Neutrophilic reversible allograft dysfunction (NRAD) and restrictive allograft syndrome (RAS)

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Summary

Lung transplantation is currently considered as an ultimate life-saving treatment for selected patients suffering from end-stage pulmonary disease. Long-term survival, however, is hampered by chronic rejection, or chronic lung allograft dysfunction (CLAD). Recently, various phenotypes within CLAD have been identified, challenging the established clinical definition of Bronchiolitis Obliterans Syndrome (BOS). Some patients with presumed BOS for instance, demonstrate an important improvement in FEV₁ after treatment with azithromycin. These patients are characterized by the presence of excess ($\geq 15\%$) BAL neutrophils, in absence of concurrent infection. This phenotype of CLAD has been redefined as neutrophilic reversible allograft dysfunction (NRAD) and these patients generally have a very good prognosis after diagnosis. Another group of patients with CLAD develop a restrictive rather than an obstructive pulmonary function defect (defined as a decline in total lung capacity of at least 10%) and demonstrate persistent interstitial and ground glass opacities on chest CT scan. This phenotype is called restrictive allograft syndrome (RAS) and patients with RAS have a much worse prognosis after diagnosis. In this review, we will further discuss both of these CLAD phenotypes that do not fit the classical definition of BOS. Potential pathophysiological mechanisms, etiology, diagnosis, prognosis and treatment will be discussed.

Introduction

Lung transplantation (LTx) has become an established treatment option for patients with end-stage pulmonary diseases like COPD, cystic fibrosis, pulmonary fibrosis, pulmonary arterial hypertension, ... Survival after LTx, however, remains rather poor as only 55% of patients survive more than 5 years according to the registry of the International Society for Heart and lung Transplantation (ISHLT) database (1). Early post-operative complications include primary graft dysfunction (2), infections, suture stenosis (3), acute rejection (4) and lymphocytic bronchiolitis (LB) (5). Long-term survival is limited by infections and the development of chronic rejection or chronic lung allograft dysfunction (CLAD), which may clinically manifest as Bronchiolitis Obliterans Syndrome (BOS), an obstructive decline in FEV₁ of at least 20% compared to the best post-operative value in the absence of other identifiable factors like acute rejection, suture stenosis, infection, ... It was the general belief that BOS was characterized by: A/ neutrophilic airway inflammation, B/ largely irreversible and persistent obstructive pulmonary function decline, and C/ a fibroproliferative narrowing of the bronchioles (6). As diagnostic yield of transbronchial biopsies is very low (7), a decline in FEV₁ of at least 20% is regarded as the hallmark of chronic rejection in the absence of other identifiable causes. There is now accumulating evidence that not every persistent decline in FEV₁ is compatible with this definition and as a consequence all over the world the term CLAD has been introduced, although up to now this term has not clearly been defined. Nevertheless, there is growing awareness that this terminology may be better to identify a chronic, persistent decline in FEV₁ after LTx. As a consequence, this review will focus on 2 different non-BOS phenotypes of CLAD. More specifically, we will focus on neutrophilic reversible allograft dysfunction (NRAD), characterized by an increase in FEV₁ of at least 10% after azithromycin treatment on the one hand, and the recently identified restrictive allograft syndrome (RAS) on the other hand.

Neutrophilic reversible allograft dysfunction

Neutrophils

Digiovine et al. were the first to demonstrate the presence of excess BAL neutrophils during chronic rejection (8), which was further corroborated by Riise et al. who demonstrated that neutrophil infiltration and activation plays a role in the development of BOS. Convincing evidence of neutrophil activation was demonstrated in BAL fluid and in transbronchial biopsies obtained from lung transplanted patients with BOS, moreover BAL fluid levels of IL-8 and neutrophils significantly increased in patients diagnosed with BOS (9). Thereafter, more and more studies corroborated the association between excess BAL neutrophilia and the development of chronic rejection and survival after lung transplantation (10-12). Moreover, the presence of BAL neutrophils at 3 months and 1 year after transplantation proved to be prognostic for the later development of BOS (13). Neutrophils were thought to be responsible for the development of obliterative bronchiolitis (OB) via secretion of matrix metalloproteinases (14), different chemokines and growth factors and oxidative stress (15), causing damage to the respiratory epithelium, leading to an excess repair process with proliferation of fibroblasts and finally established OB.

Neomacrolides

The introduction of the neomacrolide antibiotic azithromycin, however, has led to a complete paradigm shift. Gerhardt et al. were the first to use low-dose azithromycin (250 mg three times a week) as an add-on treatment to conventional immunosuppressive therapy in six patients with BOS after LTx. FEV₁ improved in five of these six patients by a mean of 17.1% or 0.50 L over a 4-month period (16). This was later on corroborated by several other groups (17-20), all reporting an increase in FEV₁ in at least a subset of patients, clearly illustrating the beneficial effect of azithromycin in established BOS. However, not all studies reported a clear beneficial effect of azithromycin. Shitrit et

al. for instance, demonstrated that the overall FEV₁ of 11 LTx patients (BOS 3: n=1; BOS 2: n=6; BOS 1: n=4) had decreased by 1% over 4 months and 2% after 10 months of additional azithromycin treatment. Azithromycin therapy also did not lead to any improvement of BOS status, however, it could at least arrest the progression of the disease (21). The reason why not all patients responded to azithromycin treatment was not clear at first. In fact, several possible reasons were raised such as a change in calcineurin trough level, the initial BOS stage at the start of azithromycin treatment, colonisation of the airways with Pseudomonads,... (16-19). Later on, however, it became clear that patients who responded to azithromycin, had high BAL IL-8 levels and neutrophilia at the time of diagnosis. Moreover, there was a good correlation between the initial BAL neutrophilia and the FEV₁ response after 3 months of treatment (22), a finding that was later corroborated by Gottlieb et al. (17). The observed discrepancy between responders and non-responders disclosed a dichotomy within chronic rejection after lung transplantation. The first phenotype displays a high % of neutrophils in BAL (> 15%), develops rather early after LTx and displays an improvement in FEV₁ of at least 10% after 3 to 6 months of azithromycin treatment. This phenotype was called neutrophilic reversible allograft dysfunction (NRAD) or neutrophilic CLAD (nCLAD). This is in big contrast with the other phenotype, which lacks high BAL neutrophilia (< 15%), develops later and does not respond to azithromycin therapy and was denominated at that moment as fibroproliferative Bronchiolitis Obliterans Syndrome (fBOS) (23,24). A randomized double blind placebo-controlled trial recently showed that azithromycin started at discharge from the hospital after LTx could prevent the development of CLAD. The azithromycin group also displayed a better FEV₁, lower BAL neutrophils and lower systemic C-reactive protein (CRP) levels compared to the placebo group (25).

At this moment it is still unclear whether a NRAD episode any time during follow-up is a risk factor for the later development of BOS, but it has been shown that NRAD is at least a risk factor for long-time survival after LTx (26). Azithromycin is not the only macrolide used in post-transplant care, some groups prefer to use clarithromycin, which results in a similar effect in a similar way (responders vs.

non- responders) (27). Caution is needed when using clarithromycin, as it affects the calcineurin trough levels whereas azithromycin does not (28,29).

Mechanisms of action of azithromycin

We assume that both the antimicrobial and the anti-inflammatory actions contribute to the beneficial effect of neomacrolide antibiotics. The exact cellular mechanisms are beyond the scope of this review and have already extensively been described previously (30). Some aspects, however, are important to mention. A human BAL study looking at the expression of 32 different proteins in BOS, NRAD and control patients at the time of diagnosis of CLAD showed that in established NRAD patients MCP-1 , RANTES, IL-1 β , IL-8 , TIMP-1, MMP-8 , MMP-9 , HGF, MPO and bile acid concentrations were upregulated, while only PDGF-AA was downregulated compared to control patients (31). Moreover almost all these proteins were correlated with BAL neutrophilia. None of the proteins were different between azithromycin non-responsive BOS or fBOS and control patients. This is in sharp contrast with previously published data showing that these proteins are indeed involved in the development of BOS (32-34). This further illustrates the importance for adequate phenotyping of BOS. Moreover, one should keep this in mind when looking at previous research as all this work was performed with the understanding of BOS at that time, making interpretation of these results very difficult as we now know that BOS actually represents different phenotypes. An interesting future direction of research is to see whether azithromycin is able to decrease all these proteins to levels comparable to stable patients. It has for instance recently been shown that MMP-9 gelatinase activity is increased in BAL of NRAD patients and following 3-6 months of azithromycin therapy the MMP-9 gelatinase activity significantly decreased. More importantly, these decreased levels were still higher compared to stable patients, which might indicate an ongoing matrix remodeling process, potentially leading to the later development of chronic rejection (35). An intriguing hypothesis is that IL-17, an important pro-inflammatory cytokine and indirect chemo-attractant of neutrophils, is a key

player in NRAD. Evidence derived from either human or mice studies already implicated IL-17 in the pathogenesis of chronic rejection (36-38). *In vitro* data in human airway smooth muscle cells and epithelial cells shows that IL-17 can induce IL-8, a major neutrophil chemo-attractant, but more importantly that azithromycin is able to reduce the IL-17 induced IL-8 production (39,40,40). Further unpublished results show that the number of IL-17 positive lymphocytes in the submucosa increase in NRAD compared to control and azithromycin non-responsive patients. After 3 to 6 months of azithromycin therapy the number of IL-17 positive cells significantly decreased to control levels.

It remains to be added that during azithromycin treatment for NRAD, there may be a recurrence of BAL neutrophilia with or without concurrent FEV₁ decline, the significance and prognosis of which remain to be further elucidated.

Lymphocytic bronchiolitis and NRAD: different entities or not?

Lymphocytic bronchiolitis (LB) is characterized by a lymphocytic infiltration of the airway submucosa and the epithelium and is graded according to the extent of the infiltrate (41). LB is often accompanied by a decrease in pulmonary function (42). Glanville et al. showed that LB is a risk factor for the later development of BOS (43). Regarding treatment of LB, there is conflicting evidence showing that inhaled steroids might bring some relief. One study indeed shows an increase in FEV₁ after treating LB with inhaled steroids (44), while another study could not demonstrate a beneficial effect (42).

There may actually be a lot of similarities between LB and NRAD. As described above, LB is characterized by a lymphocytic infiltrate in the airway submucosa but a study by Chambers et al. also noticed the presence of a lymphocytic infiltrate in the bronchial/bronchiolar epithelium of established BOS patients. Moreover the number of T-cells in this infiltrate correlated with the BAL neutrophilia (45). BAL neutrophilia is indeed the most typical characteristic of NRAD patients, but BAL

neutrophilia can also be found during episodes of LB. Vos et al. indeed demonstrated that the % BAL neutrophils is significantly higher during LB compared to control patients and patients suffering from acute perivascular rejection (46). Lastly, when comparing CT scans of LB patients with azithromycin responsive patients, we observe very similar patterns: centrilobular nodules and tree in bud, which resolve after 2-6 months of azithromycin therapy (47). It is our believe that some episodes of LB, especially when accompanied by BAL neutrophilia, may result in a decline in FEV₁ similar to NRAD. Moreover it seems likely that a negative score for B-grade rejection at diagnosis of NRAD is simply a matter of sampling error as biopsies are very small and can give a skewed image (48). We are currently recruiting patients with isolated LB, not yet treated with azithromycin, in an open label trial with azithromycin to establish its true effect in this situation. (ClinicalTrials.gov Identifier: NCT01109160). In figure 1, we illustrate the FEV₁ evolution of a patient with biopsy-proven LB (patient 1) and one with NRAD (patient 2). Both were only treated with additional azithromycin and show a comparable increase in FEV₁. Since we know that high doses of steroids hardly result in any improvement of FEV₁ in patients with isolated LB (42), this particular situation may therefore represent another beneficial effect of neo-macrolides.

Figure 1 Legend

- A. FEV₁ evolution of patient 1 (double lung transplant for emphysema) suffering from LB grade B1R with 64.8% of neutrophils in the BAL fluid. At that time there was a single FEV₁ measurement reaching the threshold of BOS1. Following therapy with azithromycin (red arrow) the FEV₁ fully recovered.
- B. FEV₁ evolution of patient 2 (double lung transplant for emphysema, suffering from NRAD. After 18 months the patients experienced a persistent decrease (at least 3 weeks) in FEV₁ compatible with BOS. A biopsy at that time could not demonstrate perivascular or peribronchiolar rejection. BAL showed 57.6% neutrophils. Azithromycin was started (red arrow), leading to a complete restoration of the FEV₁, resulting in BOS 0 again.

Restrictive allograft syndrome

Diagnosis and prognosis

Recently, the Toronto group introduced the term 'restrictive allograft syndrome' (RAS) for patients suffering from a persistent decline in FEV₁ (> 20% compared with the best postoperative values) and an associated restrictive pulmonary function defect, which they defined as a decline in total lung capacity (TLC) >10% compared to baseline (49). As TLC is not routinely performed in most centers, Verleden et al. also used the FVC and FEV₁/FVC ratio as a surrogate marker. If the latter remains stable while the FEV₁ drops, this may also point to a restrictive pulmonary function (26). In that respect, Sato demonstrated a good correlation between FEV₁/FVC ratio and TLC (49).

This syndrome develops in about 25-35% of all lung transplant patients suffering from CLAD. Moreover, these RAS patients demonstrated a lower survival rate compared with BOS patients. The median survival after diagnosis of RAS was 8 months in the Leuven cohort, while the Toronto cohort showed a median survival of 16 months, which was considerably lower compared to patients with an obstructive pulmonary function defect (hence typical BOS patients, 35 months in Leuven and 46 in Toronto) (26,49). CT scan in RAS patients shows more interstitial opacities, ground glass opacities, upper lobe dominant fibrosis and honeycombing compared to patients with BOS, which show more signs of airtrapping (49).

Presently, the pathophysiological mechanisms leading to RAS remain elusive. In the Toronto cohort, 12 of 29 patients (41%) had a positive identification of a wide range of micro-organisms in either BAL or sputum at diagnosis, pointing to a possible infectious trigger that might lead to an excessive fibrotic reaction, resulting in a lung with end-stage fibrosis (49).

Although the terminology may be new, the syndrome seems to have been described previously in autopsy studies. In a report by Martinu et al, studying 12 retransplant lungs, at least 3 patients may now be identified as having RAS with pathology reports showing interstitial fibrosis and radiology showing ground glass opacities, intralobular thickening and areas of fibrosis (50). Moreover, interstitial changes on transbronchial biopsies are already reported for a long time and seem to occur

more frequently later on during follow-up (51). In chronic graft versus host disease with pulmonary manifestation after bone marrow or stem cell transplantation, a similar phenomenon has already been described. This syndrome is denominated as pleuroparenchymal fibro-elastosis and is a very rare complication. Patients manifested with dyspnoea, CT showed pleural thickening, interstitial fibrosis and mosaic patterns, while the pathology demonstrated obliterative bronchiolitis combined with patchy zones of intra-alveolar fibrosis (52,53). Unfortunately no detailed pulmonary function tests were performed. In this small case series prognosis was also bad, similar to RAS. The similarities between RAS and pleuroparenchymal fibro-elastosis are hence very striking and might represent a similar pathophysiological mechanism. This is also noted by the Toronto group as they reported the great similarities between the 2 conditions. One of the most remarkable findings of their study is the presence of OB in RAS patients (14/16 patients, 87.5%), which indicates that RAS might in fact be a manifestation of chronic rejection (54). Pathological analysis of an explant lung at re-transplantation for RAS indeed shows obliteration of the airway (figure 2A) with surrounding fibrosis of the alveoli. Masson's trichrome staining is able to demonstrate accumulation of granulation tissue within the airway lumen and within the parenchyma a dens area of fibrosis is seen (figure 2B).

Further research will indicate whether the definition of RAS will stand and whether more different phenotypes of RAS will be defined. In this respect, there may indeed be differences in radiological presentations as some patients do have extensive apical pleural fibrosis, whereas others rather develop interstitial changes throughout the lung, although most apparent in the upper lobes. In this respect, the paper by Pakhale et al (48) describing upper lobe fibrosis probably represents the first clinical presentation of RAS.

BOS and RAS may also co-exist, as some patients first develop a classical BOS (obstructive decline in FEV1, no decline in TLC) and only later on develop characteristics of RAS (decline in TLC $\geq$ 10%) with the appearance of interstitial infiltrates on CT scan.

Does this all represent the same manifestation of the disease or are different pathophysiological mechanisms responsible for this? Most importantly, will this have an influence on the survival of the

patients? What about other manifestations of chronic rejection, not strictly fitting the definition of BOS or RAS such as follicular bronchiolitis (55), exudative bronchiolitis (56)? This all remains to be further investigated.

RAS and Pulmonary function

Woodrow was the first to introduce the term restrictive BOS, based on a decrease of the forced vital capacity (FVC) of at least 20% compared to baseline. This study, however, could not demonstrate a survival disadvantage for the patients suffering from a restrictive pulmonary function, although a trend was seen, probably due to exclusion of patients with persistent infiltrates, who most likely also suffered from a restrictive pulmonary function (57).

The studies, documenting and describing RAS patients after lung transplantation, used different criteria to diagnose a restrictive pulmonary function. Sato et al defined RAS, along with an FEV₁ decline of at least 20%, as an additional TLC decline of at least 10% compared to baseline. Verleden et al. also incorporated the tiffeneau index to describe restriction and Woodrow, as discussed above, used an FVC decline of at least 20% (25, 45, 51). Further research will show if there is an easier method to diagnose RAS as TLC measurements are not routinely performed in most centers, although it is advised now to do so. An easier method to diagnose RAS would surely benefit an earlier and a more accurate diagnosis. If this would prove to be ineffective, then more extensive pulmonary function testing will need to be performed more often in the routine transplant patient follow-up.

Two different types of RAS patients are illustrated in figure 3. One patient shows a very fast evolution towards re-transplantation, while the other patient shows a rather slow evolution.

Risk factors

The only identified risk factor up to now for the later development of RAS is late onset diffuse alveolar damage (DAD occurring later than 3 months after LTx) as 58% of RAS patients suffered from an episode of late onset DAD (58). The problem, however, is that only approximately 30% of patients

with chronic rejection suffer from RAS, making it very difficult to do single-center studies. Multi-center studies are necessary to accurately define risk factors for RAS. Perhaps similar risk factors as compared to BOS (A grade rejection, LB, CMV infection, pseudomonas colonization, gastro-oesophageal reflux, ...) will prove to be important for the later development of RAS (59). Some new risk factors may arise such a genetic predisposition towards the development of RAS. Interesting in that perspective is the fact that a MUC5B polymorphism has been demonstrated to be an important genetic predictor for Idiopathic Pulmonary Fibrosis (IPF) (60), which may in some way resemble RAS development. This has hitherto not been investigated in the development of RAS.

Since serum KL-6 levels, a marker of fibroblast proliferation, are increased in RAS patients compared to BOS patients (61), this may strengthen this hypothesis as KL-6 is also a marker for IPF or CF, diseases characterized by interstitial fibrosis (62,63). The patient cohort was, however, rather small, and future studies might be able to corroborate the potential role of this intriguing marker.

In the Toronto experience, neither age (donor/receptor), type of transplantation, original diagnosis for LTx, CMV (mis-)match and acute rejection proved to be risk factors for the later development of RAS (49).

Treatment

As the concept of RAS is rather new, no valid treatment has been proposed yet. At this moment the influence of conventional BOS treatment options like total lymphoid irradiation (64), photophoresis (65) and montelukast (66) is not clear. In our experience azithromycin does not bring relief to these patients. Perhaps we should look outside the field of lung transplantation and extend our horizon to IPF, a disease with much similarities to RAS as the etiology of the disease is unknown, radiology looks similar (consolidation zones, reticular patterns) and histology shows end-stage fibrosis. A beneficial effect of pirfenidone has been shown to slow down the decline in FVC in patients with established IPF (67). Pirfenidone suppresses TNF- α production and influences the production of TGF- β (68). Moreover, in vitro studies showed that pirfenidone was able to slow down proliferation of human

lung fibroblast cells (69). Even within the lung transplant setting, there is some evidence that pirfenidone might bring relief, although this evidence is mostly based on animal studies. Both in a murine heterotopic tracheal transplantation model (70,71) and a rat orthotopic lung transplantation model, pirfenidone has been shown to reduce fibrosis (72). At this moment, however, the only treatment that can bring relief in well-selected patients is re-transplantation. Another drug that might be of help is the CD52 antagonist alemtuzumab (Campath-1H). CD52 is a protein expressed on B-cells, lymphocytes, dendritic cells and monocytes and was first used in lung transplantation to treat recurrent acute rejection episodes, which were untreatable with conventional treatment protocols (73), with promising results. A further study used alemtuzumab in treatment of both refractory acute rejection (n=12) and bronchiolitis obliterans syndrome (n=10), showing improvement in BOS stage in 4 patients, stabilization in 3 and further deterioration in the 3 other patients (74). Indications that it can be used in RAS came from 2 recent reports. Treatment with alemtuzumab in a patient suffering from pulmonary chronic graft versus host disease after bone marrow transplantation showed impressive improvement in pulmonary condition. The initial radiology, resembling RAS, shows patchy consolidation and diffuse reticulonodular areas, which completely resolved after 20 days of treatment (75). Alemtuzumab further improved lung function in 4 patients who developed interstitial lung injury with alveolar septal fibrosis, typical hallmarks of RAS patients (76).

Conclusion

Phenotyping CLAD has very important implications towards clinical practice. NRAD patients are most easily to diagnose using BAL fluid and these patients will recover following azithromycin treatment (as per definition) and have a very good prognosis. RAS patients are diagnosed using TLC and CAT-scans and have a much worse prognosis. Unless other treatments become available to treat RAS, this implicates that possible re-transplantation should be considered quickly after diagnosis as prognosis is bad. Moreover phenotyping CLAD also has important scientific implications as most previous studies used a pool of patients suffering from BOS/CLAD whereas it is now presumed that each

phenotype has its own characteristics and possibly different risk factors, which implicates that accurate phenotyping is necessary for valid scientific research. An overview of different characteristics representing the different CLAD phenotypes is given in table 1. Only by adequate phenotyping patients, a better long term survival can be achieved. The future will probably lie in an individualized therapy depending on the type of CLAD that the patient is suffering from. This is probably the only hope to win the battle against chronic rejection after lung-transplantation and to finally have a long term survival that matches other solid-organ transplants.

Figure 2

- FEV₁ evolution of patient 1 with RAS. 9 years after initial double lung transplantation for cystic fibrosis, the patient develops a very rapid irreversible decline in FEV₁. Red line indicates a decline of 20%.
- TLC evolution of RAS patient 1. When the FEV₁ starts declining a decrease in TLC >10% was observed compatible with the definition of RAS. Red line indicates a decline in TLC of 10%.
- CAT scan of patient 1 at diagnosis of RAS. CAT shows ground glass opacities with accompanying zones of consolidation.
- FEV₁ evolution of patient 2 with RAS. 4 years after initial double lung transplantation for α 1-anti trypsin deficiency, the patient develops a slow decline in FEV₁. Red line indicates a decline of 20%.
- Tiffeneau index evolution of patient 2 as no TLC measurements before development of RAS are available. As the FEV₁ declines, the Tiffeneau index does not drop below the baseline of 0.7 compatible with a restrictive pulmonary function decline.
- CAT scan of patient 2, 2 years after diagnosis of RAS, showing severe pleural and septal thickening.

Figure 3

A) H&E staining of an explanted lung of a RAS patient at re-transplantation. A dense accumulation of granulation tissue is seen within the airway lumen. The airway parenchyma is partially fibrosed resulting in very dense tissue. Black arrow points to the airway wall.

B) Masson- trichrome staining on an explanted lung of a RAS patient at re-transplantation. The airway lumen is filled with additional collagen. Within the airway parenchyma a dense collagen positive infiltrate is seen. Black arrow points to the airway wall, dashed arrow points to fibrosis within the parenchyma.

	NRAD	BOS	RAS
Inflammation (BAL)	neutrophilic airway inflammation	no airway inflammation	airway inflammation?
Clinically	coarse crackles and increased sputum production	no coarse crackles, minimal sputum	Minimal coarse crackles
Time of onset	mostly early onset after transplantation	onset late after transplantation	Onset late after transplantation
Survival	very good	fairly good	bad
Histology	initially inflammatory ends up in pure fibrosis	OB lesions, healthy parenchyma	parenchymal fibrosis, thickened septa and pleura
Radiology	bronchiectasis, airway wall thickening, mucus plugging , centrilobular nodules	air trapping and mosaic attenuation	consolidation, reticular pattern, persistent infiltrate
Azithromycin	reversible (effective)	irreversible (ineffective)	irreversible
Pulmonary function	obstructive	obstructive	restrictive (TLC decline >10%), increasing tiffeneau index

Table 1: proposal of different characteristics in NRAD, BOS and RAS patients (partially adapted from (23))

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