several measures are therefore used as surrogate. Glomerular hyperfiltration is sometimes defined as an increased filtration fraction. However, measurement of filtration fraction by infusion of exogenous tracers such as iothalamate and hippuran may be inaccurate. It may lead to overestimation of filtration fraction, especially when tubular function is compromised, as in ADPKD. Glomerular hyperfiltration is therefore more commonly defined as the loss of kidney function reserve capacity, i.e. the impairment of the kidney to increase GFR in response to stimuli such as dopamine. In chapter 6, we investigated in a cross-sectional study of 150 ADPKD patients with a broad range of kidney function, whether there was a loss of kidney function reserve capacity compared to age and sex matched healthy controls. This study showed that ADPKD patients at a young age, despite having enlarged kidneys, have a GFR that is comparable to that of healthy controls at similar age. Remarkably, ADPKD patients in this age group had a normal level of kidney function reserve capacity as well as patients in early CKD stages. In older age groups and at later CKD stages, kidney function reserve capacity was lower compared to healthy controls. Hyperfiltration, measured as loss of kidney function reserve capacity,
therefore not be used as an early biomarker of disease severity. Filtration fraction, another surrogate measure of hyperfiltration, was also not elevated at young age and early CKD stages. Taken together, these results suggest that there may be no hyperfiltration in early stage ADPKD.

Somatostatin, a naturally occurring hormone, has multiple effects in the kidney including inhibition of cAMP production\textsuperscript{13} and inhibition of proliferation of renal cells\textsuperscript{14-16}. Since elevated cAMP is one of the pivotal detrimental factors in the pathophysiology of ADPKD\textsuperscript{17}, it is hypothesized that somatostatin may have favorable effects in ADPKD. Indeed, randomized clinical trials suggest that systemic administration of somatostatin analogues may be beneficial in slowing disease progression in ADPKD\textsuperscript{18-20}. Given these findings, we hypothesized that endogenous, systemic somatostatin levels are involved in the pathophysiology of ADPKD and therefore is associated with urinary cAMP, disease severity and progression. Furthermore, we hypothesized that administration of somatostatin analogues may down-regulate systemic somatostatin, and that the degree of systemic somatostatin down-regulation may reflect therapy efficacy. We showed in \textit{chapter 7}, that fasting plasma levels of endogenous somatostatin were not associated with urinary cAMP excretion, or measured GFR and TKV (n=127), nor with annual change in measured GFR and TKV (n=97) during a follow-up of 3.8 ± 1.3 years. This, however, does not necessarily imply that somatostatin is not involved in the pathophysiology of ADPKD. The systemic plasma concentration of endogenous somatostatin, may be too low to trigger the renal somatostatin receptors and may not reflect the active somatostatin concentration at renal tissue level, since it is suggested that somatostatin acts in an autocrine/paracrine manner. When lanreotide was administered in 25 ADPKD patients, that participated in a clinical trial, there was a decrease in plasma level of endogenous somatostatin. It may be that this decline in plasma somatostatin levels during administration of lanreotide reflects the extent to which somatostation receptors are triggered, and thus indirectly reflects efficacy of lanreotide treatment in ADPKD patients. Plasma somatostatin levels may then be used to monitor therapy efficacy in clinical care and may help in the decision to continue, stop or intensify lanreotide treatment in patients with ADPKD.

We and other study groups have investigated somatostatin analogues as a possible therapeutic option in ADPKD\textsuperscript{20,23-28}. To understand the possible mechanism of action of somatostatin we gave an overview of the complex physiology of somatostatin in \textit{chapter 8}. In this chapter, we also discussed the results of studies with somatostatin analogues.
analogues in ADPKD. Although both preclinical and clinical studies suggested beneficial effects of somatostatin analogues in the treatment of ADPKD, there may be no role for somatostatin analogues to preserve kidney function in ADPKD. This conclusion was based on the results of the most recent and largest of the randomized controlled trials that have been performed with somatostatin analogues. This DIPAK-1 study showed convincing evidence that lanreotide does not slow the rate of renal function decline in later stage ADPKD, defined as an eGFR of 30-60 ml/min/1.73 m$^2$ \(^{28}\). Results of ongoing clinical trials with somatostatin analogues should be awaited to draw definitive conclusions, because results may be different with other somatostatin analogues or in early ADPKD. The DIPAK-1 study did show that somatostatin analogues reduce the growth rate of total kidney volume and liver volume. Given these results it seems prudent to reserve treatment with somatostatin analogues to only ADPKD patients with symptoms related to increased intra-abdominal volume.

The vasopressin V2 receptor antagonist tolvaptan has recently been granted marketing authorization in Europe, the US and other countries around the globe as the first therapy to slow the rate of renal function decline in adult patients with ADPKD and rapidly progressive disease\(^{22,28}\). Tolvaptan blocks the vasopressin V2 receptor in the distal nephron and collecting duct, which results in inhibition of AC activity and in turn a decrease in cAMP levels, which slow ADPKD progression. Interestingly, somatostatin analogues also have the ability to lower cAMP levels by inhibiting AC activity. It may therefore be that there is an interaction between the vasopressin and somatostatin pathway. If somatostatin interacts with the vasopressin pathway an effect on renal water handling may be expected. Interestingly, older studies have indeed suggested involvement of somatostatin in renal water handling, causing either a diuretic or an antidiuretic effect, dependent on vasopressin levels\(^{29-32}\). In line, a more recent study observed a lower urine volume in PKD1 mice receiving a combination of a somatostatin analogue and tolvaptan in comparison to mice receiving tolvaptan alone\(^{33}\). This also suggests that there is an interaction between the somatostatin and vasopressin pathway. In chapter 9 we investigated in 305 ADPKD patients (48±7 years of age and eGFR 50±11 ml/min/1.73 m$^2$) that participated in a randomized controlled clinical trial investigating the efficacy of the somatostatin analogue lanreotide, if lanreotide has an effect on vasopressin levels and renal water handling in patients with ADPKD. We showed that there was no change in parameters of aquareisis, in patients receiving lanreotide compared to patients receiving standard care overall. According to literature the effect of somatostatin on aquareisis could possibly be dependent on levels of...
It may be that lanreotide did not have an effect on aquaresis in these subjects, because they had higher levels of vasopressin, as is often the case in ADPKD. Furthermore, in previous studies, only healthy subjects were studied who moreover, did not use medication that may affect aquaresis. Accordingly, the effect on aquaresis may become less apparent with more severe ADPKD, when tubular function is compromised and urine concentrating defects exist, or when diuretics are used. We therefore investigated if results were dependent on copeptin level (as surrogate of vasopressin), eGFR (as marker of disease severity) or use of diuretics. We found that results were dependent on eGFR and showed that lanreotide lowered free water clearance in patients with an eGFR >50 ml/min/1.73m². This effect seemed to be a direct effect of lanreotide and not mediated by vasopressin as copeptin levels did not change in this patient group. As treatment with somatostatin analogues are currently reserved for ADPKD patients with symptoms related to an increased intra-abdominal volume, combination therapy with tolvaptan may be indicated in some patients. If somatostatin analogues and tolvaptan are then co-prescribed, the aquaretic side effects of tolvaptan may be inhibited in patients with a preserved kidney function.

Tolvaptan was approved by the European Medicine Agency (EMA) in May 2015 as the first therapy to slow the rate of renal function decline in adult patients with ADPKD. This approval was based on the results of TEMPO 3:4 trial, a randomized controlled clinical trial, were the efficacy of tolvaptan was tested in 1445 ADPKD patients with relatively early stage disease (estimated creatinine clearance (Cockroft-Gault) ≥60 ml/min)\(^2\). According to the EMA label, tolvaptan ‘is indicated to slow the progression of cyst development and renal insufficiency of ADPKD in adults with CKD stages 1-3 at initiation of treatment with evidence of rapidly progressing disease’. This indication needed clarification at which CKD stage and age patients were qualified for treatment and how ‘evidence of rapidly progressing disease’ is defined. Therefore the ERA-EDTA working groups on inherited kidney disorders (WGIKD) and European renal best practice (ERBP) provided a recommendation on how to use tolvaptan for ADPKD in clinical care. The WGIKD and ERBP recommended to only prescribe tolvaptan in patients with CKD stages 1-3a (i.e. eGFR>45 ml/min/1.73m²), because information on the benefit-to-risk ratio of tolvaptan in patients with CKD stage 3b (i.e. eGFR 30-45 ml/min/1.73m²) was too limited to warrant treatment. Furthermore, they proposed a hierarchical decision algorithm to select patients with rapidly progressive disease that are eligible for tolvaptan treatment. This resulted in the recommendation not to start tolvaptan in patients aged >50 years, because patients >50 years who still...
have an eGFR >45 ml/min/1.73m², have a high probability of slowly progressive disease. Recently, the results of another large clinical trial with tolvaptan have become available (REPRISE study), which included patients with later stage ADPKD (eGFR (CKD-EPI) 25-65 ml/min/1.73m²)\(^2\). With these new results and now clinical experience with tolvaptan has been acquired, the position statement by the WGIKD and ERBP needed to be updated. In chapter 10 we proposed an updated recommendation for the use of tolvaptan in clinical care and show what the consequences of this update are in terms of number of patients eligible for tolvaptan. Because the REPRISE study showed a beneficial effect of tolvaptan in patients with later stage ADPKD and showed a comparable safety profile of this drug as seen in the TEMPO 3:4 trial, the recommendation to prescribe tolvaptan can be extended to patients with CKD stage 3b (i.e. eGFR 30-45 ml/min/1.73m²). The lower eGFR limit can now be extended to 30 ml/min/1.73m² for initiation of tolvaptan and the upper age limit to 55 years of age. We had clinical information available of 386 ADPKD patients and showed that the percentage of patients eligible for tolvaptan increased from 20% to 27% when applying these new recommendations. Based on experiences from clinical care, the hierarchical decision algorithm to select patients with rapidly progressive disease for tolvaptan treatment could be simplified. Lastly, with the clinical information available of 386 ADPKD patients, we provided evidence that selection based on eGFR and age alone is already a sensitive parameter to select patients with rapidly progressive disease. The decision algorithm may therefore be changed to an algorithm largely focused on CKD stage by age.

**DISCUSSION AND FUTURE PERSPECTIVES**

The last decade many studies have focused on TKV as a predictor of disease progression in patients with ADPKD\(^4,38-42\). This had led the FDA and EMA to accept TKV as a prognostic biomarker to enrich clinical trials for ADPKD patients with rapid disease progression. TKV has therefore grown to be one of the most important prognostic biomarkers in ADPKD. Also in clinical care, TKV may be used to select patients for disease modifying treatment. However, measurement of TKV by the gold standard MRI based manual tracing method is too time consuming for use in clinical care and therefore TKV estimation methods have been developed. We showed that especially the ellipsoid\(^4\) method would be suitable for clinical care. This finding has been corroborated by others\(^4\). This method may also be used for clinical trials, however the gold standard
manual tracing method had a slightly lower intra- and interreader variability in our study compared to the estimation methods and thus is probably more precise. In clinical trials kidney growth is often used to assess therapy efficacy of the studied drug and therefore precision of TKV is relatively more important for clinical trials than for clinical care. In addition, the manual tracing method can also be used to measure liver volume in contrast to the estimation methods. So especially when therapies are investigated that may affect both the hepatic as the renal phenotype, the manual tracing methods may still be the preferred method. Since the first therapeutic disease modifying treatment for ADPKD has become available we now indeed use the ellipsoid method to estimate TKV and to select patients for treatment in clinical care. Yet, a MRI is needed which is a relative expensive procedure. We showed that the single shot T2 weighted MRI technique results in higher quality images compared to the T1 weighted technique. Therefore less often an extra MRI is needed for volume measurement, which will save costs and lower patient burden. In this respect, we recommend to only scan the T2 weighted sequence, which will in addition save scanning time.

In this thesis, we showed that relatively inexpensive and easy to measure urinary tubular damage and inflammation markers are promising to be used as a prognostic biomarker. We showed in 2 independent cohorts of ADPKD patients that especially β2MG and MCP-1 were associated with annual change in eGFR and could predict rapidly progressive disease independent of TKV or PKD mutation. These inexpensive biomarkers (around €3-4 per sample) may therefore be used instead of an expensive MRI (in the Netherlands €300 without TKV measurement) or PKD mutation analysis (in the Netherlands €1675) for risk prediction and treatment selection in patients with ADPKD. These results have to be corroborated in a larger independent cohort after which efforts have to be made to incorporate these biomarkers in clinical care, preferably as a risk score including other clinical relevant parameters (e.g. age, sex and eGFR).

Although eGFR may not be a reliable prognostic biomarker, especially in early stage ADPKD, results from this thesis suggest that eGFR may possibly perform better than previously assumed. It was assumed that GFR stays stable in early stages of the disease after which it progressively declines, because of compensatory hyperfiltration of remnant nephrons. In our clinical experience, we first of all do not observe this phenomenon, and see a rather linear decline in eGFR in these patients. However, this can be due to the fact that the UMCG is a tertiary referral hospital, with relatively more
patients with later and more severe disease. Yet, we also have experienced ADPKD patients with preserved kidney function and markedly increased kidney function during pregnancy. It is known that pregnancy physiologically leads to hyperfiltration and if patients with ADPKD hyperfilter prior to a decline in GFR, this phenomenon should not be observed in ADPKD. We showed in this thesis, in chapter 6, that this hypothesis may indeed be rejected. The results from this chapter also suggest that kidney function does not remain stable for decades in ADPKD as kidney function was already diminished in patients 30-39 years of age. However, such conclusions cannot be made based on this chapter because of the cross-sectional nature of this study. Longitudinal studies should be performed to confirm this observation. Recently, Brosnahan et al. studied patterns of kidney function decline in 929 ADPKD patients of the HALT-PKD trials (Study A, with younger patients included with preserved eGFR and Study B with older patients included with reduced eGFR)45. They showed that the majority of patients (62.5% in Study A and 81% in Study B) had a linear progressive loss of kidney function during up to 8 years of follow-up. A non-linear decline was observed only in 22% in Study A and 13% in Study B and also in many different shapes; periods of slow or no eGFR decline could be followed by periods of faster decline and vice versa. These findings thus confirm our observations from this thesis and our clinical experience. A stable eGFR for decades followed by progressive loss of kidney function is therefore not a general phenomenon in patients with ADPKD. Moreover, studies with other renal diseases described nonlinear trajectories in up to 40% of patients46-49, so a nonlinear decline may be even less of a problem in this patient group compared to others. Therefore, eGFR is probably not such a poor predictor of prognosis in patients with ADPKD as always assumed. In line, in this thesis we showed that categorizing 386 ADPKD patients from the UMCG according to eGFR by age resulted in similar conclusions as whether a patient has rapidly progressive ADPKD compared to historical eGFR decline and the Mayo htTKV classification. However, at young age when there has not yet been a decline in renal function, or when eGFR is not reliable (for example in patients with increased or decreased muscle mass), risk classifications are still needed.

The main renal outcome of ADPKD is end stage kidney disease. Since ADPKD is a slowly progressing disease it is not feasible to take end stage kidney disease as an outcome measure in clinical trials. Therefore surrogates are used to predict a patients risk for progression to end stage kidney disease. Because eGFR was assumed to remain stable in the first decades of ADPKD, kidney function decline was thought not
to be a reliable surrogate. The rate of TKV growth has therefore been widely used as a primary outcome to assess therapy efficacy in clinical trials instead. However, in some studies, as was the case for the DIPAK-1 trial, the effect of therapy on eGFR and TKV are divergent\textsuperscript{22,28,50}. Some state that this divergent effect is mainly caused by trial design, suboptimal dosing of the treatment agent and substantial pharmacologic side effects. We however believe that results with GFR and TKV may be unrelated. Although multiple studies have shown that growth in TKV is well associated with a decline in GFR, there is a large interindividual variability in this association. This is in line with our results from chapter 10. In this chapter we showed that many patients with a relatively preserved kidney function at older age, which indicates that these patients have slowly progressive disease, were classified as Mayo class 1C/D/E which would suggest that these patients have rapidly progressive disease. Furthermore, in this thesis none of the urinary biomarkers studied were associated with growth in TKV, whereas several were associated with kidney function decline. This suggests that there are more and other factors associated with a decline in GFR than growth in TKV alone, like inflammation and fibrosis. This emphasizes that one should be cautious with using TKV both as a biomarker of therapy efficacy and as sole biomarker to predict prognosis.

Currently no markers are available to assess on a short-term basis whether treatment is effective in the long-term for the renal phenotype of ADPKD. Therefore there are no recommendations available on how to determine therapy efficacy in patients with ADPKD in clinical care\textsuperscript{43}. In previous research conducted by our research group we measured urinary tubular damage markers that decreased during short term treatment with tolvaptan in 27 patients\textsuperscript{51}. We showed in this thesis that tubular damage markers are also associated with renal function decline on the long term and tolvaptan was found to be effective to slow renal function decline in patients with ADPKD\textsuperscript{21,22}. In line, we found no decrease in urinary tubular damage markers during short term treatment with lanreotide (unpublished work) and lanreotide was found not to be effective to slow renal function decline in patients with ADPKD\textsuperscript{28}. These markers have therefore the potential to serve both as prognostic and as therapy efficacy markers. Yet, no studies have been performed that confirmed these results. Therefore future studies should focus on finding associations between tolvaptan induced short term changes in urinary tubular damage markers and long-term outcome. These markers may then be of help to choose whether to stop, continue, or intensify therapy in clinical care.
In this thesis we discussed that somatostatin analogues may, for now, only be used in ADPKD patients with symptoms related to increased intra-abdominal volume. An increase in total liver volume appears to have the largest contribution to these symptoms in patients with ADPKD\textsuperscript{52}. Currently, patients are treated with a somatostatin analogue when total liver volume is already enlarged and symptoms are already present. As far as we are informed, no prognostic biomarkers are available to predict which patients will have symptoms related to an enlarged liver and therefore patients cannot be treated preventatively. Furthermore, when patients are treated with lanreotide for the hepatic phenotype in the Netherlands, assessment of the effect of therapy is performed by assessment of liver volume and quality of life questionnaires only after 1.5 years of treatment. Like the situation for the renal phenotype, there are also no short-term efficacy parameters available in clinical care for treatment of the hepatic phenotype. In this thesis we showed that circulating levels of endogenous somatostatin was not associated with disease severity and progression in relation to the renal phenotype. This could be explained as somatostatin is mainly produced at local sites of action and plasma levels of somatostatin may therefore not represent levels at the renal tissue level. Yet, it may represent levels at the hepatic tissue level as circulating concentration of somatostatin mainly consist of somatostatin secreted from the gastro-intestinal tract\textsuperscript{53-56}. In this thesis we did not have data available to relate plasma somatostatin levels to growth in liver volume, but we did show that plasma somatostatin declined after 3 months of treatment with lanreotide. Future studies should therefore investigate if plasma somatostatin is associated with growth in liver volume and if change in plasma somatostatin is associated with long-term therapy efficacy.

In conclusion, now the first therapeutic options to slow ADPKD progression have emerged, questions arise in clinical care as whom to treat and when to stop, continue, or intensify therapy. Prediction of disease progression and therapy response form the key to answer these questions. The studies described in this thesis have contributed to deliver that key, but the door is not yet fully unlocked.
REFERENCES


General discussion and future perspectives